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Exome sequencing of 20,979 individuals with epilepsy reveals shared and distinct ultra-rare genetic risk across disorder subtypes

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Identifying genetic risk factors for highly heterogeneous disorders such as epilepsy remains challenging. Here we present, to our knowledge, the largest whole-exome sequencing study of epilepsy to date, with more than 54,000 human exomes, comprising 20,979 deeply phenotyped patients from multiple genetic ancestry groups with diverse epilepsy subtypes and 33,444 controls, to investigate rare variants that confer disease risk. These analyses implicate seven individual genes, three gene sets and four copy number variants at exome-wide significance. Genes encoding ion channels show strong association with multiple epilepsy subtypes, including epileptic encephalopathies and generalized and focal epilepsies, whereas most other gene discoveries are subtype specific, highlighting distinct genetic contributions to different epilepsies. Combining results from rare single-nucleotide/short insertion and deletion variants, copy number variants and common variants, we offer an expanded view of the genetic architecture of epilepsy, with growing evidence of convergence among different genetic risk loci on the same genes. Top candidate genes are enriched for roles in synaptic transmission and neuronal excitability, particularly postnatally and in the neocortex. We also identify shared rare variant risk between epilepsy and other neurodevelopmental disorders. Our data can be accessed via an interactive browser, hopefully facilitating diagnostic efforts and accelerating the development of follow-up studies.

Epilepsy is a group of heterogeneous disorders, characterized by an enduring predisposition to generate epileptic seizures[1]. Epilepsy has a prevalence of 4–10 per 1,000 individuals worldwide, making it one of the most common neurological conditions[2]. The role of genetic contributions to epilepsy has been long recognized[3,4], yet delineating the full range of genetic effects on the epilepsies remains a core challenge.

Whole-exome sequencing (WES) has proven effective in gene discovery for Mendelian disorders, including familial and severe epilepsy syndromes. An increasing number of genes have been implicated in developmental and epileptic encephalopathies (DEEs (Mendelian Inheritance in Man (MIM): 308350)), a severe group of epilepsies characterized by early-onset, intractable seizures and developmental delay. In contrast, genes discovered for the milder, more common forms of epilepsies—genetic generalized epilepsy (GGE (MIM: 600669)) and non-acquired focal epilepsy (NAFE (MIM: 604364 and 245570)) characterized by generalized and focal seizures, respectively—remain

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scarce[5]. Most discoveries have been based on familial cases with limited sample sizes and/or hypothesis-driven approaches that are focused on one or a few predefined candidate genes[6,7]. Hypothesis-free, large-scale WES analyses have only recently been enabled and expanded through global consortia efforts[6,8–10].

Here we present, to our knowledge, the largest WES analysis of epilepsy to date, from the Epi25 Collaborative, a global collaboration committed to sequencing and deep phenotyping 25,000 individuals with epilepsy. Our previous data collection and analysis of approximately 17,000 and approximately 29,000 individuals in case–control cohorts revealed rare coding variants that confer risk for all three major subtypes of non-lesional epilepsies (DEEs, GGE and NAFE)[9,10]. In the present study, we expand the evaluation to approximately 54,000 individuals, comprising 20,979 cases and 33,444 matched controls spanning six genetic ancestries, with improved power for detecting 'ultra-rare' variant (URV) association. We applied a hypothesis-free approach to test for association between URVs (single-nucleotide variants (SNVs) and short insertions and deletions (indels)) and case–control status, at individual gene level, gene set level and exome wide, and we pursued these analyses separately for each subtype of epilepsy. With the enlarged sample size, we discovered exome-wide significant genes for different epilepsies, identifying both shared and distinct rare variant risk factors. Integrating these findings with associations implicated by copy number variants (CNVs) and genome-wide association study (GWAS), we identified convergence of different types of genetic risk factors in the same genes. Spatiotemporal brain transcriptome analysis of WES-implicated and GWAS-implicated genes shows consistent expression patterns highlighting the neocortex and the postnatal development period. More broadly, comparing results to other large-scale WES studies, we provide substantial evidence for an overlapping rare variant risk between epilepsy and other neurodevelopmental disorders (NDDs). Together, our WES analysis at the unprecedented scale makes an important step forward in discovering rare variant risk underlying a spectrum of epilepsy syndromes and offers a valuable resource for generating hypotheses about syndrome-specific etiologies.

Results

Study overview

We performed WES and harmonized variant detection of an initial dataset of over 70,000 epilepsy-affected and control individuals recruited across 59 sites globally. After stringent quality control (QC; Methods), we included a total of 20,979 individuals with epilepsy and 33,444 controls without known neurological or neuropsychiatric conditions in our URV association analysis, roughly doubling the sample size in our last release of the Epi25 WES study[10]. The samples were predominantly of European genetic ancestries (76.6% non-Finnish and 2.7% Finnish), with smaller proportions of African (7.7%), East Asian (5.3%), South Asian (1.1%) and Admixed American (6.6%) genetic ancestries. Epilepsy cases were matched with controls of the same genetic ancestry, and samples were pooled for a joint burden analysis (Supplementary Tables 1–3 and Supplementary Figs. 1–5).

In the primary analysis, we evaluated the excess of ultra-rare, deleterious SNVs and indels—protein-truncating/damaging missense (MPC[11] score ≥ 2) variants observed at no more than five copies among the entire dataset—in individuals with epilepsy compared to controls, using a Firth logistic regression model with adjustment for sex and genetic ancestry (Methods). We performed the association analyses separately for each epilepsy subtype, including 1,938 individuals with a diagnosis of DEE, 5,499 with GGE and 9,219 with NAFE, as well as for all epilepsy-affected individuals combined (including an additional 4,323 with other epilepsy syndromes). Stringent Bonferroni correction was applied to adjust for 18,531 consensus coding sequence (CCDS) genes and 5,373 gene sets, each multiplied by eight case–control comparisons across four epilepsy groups and two variant classes. To ensure that our model was well calibrated, we used ultra-rare synonymous variants as a negative control for all tests (Extended Data Fig. 1). In a similar fashion, we performed CNV discovery and burden analysis on the same dataset (Methods). Moreover, we performed secondary subgroup burden analyses, splitting the data by genetic ancestry and sex. We focused on the primary, most-powered analyses in our main Results section and present the secondary analyses in the Extended Data.

Gene-based burden identifies exome-wide significant genes

For gene discovery, we tested the burden of URVs in each protein-coding gene, across all three epilepsy subtypes and all-epilepsy combined (Supplementary Data 1). In the analysis of protein-truncating URVs in DEEs, we identified five genes at exome-wide significance (Fig. 1a, Table 1a and Methods): *NEXMIF* ((MIM: 300524), log(odds ratio (OR)) = 6.7, $P < 2.2 \times 10^{-16}$); *SCN1A* ((MIM: 182389), log(OR) = 4.1, $P = 6.3 \times 10^{-9}$); *SYNGAP1* ((MIM: 603384), log(OR) = 4.2, $P = 5.9 \times 10^{-8}$); *STX1B* ((MIM: 601485), log(OR) = 4.5, $P = 2.3 \times 10^{-7}$); and *WDR45* ((MIM: 300526), log(OR) = 5.5, $P = 2.4 \times 10^{-7}$). All five are well-established epilepsy genes, reviewed by the Genomic Medicine Service (GMS) Genetic Epilepsy Syndromes panel[12] with diagnostic level of evidence. *NEXMIF* and *SCN1A* were consistently the top genes in our prior Epi25 analyses[9,10]; the other three genes for the first time surpassed the exome-wide significance threshold. The sixth-ranked gene—*ANKRD11* (MIM: 611192), which approaches exome-wide significance (log(OR) = 3.9, $P = 1.2 \times 10^{-6}$)—emerged as a novel candidate for DEEs.

Analysis of protein-truncating URVs in NAFE showed the strongest association evidence for *DEPDC5* ((MIM: 614191), log(OR) = 2.6, $P < 2.2 \times 10^{-16}$; Fig. 1a and Table 1a), which encodes part of the GATOR1 complex, a repressor of the mTORC1 pathway that has been prominently associated with focal epilepsies[7]. The other two components of the GATOR1 complex, *NPRL3* (MIM: 600928) and *NPRL2* (MIM: 607072), were also among the top associations (*NPRL3*: log(OR) = 2.9, $P = 1.4 \times 10^{-6}$; *NPRL2*: log(OR) = 2.8, $P = 3.6 \times 10^{-5}$), and they together manifested the strongest burden in the subsequent gene-set-based analysis. Notably, *DEPDC5* was the only exome-wide significant hit in the earlier Epi4K WES study of familial NAFE cases[8]; the expanded inclusion of non-familial cases in our cohort implicates *DEPDC5* in both familial and non-familial cases (Supplementary Data 2), reinforcing the notion that sporadic and familial forms of epilepsy have shared genetic risk.

No genes surpassed the exome-wide significance threshold in GGE. Three genes remained significant when we combined all epilepsy subtypes (Fig. 1a): *DEPDC5* (log(OR) = 2.1, $P = 3.4 \times 10^{-15}$), *NEXMIF* (log(OR) = 4.1, $P = 6.3 \times 10^{-9}$) and *SCN1A* (log(OR) = 2.7, $P = 3.5 \times 10^{-8}$). The signals of enrichment became slightly attenuated compared to the epilepsy subtype-specific analysis, which may reflect the genetic and etiological heterogeneity of different epilepsies.

In comparison to protein-truncating URVs, burden analysis of damaging missense URVs did not identify individual genes at exome-wide significance. Nevertheless, the top associations captured known epilepsy genes—notably, the *SLC6A1* (MIM: 137165) and *GABRB3* (MIM: 137192) genes, both involved in the GABAergic pathway[9] and showing enrichment across multiple epilepsy subtypes (Fig. 1b). Most of the previously implicated variants in these two genes were also missense[13,14], and we discovered an additional 24 and 26 damaging missense URVs in *SLC6A1* and *GABRB3*, respectively, increasing the existing candidates by approximately 50% (Supplementary Data 3). Another top hit, *KDM4B* (MIM: 609765), was found specifically associated with DEEs, which has not been previously reported.

Gene-set-based burden facilitates biological interpretation

To further investigate biologically relevant pathways associated with epilepsy, we performed burden tests at a gene set level. Different from our prior Epi25 analyses, which focused on a few prioritized gene sets[15], we systematically tested collections of gene entities that belong to a gene family[16] or encode a protein complex[17] (Supplementary Data 4 and Methods), in search for novel associations.

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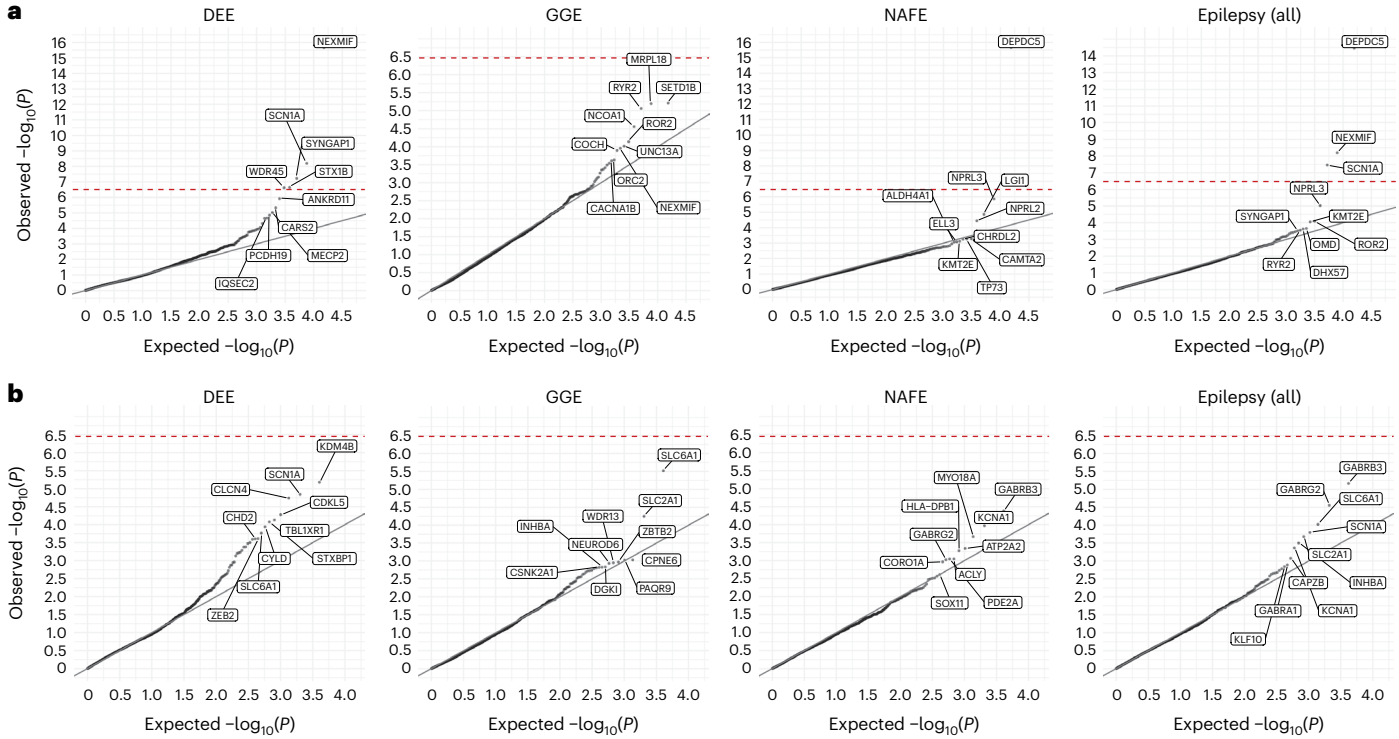

Fig. 1 | Results from gene-based burden analysis of URVs. a,b, Burden of protein-truncating (**a**) and damaging missense (**b**) URVs in each protein-coding gene with at least one epilepsy or control carrier. The observed $-\log_{10}$-transformed *P* values are plotted against the expectation given a uniform distribution. For each variant class, burden analyses were performed across four epilepsy groups—1,938 DEEs, 5,499 GGE, 9,219 NAFE and 20,979 epilepsy-affected individuals combined—versus 33,444 controls. *P* values were computed using a Firth logistic regression model testing the association between the case–control status and the number of URVs (two-sided); the red dashed line indicates exome-wide significance, $P = 3.4 \times 10^{-7}$, after Bonferroni correction (Methods). The top 10 genes with URV burden in epilepsy are labeled.

The most pronounced signal, as described in the gene-based burden of protein-truncating URVs, was from the GATOR1 complex in NAFE ($\log(OR) = 2.7$, $P < 2.2 \times 10^{-16}$; Fig. 2a and Table 1b). We identified a total of 56 distinct protein-truncating URVs in GATOR1 (Supplementary Data 5), among which 45 appeared novel according to the most recent study of epilepsy-related GATOR1 variants by Baldassari et al.[7]. In contrast to Baldassari et al., where most (>70%) GATOR1 protein-truncating variant carriers were familial, only 20% of the carriers in our study cohort had a known family history of epilepsy. Both familial and non-familial cases showed significant burden of GATOR1 protein-truncating URVs (Supplementary Data 2), highlighting the role of GATOR1 genes in the etiology of focal epilepsy.

Several strong signals emerged in the analysis of damaging missense URVs, led by well-established ion channel protein complexes and gene families (Fig. 2b and Table 1c). The top association was the $GABA_A$ receptor complex, encoded by *GABRA1* (MIM: 137160), *GABRB2* (MIM: 600232) and *GABRG2* (MIM: 137164); $(\alpha1)_2(\beta2)_2(\gamma2)$, the most abundantly expressed isoform in the brain[18], which controls the majority of inhibitory signaling in the central nervous system. The complex showed extensive enrichment across all epilepsy subtypes, recapturing the pervasive role of $GABA_A$ receptors across the spectrum of severities in epilepsy[19]. Further dissecting the signals with respect to the structural domain of the complex, we observed stronger URV burden in the transmembrane domains (TMDs) than in the extracellular domain (ECD), particularly for DEEs and GGE; and DEEs exhibited a unique signal in the second transmembrane helix lining the ion channel pore of the receptor[20] (Fig. 2c and Supplementary Data 6). These patterns collectively point to an association of damaging missense URVs in the pore-forming domain with a more severe form of epilepsy.

