De novo Variants in Neurodevelopmental Disorders with Epilepsy

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Abstract

Epilepsy is a frequent feature of neurodevelopmental disorders (NDD) but little is known about genetic differences between NDD with and without epilepsy. We analyzed *de novo* variants (DNV) in 6753 parent-offspring trios ascertained for different NDD. In the subset of 1942 individuals with NDD with epilepsy, we identified 33 genes with a significant excess of DNV, of which *SNAP25* and *GABRB2* had previously only limited evidence for disease association. Joint analysis of all individuals with NDD also implicated *CACNA1E* as a novel disease gene. Comparing NDD with and without epilepsy, we found missense DNV, DNV in specific genes, age of recruitment and severity of intellectual disability to be associated with epilepsy. We further demonstrate to what extent our results impact current genetic testing as well as treatment, emphasizing the benefit of accurate genetic diagnosis in NDD with epilepsy.

Introduction

Epilepsies, defined as recurrent, unprovoked seizures, affect about 50 million people worldwide (www.who.int, 03/2017). A significant subset of severe and intractable epilepsies starts in infancy and childhood and poses a major clinical burden to patients, families, and society[1]. Early onset epilepsies are often comorbid with neurodevelopmental disorders (NDD), such as developmental delay, intellectual disability and autism spectrum disorders (DD, ID, ASD)[2-4], while up to 26% of individuals with NDD have epilepsy, depending on the severity of intellectual impairment[4-6]. Several genes have been implicated in both NDD and epilepsy disorders[7,8]. The epileptic encephalopathies (EE) comprise a heterogeneous group of epilepsy syndromes characterized by frequent and intractable seizures that are thought to contribute to developmental regression[3,9]. Phenotypic categorisation of clinically-recognizable EE syndromes enabled identification of several associated genes[1,2,10]. However, the phenotypic spectrum of these disease genes was broader than expected[11,12], ranging from EE (e.g. *SCN1A*[13], *KCNQ2*[14]) to unspecific NDD with or without epilepsy (e.g. *SCN2A*[15], *STXBP1*[16]). While clinically distinguishable entities exist, many patients with NDD and epilepsy are not easily classified into EE syndromes[1,12]. Consequently, EE is often used synonymously with NDD with epilepsy[17]. Targeted sequencing of disease-specific gene panels is commonly used in diagnostics of epilepsies[12,18,19]. However, epilepsy gene panel designs of diagnostic laboratories differ substantially in gene content[19].

Application of a mutational model[18] to detect enrichment for *de novo* variants (DNV) has proven to be a powerful approach for identification of disease-associated genes in neurodevelopmental disorders including ID, congenital heart disease, schizophrenia and ASD[20-23]. For EE, the currently largest exome-wide DNV burden study comprised 356 parent-offspring trios of two classic EE syndromes (infantile/epileptic spasms, IS and Lennox-Gastaut syndrome, LGS) and revealed seven genes at exome-wide significance[24]. To identify genes that are significantly associated with NDD with epilepsy, we analysed 6753 parent-offspring trios of NDD, focusing on 1942 cases with epilepsy including 529 individuals with epileptic encephalopathy. We compared rates of DNV between EE, NDD with unspecified epilepsies and NDD without epilepsy to identify genetic differences between these phenotypic groups. We further investigated the potential impact of our findings on the design of genetic testing approaches and assessed the extent of therapeutically relevant diagnoses.

Results

Description of dataset

We analysed DNV in parent-offspring trios of eight published[7,20,23-27], one partly published[28] and three unpublished cohorts of in total 6753 individuals with NDD stratifying for the 1942 cases with epilepsy (Supplementary Table 1, Figure 1, Online Methods). These 1942 patients were ascertained for either EE or NDD with unspecified epilepsy (DD[21], ASD[11] with ID and ID[20]). We define those two phenotype groups as NDD_{EE} (n = 529) and NDD_{uE} (n = 1413), respectively. We later compared DNV in NDD with epilepsy (NDD_{EE+uE}) to DNV in NDD without epilepsy (NDD_{woE}, n = 4811). For genotype-phenotype comparisons, we restricted our analysis to regions that were adequately captured across different capture solutions (see Online Methods). For ASD data from the Simon Simplex Consortium[29], we included only individuals with IQ < 70 (defined as ID) as different studies have found DNV only associated with low-IQ ASD[6,30]. Individuals with NDD_{EE} were diagnosed with following specific syndromes: IS (n = 243), LGS (n = 145), electrical status epilepticus in sleep (ESES, n = 42), myoclonic-atonic epilepsy (MAE, n = 39), Dravet syndrome (DS, n = 16), unspecified EE (n = 44). Six of eight NDD cohorts (n = 6037) included individuals with as well as without epilepsy[20,23,25-27,31]. Of these, 20.3% of patients had epilepsy. In cohorts with more severe ID, a higher rate of patients had epilepsy (Spearman-Rank correlation, p-value = 0.012, rho = 0.89, Supplementary Figure S2), in line with previous literature[4,6]. We considered DNV of 1911 healthy siblings of patients with ASD as a control group.

DNV in known EE genes in patients with different NDD diagnoses

We first compared DNV in known EE genes between NDD_{EE}, NDD_{uE}, NDD_{woE} and control cohorts. We investigated missense and truncating DNV ($DNV_{mis+trunc}$) in 50 known autosomal dominant or X-linked EE genes (updated list from[19], Supplementary Table 3). We excluded DNV present in ExAC[32] to improve power, as these have been shown to confer no risk to childhood-onset NDD on a group level[33]. The frequency of $DNV_{mis+trunc}$ in EE genes was not significantly different between NDD_{EE} (13.0%±3.1, mean, 95%-CI) and NDD_{uE} (11.5%±1.8, mean, 95%-CI, p-value = 0.4, Fisher's Exact Test, Figure 1A, see Supplementary Figure S2 for individual cohorts), but was significantly greater than in NDD_{woE} (2.7%±0.5, mean, 95%-CI, p-value = 4.4×10^{-46}) and in healthy controls (0.3%±0.2, mean, 95%-CI)[20]. Within three different NDD diagnoses (ID, ASD [with and without ID], DD), we detected more DNV in EE genes in individuals with epilepsy than without epilepsy (Cochran–Mantel–Haenszel test, p-value 3.5×10^{-43}, common OR 4.6, 95%-CI: 3.7 to 5.9, Figure 2B). This suggests a markedly overlapping genetic spectrum of NDD_{EE} and NDD_{uE}. We subsequently performed DNV enrichment analyses on the combined cohort of NDD_{EE+uE}.

