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Lal, Dennis

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RESEARCH ARTICLE

Evaluation of Presumably Disease Causing SCN1A Variants in a Cohort of Common Epilepsy Syndromes

Dennis Lal1,2,3,4*, Eva M. Reinthaler5*, Borislav Dejanovic5*, Patrick May7, Holger Thiele1, Anna-Elina Lehesjoki8,9,10, Günter Schwarz2, Erik Riesz11, M. Arfan Ikram12, Cornelia M. van Duijn12, Andre G. Uitterlinden13, Albert Hofman14, Hannelore Steinböck15, Ursula Gruber-Sedlmayr16, Birgit Neophytou17, Federico Zara18, Andreas Hahn19, Genetic Commission of the Italian League against Epilepsy10, EuroEPINOMICS CoGIE Consortium10, Padhraig Gormley2,3,4, Felicitas Becker20, Yvonne G. Weber20, Maria Roberta Cilio21, Wolfram S. Kunz22, Roland Krause6, Fritz Zimprich5, Johannes R. Lemke23, Peter Nürnberg1,24, Thomas Sander1, Holger Lerche20, Bernd A. Neubauer19

1 Cologne Center for Genomics, University of Cologne, Cologne, Germany, 2 Psychiatric and Neurodevelopmental Genetics Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, United States of America, 3 Program in Medical and Population Genetics, Broad Institute of MIT and Harvard, Cambridge, Massachusetts, United States of America, 4 Stanley Center for Psychiatric Research, Broad Institute of MIT and Harvard, Cambridge, Massachusetts, United States of America, 5 Department of Neurology, Medical University of Vienna, Vienna, Austria, 6 Institute of Biochemistry, Department of Chemistry, University of Cologne, Cologne, Germany, 7 Luxembourg Centre for Systems Biomedicine (LCSB), University of Luxembourg, Esch-sur-Alzette, Luxembourg, 8 Folkhålsan Institute of Genetics, Helsinki, Finland, 9 Neuroscience Center, University of Helsinki, Helsinki, Finland, 10 Research Programs Unit, Molecular Neurology, University of Helsinki, Helsinki, Finland, 11 CeGaT GmbH—Centre for Genomics and Transcriptomics, Tübingen, Germany, 12 Departments of Epidemiology, Neurology, Radiology, Erasmus Medical Center, Rotterdam, Netherlands, 13 Department of Epidemiology, Erasmus Medical Center, Rotterdam, Netherlands, 14 Department of Internal Medicine, Erasmus Medical Center, Rotterdam, Netherlands, 15 Private Practice for Pediatrics, Vienna, Austria, 16 Department of Pediatrics, Medical University of Graz, Graz, Austria, 17 St. Anna Children’s Hospital, Department of Neuropediatrics, Vienna, Austria, 18 Laboratory of Neurogenetics and Neuroscience, Institute G. Gaslini, Genova, Italy, 19 Department of Neuropediatrics, University Medical Center Giessen and Marburg, Giessen, Germany, 20 Department of Neurology and Epileptology, Hertie Institute for Clinical Brain Research, University of Tübingen, Tübingen, Germany, 21 Departments of Neurology and Pediatrics, University of California San Francisco, San Francisco, California, United States of America, 22 Department of Epileptology, University of Bonn, Bonn, Germany, 23 Institute of Human Genetics, University of Leipzig, Leipzig, Germany, 24 Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, Cologne, Germany

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Abstract

Objective

The SCN1A gene, coding for the voltage-gated Na⁺ channel alpha subunit NaV1.1, is the clinically most relevant epilepsy gene. With the advent of high-throughput next-generation sequencing, clinical laboratories are generating an ever-increasing catalogue of SCN1A variants. Variants are more likely to be classified as pathogenic if they have already been classified as such by other laboratories.
identified previously in a patient with epilepsy. Here, we critically re-evaluate the pathogenicity of this class of variants in a cohort of patients with common epilepsy syndromes and subsequently ask whether a significant fraction of benign variants have been misclassified as pathogenic.

Methods
We screened a discovery cohort of 448 patients with a broad range of common genetic epilepsies and 734 controls for previously reported SCN1A mutations that were assumed to be disease causing. We re-evaluated the evidence for pathogenicity of the identified variants using in silico predictions, segregation, original reports, available functional data and assessment of allele frequencies in healthy individuals as well as in a follow up cohort of 777 patients.