Potential novel associations were found in two gene sets: the NSL complex (with protein-truncating URVs in *KANSL1* (MIM: 612452), *KANSL2* (MIM: 615488) and *PHF20* (MIM: 610335)) and the phosphodiesterase (PDE) gene family (with damaging missense URVs in *PDE2A* (MIM: 602658) and *PDE10A* (MIM: 610652)), associated with DEEs and NAFE, respectively. Despite the sparsity of URVs, our results broaden the potential allelic spectrum of variants that may confer risk for different subtypes of epilepsies.

Protein structural analysis characterizes missense URVs

The strong burden of damaging missense, but not protein-truncating, URVs in genes encoding ion channels suggests a pathophysiological mechanism of protein alteration rather than haploinsufficiency. Given the specialized structure of ion channels, we further characterized missense URVs at a protein structure level. We collected experimentally resolved three-dimensional (3D) structures of ion channel proteins, and we applied Rosetta[21] to assess the Gibbs free energy changes (ΔΔG, or ddG in abbreviation) of protein folding upon a particular missense URV; a decrease in Gibbs free energy of unfolding—that is, a positive ddG value—suggests a destabilizing effect of the variant on protein, and a negative value suggests a stabilizing effect. We computed ddG for a total of 1,782 missense URVs across 16 ion channel protein complexes (Supplementary Data 7 and Methods).

A positive correlation was observed between ddG and MPC (= 0.15, $P = 8.3 \times 10^{-11}$; Fig. 3a); nonetheless, incorporating ddG further stratified epilepsy association signals (Fig. 3b and Supplementary Data 8). Significant enrichment was found for both destabilizing (ddG ≥ 1 kcal mol^{-1}) and stabilizing (ddG ≤ −1 kcal mol^{-1}) URVs, suggesting a diverging molecular mechanism for these missense URVs. Dissecting the signals by protein structural domains found divergent distributions for destabilizing and stabilizing missense URVs, with the former enriched in the ECD of the complex and the latter in the TMD (Fig. 3c and Supplementary Data 9). These results may provide testable hypotheses

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Table 1 | Genes and gene sets identified by exome-wide gene burden analyses

Gene	EPI carrier	Control carrier	EPI log(OR)	EPI *P* value	DEE carrier	DEE log(OR)	DEE *P* value	GGE carrier	GGE log(OR)	GGE *P* value	NAFE carrier	NAFE log(OR)	NAFE *P* value
a, Gene-based protein-truncating URV burden													
NEXMIF	22	0	4.15	6.28×10^{-9}	15	6.65	$<2.2\times10^{-16}$	5	3.87	1.11×10^{-4}	1	1.84	2.16×10^{-1}
SCN1A	27	2	2.67	3.49×10^{-8}	9	4.14	6.32×10^{-9}	4	2.43	2.41×10^{-3}	7	2.01	4.17×10^{-3}
SYNGAP1	13	1	2.40	3.07×10^{-4}	7	4.18	5.95×10^{-8}	4	2.34	6.95×10^{-3}	2	1.91	6.41×10^{-2}
STX1B	6	0	2.63	6.87×10^{-3}	5	4.53	2.29×10^{-7}	0			0		
WDR45	5	0	2.45	2.94×10^{-2}	5	5.52	2.38×10^{-7}	0			0		
DEPDC5	66	11	2.14	3.44×10^{-15}	3	1.34	8.76×10^{-2}	1	−0.33	6.97×10^{-1}	47	2.58	$<2.2\times10^{-16}$
b, Gene-set-based protein-truncating URV burden													
GATOR1 complex	94	14	2.26	$<2.2\times10^{-16}$	4	1.33	4.83×10^{-2}	3	0.21	7.37×10^{-1}	67	2.72	$<2.2\times10^{-16}$
DEPDC5	66	11	2.14	3.44×10^{-15}	3	1.34	8.76×10^{-2}	1	−0.33	6.97×10^{-1}	47	2.58	$<2.2\times10^{-16}$
NPRL3	18	2	2.44	9.29×10^{-6}	1	1.84	1.32×10^{-1}	2	1.36	1.53×10^{-1}	12	2.90	1.35×10^{-6}
NPRL2	10	1	2.25	5.64×10^{-4}	0	1.40	4.54×10^{-1}	0	0.92	6.03×10^{-1}	8	2.78	3.62×10^{-5}
NSL complex subunit 1 paralogs*	29	7	1.64	3.56×10^{-6}	10	2.54	1.43×10^{-6}	4	1.19	3.43×10^{-2}	6	1.13	3.18×10^{-2}
KANSL1	17	4	1.62	6.80×10^{-4}	7	2.33	4.46×10^{-4}	2	1.16	1.78×10^{-1}	2	0.70	4.18×10^{-1}
KANSL1L	12	3	1.54	2.24×10^{-3}	3	2.89	6.11×10^{-4}	2	1.21	7.55×10^{-2}	4	1.45	3.06×10^{-2}
c, Gene-set-based damaging missense URV burden													
NaV1 channel family	165	170	0.45	9.74×10^{-5}	38	1.31	2.13×10^{-9}	39	0.33	7.72×10^{-2}	54	0.17	3.00×10^{-1}
SCN1A	49	37	0.85	1.60×10^{-4}	11	1.88	1.44×10^{-5}	11	0.60	9.90×10^{-2}	11	0.32	3.78×10^{-1}
SCN8A	68	71	0.39	2.98×10^{-2}	16	1.23	2.57×10^{-4}	18	0.42	1.27×10^{-1}	27	0.32	1.90×10^{-1}
SCN2A	40	49	0.29	1.88×10^{-1}	11	1.42	4.16×10^{-4}	9	0.24	5.17×10^{-1}	12	−0.08	8.14×10^{-1}
SCN3A	8	14	−0.11	8.08×10^{-1}	0	−1.15	3.78×10^{-1}	1	−0.64	4.24×10^{-1}	4	0.00	9.98×10^{-1}
$GABA_A$ receptor complex	71	29	1.30	1.04×10^{-9}	15	2.04	2.19×10^{-7}	17	1.29	5.53×10^{-5}	25	1.13	1.13×10^{-4}
GABRG2	33	13	1.31	2.79×10^{-5}	7	2.01	4.20×10^{-4}	6	1.10	3.64×10^{-2}	14	1.32	9.45×10^{-4}
GABRA1	24	12	1.10	1.28×10^{-3}	6	2.11	5.19×10^{-4}	6	1.14	1.72×10^{-2}	9	1.06	2.73×10^{-2}
GABRB2	14	4	1.56	2.17×10^{-3}	2	2.08	4.05×10^{-2}	5	2.07	2.72×10^{-3}	2	0.57	4.87×10^{-1}

a, Genes with significant burden of protein-truncating URVs. **b**,**c**, Gene sets with significant burden of protein-truncating (**b**) and damaging missense (**c**) URVs. Genes and gene sets with exome-wide significance are shown ($P<3.4\times10^{-7}$ for gene-based burden testing and $P<1.2\times10^{-6}$ for gene-set-based burden testing); asterisk indicates a *P* value very close to exome-wide significance. *P* values are reported as $<2.2\times10^{-6}$ for extremely small values below the precision threshold of the statistical software. The number of mutation carriers, log(OR) and *P* value are listed for each burden test across four epilepsy (EPI) groups.

about how ion channel dysfunction could produce a broad range of epilepsy syndromes.

CNV deletion burden converges with protein-truncating URVs

In parallel with SNVs and indels, we performed variant calling of CNVs on the same dataset (Methods). After sample QC, we examined the burden of rare CNVs in 18,963 epilepsy cases—including 1,743 DEEs, 4,980 GGE and 8,425 NAFE—versus 29,804 controls (~90% of initial; Methods). We first tested a curated set of 79 CNVs previously associated with NDDs, known as 'genomic disorders' (GDs)[22]. Four GD loci were significantly enriched in epilepsy cases: three from the common complex forms of epilepsies (16p13.11 deletion, 15q13.2-q13.3 deletion and 17q12 duplication) and one from DEEs (15q11.2-q13.1 duplication). All four loci were prominent in previous reports as predisposing to a diverse range of epilepsy syndromes[23–26].

Moreover, we evaluated CNV burden at the individual gene level; a gene was considered affected by a CNV if 10% of its coding exons were deleted or 75% were duplicated. The most significant signal was from CNV deletions in the *NPRL3* gene, with 11 deletions found in NAFE cases versus zero in controls (log(OR) = 4.1, $P = 9.4\times10^{-7}$; Supplementary Data 10). Notably, *NPRL3* was also one of the top hits implicated by protein-truncating URVs in NAFE, and jointly analyzing the two identified *NPRL3* as a new exome-wide significant gene (log(OR) = 3.8, $P = 8.1\times10^{-12}$; Fig. 4a). Among the top 10 genes with protein-truncating URV burden across four epilepsy groups, about one-third (14/40) were found affected by a CNV deletion, and the vast majority (11/14) showed enrichment in epilepsy cases (Fig. 4b). These included *DEPDC5*, which, together with *NPRL3*, reinforces a haploinsufficiency mechanism for GATOR1-related focal epilepsies (Fig. 4c). Strengthened burden was also found for potential novel genes—for example, *CARS2* (MIM: 612800) in DEEs and *NCOA1* (MIM: 602691) in GGE, both with supporting evidence from previous studies[27–29]. Analysis of CNV duplications did not show any individual genes close to exome-wide significance (Supplementary Data 10).

An expanded view of epilepsy genetic architecture

Similar to other common NDDs, the common forms of epilepsy—GGE and NAFE—have both common and rare genetic risk factors. In partnership with the International League Against Epilepsy (ILAE) Consortium on Complex Epilepsies, we recently performed the largest GWAS meta-analysis of over 29,000 individuals with common epilepsies[30], which revealed 26 genome-wide significant common risk loci (minor allele frequency (MAF) > 1%). Together with the rare variant findings in this study, they constitute an expanded view of the genetic architecture

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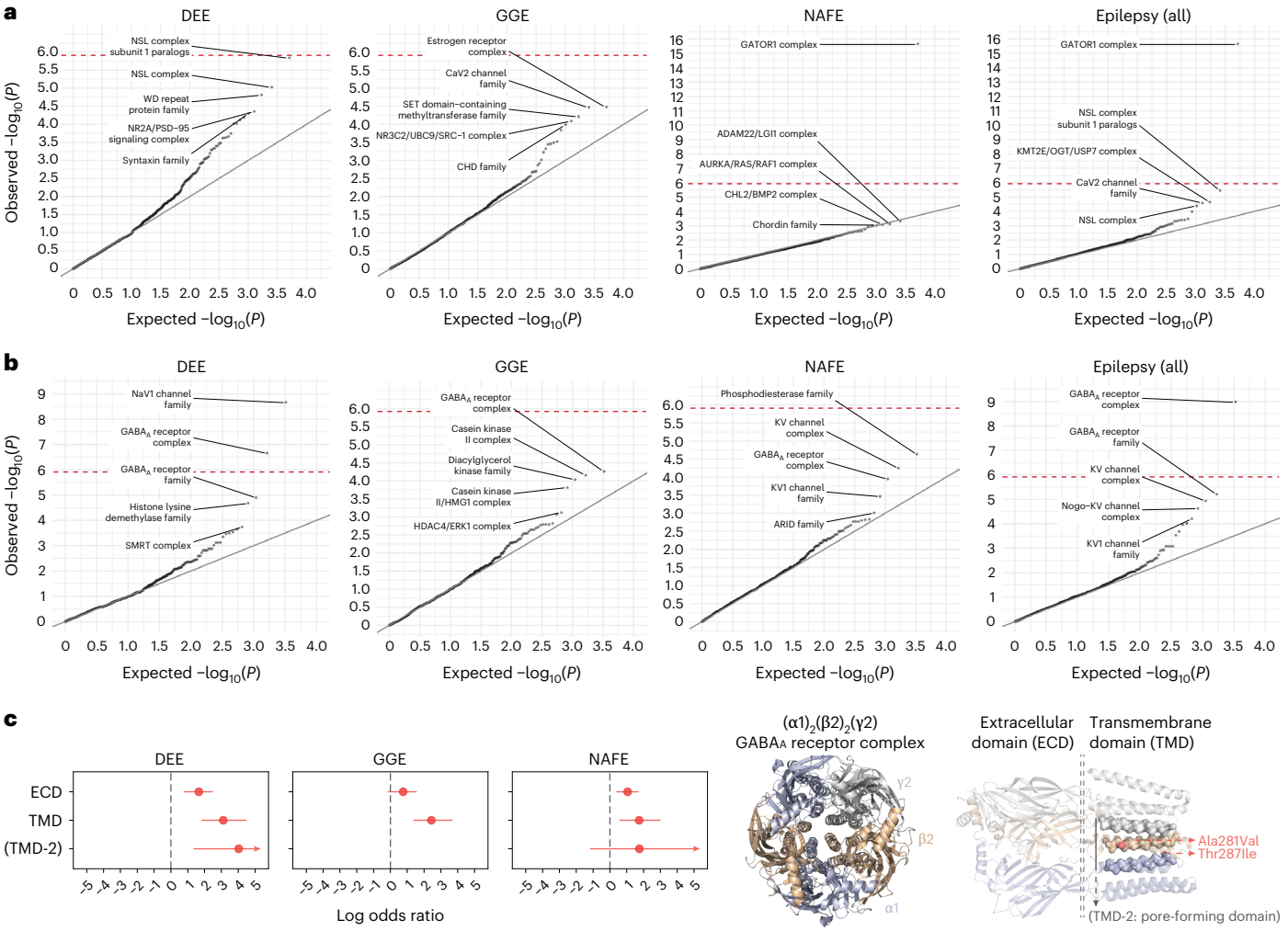

Fig. 2 | Results from gene-set-based burden analysis of URVs. a,b, Burden of protein-truncating (**a**) and damaging missense (**b**) URVs in each gene set (gene family/protein complex) with at least one epilepsy or control carrier. The observed $-\log_{10}$-transformed *P* values are plotted against the expectation given a uniform distribution. For each variant class, burden analyses were performed across four epilepsy groups—1,938 DEEs, 5,499 GGE, 9,219 NAFE and 20,979 epilepsy-affected individuals combined—versus 33,444 controls. *P* values were computed using a Firth logistic regression model testing the association between the case–control status and the number of URVs (two-sided); the red dashed line indicates exome-wide significance, $P = 1.2 \times 10^{-6}$, after Bonferroni correction (Methods). The top five gene sets with URV burden in epilepsy are labeled. **c**, Burden of damaging missense URVs in the $(\alpha1)_2(\beta2)_2(\gamma2)$ $GABA_A$ receptor complex with respect to its structural domain. Left, forest plots showing the stronger enrichment of damaging missense URVs in the TMD than in the ECD and the unique signal from DEEs in the second TMD (TMD-2) that forms the ion channel pore. The dot represents the log(OR), and the error bars represent the 95% confidence intervals of the point estimates. For presentation purposes, error bars that exceed a log(OR) of 5 are capped, indicated by arrows at the end of the error bars (see Supplementary Data 6 for exact values). Right, a co-crystal structure (Protein Data Bank ID: 6X3Z) showing the pentameric subunits of the receptor and highlighting the two protein-truncating URVs from DEEs located in the pore-forming domain.

of epilepsy (Fig. 5a). As natural selection purges deleterious variants from human populations, rarer variants exhibited larger disease effects: protein-truncating URVs (MAF < 0.005%) had the largest effect sizes (median OR = 79), pinpointing specific genes at exome-wide significance; rare CNVs (MAF < 0.1%), known for genomic disorders, also showed large effects in increasing epilepsy risk (median OR = 26); and common variants each individually had a small contribution (median OR < 1.1).