Discovery of genes with exome-wide DNV burden in NDD with epilepsy

We compared the numbers of DNV in the combined cohort of NDD with epilepsy (NDD_{EE+uE}), to the number of DNV expected by a mutational model[30] revealing global enrichment of truncating (2.3-fold, $p_{trunc} = 1 \times 10^{-47}$, Poisson Exact test, see Online Methods) and missense (1.6-fold, $p_{mis} = 2 \times 10^{-33}$) but not synonymous DNV (0.6 fold, $p_{syn} = 1.0$). We identified 33 genes with an exome-wide significant burden of $DNV_{mis+trunc}$ (Table 1), of which *KCNQ2* (n=21), *SCN2A* (n=20) and *SCN1A* (n=19) were most frequently mutated. *GABRB2* and *SNAP25* had previously no statistical evidence for disease association (see Supplementary Note). Beyond the 33 genes with exome-wide significant DNV burden, 114 genes had at least two $DNV_{mis+trunc}$ in our cohort (Supplementary Table 6). After DNV enrichment analysis, we again excluded DNV in ExAC[32] to improve specificity[33].

Collectively analysing all patients with NDD with or without epilepsy (n = 6753), we found 101 genes with exome-wide DNV burden (Supplementary Table 7). Among these 101 genes five were mutated in at least one individual with EE and at least two other individuals with epilepsy with DNV in the same variant class. Of these, *SMARCA2* *DYNC1H1* and *SLC35A2* were formerly associated with NDD with epilepsy. *KCNQ3* had previously limited association with NDD with epilepsy and *CACNA1E* had previously no statistical evidence for disease association (Genes further described in Supplementary Notes).

Table 1, <u>Genes with exome-wide DNV burden in NDD with epilepsy</u>

Gene	DNV_{trunc}		DNV_{mis}		$DNV_{mis+trunc}$
Epilepsy	yes	no	yes	no	yes+no
KCNQ2	0	1	21	3	25
SCN2A	2	12	18	5	37
SCN1A	8	0	11	0	19
CHD2	9	1	3	2	15
SYNGAP1	10	7	1	2	20
STXBP1	4	3	7	5	19
SCN8A	0	1	10	3	14
MEF2C	4	1	5	0	10
SLC6A1	2	1	7	3	13
DNM1	0	0	9	2	11
EEF1A2	0	0	8	3	11
CDKL5	2	0	6	0	8
DYRK1A	7	9	0	5	21
SMC1A	7	0	0	2	9
GABRB3	0	0	7	1	8
KIAA2022	6	0	0	0	6
ASXL3	6	12	0	0	18
WDR45	5	5	1	0	11
ARID1B	6	28	0	2	36
GNAO1	0	1	6	2	9
ALG13	0	0	6	0	6
KCNH1	0	0	6	2	8
GRIN2B	0	3	6	9	18
HNRNPU	5	2	0	1	8
PURA	3	4	2	4	13
GABRB2	0	0	5	1	6
COL4A3BP	0	0	5	4	9
MECP2	2	5	3	5	15
FOXG1	2	3	3	3	11
ANKRD11	4	28	0	2	34
SNAP25	1	0	3	0	4
DDX3X	3	19	1	11	34
IQSEC2	3	2	1	3	9

**Genes in order of decreasing numbers of $DNV_{mis+trunc}$ in NDD with epilepsy*

Phenotypic, biological and therapeutic properties of genes with DNV burden in NDD with epilepsy

We aimed to explore whether the 33 genes with DNV burden in NDD with epilepsy (NDD_{EE+uE}) were associated with specific phenotypes. Analyses of human phenotype ontology[34] (HPO) terms revealed most significant enrichment in genes associated with "epileptic encephalopathy" (see Online Methods, Supplementary Table 8). After excluding the 529 patients diagnosed with EE from the DNV enrichment analysis, the most significantly enriched HPO term was still "epileptic encephalopathy" (Bonferroni p-value 3.6×10^{-14}), confirming our previous findings (Figure 1). Per DNV-enriched gene, we plotted distribution of EE phenotypes, sex and seizure phenotypes of generalized, focal, febrile or spasms (Supplementary Figure S6 - 8).

Since the disease onset of NDD with epilepsy is typically in infancy and early childhood, we evaluated expression levels of the 33 genes with DNV burden in the developing infant brain (expression data: brainspan.org, see Online Methods). At a group level, these genes showed high levels of brain expression (Supplementary Figure S9A). The DNV-enriched genes were also substantially depleted for truncating and missense variants in the ExAC control data (Supplementary Figure S9B, S9C). Genes with at least two DNV in NDD_{EE+uE}, but no significant DNV burden showed similar patterns.

We finally evaluated if genes with $DNV_{mis+trunc}$ in NDD with epilepsy were associated with therapy. For each gene, we used criteria from the Centre for Evidence-Based Medicine (CEBM)[35] to evaluate the evidence for targeted treatments. Five of the 33 DNV-enriched genes (*SCN1A, SCN2A, SCN8A, KCNQ2, MECP2*) had evidence for therapeutic relevance (CEBM Grade of Recommendation A and B, see Online Methods, Supplementary Table 9). These five genes accounted for 28% of all $DNV_{mis+trunc}$ in the significantly implicated genes. Three additional genes (*PTEN, CACNA1A, SLC2A1*) with at least two $DNV_{mis+trunc}$, which were also known disease genes, also had therapeutic relevance according to CEBM criteria. In total 5% (84/1587) of $DNV_{mis+trunc}$ in NDD with epilepsy were in genes with therapeutic consequences. According to the guidelines of the American College of Medical Genetics (ACMG,)[36] all DNV that are not in ExAC and that are in known disease genes or genes with DNV burden in our dataset are categorized as "likely pathogenic", while we did not apply all ACMG criteria to individual DNV (see online methods).