Results and Interpretation
We identified 8 known missense mutations, previously reported as pathogenic, in a total of 17 unrelated epilepsy patients (17/448; 3.80%). Our re-evaluation indicates that 7 out of these 8 variants (p.R27T; p.R28C; p.R542Q; p.R604H; p.T1250M; p.E1308D; p.R1928G; NP_001159435.1) are not pathogenic. Only the p.T1174S mutation may be considered as a genetic risk factor for epilepsy of small effect size based on the enrichment in patients (P = 6.60 x 10^{-4}; OR = 0.32, fishers exact test), previous functional studies but incomplete penetrance. Thus, incorporation of previous studies in genetic counseling of SCN1A sequencing results is challenging and may produce incorrect conclusions.

Introduction
The SCN1A gene (MIM#182389), coding for the voltage-gated Na+ channel alpha subunit NaV1.1, is the most clinically relevant epilepsy gene. SCN1A variants are associated, at the more benign end of the disease spectrum, with the dominantly inherited genetic epilepsy with febrile seizures plus [1, 2] (GEFS+), and, at the severe end, with Dravet syndrome (DS), an epileptic encephalopathy arising from de novo SCN1A mutations in the vast majority of DS patients [3]. More rarely, SCN1A mutations are also found in other types of infantile epileptic encephalopathies [4]. Common variants in SCN1A have been associated with mesial temporal lobe epilepsy and hippocampal sclerosis with febrile seizures in a genome-wide association study [5], and in a recent meta-analysis including 8696 patients with genetic generalized-, focal-, or unclassified epilepsies [6]. In around 88% of epilepsy patients carrying rare SCN1A mutations, these arise de novo, whereas only 12% of the affected individuals inherit the mutation from a, usually unaffected, parent [4]. Patients with an identical mutation may express a broad spectrum of phenotypes even within a single large family ranging from unaffected, over GEFS+ to Dravet syndrome [7, 8]. Most functional studies of disease-associated variants showed loss-of-function effects of SCN1A mutations [9–12]. Mutations with a complete loss of function, in general, lead to more severe epilepsies [3, 13, 14].

Massive parallel sequencing studies have accelerated mutation discovery. With declining costs, more patients will be sequenced, and many newly identified mutations are expected. Determination of the functional consequences and pathogenicity is challenging and molecular studies for each variant are currently not feasible. Databases like the "The Human Gene
Mutation Database” (HGMD; http://www.hgmd.org/) constitute a comprehensive collection of mutations in genes underlying or associated with human inherited diseases. The database is routinely accessed and utilized by next generation sequencing (NGS) project researchers, human molecular geneticists, neurologists and genetic counselors.

Interpretation of genetic results is challenging, especially in multifactorial diseases like common epilepsies. The HGMD comprises more than 1000 disease-associated mutations annotated for the SCN1A gene (accessed Dec/2014). Here, we investigated a already whole-exome sequenced cohort of 448 patients with a broad range of common genetic epilepsies, for SCN1A mutations listed as disease associated in the HGMD. We re-evaluated the identified variants’ evidence for pathogenicity by in silico prediction, segregation, literature review for clinical, genetic and functional relevance and assessment of allele frequencies in healthy individuals. Finally, we investigated the phenotypic spectrum of the most reliable variant in a diagnostic epilepsy cohort.

**Patients and Methods**

**Patients and sequencing**

Patients of European and Turkish ancestry were recruited at several centers from Germany, Austria, Italy, Finland, Canada and Turkey. We included Turkish patients since SCN1A variant frequencies do not show correlation with the ethnicity when compared between Asian and European patients [4]. Samples from The Rotterdam Study [15] not specifically screened for European ethnicity, which were handled in the same way as our cases, i.e. using the same enrichment and sequencing methods, served as controls for the whole exome dataset. The controls are drawn from the population and are not evaluated for seizure disorders. Informed consent for whole exome sequencing was obtained from all participants or legal representatives respectively. The two institutions in charge of the reported analysis are the University hospitals in Tübingen (for “genetic generalized epilepsy”) and Giessen (for “genetic focal epilepsy”), Germany. Both review boards in Tübingen and Giessen approved this study.