With new discoveries expanding the allelic spectrum, we found emerging evidence for convergence between different classes of risk variants. Besides the *NPRL3* gene highlighted by the joint CNV + SNV burden analysis, three (out of 29) genes—*SCN1A*, *SCN8A* and *STX1B*—prioritized from the GWAS loci overlapped with genes enriched with URVs, contributing to an overall URV burden in GWAS genes (Fig. 5b). Further delineating the analysis to epilepsy subtypes, we observed significant enrichment for URVs from GGE in the GGE-specific GWAS genes, whereas none was observed for URVs from NAFE (Fig. 5b and Supplementary Data 11). This result suggests that the convergence of common and rare variant risk tends to be epilepsy subtype specific.

At the individual gene level, 13 of the 23 GGE GWAS risk genes showed an excess of protein-truncating URVs (Supplementary Data 11). The lead gene was *RYR2* (MIM: 180902), in which 14 protein-truncating URVs were observed in our GGE cohort (log(OR) = 1.8, $P = 8.6 \times 10^{-6}$), with the GWAS hit residing in the intronic region (rs876793). Mutations in *RYR2* are well known in the etiology of arrhythmogenic disorders[31], whereas more recent studies have reported that the same mutation can cause GGE independent of arrhythmias[32,33]. Our finding adds weight to the hypothesis that *RYR2* mutations likely constitute a neuro-cardiac calcium channelopathy[32,33], where mutant receptors may induce either arrhythmias or GGE depending on their selective expression in the heart or in the brain.

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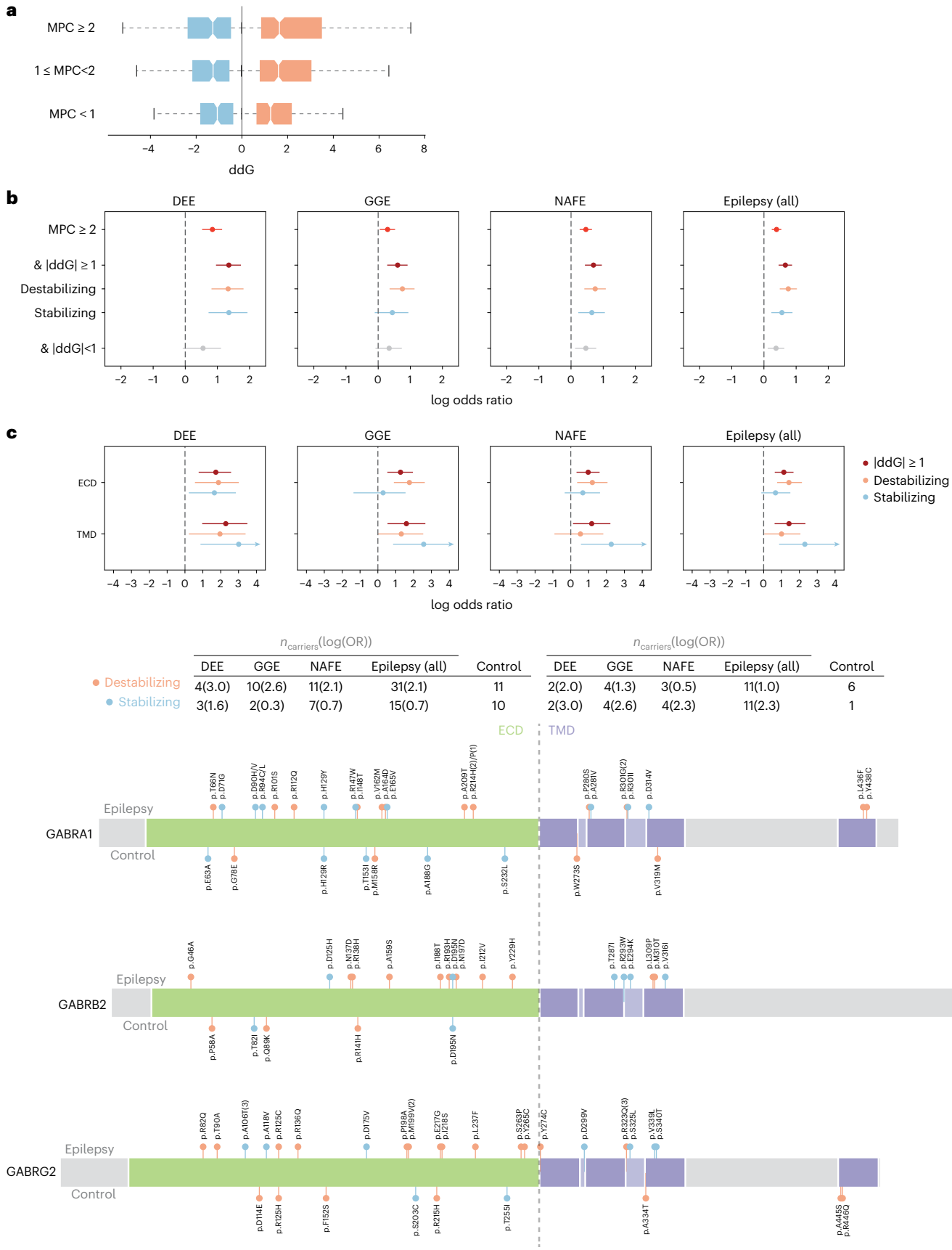

	$n_{carriers}$(log(OR))					$n_{carriers}$(log(OR))				
	DEE	GGE	NAFE	Epilepsy (all)	Control	DEE	GGE	NAFE	Epilepsy (all)	Control
Destabilizing	4(3.0)	10(2.6)	11(2.1)	31(2.1)	11	2(2.0)	4(1.3)	3(0.5)	11(1.0)	6
Stabilizing	3(1.6)	2(0.3)	7(0.7)	15(0.7)	10	2(3.0)	4(2.6)	4(2.3)	11(2.3)	1

Functional roles of candidate genes in neural circuitry

To gain more functional insights into the identified genetic associations, we surveyed the spatiotemporal expression of top candidate genes in the human brain. We first focused on 13 genes—seven with individually exome-wide significance (*NEXMIF*, *SCN1A*, *SYNGAP1*, *STXBP1*, *WDR45*, *DEPDC5* and *NPRL3*) and six from members of significant gene families or protein complexes (*NPRL2*, *SCN2A*, *SCN8A*, *GABRA1*, *GABRB2* and *GABRG2*). We analyzed the expression of each

Fig. 3 | Protein structural analysis of missense URVs in ion channel genes. **a**, Correlation between ddG and MPC in measuring the deleteriousness of missense URVs. A higher absolute ddG value suggests a more deleterious effect on protein stability; positive (orange) and negative (blue) values suggest destabilizing and stabilizing effects, respectively. Box plots show the distribution of ddG values across different MPC ranges (blue boxes: n = 232, 272 and 242 for MPC < 1, 1 ≤ MPC < 2 and MPC ≥ 2, respectively; orange boxes: n = 327, 397 and 342 for MPC < 1, 1 ≤ MPC < 2 and MPC ≥ 2, respectively). The center line represents the median (50th percentile), and the bounds of the box indicate the 25th and 75th percentiles, with the whiskers extending to the minimum and maximum values within 1.5 times the interquartile range from the lower and upper quartiles, respectively. **b**, Burden of damaging missense URVs stratified by ddG. Stronger enrichment was observed when applying |ddG| ≥ 1 to further prioritize damaging missense URVs with MPC ≥ 2. **c**, Burden and distribution of destabilizing (ddG ≥ 1) and stabilizing (ddG ≤ −1) missense URVs on the $(\alpha1)_2(\beta2)_2(\gamma2)$ $GABA_A$ receptor complex with respect to its structural domain. Top, forest plots showing the stronger enrichment of destabilizing missense URVs (orange) in the ECD and stabilizing missense URVs (blue) in the TMD. Bottom, schematic plots displaying the distribution of destabilizing and stabilizing missense URVs on $GABA_A$ receptor proteins. URVs found in epilepsy cases are plotted above the protein, and those from controls are plotted below the protein. The number of epilepsy and control carriers are listed in the table above. As shown in **b** and **c**, burden analyses were performed across four epilepsy groups—1,938 DEEs, 5,499 GGE, 9,219 NAFE, and 20,979 epilepsy-affected individuals combined—versus 33,444 controls. The dot represents the log(OR), and the error bars represent the 95% confidence intervals of the point estimates.

gene across six brain structures and nine developmental periods, using the BrainSpan[34] database (Methods). We found that the candidate genes were more highly expressed during the postnatal period than prenatally ($P = 7.0 \times 10^{-18}$; Extended Data Fig. 2), and the highest expression was found in the neocortex ($P = 7.6 \times 10^{-6}$; Fig. 6a and Methods). Consistent neocortical and developmental expression patterns were found for the 29 epilepsy risk genes discovered by GWAS[30] (Fig. 6a).

Extending the comparison to the top 20 genes enriched for deleterious URVs recapitulated the postnatal expression pattern across different subtypes of epilepsy, and, on average, genes implicated in the more severe subtype DEEs showed a higher expression level (Fig. 6b). Further quantifying the relative prenatal versus postnatal expression pattern[35] for both WES-implicated and GWAS-implicated genes, we classified 43 genes to have a prenatal expression preference and 50 genes to have a postnatal expression preference (Supplementary Data 12). Gene Ontology (GO) enrichment analysis[36] of these two gene sets identified two different functional categories: 'gene transcription regulation' for the prenatal genes and 'synaptic transmission & membrane excitability' for the postnatal genes (Fig. 6c and Supplementary Data 12). The latter showed overall stronger enrichment, in which 34 genes were mapped to a range of functional components of synaptic transmission and neuronal excitability (Fig. 6d and Methods).

For the prenatal genes with a transcriptional regulatory role, we further tested whether they regulate the postnatal genes and, thus, functionally converge at neurotransmission. We constructed a gene regulatory network between these two gene sets and asked whether there are more transcription factor (TF)–target connections between them than random. Forty connections were found (Supplementary Data 21), which was significantly higher than that of a random gene set with similar brain expression profiles (Methods). The result lends support to the hypothesis about a convergent pathophysiological effect associated with these two different categories of genes in epilepsy. In addition, the same TFs had a much larger number of targets beyond our candidate genes, which showed significant enrichment in a broader set of neurodevelopmental processes other than neurotransmission (Supplementary Data 12). Therefore, these results collectively implicate both developmental and functional changes of neural circuitry in the pathophysiology of epilepsy.

Shared rare variant risk with NDDs

Recent WES studies revealed substantial rare variant risk for NDDs. An analysis of de novo mutations in severe developmental disorders (DDs) discovered 285 genes at exome-wide significance[37], and rare variant associations in autism spectrum disorder (ASD)[22] and schizophrenia (SCZ)[38] implicated 185 and 32 genes at a false discovery rate (FDR) of 5%, respectively. To explore how these and our findings may point to common genetic etiologies, we examined the burden of URVs in these established NDD genes (Supplementary Data 13). Significant enrichment was found for all three gene sets associated with DDs, ASD and SCZ (Fig. 7a), suggesting that there is shared genetic risk of rare variation among the broader spectrum of NDDs. Nine (out of 13) epilepsy significant genes overlapped with DD/ASD genes but none with SCZ (Extended Data Fig. 3 and Supplementary Data 13), suggesting a larger genetic overlapping between epilepsy and DD/ASD than SCZ, which is in line with the high comorbidity of DD/ASD and epilepsy, in particular DEEs. Given the known genetic overlapping between DDs and ASD, we repeated the analyses on the subsets of mutually exclusive NDD genes (that is, 196 DD-only, 99 ASD-only and 22 SCZ-only genes, respectively). Although attenuated, there remained clear rare variant signals shared by epilepsy and other NDDs (Supplementary Data 13).

About one-third (136/409) of NDD genes showed nominally significant enrichment of deleterious URVs in at least one epilepsy subtype (Supplementary Data 13). The vast majority (128/136, 94.1%) were DD/ASD genes, and only one gene, *KDM6B* (MIM: 611577), was shared by all three NDD gene sets. Notably, URVs in *KDM6B* associated with epilepsy were exclusively missense (MPC ≥ 2), whereas *KDM6B* variants implicated in DDs were predominately protein truncating (Fig. 7b and Supplementary Data 13). All missense variants were clustered at the KDM6B catalytic domain (JmjC) and C-terminal helix/zinc motifs, which are important for enzyme–cofactor binding and protein stability[39]. Protein

Fig. 4 | Convergence of CNV deletions and protein-truncating URVs in gene-based burden. **a**, Joint burden of CNV deletions and protein-truncating URVs in each protein-coding gene with at least one epilepsy or control carrier. The observed $-\log_{10}$-transformed P values are plotted against the expectation given a uniform distribution. Joint burden analyses were performed on the subset of samples that passed CNV calling QC (Methods), across four epilepsy groups—1,743 DEEs, 4,980 GGE, 8,425 NAFE and 18,963 epilepsy-affected individuals combined—versus 29,804 controls; for genes that do not have a CNV deletion called, results from the burden analysis of protein-truncating URVs on the full sample set are shown. P values were computed using a Firth logistic regression model testing the association between the case–control status and the number of URVs (two-sided); the red dashed line indicates exome-wide significance, $P = 3.4 \times 10^{-7}$, after Bonferroni correction (Methods). The top 10 genes with variant burden in epilepsy are labeled. **b**, Joint burden of CNV deletions and protein-truncating URVs in the top 10 genes ranked by protein-truncating URV burden. Only genes affected by both variant types with enrichment in epilepsy (log(OR) > 0) are shown. For comparison, the burden of protein-truncating URVs (SNVs/indels; red), CNV deletions (gray) and the joint (purple) were analyzed on the same sample subset as described in **a**. The dot represents the log(OR), and the error bars represent the 95% confidence intervals of the point estimates. For presentation purposes, error bars that exceed a log(OR) of 5 are capped, indicated by arrows at the end of the error bars (see Supplementary Data 10 for exact values). **c**, Genomic location and distribution of CNV deletions and protein-truncating URVs with respect to the *NPRL3* and *DEPDC5* genes. Variants found in epilepsy cases (red) are plotted above the schematic gene plots, and those from controls (gray) are plotted below the gene. The number of epilepsy and control carriers are listed in the table above. P values were computed using a Firth logistic regression model testing the association between the case–control status and the number of URVs (two-sided).