Comparing DNV between NDD with and without epilepsy

We compared frequencies of $DNV_{mis+trunc}$ in NDD with epilepsy (NDD_{EE+uE}) to NDD_{woE} across all 107 DNV-enriched genes (logistic regression, see Online Methods). Increasing age at time of recruitment increased likelihood of epilepsy (three-year OR 1.11, 95%-CI 1.04 to 1.18, p-value = 3×10^{-3}, individual genes in Supplementary Figure S5). Sex was not associated with epilepsy status (p-value = 0.5). Individuals with DNV_{mis} were more likely to have epilepsy than individuals with DNV_{trunc} (Figure 2, OR_{mis} 2.1, 95%-CI 1.6 to 2.8, p-value 2×10^{-7}). In line with previous reports[15], we observed this pattern on a single gene level for *SCN2A* (Firth regression, OR_{mis} 23.5, 95%-CI 3.8 to 277, p-value 0.0003, Table 1). Confirming previous findings[24,37], DNV in ion channel genes were associated with epilepsy (OR 6.0, 95%-CI 3.9 to 9.2, p-value 1×10^{-16}). 83% (110/133) of DNV in ion channel genes were DNV_{mis}. However, in the subset of 910 DNV not in ion channel genes, DNV_{mis} were still associated with epilepsy (OR 1.5, p-value 0.005, 95%-CI 1.1 to 2.1), implying that the effect of DNV_{mis} on epilepsy was not entirely driven by ion channel genes. We observed a higher rate of DNV_{mis} in NDD_{EE} than in NDD_{uE}, though only with nominal significance (Fisher's exact test, OR 1.8, 95%-CI 1.04 to 3.4, p-value 0.03, Supplementary Figure S10B). Four genes were more frequently mutated in NDD with

261 epilepsy (NDD_{EE+uE}) than NDD_{woE} (Fisher's Exact Test, Figure 2A/2B, Table 1,
262 Supplementary Table 10). With the exception of *SCN1A,* frequencies of DNV were not
263 significantly different per gene between NDD_{EE} and NDD_{uE} for DNV_{mis} or DNV_{trunc}
264 (Supplementary Figure S10, Supplementary Table 11).
265
266 *Evaluation of diagnostic gene panels for epilepsy disorders*
267 Targeted sequencing of disease-specific gene panels is widely employed in diagnostics
268 of epilepsies[18,19]. We compared our results to 24 diagnostic panels for epilepsy or EE
269 (see Online Methods, full list in Supplementary Table 12). In total, the 24 different
270 panels covered 358 unique genes (81.5 ± 8.8 genes per panel, mean ± sd). Applying
271 these 24 diagnostic panels on our data set would only have detected on average 59% of
272 $DNV_{mis+trunc}$ in the 33 DNV-enriched genes (Supplementary Figure S11). However, similar
273 to most other research studies involving clinical WES[7], we cannot fully assess the extent
274 of potential pre-screening. We investigated whether genes in the 24 panels had some
275 evidence for disease association given the following features that we (and others[23,33])
276 observed in genes with DNV burden in NDD: depletion for truncating and missense
277 variants in ExAC[32] controls as well as brain expression (Online Methods, Supplementary
278 Figure S9). We restricted this analysis to autosomal dominant and X-linked acting panel
279 genes ($n_{dominant+X\text{-}linked}$ = 191, Supplementary Table 13). 95% (52/55) of panel genes that
280 had two or more $DNV_{mis+trunc}$ in our study were both constraint and brain-expressed.
281 However, only 63% (86/136) of panel genes with one or less $DNV_{mis+trunc}$ in our study
282 were constraint and brain-expressed (Fisher's exact test, OR 10.2, 95%-CI 3.0 to 53.0, p-
283 value 2.3 x 10^{-6}). We applied evidence of disease association as defined by the ClinGen
284 Gene Curation Workgroup[38], to those 50 panel genes lacking two of the criteria
285 DNV/brain expression/constraint. We found that ten of the 50 genes had no, eight had
286 limited and seven had conflicting published evidence for disease association
287 (Supplementary Table 14). Thirteen genes showed moderate, strong or definitive
288 evidence for association to entities where neither NDD nor epilepsy were major features
289 which may partly be explained by a panel design containing genes associated with
290 diseases beyond the spectrum of NDD (for further details see Online Methods and
291 Supplementary Figure S11).

Discussion

In this study, we systematically investigated DNV in NDD with and without epilepsy. In NDD with epilepsy, we could hardly distinguish individuals ascertained for epileptic encephalopathy and NDD with unspecified epilepsy on a genetic level. Thus, we conclude that these phenotype groups share a spectrum of disease genes predominantly including genes initially reported as EE genes. We identified 33 genes with DNV burden in NDD with epilepsy, of which the majority was expressed in the infant brain and depleted for functional variation in ExAC[32], as previously described for NDD genes[23,33]. We report statistically robust disease association for *SNAP25, GABRB2* and *CACNA1E*, which was previously lacking (Supplementary Notes).

We found, that individuals with DNV_{mis} were generally more likely to have epilepsy than individuals with DNV_{trunc}. This association was largely driven by ion channel genes, which confirms longstanding statements that many epilepsy disorders act as channelopathies[2,37,24]. Heterozygous DNV_{mis} have been shown to cause epilepsy via dominant negative (e.g. *KCNQ2*[39]) or gain-of-function (e.g. *SCN8A*[40]) effects on ion channels. On the individual gene level, missense variants in *SCN2A*[15] and *SCN8A*[41] were more strongly implicated in epilepsy than protein truncating variants, which we statistically confirm for *SCN2A.* Yet, we found that DNV_{mis} were also associated with epilepsy independent of ion channel genes. This may imply that alteration of protein function quantitatively plays a larger role than haploinsufficiency[42] in the pathophysiology of NDD with epilepsy compared to NDD without epilepsy. We found multiple gene sets enriched for DNV_{mis} in epilepsy compared to no epilepsy (see Supplementary Note). The majority was related to ion channels, while others related to neuronal cells (e.g. axon part, synaptic transmission). However, biological interpretation should be done with caution given that previous studies have found that many of these gene sets share a large number of underlying genes[22] and gene annotations are biased[43]. We further replicate a previous finding that the rate of epilepsy was correlated with severity of intellectual disability[4-6], implying that brain function could contribute to epileptogenesis or genetic variants cause both epilepsy and NDD. Alternatively, severe epileptic activity may also damage brain function and thereby contribute to NDD, which constitutes the original definition of EE[9,17]. This is supported by many cases of clinical regression after onset of epilepsy and improvement of NDD through seizure control.