High-throughput, targeted sequencing was performed as previously described [16] with the Nimblegen-SeqCapEZ-V244M enrichment kit on the Illumina HiSeq2000 system. For each sample, we calculated the gene-coverage of SCN1A. Only samples with a minimum of 90% of all bases in the coding region of the gene (i.e. all exons plus the first and last five intronic bases, NM_001165963.1) being covered by at least 15 reads were used. After quality control, 448 unrelated epilepsy patients (235 with genetic generalized epilepsy; 182 with rolandic epilepsy; 22 with atypical rolandic epilepsy; 4 with benign familial neonatal seizures; 4 with benign familial infantile epilepsy; 1 with benign adult familial myoclonic epilepsy; 226 males, 222 females) and 734 control individuals (435 females, 299 males) were included in the analysis. All SCN1A HGMD variants identified in the patients were validated with Sanger sequencing using standard protocols. Primers are available upon request. Rare SCN1A sequence variants, which are not annotated by HGMD, were not investigated in this study.

**HGMD annotation and assessment of the literature**

Variant annotation was performed using the HGMD Professional 2013.4–15th December 2013 Version [17]. We assessed original reports cited by the HGMD for each identified HGMD patient mutation. We re-analyzed the mutations, and if the original report used the sequence of a shorter transcript of the SCN1A gene for numbering we numbered the variant accordingly to the RefSeqGene (NM_001165963.1; which represent the longest transcript and reference protein NP_001159435.1) used in our data set.
Assessment of mutation frequency in controls

Besides our control dataset, variant frequencies were taken from the ExAC collection (http://exac.broadinstitute.org/). Note that this database also includes patients from the "Swedish Schizophrenia & Bipolar Studies". Furthermore, it is not clear if patients with mild seizure disorders have been excluded.

In silico prediction

Functional prediction scores were obtained from the dbNSFP database version 2.8 (http://sites.google.com/site/jpopgen/dbNSFP, accessed 01/2015). In total we used six prediction scores (SIFT, Polyphen-2-HVAR, Polyphen-2-HDIV, Mutation Assessor, FATHMM, LRT) and two conservation scores (GERP++, PhyloP). We did not use the dbNSFP generated ensemble scores, as they integrate the prediction of the tool Mutation Taster, which itself incorporates HMGD entries. We classified the variant as “damaging” when the majority of the tools predicted a functional effect for the variant (i.e. a minimum of 5 out of 8 tools). Topology and domain structure were taken from UniProt entry P35498.

Screening of the p.T1174S mutation in a diagnostic cohort of epilepsy

To further investigate the frequency and phenotypic spectrum of the p.T1174S mutation, we evaluated NGS panel sequencing data of epilepsy-associated genes [18] of 777 individuals provided by the CeGaT diagnostic lab (http://www.cegat.de, genes listed in S1 Table). Patients covered a broad spectrum of epilepsy phenotypes, often associated with intellectual disability.

Criteria for variant re-classification

We consider a variant as pathogenic if all of the following three criteria are met: i) The variant is statistically enriched in the patient cohort and/or absent in controls; ii) the segregation pattern analysis and the re-evaluation of original reports do not weaken the epilepsy association, iii) the variant is predicted to be pathogenic by the majority of in silico classifiers and/or molecular functional analyses supports its pathogenicity.

Results

Case vs. control HGMD analysis

We analyzed sequence data of 448 epilepsy patients and 734 controls for SCN1A variants previously reported as disease causing collected by the HGMD. Our mutation screening identified eight distinct HGMD missense mutations (Table 1: p.R27T, p.R28C, p.R604H, p.R542Q, p.T1174S, p.T1250M, p.E1308D, p.R1928G) affecting in total 17 unrelated epilepsy patients (17/448 = 3.80%) compared to six HGMD variants affecting 10 control individuals (10/734 = 1.36%). None of these patients were carrier of a rare loss of function variant in a known epilepsy gene (S2 Table). The SCN1A HGMD variant frequency was therefore slightly enriched in the patient cohort ($P = 8.30 \times 10^{-3}$). A high allele frequency in public databases would argue against pathogenicity of the identified SCN1A HGMD variants identified in our patients. In addition to our own sequenced controls we investigated the SCN1A HGMD variant frequencies further using the ExAc, a publicly accessible variant database of 60706 unrelated individuals. All eight variants were found in individuals collected in the ExAc database (Table 1). After combining our in-house control and ExAc data in a meta-analysis, only the p.T1174S variant remained significantly enriched after correction for multiple testing (Table 1) in epilepsy patients supporting p.T1174S as the only identified SCN1A genetic risk factor for epilepsy in our cohort.
Segregation analysis

We further analyzed the family segregation pattern whereby a shared segregation of the SCN1A HGMD variant and epilepsy was evaluated as support for the variant pathogenicity. For five variants detected in patients (p.T1250M, p.T1174S, p.R604H, p.R542Q, p.R28C), segregation analysis was possible in a total of nine families (Fig 1). All variants were inherited, five times maternally and three times paternally, six times from a healthy parent and only twice from an affected parent. Only the p.T1174S variant segregated with epilepsy in our investigated families (Fig 1).