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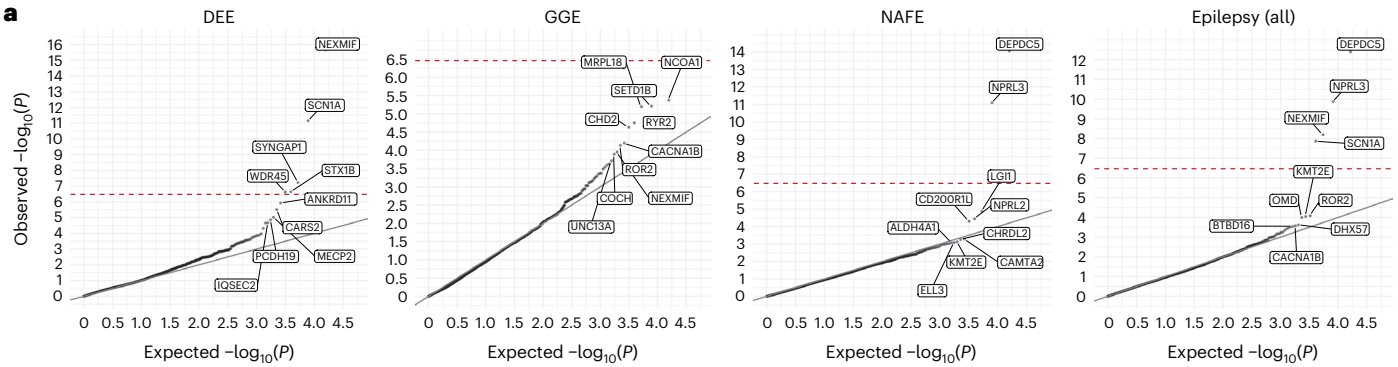

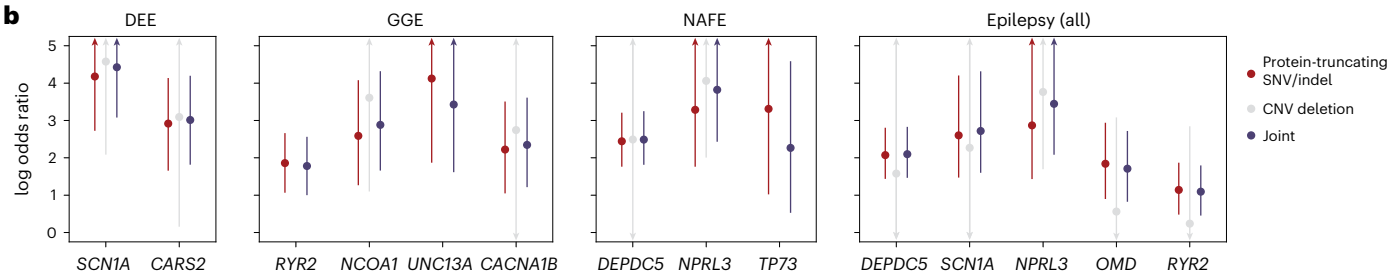

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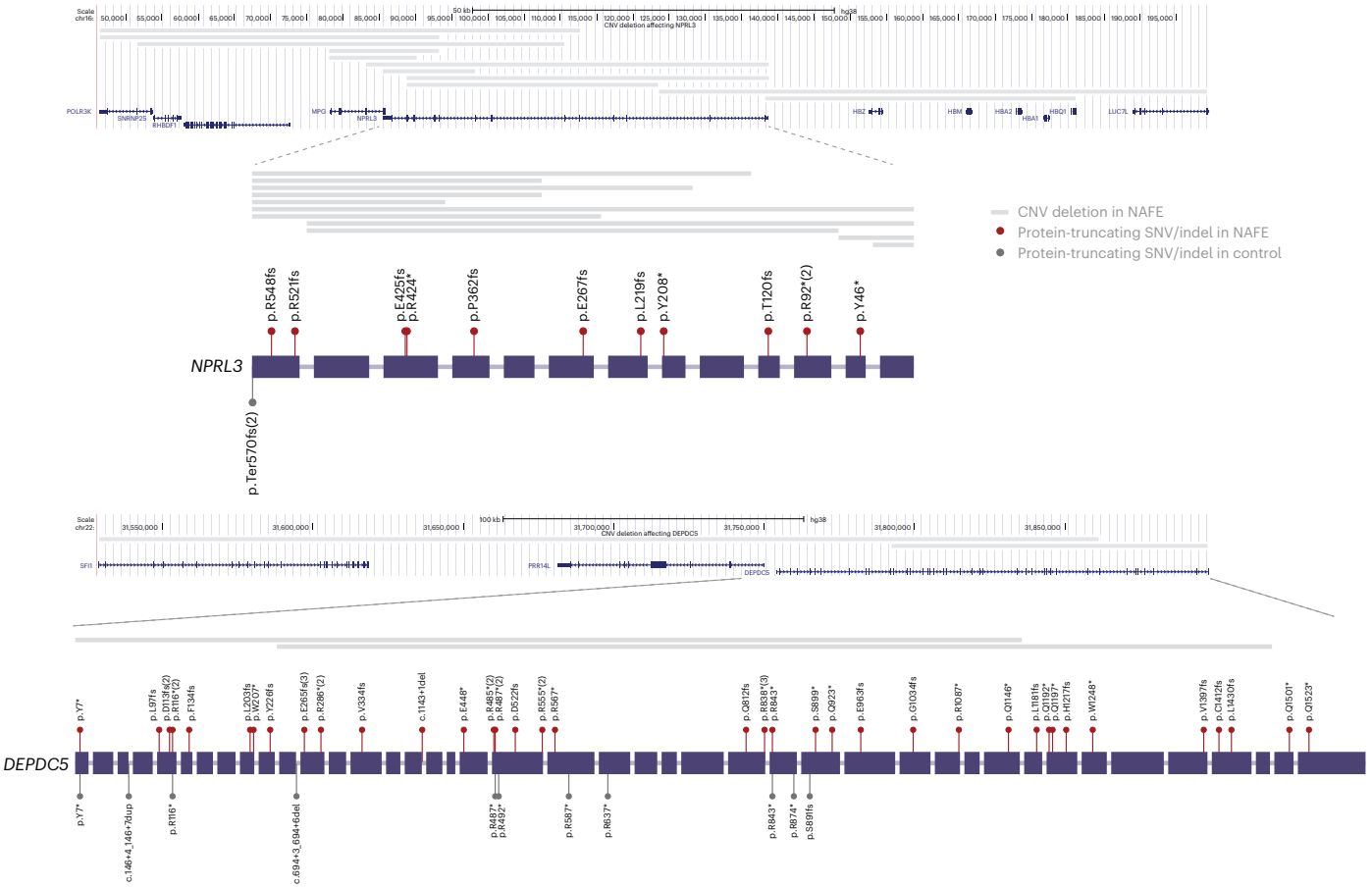
Gene	Protein-truncating SNV/indel NAFE (n = 9,219)	Protein-truncating SNV/indel Control (n = 33,444)	Protein-truncating SNV/indel log(OR)	Protein-truncating SNV/indel P value	CNV deletion NAFE (n = 8,325)	CNV deletion Control (n = 29,804)	CNV deletion log(OR)	CNV deletion P value	Joint NAFE (n = 8,325)	Joint Control (n = 29,804)	Joint log(OR)	Joint P value
NPRL3	12	2	2.9	1.4×10^{-6}	11	0	4.1	9.4×10^{-7}	21	1	3.8	8.1×10^{-12}
DEPDC5	47	11	2.6	$<2.2 \times 10^{-16}$	2	0	2.5	0.05	43	10	2.5	8.3×10^{-15}



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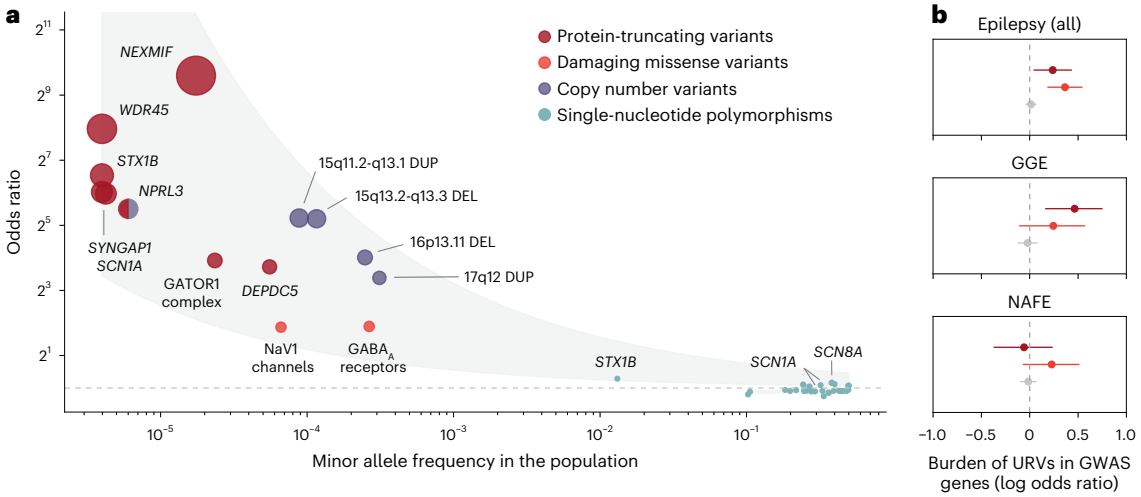

Fig. 5 | Epilepsy genetic architecture from large-scale genetic association studies. a, An allelic spectrum of epilepsy genetic risk loci. Significant risk loci identified by large-scale WES and GWA studies are shown. The OR of each risk loci (*y* axis) is plotted against the MAF in the general population (gnomAD non-neuro subset, *x* axis); for individual genes, the cumulative allele frequency (CAF) was computed, and, for gene sets, the CAF was averaged over gene members. The color and size of each dot represent the variant class and effect size (OR) of the genetic association. Bold indicates convergent findings between different variant classes. The shaded area represents the upper and lower 95% confidence intervals of the point estimates, fitted by exponential curves. **b**, Burden of URVs in genes implicated by GWAS loci. Significant enrichment was observed for URVs from epilepsy-affected individuals in 29 GWAS genes (upper: 20,979 cases versus 33,444 controls) and URVs from GGE in the 23 GGE-specific GWAS genes (middle: 5,499 GGE versus 33,444 controls) but not for URVs from NAFE in GGE GWAS genes (bottom: 9,219 NAFE versus 33,444 controls); and significance was seen only for protein-truncating (red) and damaging missense (orange) URVs but not for synonymous URVs (gray). The dot represents the log(OR), and the error bars represent the 95% confidence intervals of the point estimates. gnomAD, Genome Aggregation Database; GWA, genome-wide association.

structural analysis predicted most of these missense variants to have a destabilizing effect on the KDM6B protein (Supplementary Data 13), especially those in DDs, whereas diverging effects were observed for epilepsy and SCZ (Fig. 7b). These results suggest that, even converging in the same gene, rare variant risk may differ in its severity and/or the molecular mechanism that underlies specific phenotypes of NDDs.

Shared rare variant risk across genetic ancestries and sexes

Finally, we repeated the burden analysis within subgroups of samples by genetic ancestry and sex. Six genetic ancestral groups were classified, comprising 76.6% non-Finnish European, 7.7% African, 6.6% Admixed American, 5.3% East Asian, 2.7% Finnish and 1.1% South Asian samples (Extended Data Fig. 4a and Supplementary Data 14). The non-European subgroups remained underpowered to detect exome-wide significant genes yet showed enrichment of URVs in top genes identified in the full analysis (Extended Data Fig. 4b). Significant enrichment was found in the sets of established epilepsy genes and genes that are intolerant to genetic variation as well as in intolerant genes that are not currently linked to epilepsy (Extended Data Fig. 4c and Supplementary Data 14). These results suggest that, although there likely exists shared genetic risk underlying epilepsy across different genetic ancestries, more remains to be discovered, especially for non-European ancestral groups.

Similar patterns were observed in sex-specific burden analyses. Both female and male subgroups showed significant URV burden in known epilepsy genes, with the female subgroup exhibiting stronger enrichment in X-linked genes (Extended Data Fig. 4d and Supplementary Data 15). At the individual gene level, given the similar sample size between sexes, we directly compared their burden *P* values and found three exome-wide significant genes with sex-biased URV burden: *NEXMIF* and *SCN1A* in female and *NPRL3* in male (Extended Data Fig. 4e and Supplementary Data 15). The top hit *NEXMIF* is an X-linked gene, the loss of function of which causes NEXMIF encephalopathy and has been shown to have markedly different manifestations between females and males[40]. Different molecular mechanisms could explain the phenotypic variability[40,41]; albeit intriguing based on the X-chromosome inactivation theory, they require further investigation with functional studies.

Discussion

In what is, to our knowledge, the largest WES study of epilepsies to date, we characterized the contribution of ultra-rare genetic variation to epilepsy risk. As shown here, we aggregated deeply phenotyped epilepsy collections from across the world, sequenced them and harmonized variant detection and QC to power analysis and interpretation of the genetic data for etiological and clinical implications.

Our exome-wide burden analyses re-demonstrated the role of known epilepsy genes with improved power and discovered potential novel rare risk variants for different subtypes of epilepsies. Most associations were identified in a particular epilepsy subtype, implicating distinct genetic etiologies underlying different epilepsies. Protein-truncating URVs exhibited the strongest signal, with six individual genes surpassing the stringent exome-wide significance threshold: five genes (*NEXMIF*, *SCN1A*, *SYNGAP1*, *STX1B* and *WDR45*) were associated with the severe group of DEEs, whereas the most significant gene, *DEPDC5*, was found in NAFE. In comparison to protein-truncating URVs, analysis of damaging missense URVs remained underpowered to identify individual genes at exome-wide significance. However, strong associations emerged when we aggregated sets of genes that share common functions. The top associations were predominantly genes encoding ion channel complexes, such as Nav/Kv channels and GABA$_A$ receptors. Notably, these gene sets did not show significant enrichment of protein-truncating URVs, suggesting more diverse molecular mechanisms than haploinsufficiency. Protein structural analysis of missense URVs in these genes suggested diverging effects on ion channel protein stability. In this study, we deliberately separated the analysis of protein-truncating and damaging missense URVs, and assuming a protein-truncating-like effect for all damaging missense URVs identified no additional significant genes but weakened our analytical power (Extended Data Fig. 5).

Potential novel associations were identified in several genes and gene sets. The top candidates were predominately implicated in DEEs,

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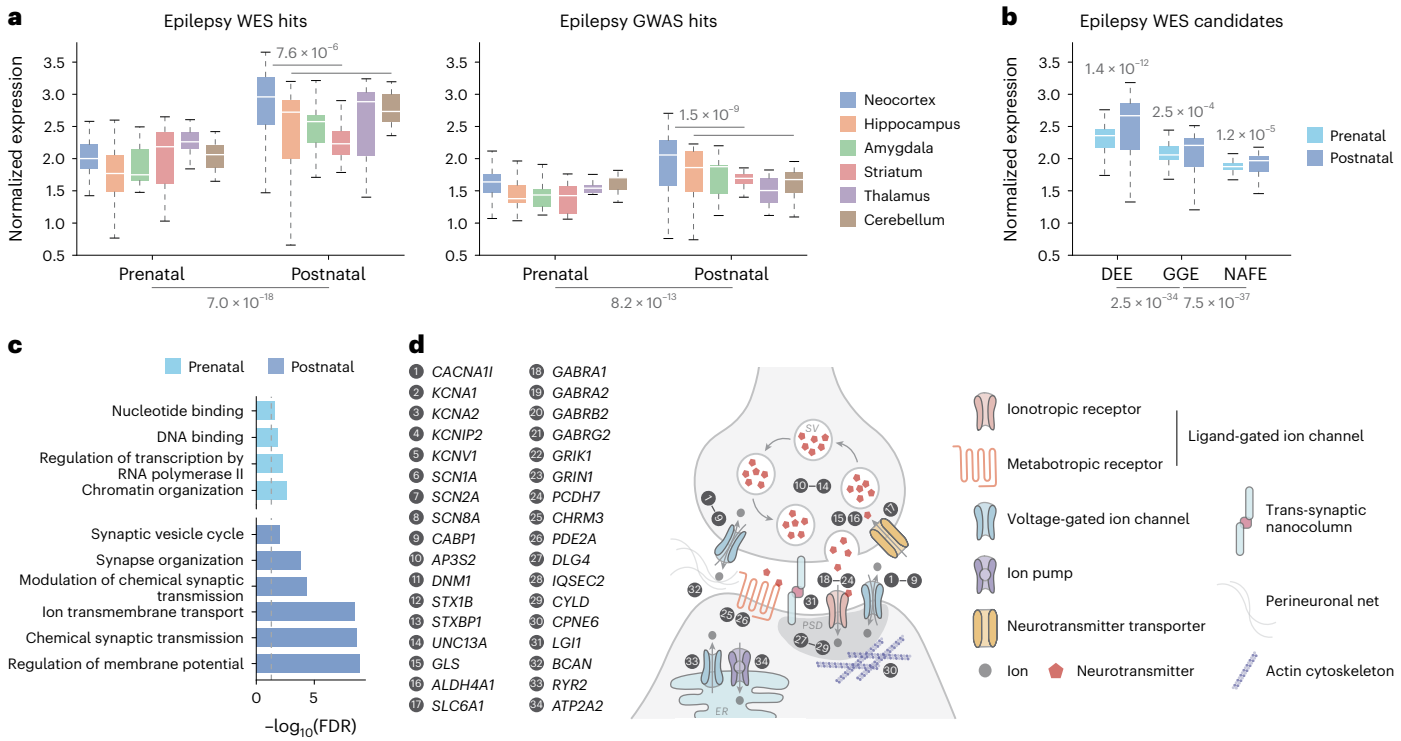

Fig. 6 | Functional analysis of candidate epilepsy genes. a,**b**, Spatiotemporal brain transcriptome analysis of exome/genome-wide significant genes identified in this WES study ($n = 13$) or our recent GWAS ($n = 29$) (**a**) and the top 20 genes enriched for deleterious URVs in each subtype of epilepsy (**b**). Candidate genes show the highest expression in the neocortex during postnatal periods. The expression values ($\log_2(\text{TPM} + 1)$) are normalized to the mean for each BrainSpan sample and then averaged by each candidate gene set. Significance was evaluated by Wilcoxon signed-rank test ($n = 162/200, 15/17, 14/19, 20/14, 13/16$ and $13/21$ for prenatal/postnatal neocortex, hippocampus, amygdala, striatum, thalamus and cerebellum samples, respectively). Box plots indicate median and interquartile range (IQR), with whiskers adding IQR to the first and third quartiles. **c**, GO terms enriched for candidate epilepsy genes with a prenatal or postnatal expression preference ($n = 43$ and $n = 50$, respectively). Vertical dashed line indicates FDR = 0.05; the full list of enriched terms is provided in Supplementary Data 12. **d**, A schematic diagram showing the distribution and function of 34 postnatal genes on neuron structures.

including protein-truncating URVs in *ANKRD11* and NSL complex and damaging missense URVs in *KDM4B*. The *ANKRD11* gene is a known causal gene for KBG syndrome[42], a rare genetic disorder characterized by a range of developmental and neurological abnormalities, including epilepsy[43]. The NSL complex genes play an important role in regulating core transcriptional and signaling networks[44], mutations in which have been associated with NDDs[45,46]. The *KDM4B* gene encodes a demethylase enzyme that regulates gene expression essential for brain development[47], and rare variants in KDM4B have been implicated in global developmental delay[48]. Collectively, these genes have an already established role in NDDs that present shared clinical characteristics with DEEs. Another new candidate, the PDE gene family, was found associated with NAFE. PDE enzymes catalyze the hydrolysis of cAMP and cGMP, two key second messengers modulating a variety of neuronal pathways[49,50]. Lastly, a noteworthy finding was the *RYR2* gene associated with GGE, which was identified by combining rare and common genetic variation, representing an example of convergent epilepsy generic risk across the allele frequency spectrum.