In NDD with epilepsy we found no genetic differences between unspecified epilepsy and EE, with the exception of *SCN1A* (Supplementary Note). Phenotypic heterogeneity has been described for the majority of EE genes[1,11], i.e. variants in the same gene could lead to a spectrum of different phenotypes. Due to pleiotropy, individuals that carry a pathogenic DNV in an EE gene and fulfil diagnostic criteria of EE may also be eligible for another NDD diagnosis and thus by chance be assigned to an ASD, DD or ID and not an EE screening cohort. In line with this hypothesis, we found typically EE-associated seizure types (e.g. epileptic spasms) in cohorts with unspecified epilepsy. Some of the diagnostic criteria for EE[1,10] may present ambiguously, leading to uncertainty in terminology[17]. Thus, 43% (21/49) of individuals diagnosed with EE in the Epi4K-E2[24] study initially presented with DD prior to seizure onset conflicting with the original definition of EE[3,17]. Clear phenotypic distinction between encephalopathic versus non-encephalopathic epilepsies may therefore be difficult. Accordingly, mechanisms that result in an encephalopathic course of a genetic NDD remain elusive.

Restricting DNA sequencing or DNA sequence analysis to panels of known disease genes is widely used in diagnosis of genetic diseases including epilepsy ([19], 100,000 genomes project [www.genomicsengland.co.uk]). We confirmed that epilepsy gene panels from

345 diagnostic laboratories differ substantially in gene content[18] with at least 25 genes with
346 low evidence for disease association (ClinGen criteria[38]). Statistically not robust gene-
347 disease associations occasionally resulted in false-positive reports of causality posing
348 challenges for correct diagnosis in research and clinical settings[11,44]. Our data provide
349 grounds for replacing genes with limited evidence by genes with higher evidence in the
350 design of gene panels for NDD with epilepsy.
351
352 Therapeutic approaches, tailored to the patient's underlying genetic variant, have
353 successfully been applied for several EE[2] including treatment with ezogabine in *KCNQ2*
354 encephalopathy[45] or ketogenic diet in *SLC2A1*-related disorders[46]. 5% of $DNV_{mis+trunc}$ in
355 our study were in eight genes (Supplementary Table 9) for which we could confirm
356 therapeutic consequences with established evidence-based medicine criteria[35]. This
357 finding reinforces the urgency of making a genetic diagnosis in NDD with epilepsy. We
358 expect that with increasing understanding of the underlying pathomechanisms, the
359 group of genetic epilepsies with relevant therapeutic consequences will continue to
360 grow.

361 # Online Methods
362
363 *Patient cohorts*
364 For this study, we ascertained 8,529 patients with the following neurodevelopmental
365 disorders (NDD): developmental delay (DD[31], n=4293), autism spectrum disorder
366 (ASD[20], n=2508), epileptic encephalopathy[24,28] (NDD_{EE}, n=529), intellectual
367 disability[23,25-27] (ID, n=1035), and epilepsy with NDD[28] (n=164). From this cohort, we
368 selected 6753 individuals, for which the presence or absence of epilepsy was
369 ascertained and of whom ca. 88% had ID (based on assumption of 81.7% ID in the DDD
370 study[47], 89.8% ID in a diagnostic cohort from AmbryGenetics[28] and 100% ID in all other
371 cohorts.) Among individuals with ASD who were phenotyped within the Simon Simplex
372 Consortium[29], we restricted our analysis to patients with ID (IQ < 70) as it has been
373 shown that DNV play only a minor role, in normal IQ ASD[6,30]. Previously sequenced trios
374 (n = 1911), from unaffected siblings of a child with ASD[20,29], served as control trios. For
375 our main analyses, we stratified this combined cohort of patients with NDD for patients
376 comorbid or primarily diagnosed with epilepsy (NDD_{EE+uE}, n=1942)[20,29]. Two EE cohorts
377 and one ID cohort comprising a combined 144 patients were not previously published;
378 one cohort was only partly published (see Supplementary Table 1). Medical doctors,
379 mostly clinical geneticists, but also neurologists, paediatricians and for ASD[29] some
380 primary care physicians reported out phenotypes, including presence of epilepsy, in all
381 patients. Our analysis is based on the assumption that medical professionals are
382 sufficiently qualified to diagnose the presence or absence of epilepsy correctly.
383
384 *Subphenotypes*
385 We obtained information on specific EE syndromes on 98% of 518/529 individuals with
386 NDD_{EE} (see main text). We obtained specific seizure types (febrile, focal, spasms,
387 generalized) for 55% (140/256) and age of seizure onset for 30% (77/256) of
388 individuals with $DNV_{mis+trunc}$ in genes with DNV burden in NDD_{EE+uE}. (See Supplementary
389 Figure S5 and S6). We did not obtain EEG data per patient. Some patients may have
390 developed epilepsy after inclusion in the study, so we ascertained age at recruitment,
391 that we obtained for 94% (1087/1157) of all individuals with NDD with $DNV_{mis+trunc}$ in
392 DNV-enriched genes (median age at recruitment: 74.8 months). We obtained age of
393 seizure onset for 30% (77/256) of individuals with epilepsy and $DNV_{mis+trunc}$ in DNV-
394 enriched genes (Supplementary Figure S5). We identified 30 individuals with potentially
395 epilepsy-relevant brain malformations (abnormalities of neuronal migration, structural
396 abnormalities of corpus callosum, midbrain, brainstem as schiz-, megal-,
397 holoprosencephaly) in individuals with $DNV_{mis+trunc}$ in DNV-enriched genes (29 from
398 DDD[29], 1 from Hamdan *et al.*[7]). 11 of them (37%) also had seizures.
399
400 *Whole exome sequencing of parent-patient trios*
401 In all cohorts, both patients and their unaffected parents underwent whole exome
402 sequencing (WES). Variants that were not present in either parent were considered *de*
403 *novo* variants (DNV). 1942 individuals with NDD with epilepsy (NDD_{EE+uE}) had 1687
404 DNV_{mis} and 396 DNV_{trunc} (i.e. stopgain, frameshift, essential splice site). 4811 individuals
405 with NDD_{woE} had 4227 DNV_{mis} and 1120 DNV_{trunc} (Supplementary Table 2, for individual
406 cohorts see Supplementary Figure S3). The study was approved by the ethics committee
407 of the University of Leipzig (224/16-ek, 402/16-ek) and additional local ethics
408 committees. A list of all published and unpublished cohorts used in this paper can be
409 found in Supplementary Table 1.
410
411 *Sequencing pipelines of previously unpublished/partly published cohorts (cohorts 8 -11)*
412 Libraries were prepared from parents' and patients' DNA, exome captured and
413 sequenced on Illumina sequencers. Raw data was processed and technically filtered