Review of the original reports

Next, we went back to the 16 original reports (1–4 cited reports for each mutation, Table 2) of all eight identified patient mutations referenced in the HGMD to summarize inheritance, segregation and available functional data. The majority of the reports do not support the disease association of the variant from a today’s perspective. None of the mutations have been described as de novo. In three reports (p.R604H, p.E1308D, p.R1928G) an additional SCN1A mutation has been reported in the HGMD referenced patient, including a stop codon mutation (p.R604H in combination with p.R1525X; p.R1928G in combination with p.L1207P, and p.E1308D in combination with p.A239V; Table 2). In another study the HGMD referred patient had two additional SCN1A mutations, one missense and one splice site acceptor variant (Table 2, p.T1250M in combination with p.R27T and c.3706-2A > G). In four families the mutation was inherited (each one family: p.R27T, p.R542Q, p.T1174S, p.E1308D; Table 2) from a healthy parent and segregation was not determined in 12 families. In contrast, only in three families the mutation segregated with the disease, one affected by the p.R604H and two by the p.T1174S variants. Furthermore, only for the p.T1174S variant functional studies have been conducted. In particular, experiments in tsA-201 cells and computational modeling of mutant properties both supported its epileptogenic effect [19].

In silico prediction analysis

Six of the eight identified variants (p.R28C, p.R542Q, p.R604H, p.T1250M, p.E1308D and p.R1928G) are predicted to have a damaging effect when both structural prediction and local conservation scores were considered (Table 3). In contrast to previous molecular studies [19]...
and our statistical enrichment and segregation studies, the p.T1174S variant was not predicted to be damaging by the majority of prediction tools (Table 3).

Replication analysis p.T1174S

Overall, after the initial re-evaluation of the identified SCN1A HGMD variants, we suggest that only the p.T1174S variant is potentially a epilepsy associated variant. The p.T1174S variant is enriched in patients, segregates with the phenotype in families and published functional data support pathogenicity of the variant (Table 2). A major factor in our variant classification is comparing the allele frequency differences between patients and controls. To further assess the frequency of p.T1174S alleles in epilepsy patients and replicate our association, we investigated an additional epilepsy cohort provided by the CeGaT diagnostic lab using targeted sequencing. We identified seven out of 777 epilepsy patients (0.90%) carrying the p.T1174S mutation. Four of the patients were diagnosed with intellectual disability and epilepsy, one patient had temporal lobe epilepsy, and two patients had epileptic seizures without further specification of the phenotype. In five of the seven patients, the mutation had been inherited from a healthy parent. In the remaining two cases, parents were not available for segregation testing. Of note, in one of the seven patients, an additional pathogenic variant was detected by detection of a de novo mutation in GRIN2B [34] (c.1619G>A, p.R540H).

Combining both epilepsy cohorts together, we have identified 13 out of 1219 patients carrying the p.T1174S mutation. A comparison of all available patient and control data confirms...
enrichment in epilepsy patients ($P = 5.68 \times 10^{-4}$; OR = 3.08, 95%-CI: 1.61–5.40, fisher exact test, 13/1219 patients vs. 21461409 controls).

**Discussion**

In the context of molecular genetic testing, it is often challenging to establish the pathogenicity of an intragenic variant. Lack of parental DNA or control individuals, absence of functional data and association with different phenotypes add to this complexity.