In addition to nominating new genes, identifying new variants in known epilepsy genes will also facilitate the characterization of specific mechanisms. Over the past 5-year efforts from Epi25 WES, there has been a steady increase in the number of deleterious URVs discovered in epilepsy panel genes (Extended Data Fig. 6a); almost all (130/134) genes with a known monogenic cause have been identified with at least one deleterious URV (Extended Data Fig. 6b), providing a valuable resource for downstream functional analysis. Although the number of damaging missense URVs increases at a higher rate than protein-truncating URVs, the number of additional genes identified with a missense URV grows more slowly (Extended Data Fig. 6b). This pattern reflects an accumulation of missense URVs in the same set of genes, highlighting the need of effective approaches to characterize the specific functional consequences of these missense variants.

Compared to our prior URV results[10], the top genes that maintained or obtained stronger association in this enlarged study are known epilepsy genes (Extended Data Fig. 6c). This trend demonstrates a high replicability of existing gene findings and, likewise, calls for larger sample sizes to confirm the present results. Moreover, since our last Epi25 study, we began to include diverse genetic ancestry samples in our primary analysis (Extended Data Fig. 6d), and, in the present study, we performed the first genetic ancestry-specific subgroup analyses (Extended Data Fig. 4). There was an overall trend of shared rare variant risk across ancestry groups, yet delineating differences in individual genes still awaits larger sample sizes. The power of identifying significant genes depends on both our ability to detect ultra-rare variants and the effect sizes of these variants in increasing epilepsy risk. At the current sample size, we have approximately 80% power to detect strong gene burden with a large effect size of OR > 8 (Extended Data Fig. 6e and Methods). Larger sample sizes are required to detect smaller effect sizes. With the current case:control ratio, we will need approximately 58,000 cases to achieve 80% power for detecting moderate gene burden (OR ≥ 3) and approximately 462,000 cases for weak gene burden with OR = 1.5. In addition, increasing the control-to-case ratio will also enhance power. With the same number of cases, doubling the controls will yield up to a 20% power increase, and quadrupling the controls will provide us with nearly 100% power to detect strong gene burden (Extended Data Fig. 6f and Methods). Existing epilepsy panel genes are largely dominated by DEE genes, and these genes exhibited substantially stronger burden for URVs from DEEs than the other two

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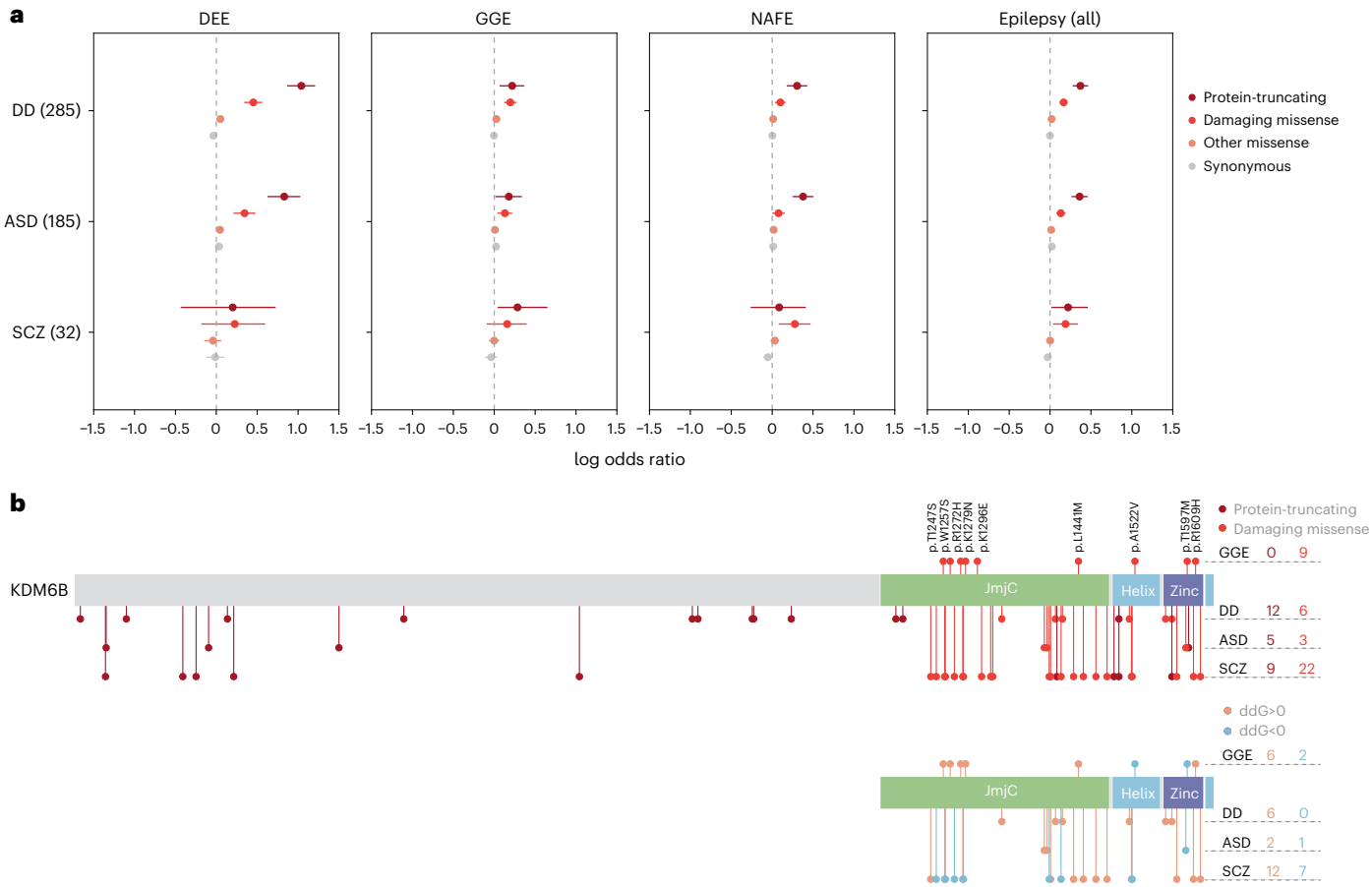

Fig. 7 | Shared rare variant risk between epilepsy and other NDDs. a, Burden of URVs in genes implicated by WES of severe DDs ($n = 285$), ASD ($n = 185$) and SCZ ($n = 32$). Burden analyses were performed across four variant classes and four epilepsy groups—1,938 DEEs, 5,499 GGE, 9,219 NAFE and 20,979 epilepsy-affected individuals combined—versus 33,444 controls. Overall, DD/ASD-associated genes show stronger enrichment of epilepsy URVs than SCZ. The dot represents the log(OR), and the error bars represent the 95% confidence intervals of the point estimates. **b**, Distribution of rare variants from GGE and other NDDs on the KDM6B protein. Top, a schematic protein plot displaying the distribution of protein-truncating (darker red) and damaging missense (lighter red) variants on KDM6B. Bottom, a schematic protein plot displaying the distribution of damaging missense variants with a likely destabilizing (ddG > 0; orange) and stabilizing (ddG < 0; blue) effect on KDM6B. In both plots, variants found in GGE are plotted above the protein, and those from other NDDs are plotted below the protein (in the order of DD, ASD and SCZ as labeled); the number of variant carriers is listed accordingly on the right.

epilepsy subtypes (Extended Data Fig. 6g). Excluding epilepsy panel genes, we found significant residual burden of URVs in genes that are intolerant to genetic variation (Extended Data Fig. 6g). Collectively, these findings suggest that the discovery of rare variant risk for epilepsy is far from saturation.

A promising strategy to accelerate gene discovery is to integrate URVs and other genetic variants. As presented in this study, the timeliness of our WES and GWAS efforts has resulted in the discovery of a wide allelic spectrum of epilepsy genetic risk factors, comprising different types of genetic variation, varying in size and/or frequency, each contributing to uncovering part of the complex genetic architecture of epilepsy. Currently missing pieces include balanced structural variants (for example, inversions and translocations not detected in our CNV calls) and mitochondrial variants. Both have been recognized as sources of epilepsy genetic risk[51,52], and larger-scale studies are required to characterize genome-wide patterns of these variants. Given the growing evidence that different genetic risk factors converge at least partially in the same genes, we think that an extended model that jointly analyzes these variants would likely provide the most informative results beyond any single approach. Overall, the ongoing sequencing and genotyping efforts, together with the ever-increasing scale of genetic association studies, will continue to expand and refine understanding of the genetic architecture of epilepsy, delineate specific underlying pathophysiological processes and hopefully enable a move toward more targeted treatment approaches.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41593-024-01747-8.

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Methods

Study design and participants

We collected DNA and detailed phenotyping data of individuals with epilepsy from 59 participating Epi25 sites in Europe, North America, Australasia and Asia (Supplementary Table 1). In total, we analyzed 20,979 epilepsy cases—including 1,938, 5,499 and 9,219 individuals with DDEs, GGE and NAFE, respectively, and 4,323 with other epilepsies (mostly lesional focal epilepsy (2,495)) and febrile seizures ((FS)/FS+ (327))—and 33,444 controls. Control individuals were aggregated from a subset of Epi25 sites, local collections at the Broad Institute or the database of Genotypes and Phenotypes (dbGaP) and were not screened for neurological or neuropsychiatric conditions (Supplementary Table 2).

Phenotyping procedures

Epilepsies were diagnosed by epileptologists on clinical grounds (see below for specific criteria for DEEs, GGE and NAFE) in accordance with the ILAE classification at the time of diagnosis and recruitment[1,9,10]. Phenotyping data were entered into the Epi25 data repository (https://github.com/Epi25/epi25-edc) via case record forms hosted on the REDCap platform[53]. The data fields do not contain protected health information. Data collected from previous coordinated efforts with phenotyping on databases (for example, the Epilepsy Phenome/Genome Project[54] and the EpiPGX project (http://www.epipgx.eu)) were integrated via scripted transformations. All phenotyping data underwent review for uniformity among sites and QC by automated data checking and manual review as required; the process was overseen by a phenotyping committee with clinical expertise.

Epilepsy case definitions

Epilepsy diagnoses and classification for Epi25 were described previously[9,10]. In brief, diagnosis of DEEs required severe refractory epilepsy of unknown etiology, with developmental plateau or regression, and with epileptiform features on electroencephalogram (EEG). Diagnosis of GGE required a history of generalized seizure types (generalized tonic-clonic, absence or myoclonic seizures) with generalized epileptiform discharges on EEG; exclusion criteria included focal seizures, moderate-to-severe intellectual disability and epileptogenic lesions on neuroimaging if available. Diagnosis of NAFE required a history of focal seizures with either focal epileptiform discharges or normal findings on EEG; exclusion criteria included primary generalized seizures, moderate-to-severe intellectual disability and neuroimaging lesions (except hippocampal sclerosis).

Informed consent

Adult participants, or the legal guardian of child participants, provided signed informed consent at participating centers based on the local ethical requirements at the time of collection. To be included in the study, consent was required not to exclude data sharing. Consent forms for samples collected after 25 January 2015 required specific language according to the National Institutes of Health's Genomic Data Sharing Policy. Before sequencing at the Broad Institute of MIT and Harvard, the Mass General Brigham (MGB) institutional review board (IRB) provided approval for secondary use of each of the Epi25 cohorts, after reviewing the consent forms from each cohort (MGB protocol number: 2012P000788). The Broad Institute's Office of Research Subjects Protection (ORSP) provided approval for each cohort (ORSP approval number: ORSP-1733). For deposition of data into AnVIL, we obtained Data Use Limitations letters for each cohort from the original IRBs and Institutional Certifications from the Broad Institute's ORSP.

WES data generation

All samples were sequenced at the Broad Institute of MIT and Harvard on Illumina HiSeq X or NovaSeq 6000 platforms with 150-bp paired-end reads. Exome capture was performed using multiple kits: Illumina Nextera Rapid Capture Exomes or TruSeq Rapid Exome enrichment kit (target size, 38 Mb) and Twist Custom Capture (target size, 37 Mb). Sequence data in the form of BAM files were generated via the Picard data processing pipeline, and well-calibrated reads were aligned to the human reference GRCh38. Variants were jointly called across all samples via the Genome Analysis Toolkit (GATK) best practices pipeline[55] and were annotated using Variant Effect Predictor (VEP)[56] with custom annotations, including the Loss-Of-Function Transcript Effect Estimator (LOFTEE)[57] and MPC (missense badness, PolyPhen-2 and regional constraint)[11], using Hail.[58]

Variant and sample QC

Initial variant QC criteria included the following: (1) genotype quality (GQ) ≥ 20; (2) read depth (DP) ≥ 20; (3) allele balance (AB) ≥ 0.2 and AB ≤ 0.8; (4) passing the GATK Variant Quality Score Recalibration (VQSR) filter; (5) residing in GENCODE coding regions that were well covered by both capture platforms, where 80% of the Illumina or Twist sequenced samples had at least 10× coverage; and (6) outside of the low-complexity regions (LCRs)[59]. Additional variant QC was applied after sample QC (see below for details): (1) call rate ≥ 0.98; (2) case–control call rate difference ≤ 0.02; and (3) Hardy–Weinberg equilibrium (HWE) test $P \geq 10^{-6}$.

Sample QC criteria, on the basis of all sequenced samples and the initial quality controlled variants, included the following: (1) mean call rate ≥ 0.90; (2) mean GQ ≥ 57; (3) mean DP ≥ 25; (4) freemix contamination estimate ≤ 2.5%; (5) percent chimeric reads ≤ 2%; and (6) genetically imputed sex matching with self-reported sex. We performed principal component analysis (PCA) to classify samples into genetic ancestral groups, using a random forest model trained on the 1000 Genomes data; samples with a probability ≥ 0.9 to be one of the six populations—Non-Finnish European (NFE), Finnish (FIN), African (AFR), East Asian (EAS), South Asian (SAS) and Admixed American (AMR)—were retained. Within each ancestral group, we examined cryptic relatedness based on identity-by-descent (IBD) estimates and excluded one sample from each pair of related individuals with an IBD > 0.2. Additional sample QC was applied on a population-specific and cohort-specific basis, which excluded outliers with >4 s.d. from the mean of (1) transition/transversion ratio, (2) heterozygous/homozygous ratio and (3) insertion/deletion ratio. To control for residual population stratification, we further excluded samples and/or cohorts that show extreme counts of synonymous singletons. The number of samples that passed QC at each step is detailed in Supplementary Table 3.