414 with established pipelines at the respective academic or diagnostic laboratories. DNV
415 data from all cohorts was re-annotated for this study (see below). Specific pipelines of
416 cohorts 10 to 14 are described below.
417
418 Cohort 8 (Ambry Genetics):
419 Diagnostic WES was performed on parent-offspring trios at Ambry Genetics (Aliso Viejo,
420 CA) in 216 individuals with a history of seizures who have been previously described[28].
421 Genomic DNA extraction, exome library preparation, sequencing, bioinformatics
422 pipeline, and data analyses were performed as previously described[48]. Briefly, samples
423 were prepared and sequenced using paired-end, 100 cycle chemistry on the Illumina
424 HiSeq 2500 sequencer. Exome enrichment was performed using either the SureSelect
425 Target Enrichment System 3.0 (Agilent Technologies) or SeqCap EZ VCRome 2.0 (Roche
426 NimblGen). The sequencing reads were aligned to human reference genome (GRCh37)
427 and variants were called by using CASAVA software (Illumina). The following variants
428 filters were applied to generate a list of high confident de novo variant calls: 1) mutation
429 base coverage >= 20x in all members of the trio; 2) heterozygous read ratio in probands
430 >30% and <80%; 3) heterozygous read ratio in parents <2%; 4) genotype quality cutoffs
431 SNV > 100 and indels > 300 and 5) exclusion of known sequencing artefacts (based on
432 Ambry Genetics' internal databases).
433
434
435 Cohorts 9 (EuroEPINOMICS RES) and 10 (DFG atypical EE):
436 Exonic and adjacent intronic sequences were enriched from genomic DNA using the
437 NimbleGen SeqCap EZ Human Exome Library v2.0 enrichment kit. WES was performed
438 using a 100bp paired-end read protocol due to the manufacturer's recommendations on
439 an Illumina HiSeq2000 sequencer by the Cologne Center for Genomics (CCG), Cologne,
440 Germany. Reads were mapped on the human hg19 reference genome (bwa-aln software,
441 bio-bwa.sourceforge.net/). The UnifiedGenotyper (GATK,
442 www.broadinstitute.org/gatk/) and Mpileup (Samtools,
443 http://samtools.sourceforge.net/) software were used to call variants. The paired
444 sample feature from the DeNovoGear software was further used to examine potential de
445 novo mutations in twin pairs. Data analysis and filtering of mapped target sequences
446 was performed with the 'Varbank' exome and genome analysis pipeline v.2.1
447 (unpublished; https://varbank.ccg.uni-koeln.de). In particular, we filtered for high-
448 quality (coverage of more than six reads, fraction of allele carrying reads at least 25%, a
449 minimum genotype quality score of 10, VQSLOD greater than -8) and rare (Caucasian
450 population allele frequency < 0.5%) variations on targeted regions + flanking 100bp. In
451 order to exclude pipeline specific artifacts, we also filtered against an in-house cohort of
452 variations, which were created with the same analysis pipeline.
453
454 Cohort 11 (University of Leipzig):
455 Exome capture was carried out with Illumina's Nextera Rapid Capture Exome Kit
456 (Illumina, Inc., San Diego, CA, USA). WES was on an NextSeq500 or HiSeq4000
457 sequencer (Illumina, Inc.) to 2 × 150bp reads at the Centogene AG, Rostock, Germany.
458 Raw sequencing reads were converted to standard fastq format using bcl2fastq software
459 2.17.1.14 (Illumina, Inc.), and fed to a pipeline at Centogene AG based on the 1000
460 Genomes Project (1000G) data analysis pipeline and GATK best practice
461 recommendations. Sequencing reads were aligned to the GRCh37 (hg19) build of the
462 human reference genome using bwa-mem (bio-bwa.sourceforge.net/). In addition to
463 GATK HaplotypeCaller (www.broadinstitute.org/gatk/)), variant calling was performed
464 with freebayes (https://github.com/ekg/freebayes) and samtools
465 (http://samtools.sourceforge.net/). Quality filtering of sequencing reads in both parents
466 and children was done according to the following criteria: read depth > 20, quality > 500,
467 frequency of alternative allele between 30 and 70% for the child and not present in the