### Table 2. Summary of previous studies referenced at the HGMD specific for each identified variant.

| Mutation | Previous Phenotype | Previous inheritance | Comment | Ref | Phenotypes in this study
|----------|--------------------|----------------------|---------|----|-------------------------|
| p.R1928G | GEFS+ | Not determined | - | [1] | 1x CAE
| Control | | | - | [1] | |
| SMEI | | Not determined | Patient carried an additional p.L1207P mutation in SCN1A | [20] | |
| p.E1308D | FC | Not determined | - | [21] | 1x JAE
| SMEI | | Inherited from healthy father | Patient carried an additional m.p.A239V mutation in SCN1A | [22] | |
| p.T1250M | DS | Not determined | Patient has an additional SCN1A missense mutation (p.Arg27Thr) and one variant predicted as splice Site acceptor mutation (c.3706-2A > G) | [23] | 1x RE
| | | | | |
| p.T1174S | GEFS+ | | - | [21] | |
| | DS + Migraine | Maternal inherited | Patient has DS, mother has migraine with aura | [24] | 1x GTCS, 2x RE, 1x SMEI Not determined | BFNE, 1x CAE, 1x JAE
| SMEI | Not determined | | - | [25] | |
| | | | | |
| | Seizures and hemiplegic migraine | Large family: all mutation carriers had epilepsy or migraine, 3x migraine with aura, 1 migraine without aura and benign occipital epilepsy, 2x migraine with aura and benign occipital epilepsy = > inherited | Functional studies, interpretation = modulation of the properties of T1174S can lead to a switch between overall gain and loss of function, consistent with a switch between promigraine end epileptogenic effect and, thus, with coexistence of epileptic and FHM phenotypes in the same family. | [19] | |
| MAE | Inherited from healthy mother | | - | [26] | |
| FHM | Not determined | | - | [27] | |
| p.R604H | Intractable Epilepsy | Not determined | | [28] | 2x JME;
| FS, FS+ | Inherited all affected | With a single family, All three mutation carrier had FS or FS+ | [29] | 1x CAE, 1x RE, 1x EOAE |
| DS | Not determined | Patient carries also a SCN1A Stop codon mutation (p.R1525X) | [30] | |
| p.R542Q | JME | Not determined | - | [31] | 1x RE
| Familial autism | Inherited from healthy father | A sib with autism is affected and carries the mutation as well | [32] | |
| ICE | Not determined | | - | [28] | |
| p.R28C | GEFS+ | Not determined | - | [33] | 1x JAE
| p.R27T | GEFS+ | Inherited from healthy father | - | [22] | 1x JME

Abbreviations: GEFS+ = genetic epilepsy with febrile seizures plus; SMEI = severe myoclonic epilepsy of infancy; FC = febrile convulsion; DS = Dravet Syndrome; MAE = Myoclonic atatic epilepsy (Doose syndrome); FHM = Familial hemiplegic migraine; FS = febrile seizures; JME = juvenile myoclonic epilepsy, ICE = Intractable childhood epilepsy; GGE syndromes: CAE: childhood absence epilepsy, JAE: juvenile absence epilepsy, JME: juvenile myoclonic epilepsy, EGMA: epilepsy with generalized tonic-clonic seizures predominantly on awakening, EGTCs: epilepsy with generalized tonic-clonic seizure

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### Table 3. Functional prediction and conservation scores of the 8 HMGD Variations.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>SIFT</th>
<th>Polyphen2_HDIV</th>
<th>Polyphen2_HVAR</th>
<th>LRT_pred</th>
<th>MutationAssessor</th>
<th>FATHMM</th>
<th>GERP++ rankscore</th>
<th>PhyloP46way_primate rankscore</th>
<th>predicted outcome (n of tools)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.R27T</td>
<td>T</td>
<td>B</td>
<td>B</td>
<td>N</td>
<td>L</td>
<td>D</td>
<td>0.589</td>
<td>0.943</td>
<td>functionally neutral (3/8)</td>
</tr>
<tr>
<td>p.R28C</td>
<td>D</td>
<td>P</td>
<td>B</td>
<td>D</td>
<td>M</td>
<td>D</td>
<td>0.912</td>
<td>0.943</td>
<td>damaging (7/8)</td>
</tr>
<tr>
<td>p.R542Q</td>
<td>D</td>
<td>B</td>
<td>B</td>
<td>D</td>
<td>L</td>
<td>D</td>
<td>0.997</td>
<td>0.943</td>
<td>damaging (5/8)</td>
</tr>
<tr>
<td>p.R604H</td>
<td>T</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>M</td>
<td>D</td>
<td>0.772</td>
<td>0.741</td>
<td>damaging (7/8)</td>
</tr>
<tr>
<td>p.T1174S</td>
<td>T</td>
<td>B</td>
<td>B</td>
<td>N</td>
<td>N</td>
<td>D</td>
<td>0.789</td>
<td>0.430</td>
<td>functionally neutral (2/8)</td>
</tr>
<tr>
<td>p.T1250M</td>
<td>T</td>
<td>D</td>
<td>B</td>
<td>D</td>
<td>M</td>
<td>D</td>
<td>0.534</td>
<td>0.711</td>
<td>damaging (6/8)</td>
</tr>
<tr>
<td>p.E1308D</td>
<td>T</td>
<td>D</td>
<td>P</td>
<td>D</td>
<td>N</td>
<td>D</td>
<td>0.802</td>
<td>0.533</td>
<td>damaging (6/8)</td>
</tr>
<tr>
<td>p.R1928G</td>
<td>T</td>
<td>B</td>
<td>B</td>
<td>D</td>
<td>M</td>
<td>D</td>
<td>0.638</td>
<td>0.697</td>
<td>damaging (5/8)</td>
</tr>
</tbody>
</table>