Exome-wide burden analysis

To evaluate the excess of rare, deleterious protein-coding variants in individuals with epilepsy, we performed burden analysis across the entire exome, at both an individual gene level and a gene set level. URVs were defined as variants observed no more than five copies among the combined case–control cohort, which corresponded to a MAF < 0.005%. Deleterious variants were defined and categorized into two classes: (1) protein truncating annotated by LOFTEE and (2) damaging missense with an MPC score ≥ 2. We tested the burden of each URV class by regressing the case–control status on the URVs aggregated across a target gene or gene set in an individual, using a Firth regression model adjusting for sex and ancestry (the PCA-predicted genetic ancestral group and the top 10 principal components (PCs)). We further included the exome-wide count of synonymous singletons as an additional covariate to better control for residual population stratification not captured by PCs[9].

We performed the burden analyses for each of the three major epilepsy subtypes—DEEs, GGE and NAFE—and for all epilepsy-affected individuals combined. At the individual gene level, we tested all protein-coding genes with at least one epilepsy or control carrier (protein truncating: n = 15,083, 15,236, 15,398 and 15,903 for the analysis of DEEs, GGE, NAFE and all-epilepsy combined, respectively; damaging

missense: n = 4,013, 4,057, 4,105 and 4,194; synonymous: n = 17,460, 17,463, 17,465 and 17,472). At the gene set level, we tested collections of gene entities that belong to the same gene family[16] or encode a particular protein complex[17] and have at least one epilepsy or control carrier (protein truncating: n = 5,080, 5,070, 5,091 and 5,126 for the analysis of DEEs, GGE, NAFE and all-epilepsy combined, respectively; damaging missense: n = 3,256, 3,279, 3,298 and 3,343; synonymous: n = 5,209). Exome-wide significance was determined by Bonferroni correction accounting for 18,531 CCDS genes or 5,373 gene sets—across four epilepsy groups and two variant classes—at $P = 3.4 \times 10^{-7}$ and $P = 1.2 \times 10^{-6}$ for the gene-based and gene-set-based burden analysis, respectively.

Protein structural analysis

We applied a metric[21] that assesses the change in Gibbs free energy (ddG) of protein folding induced by a mutation to characterize missense URVs identified in ion channel genes. In total, we computed ddG for 1,782 missense URVs on 16 ion channel protein complexes with experimentally resolved 3D structures available (Supplementary Data 7). A positive ddG value suggests a decrease in Gibbs free energy of protein unfolding—that is, a destabilizing effect of the mutation on protein—and a negative ddG value suggests a stabilizing effect. In the relevant burden analysis, we used $|ddG| \geq 1$ kcal mol^{-1} to prioritize variants that are likely to cause a change in protein stability.

CNV calling and burden analysis

To call CNVs from the raw exome data, GATK-gCNV[60] was used. In brief, GATK-gCNV is a Bayesian CNV caller, which adjusts for biases (that is, GC content) introduced through capture kits and sequencing while simultaneously accounting for systematic and technical differences. The raw sequencing files were compressed into counts and used as input across the annotated exons, and a subsequent PCA-based method was used on the observed read counts to differentiate capture kits. This was followed by a hybrid distance-based and density-based clustering approach to curate batches of samples to process in parallel. Then, the caller was iteratively run for each batch, and metrics produced by the Bayesian model were used to account for positive predictive value and sensitivity. GATK-gCNV exome QC filters were previously benchmarked in 8,439 matching genome and exome samples, as described in ref. 22.

Samples where GATK-gCNV made more than 100 unfiltered calls or more than 10 filtered calls were considered outlier samples and were removed. This resulted in 48,767 samples (~90% of initial) for the downstream burden analysis, which comprises 18,963 epilepsy cases (including 1,743 DEEs, 4,980 GGE and 8,425 NAFE) and 29,804 controls. To mitigate false positives, we used previously benchmarked filtering thresholds, where CNVs had to span more than four callable exons and had a site frequency less than 0.1% and a quality score of more than 200. In the gene-based burden analysis of CNVs, we considered CNVs to affect a gene if 10% or more of the non-redundant exon base pairs overlapped with the deletion (n_{gene} = 4,213, 4,417, 4,733 and 6,045 for the analysis of DEEs, GGE, NAFE and all-epilepsy combined, respectively) or if 75% or more of the non-redundant exon base pairs overlapped with the duplication (n_{gene} = 7,064, 7,282, 7,564 and 8,793 for the analysis of DEEs, GGE, NAFE and all-epilepsy combined, respectively). When evaluating the joint burden of CNV deletions and protein-truncating SNVs/indels, only the subset of samples passing CNV calling QC was considered.

Brain transcriptome analysis

The spatiotemporal transcriptome of the human brain was obtained from BrainSpan[34], which contains 524 bulk RNA sequencing (RNA-seq) samples collected from 26 brain regions of 42 human subjects. Gene expression values were log-transformed ($\log_2$(transcripts per million (TPM)) + 1) and centered to the mean expression level for each sample. Epilepsy candidate genes identified by this study or our recent GWAS were selected for each sample, and their average centered expression values were calculated and compared by region and developmental time, using Wilcoxon signed-rank test. Six brain regions and nine developmental periods were defined based on the oncology described in the original study: neocortex (including orbital prefrontal cortex (OFC), dorsolateral prefrontal cortex (DFC), ventrolateral prefrontal cortex (VFC), medial prefrontal cortex (MFC), primary motor M1 cortex (M1C), primary somatosensory S1 cortex (S1C), posterior inferior parietal cortex (IPC), primary auditory A1 cortex (A1C), superior temporal cortex (STC), inferior temporal cortex (ITC) and primary visual V1 cortex (V1C)), hippocampus (HIP), amygdala (AMY), striatum (STR), thalamus (MD) and cerebellum (CB); early fetal (8–12 post-conceptional weeks (PCWs)), early midfetal (13–18 PCWs), late midfetal (19–23 PCWs), late fetal (24–37 PCWs), infancy (4–11 months), early childhood (1–5 years), childhood (6–11 years), adolescence (12–19 years) and adulthood (≥20 years).

GO enrichment analysis

We surveyed functional enrichment for candidate epilepsy genes with a prenatal or postnatal expression pattern (n = 43 and n = 50, respectively) via the GO enrichment analysis portal (http://geneontology.org/). Enrichment was evaluated by a Fisher's exact test across 43,008 GO terms (2023-05 release)[36], and significant terms with an FDR of 0.05 were reported. We manually reviewed and classified the top significant terms into two main functional categories: (1) 'gene transcription regulation', which included 32 of 43 prenatal genes from GO:0006325 chromatin organization, GO:0006357 regulation of transcription by RNA polymerase II, GO:0003677 DNA binding and GO:0000166 nucleotide binding; (2) 'neuronal transmission', which included 34 of 50 postnatal genes from GO:0042391 regulation of membrane potential, GO:0007268 chemical synaptic transmission, GO:0034220 monoatomic ion transmembrane transport, GO:0050804 modulation of chemical synaptic transmission, GO:0050808 synapse organization, GO:0099504 synaptic vesicle cycle, GO:0007267 cell–cell signaling, GO:0005509 calcium ion binding and GO:0006536 glutamate metabolic process. Full results of GO enrichment analysis are provided in Supplementary Data 12.

Gene regulatory network analysis

To examine whether genes with a prenatal expression preference and genes with a postnatal expression preference are connected through gene regulation, we constructed a TF–target network by systematically searching for regulatory targets of the prenatal genes and evaluated whether the postnatal genes are overrepresented in these targets. We used the ChEA3 (ref. 61) database to obtain TF–target connections (determined by ChIP-seq experiments and manually curated individual TF studies) and co-expression of TFs with other genes based on RNA-seq data in the human brain. In total, we found target data for 10 of 43 prenatal genes, which resulted in a network of 10,560 TF–target connections with 7,792 genes as nodes and directed edges as regulation relationships. Forty connections were found between 10 TF prenatal genes and 26 postnatal genes (Supplementary Data 12). To assess the significance of the observed connectivity, we performed 10,000 random draws of gene sets matching the postnatal genes with respect to postnatal expression levels and prenatal–postnatal expression preference. An empirical P value was computed as the proportion of random gene sets with greater or equal connectivity compared to the postnatal gene set.

Power estimation

We estimated the power of gene burden testing as the percentage of genes to be detected with sufficient number of protein-truncating URVs to obtain $P \leq 0.05$ by Fisher's exact test, and we benchmarked the power by different effect sizes (OR ranges from 1.5 to 8) and case:control ratios (ranges from 1:1.6 (in this study) to 1:3.2 and 1:6.4). For a given effect size and case:control ratio, the minimum numbers of URVs in cases and controls were determined, and the number of samples required

to discover corresponding total number of URVs was estimated using a linear model of log(number of URVs) ~ log(number of samples) from downsampling the WES dataset in this study.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

We provide summary-level data at the variant and gene level in an online browser for visualization and download (https://epi25.broadinstitute.org/). There are no restrictions on the aggregated data released on the browser. Full results from the exome-wide burden analysis are also available in Supplementary Data 1 and 4. WES data from Epi25 cohorts are available via the NHGRI's controlled-access AnVIL platform (https://anvilproject.org/; dbGaP accession number: phs001489). Data availability of non-Epi25 control cohorts is provided in the Supplementary Information. Source data are provided with this paper.

Publicly available datasets analyzed in this study include:

Gene family: https://zenodo.org/records/3582386

CORUM protein complexes: https://mips.helmholtz-muenchen.de/corum/

Protein Data Bank: https://www.rcsb.org/

(Structure analyzed in Fig. 3c: https://www.rcsb.org/structure/6x3z)

BrainSpan: https://www.brainspan.org/

Gene Ontology: https://geneontology.org/

ChEA3: https://maayanlab.cloud/chea3/

Code availability

No custom code was used in this study. For sequence data generation, we used GATK version 3.4 and version 3.6 (GATK nightly-2015-07-31-g3c929b0, 3.4-89-ge494930 and 3.6-0-g89b7209), Picard version 1.1431 and VerifyBamID version 1.0.0. Sample and variant QC was performed using functions in Hail 0.1 and 0.2 (website: https://www.hail.is; documentation: https://hail.is/docs/0.1/ and https://hail.is/docs/0.2/; GitHub repository: https://github.com/hail-is/hail). Variant annotation was performed using the Ensembl Variant Effect Predictor (VEP) version 85 tool as implemented in Hail 0.1 with the LOFTEE annotation provided as default (https://github.com/konradjk/loftee/tree/27b0040f524348baa7f3257f1ce58993529e09ef). For phenotyping data, case record forms were hosted on the REDCap platform version 14 and entered into the Epi25 data repository (https://github.com/Epi25/epi25-edc). For gene burden analysis, we used the R (version 3.6.1) package logistf version 1.26.0 (https://cran.r-project.org/web/packages/logistf/index.html) to implement the Firth regression model. Additional processing and visualization were performed using R functions in the tidyverse library version 1.3.0 (https://www.tidyverse.org/packages/).

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Author contributions

All authors contributed to patient phenotyping data and sample collection or to analyses. Roles in specific committees of the project are listed in the supplementary materials.

Competing interests

B.M.N. is a member of the scientific advisory boards of Deep Genomics and Neumora. No other authors have competing interests to declare.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41593-024-01747-8.

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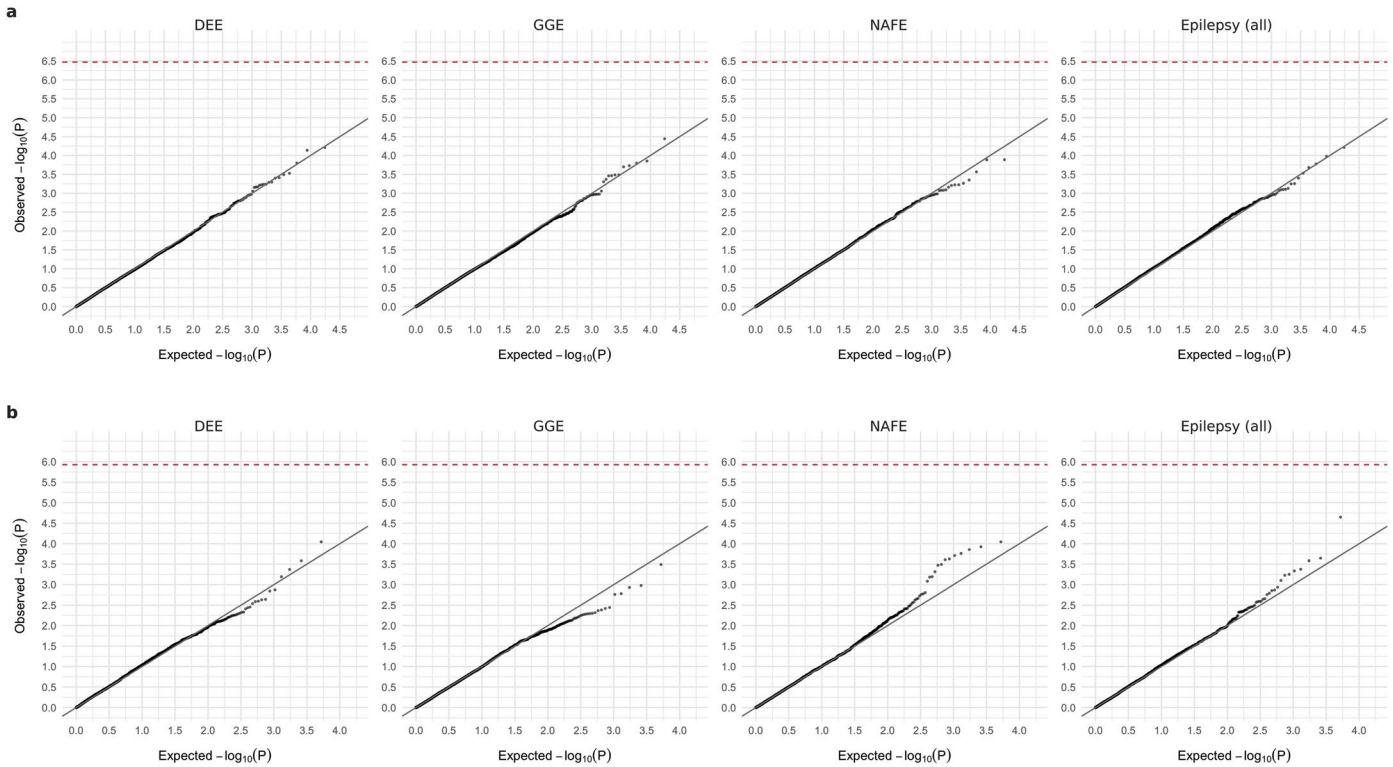

Extended Data Fig. 1 | Results from burden analysis of synonymous URVs. **a**,**b**, Burden of synonymous URVs at the individual-gene (**a**) and the gene-set (**b**) level. The observed $-\log_{10}$-transformed *P* values are plotted against the expectation given a uniform distribution. Burden analyses are performed across four epilepsy groups – 1,938 DEEs, 5,499 GGE, 9,219 NAFE, and 20,979 epilepsy-affected individuals combined – versus 33,444 controls. *P* values are computed using a Firth logistic regression model testing the association between the case-control status and the number of URVs (two-sided); the red dashed line indicates exome-wide significance $P = 3.4 \times 10^{-7}$ after Bonferroni correction (see Methods).