468 parents, frequency < 1% in internal database, variant called by at least two different
469 genotype callers.
470
471 *False positive rates of DNV*
472 In cohorts 1 to 4, all DNV were validated by Sanger sequencing to eliminate false
473 positive calls. In cohorts 5 to 7, through random selection of variants for Sanger
474 validation, the false positive rate was estimated to be approximately 1.4% and < 5 %,
475 respectively. In the clinical cohorts 8 to 11, variants defined as variants worth reporting
476 back to patients (variants of unknown significance or [likely] pathogenic) are normally
477 validated by Sanger sequencing. With this experience, false discovery rates in these
478 cohorts were estimated to be < 5% (personal communications).
479
480 *Annotation and Filtering*
481 DNV files were generated and quality-filtered by the individual groups. All DNV were re-
482 annotated with the following pipeline. Variants were annotated with Ensembl's Variant
483 Effect Predictor (http://grch37.ensembl.org/Homo_sapiens/Tools/VEP) of version 82
484 using database 83 of GRCh37 as reference genome. Per variant, the transcript with the
485 most severe impact, as predicted by VEP, was selected for further analyses. The
486 decreasing order of variant impacts was HIGH, MODERATE, MODIFIER, LOW. Only
487 protein – altering DNV (DNV_{mis} or DNV_{trunc} [premature stop codon, essential splice site,
488 frameshift]) were included in further analyses. Synonymous DNV (DNV_{syn}) were
489 analysed as a negative control, as most DNV_{syn} have no effect on amino acid sequence in
490 the protein. Variants that were present in ExAC[32], an aggregation of 60,706 exome
491 sequences from adult individuals without severe childhood-onset diseases, were
492 excluded after DNV enrichment, as these have been shown to convey no detectable risk
493 to NDD on a group level[33]. For DNV rates per cohort see Supplementary Figure S2. We
494 did not investigate pathogenicity of individual DNV according to the guidelines of the
495 American College of Medical Genetics (ACMG). However, ACMG criteria PS2 (de novo
496 occurrence, with maternity and paternity confirmed) and PM2 (absence from controls)
497 apply to all DNV in our cohort. The combination of PS2 and PM2 classifies a variant as at
498 least "likely pathogenic". ACMG criteria are only applicable to variants in disease
499 associated genes[36]. Therefore, all DNV in known disease genes and genes with genome-
500 wide DNV burden in our dataset are presumed likely pathogenic DNV.
501
502
503 *Harmonization of different cohorts*
504 The core analysis of our study is the enrichment of $DNV_{mis+trunc}$ compared to expectation
505 by a mutational model in individuals with NDD_{EE+nsE}. For this analysis, we were
506 conservative in assuming that every gene was well captured across all cohorts. However,
507 when comparing DNV burden across different phenotypes we aimed to separate
508 technical from biological differences with the following methods. In exome sequencing,
509 different capture solutions capture specific exonic regions with different efficiencies.
510 These differences have shown to be quite stable within and across different samples of
511 the same capture kits[49]. We therefore generated a list of exons that displayed consistent
512 high coverage across different capture solutions. We collected published and internal
513 data aiming for the highest possible variety of capture kits using 3,000 samples of 5
514 different capture kits, including NimbleGen SeqCap v2 and v3, Agilent SureSelect v2, v3,
515 and v5). We generated a list of exons where at least 80% of all samples had at least 10x
516 coverage. We excluded the oldest capture kits before calculating the high coverage
517 exons as well as excluding the two oldest cohorts [26,27] from our list of DNV. Restricting to
518 high coverage regions resulted in a loss of ca. 11% of DNV in DNV-enriched genes. We
519 consequently performed all genotype phenotype comparisons across cohorts (Figures
520 1A, 2, Supplementary Figures S6-10) with this restricted DNV set. Further, we compared
521 the frequency of DNV_{syn} across all cohorts and excluded cohorts of which DNV_{syn} were

522 not available. In the subset of DNV in high coverage exons, rates of supposedly neutral
523 DNV_{syn} were not different between individuals with and without epilepsy (Poisson Exact
524 test, p-value = 0.48, RR=0.99), NDD_{uE} and NDD_{EE} (p-value = 0.65, RR= 0.94) or NDD and
525 controls (p-value = 0.58, RR=0.99). The frequency of $DNV_{mis+trunc}$ was also not different
526 between individuals with and without epilepsy (p-value=0.5, RR=1.02). Our chances to
527 identify $DNV_{mis+trunc}$ in EE genes in the epilepsy cohort were therefore not inflated by a
528 higher baseline rate of $DNV_{mis+trunc}$ in comparison to NDD_{woE}. We reannotated all DNV in
529 the same way as described above.
530
531 *Statistical analysis*
532 All statistical analyses were done with the R programming language (www.r-
533 project.org). Fisher's Exact Test for Count Data, Wilcoxon rank sum test, Poisson Exact
534 Test, Cochran-Mantel-Haenszel test, logistic regression, Firth regression, Spearman
535 correlation, Welch two-sided t-test and calculation of empirical p-values were
536 performed as referenced in the results. For datasets assumed to be normally distributed
537 after visual inspection, mean and standard deviation (sd) are written as mean ± sd.
538 When performing Poisson Exact Tests, we reported effect size as rate ratio (RR), which
539 is the quotient of the two rates compared in the test. For Fisher's Exact Test and logistic
540 regression analyses, we reported odds ratios (OR). 95% confidence intervals were
541 abbreviated as 95%-CI. The R code used to perform the statistical analyses and figures is
542 available upon request.
543
544 *DNV enrichment analyses*
545 To identify genes with a significant DNV burden, we compared numbers of observed
546 with numbers of expected missense, truncating and synonymous DNV per gene using an
547 established framework of gene-specific mutation rates[30]. The analysis was done with the
548 R package denovolyzer[50], that compares observed versus expected DNV using a Poisson
549 Exact test. We corrected the obtained p-values with the Bonferroni method for the
550 number of genes for which gene specific mutation rates[30] were available (n= 18225) and
551 six tests resulting in a p-value significance threshold of $5x10^{-7}$. Genes that passed that
552 significance threshold for either missense, truncating or both missense plus truncating
553 DNV were considered genes with an exome-wide DNV burden. To compare DNV
554 between disease groups, DNV enrichment analyses were carried out in the cohort of all
555 patients with NDD (n=6753) as well as in patients with epilepsy (NDD_{EE+uE}, n=1942) and
556 without epilepsy (NDD_{woE}, n=4811), but only genes with a $DNV_{mis+trunc}$ burden in the NDD
557 with epilepsy cohort and the combined NDD cohort were reported.
558
559 *HPO enrichment analyses*
560 Significantly enriched Human phenotype ontology (HPO) terms were computed with the
561 R package of g:Profiler[34], using ordered enrichment analysis on significance-ranked
562 proteins (see Supplementary Table 8). Different gene sets were queried using the
563 background gene set of all 18225 genes for which gene specific mutation rates were
564 available[30]. Only terms that were statistically significant with a Bonferroni corrected p-
565 value < 0.01 were reported, as our negative controls (genes with at least two $DNV_{mis+trunc}$
566 in healthy control) were not enriched for any functional categories below this p-value.
567
568 *Therapeutic relevance*
569 To assess if DNV in our cohort were in genes of therapeutic relevance, we searched the
570 literature for treatment recommendations for all established disease genes with at least
571 two $DNV_{mis+trunc}$ in our NDD with epilepsy cohort. We rated the publications with the
572 standardized score of the Oxford Centre for Evidence-Based Medicine[35]. We only
573 reported and considered genes for which at least one treatment recommendation
574 achieved level of evidence of II or higher. For a list of all genes and levels of evidence see
575 Supplementary Table 9.