T = tolerated, D = damaging, B = benign, P = possibly damaging, N = neutral, L = predicted non-functional low, M = predicted functional medium, N = predicted non-functional neutral. For GERP++ and PhyloP rankscores a cutoff of 0.5 was used in order to rank the variant site as conserved. The variant was classified as “damaging” when the majority of tools predicted a functional effect (damaging, possibly damaging, predicted functional-damaging) and classified as “functionally neutral” when the majority of the tools predicted no functional effect (neutral, tolerated, benign, non-functional).
In this study, we investigated the liability of SCN1A variants that were previously classified as pathogenic, in a cohort of common epilepsy syndromes. We detected an enrichment of HGMD annotated variants in our epilepsy patient cohort compared to controls ($P = 8.30 \times 10^{-3}$). All identified HGMD SCN1A variants in our patients have also been detected in control individuals arguing against completely penetrant causal mutations. None of the variants were localized in the transmembrane regions of the protein (S1 Fig), where mutations are associated with more severe channel dysfunctions [13]. We considered seven, out of the eight (p.R27T, p.R28C, p.R542Q, p.R604H p.T1250M, p.E1308D, p.R1928G) analyzed HGMD variants, as obvious benign based on the original reports, the high frequency in control individuals and missing or negative segregation and functional results (Tables 1 and 2; Fig 1).

In contrast to the other mutations classification of the p.T1174S mutation is less trivial. On the one hand, support as a potential genetic risk factor for epilepsy is based on several reasons. Firstly, the p.T1174S variant is overrepresented in epilepsy patients ($P = 5.68 \times 10^{-4}$). Secondly, it segregates with the disease in our, and, previously analyzed families (Table 2, Fig 1). It was previously described in patients diagnosed with severe myoclonic epilepsy of infancy (inheritance not determined), Dravet syndrome (inherited from a mother with migraine), myoclonic atonic epilepsy (inherited from a healthy mother), and repetitively with familial hemiplegic migraine (FHM) [24, 27]. In our cohort, the p.T1174S mutation was found in patients with rolandic epilepsy, childhood absence epilepsy, juvenile absence epilepsy, benign familial neonatal seizures epilepsy and patients with a single generalized tonic-clonic seizure. In the diagnostic cohort we found patients with unclassified epilepsy with intellectual disability as well as temporal lobe epilepsy. Additionally, another family with the p.T1174S mutation has been identified (personal communication with Arvid Suls, University of Antwerp). In this family the mother carries the variant, as do the six elder siblings of seven. Two of these six carriers are affected by epilepsy with myoclonic seizures. Finally, previous in vitro electrophysiological recordings of the mutant p.T1174S Nav1.1 channel support the variant pathogenicity [19]. The molecular analysis revealed two divergent effects: a positive shift of the activation curve and deceleration of recovery from fast inactivation—consistent with a loss of function, and an increase of persistent current (I(NaP))—consistent with a gain of function [19]. On the other hand, the p.T1174S variant was only predicted to be damaging by one in silico program (FATHMM, Table 3) and was inherited by unaffected parent in the replication cohort five out of seven times.