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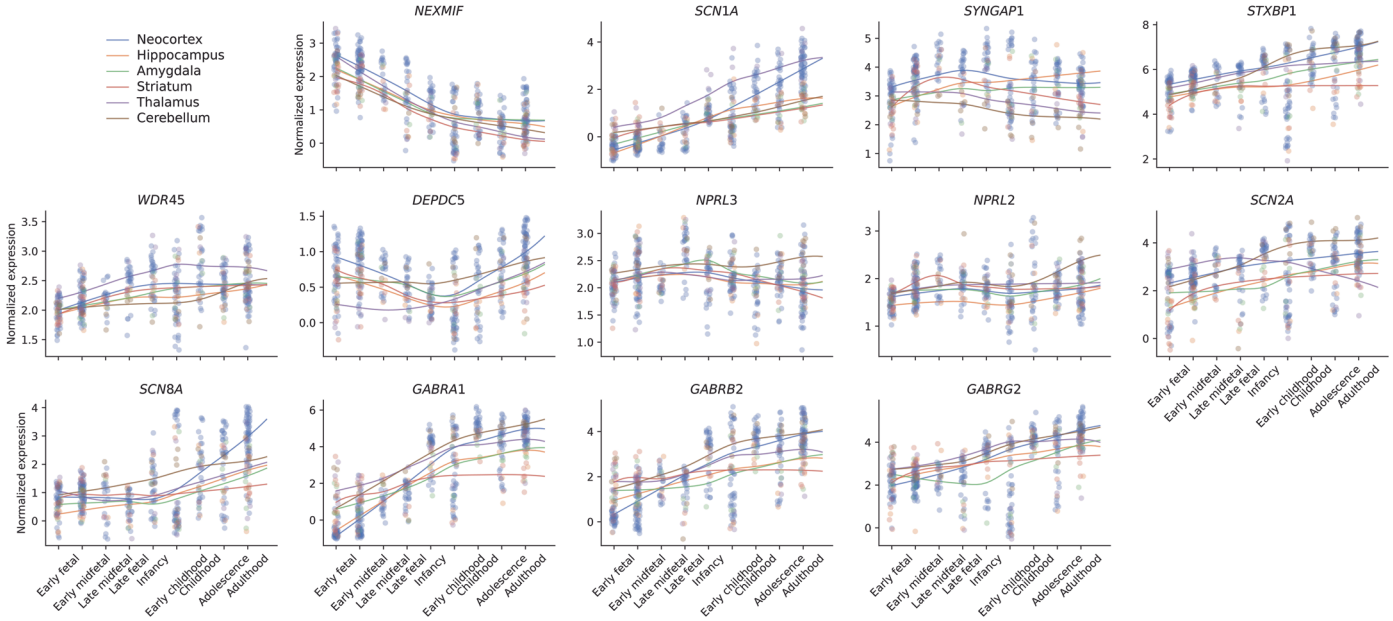

Extended Data Fig. 2 | Spatiotemporal expression of 13 exome-wide significant genes in the human brain. Expression values (log_2[TPM + 1]) are normalized to the mean for each BrainSpan sample; each dot represents the expression value of a particular gene in a sample collected in a particular brain region and developmental time (from early fetal to adulthood: N = 47/5/5/9/5/4, 69/6/6/7/5/4, 19/2/1/2/1/2, 27/2/2/2/2/3, 30/2/3/2/3/3, 41/3/4/3/4/5, 30/3/3/1/1/3, 36/3/3/2/2/4, and 63/6/6/6/6/6 neocortex/hippocampus/amygdala/striatum/thalamus/cerebellum samples, respectively). LOESS smooth curves are plotted for each brain region across developmental time.

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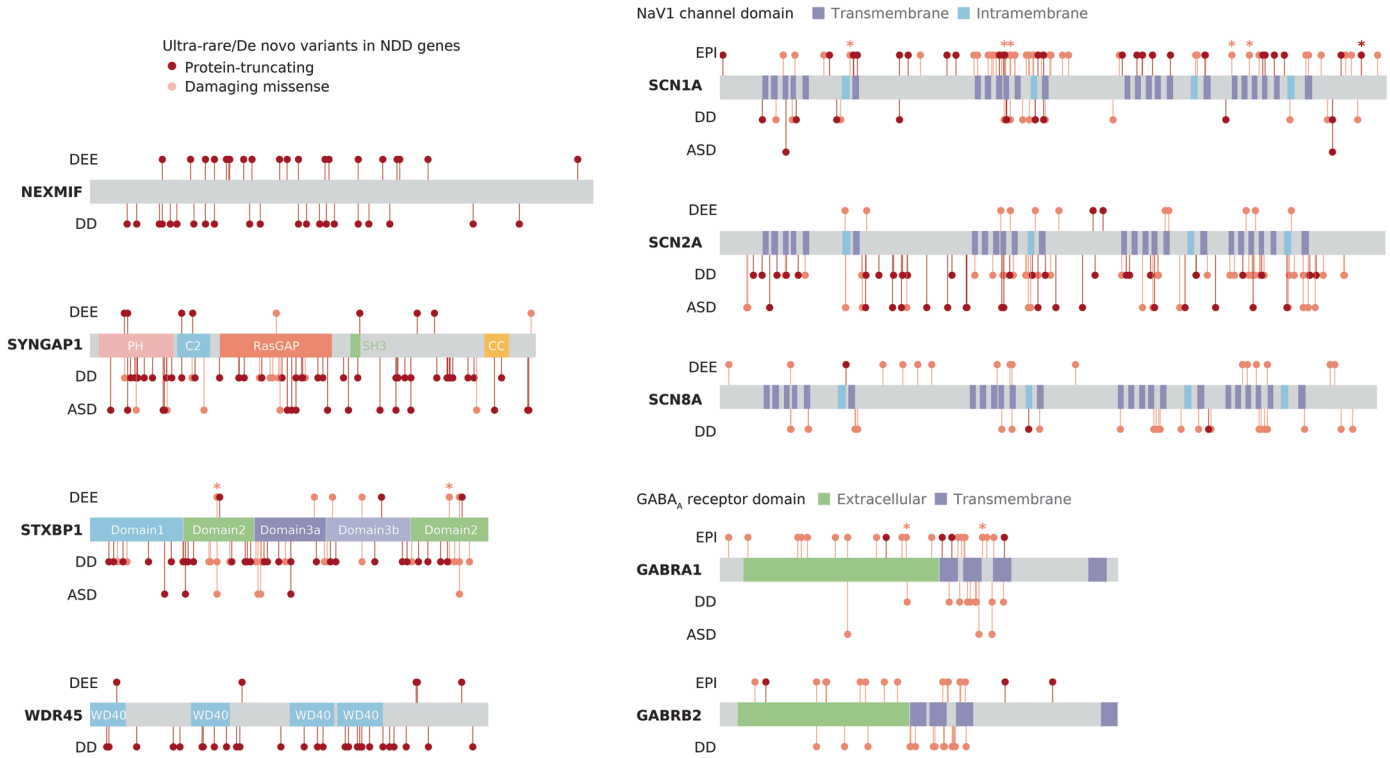
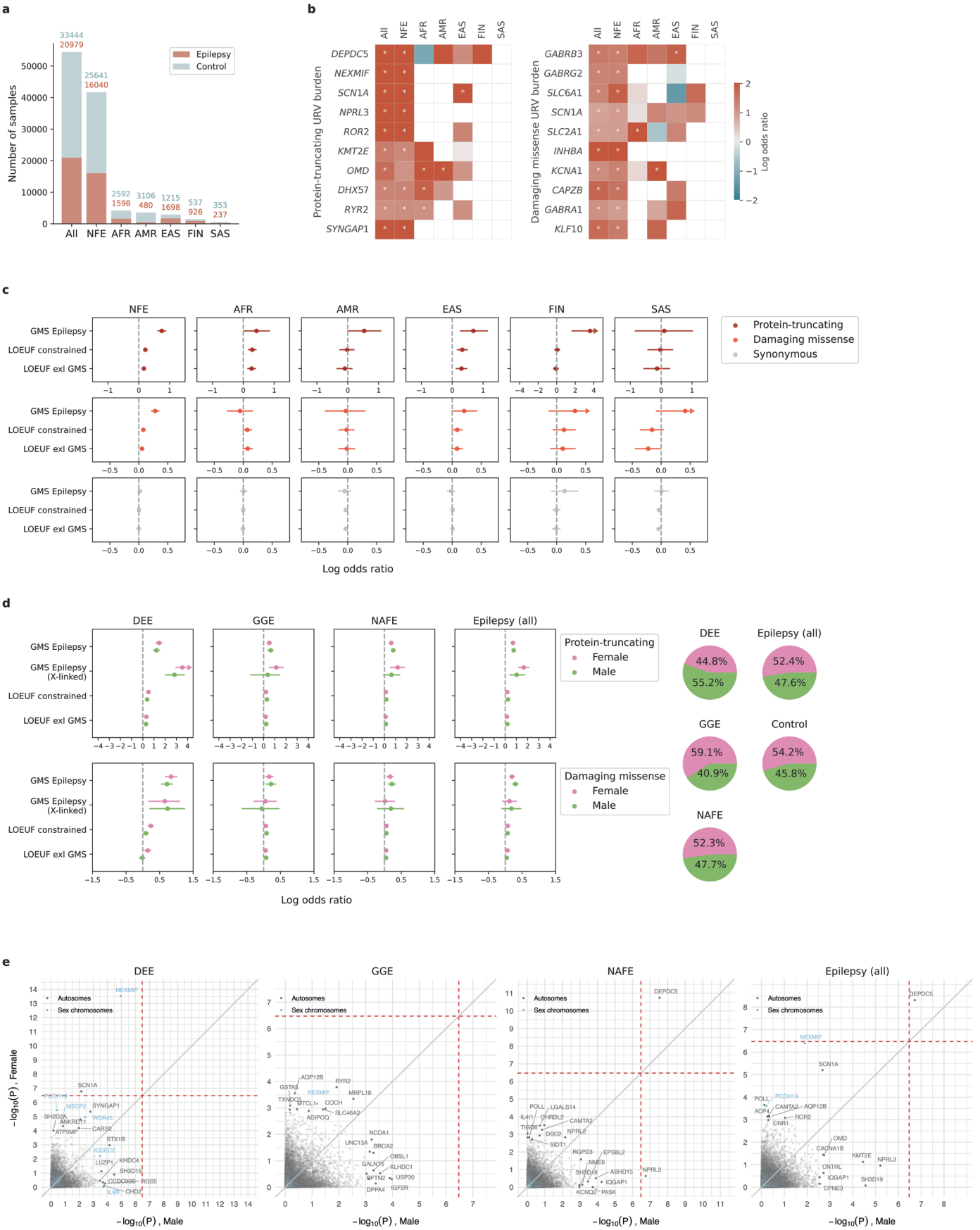


Extended Data Fig. 3 | Distributions of URVs from this study and de novo variants from other NDD studies on the same genes. Schematic protein plots of nine genes that are significant in both our epilepsy cohort (DEE: developmental and epileptic encephalopathy; EPI: all-epilepsy combined) and previous large-scale WES studies of severe developmental disorders (DD) and/or autism spectrum disorder (ASD) are shown. Asterisk indicates recurring URVs in epilepsy; recurring de novo variants in DD/ASD as well as detailed variant information are provided in Supplementary Data 13.

https://doi.org/10.1038/s41593-024-01747-8



Extended Data Fig. 4 | See next page for caption.

https://doi.org/10.1038/s41593-024-01747-8

Extended Data Fig. 4 | Results from genetic ancestry- and sex-specific burden analyses. a, The numbers of epilepsy cases (orange) and controls (blue) by genetic ancestry. **b**, Comparison of protein-truncating (left) and damaging missense (right) URV burden in the top ten genes from the primary analysis ('All') across genetic ancestry subgroups. Red color indicates enrichment in cases (log[OR] > 1), with an asterisk indicating nominal significance ($P \leq 0.05$; see Supplementary Data 14 for exact *P* values). *P* values are computed using a Firth logistic regression model testing the association between the case-control status and the number of URVs (two-sided). **c**, Genetic ancestry-specific burden of URVs in established epilepsy genes (N = 171 curated by the Genetic Epilepsy Syndromes [GMS] panel with a known monogenic/X-linked cause), constrained genes (N = 1,917 scored by the loss-of-function observed/expected upper bound fraction [LOEUF] metric as the most constrained 10% genes), and constrained genes excluding established epilepsy genes (N = 1,813). Overall, different ancestral groups show at least partially shared burden of deleterious URVs in these gene sets. In **a**-**c**, NFE: Non-Finnish European (N_{case}=16,040, $N_{control}$ = 25,641), AFR: African (N_{case}=1,598, $N_{control}$ = 2,592), AMR: Ad Mixed American (N_{case}=480, $N_{control}$ = 3,106), EAS: East Asian (N_{case}=1,698, $N_{control}$ = 1,215), FIN: Finnish (N_{case}=926, $N_{control}$ = 537), SAS: South Asian (N_{case}=237, $N_{control}$ = 353). **d**, Sex-specific burden of URVs in established epilepsy genes. Burden analyses are performed for three gene sets described in **c**, with an additional set of 37 X-linked GMS epilepsy genes, across four epilepsy groups (female: N_{DEE} = 811, N_{GGE} = 4,807, N_{NAFE} = 3,511, $N_{EPI(all)}$=11,372, $N_{control}$ = 18,144; male: N_{DEE} = 997, N_{GGE} = 2,579, N_{NAFE} = 4,395, $N_{EPI(all)}$=10,397, $N_{control}$ = 15,302). There is an overall trend of shared URV burden between female and male subgroups in these gene sets. In **c** and **d**, the dot represents the log odds ratio and the error bars represent the 95% confidence intervals of the point estimates. For presentation purposes, error bars that exceed a large log odds ratio value are capped, indicated by arrows at the end of the error bars (see Supplementary Data 14 and 15 for exact values). **e**, Comparison of sex-specific burden of protein-truncating URVs at level of the individual genes. For each gene, the $-\log_{10}$-transformed *P* value from the female subgroup analysis (y-axis) is plotted against that from the male subgroup analysis (x-axis). Top ten genes with URV burden in epilepsy are labeled for each subgroup, with genes on the sex chromosomes colored in blue. The red dashed line indicates exome-wide significance $P = 3.4 \times 10^{-7}$ after Bonferroni correction.