576
577 *Acquisition and processing of brain gene expression data*
578 We downloaded the Developmental Transcriptome dataset of 'BrainSpan: Atlas of the
579 Developing Human Brain' (www.brainspan.org, funded by ARRA Awards
580 1RC2MH089921-01, 1RC2MH090047-01, and 1RC2MH089929-01, 2011). The atlas
581 includes RNA sequencing data generated from tissue samples of developing postmortem
582 brains of neurologically unremarkable donors covering 8 to 16 brain structures. We
583 extracted brain expression data from the 5 donors that were infants aged 0 to 12
584 months. Per gene, we obtained the median RPKM value of all infant individuals and
585 across brain regions. In all calculations and figures gene expression values are displayed
586 as median (log2 + 1)-transformed RPKM values. We defined infant brain gene
587 expression as median (log2 + 1)-transformed RPKM value > 1. More details about tissue
588 acquisition and sequencing methodology can be found in the BrainSpan website's
589 documentation.
590
591 *Evaluation of genes' intolerance to protein altering variants*
592 We assessed individual gene tolerance to truncating or missense variants in the general
593 population with the pLI score (probability of being loss-of-function intolerant) and
594 missense z-score. These scores indicate depletion of truncating and missense variants in
595 ExAC[32] (60,706 individuals without childhood onset diseases), respectively. We used
596 gene constraint cut-offs >0.9 for pLI and >3.09 for missense-z scores as recommended
597 by the score developers[32]. We calculated empirical p-values to evaluate if pLI scores of
598 exome-wide and nominally DNV-enriched genes were significantly higher compared to
599 pLI scores of random gene sets as described in[23]. Briefly, we computed the expected pLI
600 for a given gene set with size n by randomly drawing 1,000,000 gene sets with size n
601 from the total 18,225 pLI annotated genes. We computed, how many times the median
602 pLI score of randomly sampled gene sets would exceed the median pLI of the gene set
603 under investigation. To that number we added 1 and divided by the number of total
604 samplings +1 to obtain the empirical p-value.
605
606 *Comparing DNV in NDD_{EE}, NDD_{uE} and NDD_{woE}*
607 We investigated $DNV_{mis+trunc}$ in $NDD_{EE+uE+woE}$ across all 107 genes that were DNV-
608 enriched in NDD_{EE+uE}, NDD_{woE} and/or $NDD_{EE+uE+woE}$. We restricted our analysis to DNV not
609 in ExAC[23] and in high coverage regions. To investigate, if age at time of recruitment, sex
610 or variant class (DNV_{mis}/DNV_{trunc}) influenced the presence of epilepsy, we tested them as
611 covariates in a logistic regression model with epilepsy as response variable. We aimed
612 to explore, whether DNV in NDD with epilepsy might be associated with ion channels
613 compared to NDD without epilepsy, as it is a long-established hypothesis, that many
614 epilepsies are channelopathies[37]. We extracted a comprehensive gene set of 237 known
615 ion channel genes from 1766 previously described[22] curated gene sets derived from
616 public pathway databases and publications (see Supplementary Note). To investigate if
617 ion channel genes were associated with epilepsy we included annotation as ion channel
618 gene as a categorical predictor in the logistic regression model. We used Firth
619 regression to assess the effect of variant class on the presence of epilepsy for individual
620 genes. We used Fisher's Exact test to compare frequencies of DNV per gene between
621 phenotype groups. To account for multiple testing, we corrected p-values for the
622 number of tests performed (Bonferroni method).
623
624 *Diagnostic gene panels for epileptic encephalopathy/ comprehensive epilepsy from 24*
625 *academic/ commercial providers*
626 We set out to compare our results to diagnostic gene panels for epileptic
627 encephalopathy of international commercial and academic providers. We searched the
628 Genetic Testing Registry (GTR)[51] of NCBI (date: 01/2017) for providers of tests for
629 "Epileptic encephalopathy, childhood-onset" and identified 16 diagnostic epilepsy

630 panels. We excluded 3 panels with < 20 or > 200 genes and added 11 additional
631 diagnostic providers not registered at GTR to evaluate 24 diagnostic panels targeting
632 epilepsy in general (n=11) or EE specifically (n=13). The gene content covered in each of
633 the 24 gene panels can be found in Supplementary Table 11. Gene lists were freely
634 available for download at the respective providers' websites. For each of the 33 genes
635 with DNV burden in NDD with epilepsy, we calculated to what proportion they were
636 included in 24 commercial or academic providers of gene panels for epileptic
637 encephalopathy/comprehensive epilepsy. For each gene, we then multiplied the
638 percentage of inclusion in any of the 24 panels by the total number of $DNV_{mis+trunc}$ of that
639 gene in the cohort of 1942 individuals with NDD_{EE+uE}.
640 We investigated if there were genes in the 24 diagnostic gene panels without evidence
641 for implication in NDD with epilepsy. We focused on 191 dominant or X-linked panel
642 genes (listed in Supplementary Table 14). We tested these genes for three criteria of
643 association with NDD with epilepsy: Firstly, if genes had at least two $DNV_{mis+trunc}$ in our
644 study; secondly, whether genes were expressed in the infant brain defined by a median
645 RPKM of all samples and brain regions > 1; thirdly, whether genes had a pLI > 0.9 or
646 missense z-score > 3.09 indicating intolerance to truncating or missense variants[32]. We
647 intersected these lists to nominate genes that did not display features of DNV-enriched
648 genes in this study. On these genes we applied ClinGen criteria[38] for gene-disease
649 association.
650
651 *Data availability*
652 The authors declare that all data used for computing results supporting the findings of
653 this study are available within the paper and its supplementary information files. Raw
654 sequencing data of published cohorts are referenced at the respective publications. Raw
655 sequencing data of cohort EuroEPINOMICS RES have been deposited in the European
656 Genome-phenome Archive (EGA) with the accession code EGAS00001000048
657 (https://www.ebi.ac.uk/ega/datasets/EGAD00001000021). Raw sequencing data of
658 cohort 10 (DFG atypical EE) will be deposited in a public repository after finalization of
659 the individual project.
660