Besides the unclear role of the p.T1174S variant, the majority of our investigated SCN1A HGMD variants cannot be classified as clearly pathogenic. Based on our results, we assume that a significant fraction of patients diagnosed with pathogenic SCN1A mutations may actually not carry an SCN1A variant of relevance. The role of SCN1A missense mutations in the pathogenesis of common epilepsies may thus be overstated (in general and e.g. studies in Table 2). Previous conclusions were frequently based on comparisons of allele frequencies between patients and small control datasets often without molecular follow up of the variant (Table 2). By accessing large gene mutation databases of non-epileptic individuals (e.g. http://exac.broadinstitute.org) we show that previous studies have been underpowered. In order to draw a definitive conclusion about pathogenicity for variants in common epilepsy syndromes, which are also present in healthy individuals, functional studies are mandatory (e.g. functional characterization of patient neurons derived form induced pluripotent cells).

The conclusions drawn from our study can be translated to other fields of research. Several prediction tools [35–39] evaluating the pathogenic potential of DNA sequence alterations are trained with variant lists extracted from the HGMD. Given that the disease association of the annotated SCN1A variants have to be questioned, it is likely that a similar picture can be seen for other complex diseases and genes. Simply extracting HGMD variants as training sets might bias prediction algorithms and machine learning approaches.
Overall, we highlight the ambiguities of variant classification in common epilepsy syndromes and emphasize that the majority of SCN1A variants could not be re-classified as pathogenic. Furthermore, our results warrant careful assessment of variants previously reported in small cohort studies.

Supporting Information

S1 Fig. SCN1A domain organization and mutations. The schematic diagram showing the domain organization of the alpha subunit of the voltage-gated sodium ion channel coded by the SCN1A gene and the positions of the missense mutations (shown as orange circles). The complex consists of four homologous domains (I-IV), each containing six transmembrane segments (S1-S6). IQ indicates the localization of the IQ calmodulin-binding motif.

S1 Table. List of genes included in the gene panel.

S2 Table. Rare loss of function variants in 17 epilepsy patients with known HGMD SCN1A variants.