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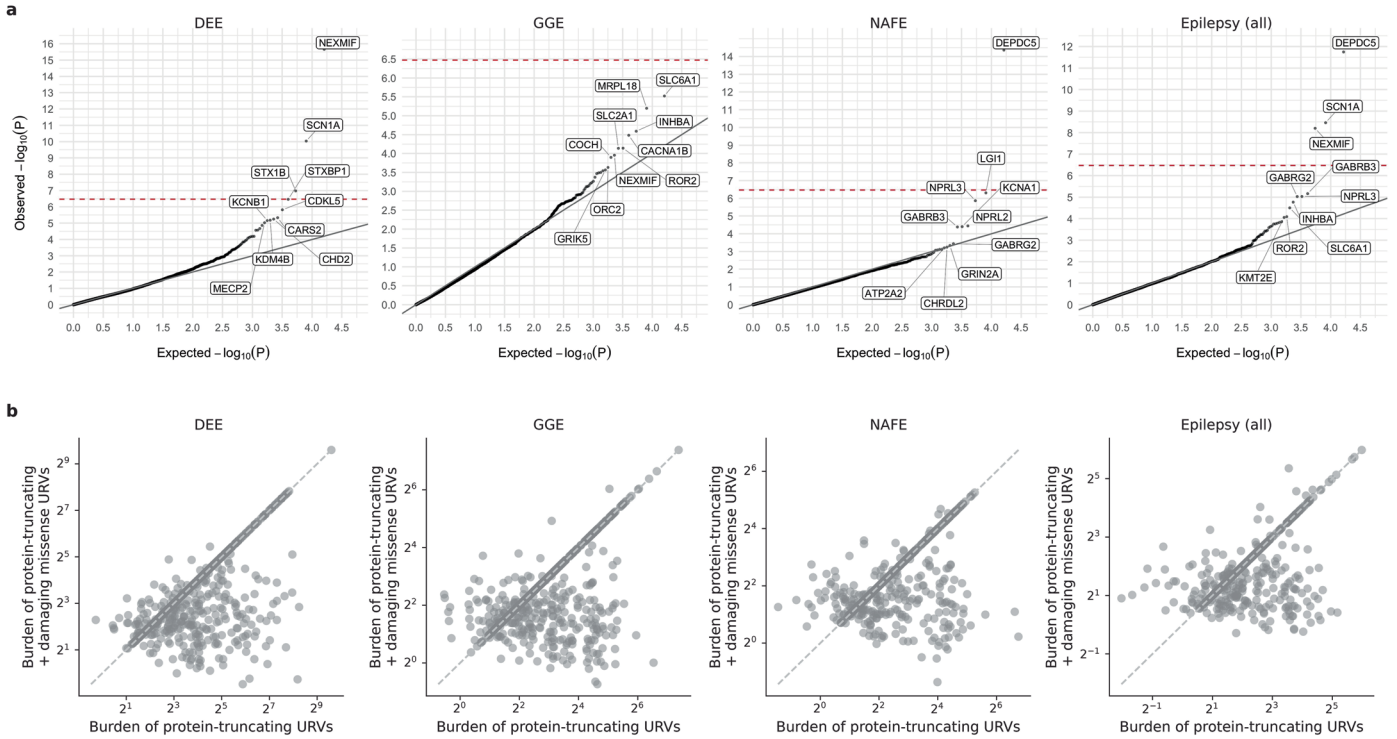
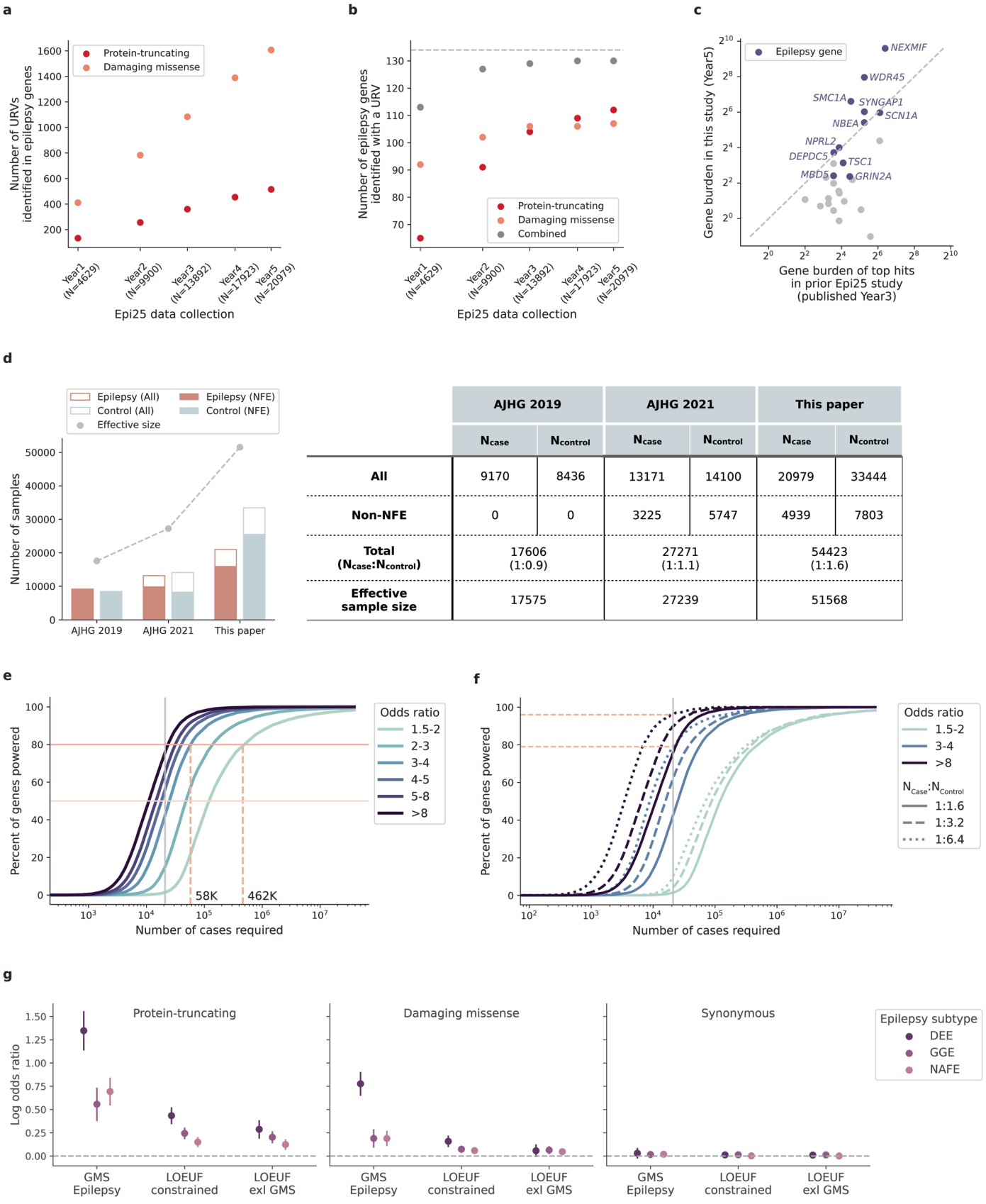


Extended Data Fig. 5 | Results from burden analysis of protein-truncating and damaging missense URVs combined. a, Joint burden of protein-truncating and damaging missense URVs at the individual-gene level. The observed −log10-transformed *P* values are plotted against the expectation given a uniform distribution. Burden analyses are performed across four epilepsy groups – 1,938 DEEs, 5,499 GGE, 9,219 NAFE, and 20,979 epilepsy-affected individuals combined – versus 33,444 controls. *P* values are computed using a Firth logistic regression model testing the association between the case-control status and the number of URVs (two-sided); the red dashed line indicates exome-wide significance $P = 3.4 \times 10^{-7}$ after Bonferroni correction (see Methods). **b**, Comparison of the joint burden in **a** with the burden of protein-truncating URVs. The odds ratio (OR) of protein-truncating plus damaging missense URVs (y-axis) and that of protein-truncating URVs alone (x-axis) are compared. Each dot represents a gene with nominally significant enrichment (OR > 0 and $P \leq 0.05$) of either protein-truncating URVs or the two variant classes combined.

https://doi.org/10.1038/s41593-024-01747-8



	AJHG 2019		AJHG 2021		This paper	
	N_{case}	$N_{control}$	N_{case}	$N_{control}$	N_{case}	$N_{control}$
All	9170	8436	13171	14100	20979	33444
Non-NFE	0	0	3225	5747	4939	7803
Total (N_{case}:$N_{control}$)	17606 (1:0.9)		27271 (1:1.1)		54423 (1:1.6)	
Effective sample size	17575		27239		51568	

Extended Data Fig. 6 | See next page for caption.

https://doi.org/10.1038/s41593-024-01747-8

Extended Data Fig. 6 | URV discovery and burden results across Epi25 data collection. a, Increase in the number of protein-truncating and damaging missense URVs discovered in epilepsy genes with a known monogenic cause. **b**, Increase in the number of monogenic epilepsy genes identified with a protein-truncating or damaging missense URV. In **a** and **b**, variant/gene count is plotted against the year of Epi25 data collection; the total number of epilepsy cases analyzed in each year is indicated in parenthesis. **c**, URV burden of previously top-ranked genes in this study. The odds ratio of protein-truncating URVs in genes from this study (y-axis) and the prior Epi25 publication (x-axis) are compared. Each dot represents one of the top ten genes implicated by our previous burden analysis (across three epilepsy subtypes). Genes with a known monogenic/X-linked cause are labeled and colored in purple. **d**, Increase in the total, non-European ancestry, and effective sample size in this study over our previous publications. The effective sample size is computed as $4/(1/N_{case} + 1/N_{control})$. **e**,**f**, The sample size required for well-powered gene burden testing. The percentage of genes powered to detect significant URV burden (Fisher's exact $P \leq 0.05$) at different effect sizes (**e**) and case:control ratios (**f**) is shown as a function of log-scaled sample size of epilepsy cases. Lighter color indicates smaller effect size (weaker burden), which requires a larger sample size to detect. The gray vertical line indicates the current sample size of 20,979 cases. In **e**, horizontal lines indicate 80% and 50% detection power, and vertical dashed lines indicate the estimated number of cases required to achieve 80% at the benchmarked effective sizes. In **f**, dashed and dotted curves indicate power estimation with increased control:case ratios from 1.6 (in this study) to 3.2 and 6.4, respectively; horizontal lines indicate the estimated power achieved by doubling and quadrupling the number of controls at the current sample size of cases. **g**, Epilepsy subtype-specific burden of URVs in established epilepsy genes (N = 171 curated by the Genetic Epilepsy Syndromes [GMS] panel with a known monogenic/X-linked cause), constrained genes (N = 1,917 scored by the loss-of-function observed/expected upper bound fraction [LOEUF] metric as the most constrained 10% genes), and constrained genes excluding established epilepsy genes (N = 1,813). Burden analyses are performed across three epilepsy subtypes – 1,938 DEEs, 5,499 GGE, and 9,219 NAFE – versus 33,444 controls. Protein-truncating and damaging missense URVs from DEEs exhibit the strongest enrichment in epilepsy panel genes, while all epilepsy subtypes show significant enrichment in constrained genes even after excluding the panel genes. No enrichment is observed for synonymous URVs. The dot represents the log odds ratio and the error bars represent the 95% confidence intervals of the point estimates.

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Reporting Summary

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
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☐	☒ A description of all covariates tested
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☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	No software was used for the collection of data, as this was an opportunistic study.
Data analysis	No custom code was used in this study. For sequence data generation, we used GATK v3.4 and v3.6 (GATK nightly-2015-07-31-g3c929b0, 3.4-89-ge494930, and 3.6-0-g89b7209), Picard version 1.1431, and VerifyBamID version 1.0.0. Sample and variant QC was performed using functions in Hail 0.1 and 0.2 (website: https://www.hail.is; documentation: https://hail.is/docs/0.1/ and https://hail.is/docs/0.2/; GitHub repository: https://github.com/hail-is/hail). Variant annotation was performed using the Ensembl Variant Effect Predictor (VEP) v85 tool as implemented in Hail 0.1 with the LOFTEE annotation provided as default (https://github.com/konradjk/loftee/tree/27b0040f524348baa7f3257flce58993529e09ef). For phenotyping data, case record forms were hosted on the REDCap platform version 14, and entered into the Epi25 Data repository (https://github.com/Epi25/epi25-edc). For gene burden analysis, we used the R (v3.6.1) package logistf version 1.26.0 (https://cran.r-project.org/web/packages/logistf/index.html) to implement the Firth regression model. Additional processing and visualization was performed using R functions in the tidyverse library version vl.3.0 (https://www.tidyverse.org/packages/).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

We provide summary-level data at the variant and gene level in an online browser for visualization and download (https://epi25.broadinstitute.org/). There are no restrictions on the aggregated data released on the browser. Full results from the exome-wide burden analysis are also available in Supplementary Data 1 and 4. WES data from Epi25 cohorts are available via the NHGRI's controlled-access AnVIL platform (https://anvilproject.org/; dbGaP accession phs001489). Data availability of non-Epi25 control cohorts is provided in Supplementary Information. Source data are provided with this paper.

Publicly available datasets analyzed in this study include
Gene family: https://zenodo.org/records/3582386
CORUM protein complexes: https://mips.helmholtz-muenchen.de/corum/
Protein Data Bank: https://www.rcsb.org/
(Structure analyzed in Fig.3c: https://www.rcsb.org/structure/6x3z)
BrainSpan: https://www.brainspan.org/
Gene Ontology: https://geneontology.org/
ChEA3: https://maayanlab.cloud/chea3/

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	The term 'sex' was used to indicate biological attribute. The method of sex inference is described Supplementary Information; there are in total 37,104 females and 32,528 males inferred the initial dataset, and 29,144 females and 25,279 males were included in the downstream analysis after sample QC as well as with permissions for public release of aggregate data. Sex-specific analyses were performed in this study for sex-specific genetic associations.
Reporting on race, ethnicity, or other socially relevant groupings	Genetic ancestry was defined by principal component analysis (PCA), using a random forest model trained on the 1000 Genomes data; samples with a probability !0.9 to be one of the six populations – Non-Finnish European (NFE), Finnish (FIN), African (AFR), East Asian (EAS), South Asian (SAS), Ad Mixed American (AMR) – were retained. In our burden analyses, we controlled for confiding factors from genetic ancestry by using the PCA-predicted genetic ancestral group and the top ten PCs as covariants in the Firth regression model.
Population characteristics	Table S1 and S2 summarized contributing collections along with the number of samples sequenced; full details of each individual participating cohort was provided in Supplementary Subjects and Methods. Epilepsies were diagnosed by epileptologists on clinical grounds in accordance with the International League Against Epilepsy (ILAE) classification at the time of diagnosis and recruitment. Phenotyping data were entered into the Epi25 Data repository (https://github.com/Epi25/epi25-edc) via case record forms hosted on the REDCap platform. Epilepsy case definitions were described in details in Methods of the manuscript. In total, we analyzed 20,979 epilepsy cases – including 1,938 individuals diagnosed with developmental and epileptic encephalopathies (DDEs), 5,499 with genetic generalized epilepsy (GGE), 9,219 with nonacquired focal epilepsy (NAFE), and 4,323 with other epilepsies (mostly lesional focal epilepsy [2,495] and febrile seizures [FS]/FS+ [327]) – and 33,444 controls. Control individuals were aggregated from a subset of Epi25 sites, local collections at the Broad Institute, or dbGaP and were not screened for neurological or neuropsychiatric conditions. 25,279 individuals (9,978 cases) had "XY" as their imputed sex, and 29,144 individuals (11,001 cases) had "XX" as their imputed sex. The number of individuals in each genetic ancestry group include: 41,681 non-Finnish European (NFE), 2,913 East Asian (EAS), 4,190 African (AFR), 1,463 Finnish (FIN), 3,586 Admixed American (AMR), and 590 South Asian (SAS). The counts in each stratum (by case and control status) is fully reported in Table S3. As described in the above sections, PCA-predicted genetic ancestral group, the top ten PCs, and imputed sex were used as covariates in our burden analyses.
Recruitment	The Epi25 collaborative (Epi25) predefined phenotype criteria for cases and controls, while the specific recruitment was carried out by each contributing study. The ascertainment strategies in Epi25 are presented in detail in Methods and Supplementary Information. To ensure compatibility with Epi25 definitions, we included samples with an epilepsy diagnosis in our analysis (see above for specific/breakdown numbers). In in Supplementary Subjects and Methods, we highlighted publications that specifically describe the ascertainment strategies of each collection.
Ethics oversight	Ethics committee of Mass General Brigham gave ethical approval for this work. All relevant ethical guidelines have been followed, and any necessary IRB and/or ethics committee approvals have been obtained. All necessary patient/participant consent has been obtained and the appropriate institutional forms have been archived, and that any patient/participant/sample identifiers included were not known to anyone (e.g., hospital staff, patients or participants themselves) outside the research group so cannot be used to identify individuals.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample size was not predetermined in this study: we aggregated all available exome data from collections that included individuals with a diagnosis of epilepsy. This is the largest sequencing study of epilepsy to date.
Data exclusions	We described sample ascertainment in detail in the Methods and Supplementary Information. There, we additionally described the criteria for which samples and variants were excluded in our study (see sections on Sample and Variant QC). Samples and variants were excluded if they failed quality control metrics. We included only cases with a clear diagnosis of epilepsy, and controls without a known diagnosis of neurological or neuropsychiatric conditions.
Replication	We performed exome-wide genetic association analysis for gene discovery, using a hypothesis-free approach We observed concordance in genes implicated in previous studies, but we did not attempt to reproduce any findings in a separate dataset, as no other data set of comparable size exists.
Randomization	Randomization was not applicable/performed because there was no allocation of samples to experimental groups in our observational genetic study. Case and control status of samples were assigned by clinicians and investigators of contributing collections. We controlled for confounding factors by incorporating relevant covariates in our main analysis.
Blinding	Blinding was not relevant to our study, as the genotype and phenotype data is determined/defined externally and could not be influenced by the analyst or during our aggregation steps.

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n/a	Involved in the study
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Methods

n/a	Involved in the study
☒	☐ ChIP-seq
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Seed stocks	*Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.*
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Authentication	*Describe any authentication procedures for each seed stock used or novel genotype generated. Describe any experiments used to assess the effect of a mutation and, where applicable, how potential secondary effects (e.g. second site T-DNA insertions, mosiacism, off-target gene editing) were examined.*