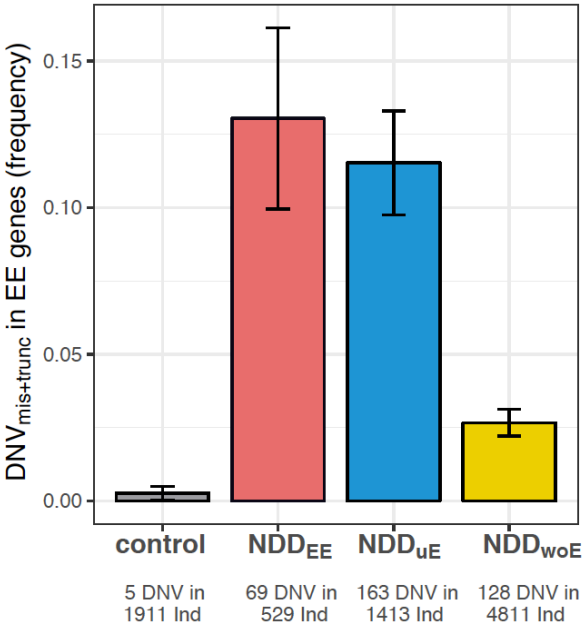
661 Figures

662

663 **Figure 1.** $DNV_{mis+trunc}$ in EE genes in different cohorts of NDD. **A**, The proportion of
664 $DNV_{mis+trunc}$ in EE genes is not significantly different between patients with NDD_{EE} (red)
665 and NDD_{uE} (blue), but higher than NDD_{woE} (yellow) or healthy controls (grey). Cohort
666 size is given as number of individuals (Ind). **B**, Proportion of DNV in EE genes in patients
667 with versus without epilepsy across different NDD (DD, ASD, ID). P-values are plotted
668 next to respective odds ratios (red dots), while 95%-CI are shown as yellow bars
669 (Fisher's exact test for individual cohorts, Cochran-Mantel-Haenszel test for combined
670 cohorts).

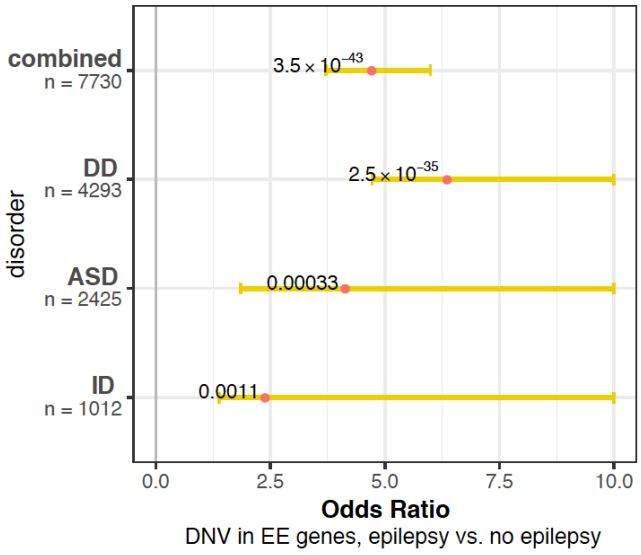
671

672 **A**,



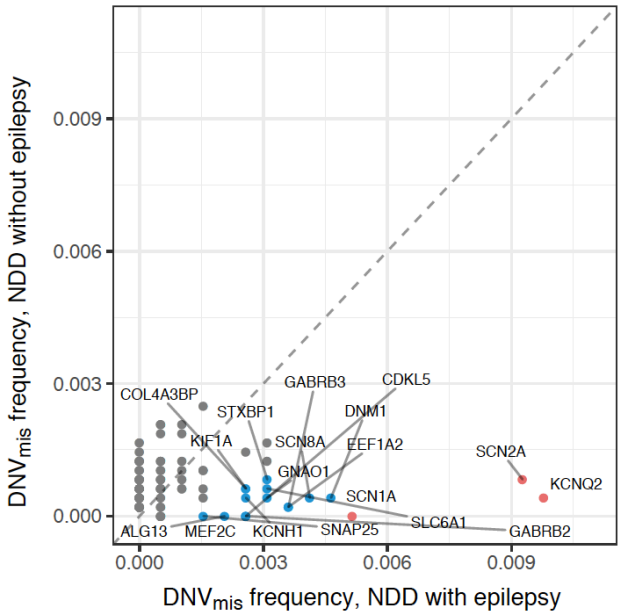
673

674 **B**,

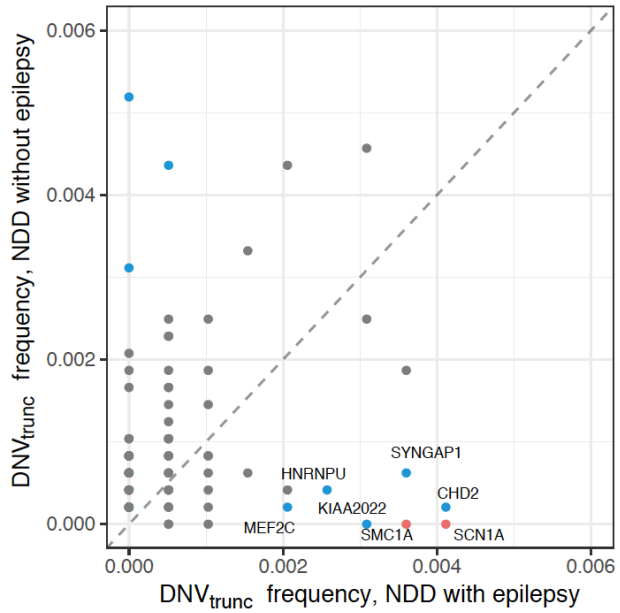


675

676 **Figure 2.** DNV in patients with epilepsy (NDD_{EE+uE}) versus without epilepsy (NDD_{woE})
677 in 107 genes with significant DNV burden. **A,** DNV_{mis}, **B**, DNV_{trunc}. Genes with different
678 frequencies are labeled (method: Fisher's Exact test, blue: nominal significance, p-value
679 < 0.05, red: significant after correcting for 266 tests). The dotted line represents equal
680 frequency of DNV in NDD with and without epilepsy.
681
682 **A,**



683
684 **B,**



685
686

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688

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