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Contributors

EuroEPINOMICS CoGIE Consortium members are: Consortium leader: Holger Lerche (holger.lerche@uni-tuebingen.de); Aarno Palotie, Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridgeshire, UK; Institute for Molecular Medicine Finland, University of Helsinki, Helsinki, Finland; Program in Medical and Population Genetics and Genetic Analysis Platform, The Broad Institute of MIT and Harvard, Cambridge, USA; Anna-Elina Lehesjoki, Folkhälsoan Institute of Genetics, Helsinki, Finland; Neuroscience
Center, University of Helsinki, Helsinki, Finland; Research Programs Unit, Molecular Neurology, University of Helsinki, Helsinki, Finland; **Ann-Kathrin Ruppert**, Cologne Center for Genomics, University of Cologne, Cologne, Germany; **Arvid Suls**, Neurogenetics group, Department of Molecular Genetics, VIB, Antwerp, Belgium; Laboratory of Neurogenetics, Institute Born-Bunge, University of Antwerp, Antwerp, Belgium; **Auli Siren**, Outpatient Clinic for Persons with Intellectual Disability, Tampere University Hospital, Tampere, Finland; **Birgit Neophytou**, St. Anna Children’s Hospital, Department of Neuropediatrics, Vienna, Austria; **Bobby Kooleman**, Department of Genetics, Center for Molecular Medicine, University Medical Center Utrecht, The Netherlands; **Dennis Lal**, Cologne Center for Genomics, University of Cologne, Cologne, Germany; **Edda Haberlandt**, Department of Pediatrics, Medical University of Innsbruck, Innsbruck, Austria; **Felicitas Becker**, Department of Neurology and Epileptology, Hertie Institute for Clinical Brain Research, University of Tübingen, Tübingen, Germany; **Fritz Zimprich**, Department of Neurology, Medical University of Vienna, Vienna, Austria; **Gabriel M Ronen**, Department of Pediatrics, McMaster University, Hamilton, Ontario, Canada; **Hande Caglayan**, Department of Molecular Biology and Genetics, Bogazici University, Istanbul, Turkey; **Helle Hjalgrim**, Danish Epilepsy Centre, Dianalund, Denmark; **Ingo Helbig**, University Medical Center Schleswig-Holstein, Christian-Albrechts University, Kiel, Germany; **Holger Thiele**, Cologne Center for Genomics, University of Cologne, Cologne, Germany; **Ingo Helbig**, University Medical Center Schleswig-Holstein, Christian-Albrechts University, Kiel, Germany; **Janine Altmüller**, Cologne Center for Genomics, University of Cologne, Cologne, Germany; **Julian Schubert**, Department of Neurology and Epileptology, Hertie Institute for Clinical Brain Research, University of Tübingen, Tübingen, Germany; **Kamel Jabbari**, Cologne Center for Genomics, University of Cologne, Cologne, Germany; **Kate Everett**, Cardiovascular and Cell Sciences Research Institute, St George’s University of London, London, UK; **Martha Feucht**, Department of Pediatrics, Medical University of Vienna, Vienna, Austria; **Martina Balestri**, Division of Neurology, Bambino Gesu’ Children’s Hospital and Research Institute, Rome, Italy; **Michael Nothnagel**, Cologne Center for Genomics, University of Cologne, Cologne, Germany; **Pasquale Striano**, Pediatric Neurology and Muscular Diseases Unit, Department of Neurosciences, Rehabilitation, Ophthalmology, Genetics, Maternal and Child Health, University of Genoa, G. Gaslini Institute, Genoa, Italy; **Patrick May**, Luxembourg Centre for Systems Biomedicine, University of Luxembourg, Esch-sur-Alzette, Luxembourg; **Peter Nürnberg**, Cologne Center for Genomics, University of Cologne, Cologne, Germany; **Rikke S Møller**, Danish Epilepsy Centre, Dianalund, Denmark, Institute for Regional Health Services, University of Southern Denmark, Odense, Denmark; **Rima Nabbout**, Centre de Reference Epilepsies Rares, Inserm U1129, Neuropediatrics Department, Necker-Enfants Malades Hospital, APHP, Paris Descartes University, CEA, Orsay, France; **Roland Krause**, Luxembourg Centre for Systems Biomedicine, University of Luxembourg, Esch-sur-Alzette, Luxembourg; **Rudi Balling**, Luxembourg Centre for Systems Biomedicine, University of Luxembourg, Esch-
sur-Alzette, Luxembourg; Stephanie Baulac, Inserm U 1127, CNRS UMR 7225, Sorbonne Universités, UPMC Univ Paris 06 UMR S 1127, Institut du Cerveau et de la Moelle épinière, ICM, F-75013, Paris, France; Thomas Sander, Cologne Center for Genomics, University of Cologne, Cologne, Germany; Ursula Gruber-Sedlmayr, Department of Pediatrics, Medical University of Graz, Graz, Austria; Wolffram Kunz, Department of Epileptology and Life & Brain Center, University of Bonn Medical Center, Bonn, Germany; Yvonne G. Weber, Department of Neurology and Epileptology, Hertie Institute for Clinical Brain Research, University of Tübingen, Tübingen, Germany

Italian League against Epilepsy members are: Consortium leader: Amedeo Bianchi (bianchi.epigenet@tin.it); Amedeo Bianchi, Centro epilessia, UO Neurologia, Ospedale San Donato Arezzo, Italy; Angela La Neve, Neurology Clinic, University of Bari, 70124 Bari, Italy Pasquale Striano, Department of Neuroscience, Rehabilitation, Ophthalmology, Genetics, Maternal and Child Health, University of Genoa and Giannina Gaslini Institute, 16148 Genova, Italy Carlo Minetti, Department of Neuroscience, Rehabilitation, Ophthalmology, Genetics, Maternal and Child Health, University of Genoa and Giannina Gaslini Institute, 16148 Genova, Italy Capovilla Giuseppe, Department of Child Neuropsychiatry, C. Poma Hospital, Mantova, Italy

Author Contributions
Conceived and designed the experiments: DL EMR BD BAN. Performed the experiments: DL EMR BD. Analyzed the data: DL EMR BD ER JRL. Contributed reagents/materials/analysis tools: A-EL MAI CMvd AGU A. Hofman HS UGS BN F. Zara A. Hahn PM HT GS FB YW MRC WK FZ AU ER JRL [Genetic Commission of the Italian League against Epilepsy] [EuroE-PINOMICS CoGIE Consortium]. Wrote the paper: DL EMR BD RK FZ JRL PN TS HL BAN PG. Revising and reviewing the manuscript: DL EMR BD PM HT A-EL GS ER MAI CMvd AGU A. Hofman HS UGS BN F. Zara A. Hahn PG FB YW MRC WK RK F. Zimprich JRL PN TS HL BAN.

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