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RESEARCH ARTICLE

Genotype–phenotype associations in 1018 individuals with *SCN1A*-related epilepsies

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Abstract

Objective: *SCN1A* variants are associated with epilepsy syndromes ranging from mild genetic epilepsy with febrile seizures plus (GEFS+) to severe Dravet syndrome (DS). Many variants are de novo, making early phenotype prediction difficult, and genotype–phenotype associations remain poorly understood.

Methods: We assessed data from a retrospective cohort of 1018 individuals with *SCN1A*-related epilepsies. We explored relationships between variant characteristics (position, in silico prediction scores: Combined Annotation Dependent Depletion (CADD), Rare Exome Variant Ensemble Learner (REVEL), *SCN1A* genetic score), seizure characteristics, and epilepsy phenotype.

Results: DS had earlier seizure onset than other GEFS+ phenotypes (5.3 vs. 12.0 months, $p < .001$). In silico variant scores were higher in DS versus GEFS+ ($p < .001$). Patients with missense variants in functionally important regions (conserved N-terminus, S4–S6) exhibited earlier seizure onset (6.0 vs. 7.0 months, $p = .003$) and were more likely to have DS (280/340); those with missense variants in nonconserved regions had later onset (10.0 vs. 7.0 months, $p = .036$) and were more likely to have GEFS+ (15/29, $\chi^2 = 19.16$, $p < .001$). A minority of protein-truncating variants were associated with GEFS+ (10/393) and more likely to be located in the proximal first and last exon coding regions than elsewhere in the gene (9.7% vs. 1.0%, $p < .001$). Carriers of the same missense variant exhibited less variability in age at seizure onset compared with carriers of different missense variants for both DS (1.9 vs. 2.9 months, $p = .001$) and GEFS+ (8.0 vs. 11.0 months,

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$p = .043$). Status epilepticus as presenting seizure type is a highly specific (95.2%) but nonsensitive (32.7%) feature of DS.

Significance: Understanding genotype–phenotype associations in *SCN1A*-related epilepsies is critical for early diagnosis and management. We demonstrate an earlier disease onset in patients with missense variants in important functional regions, the occurrence of GEFS+ truncating variants, and the value of in silico prediction scores. Status epilepticus as initial seizure type is a highly specific, but not sensitive, early feature of DS.

KEYWORDS

Dravet syndrome, GEFS+, genotype–phenotype associations, *SCN1A*, severe myoclonic epilepsy of infancy

1 | INTRODUCTION

Pathogenic variants in *SCN1A*, the gene coding for the alpha-1 subunit of the voltage-gated sodium channel, are associated with a range of epilepsy syndromes from relatively mild phenotypes in the genetic epilepsy with febrile seizures plus (GEFS+) spectrum to Dravet syndrome (DS), a severe developmental and epileptic encephalopathy.[1–4] DS usually presents at approximately 5–6 months of age with prolonged, febrile and afebrile, hemiclonic or generalized clonic seizures. From age 9 months to 4 years other seizure types, including myoclonic, absence, and focal seizures, develop.[5,6] Typical antiseizure medications have limited efficacy, and sodium channel blockers are associated with worse outcomes.[7] From age 2 years, cognitive, behavioral, and motor development becomes significantly impaired. Epilepsies within the less severe GEFS+ spectrum also present early in life[8]; however, cognitive development is normal, and many cases have a positive family history.

Within *SCN1A*, pathogenic missense variants account for 45%–55% of DS phenotypes, with the remainder consisting of protein-truncating variants (PTVs), copy number variants, and other mutation types.[9,10] More than 90% of GEFS+ variants are missense.[11] Truncating variants lead to haploinsufficiency,[12] mediated by nonsense-mediated decay (NMD) of mRNA containing premature stop codons causing total loss of function (LOF). Missense variants have varied functional effects, with those causing complete LOF likely resulting in DS and those causing partial LOF being associated with milder GEFS+ phenotypes.[13,14] Functional studies are time-consuming and costly, so accurate functional information is only available for a small proportion of the thousands of variants associated with DS and GEFS+.[15,16] Disease-associated PTVs are more evenly distributed across the gene, whereas missense variants are relatively confined to regions that code for sections important for channel function, namely, the S4 voltage sensor and S5–S6 pore regions.[11,17] *SCN1A* is mainly expressed in cortical interneurons, and the predominant disease mechanism in *SCN1A*-related epilepsies is reduced function in inhibitory interneurons shifting the balance toward increased neuronal excitation.[18] Several in silico tools provide probabilistic information on whether novel variants are likely to be disease-causing or benign; however, these tools do not differentiate between phenotypes or predict severity in allelic or spectrum disorders such as GEFS+ and DS.[19–23] Our recently developed *SCN1A* prediction model builds on previous work[24,25] and, by utilizing machine learning algorithms, supports clinical judgment in predicting the likelihood of DS or milder GEFS+ phenotypes.[6]

Key points

- We report genotype–phenotype analysis of an international cohort of 1018 individuals with *SCN1A*-related epilepsies
- Missense variants in functionally important regions are more often associated with DS, whereas those in nonconserved distal and linker regions are more often associated with less severe GEFS+ phenotypes
- Truncating variants at the very beginning or the very end of the *SCN1A* gene are rare and more likely to be associated with less severe GEFS+ phenotypes
- Identical missense variants share more similar phenotypic features
- Status epilepticus as the initial seizure type is a highly specific, but not sensitive, feature of DS

The majority of disease-associated *SCN1A* variants occur de novo, and despite recent advances genotype–phenotype associations are still poorly understood. This study assesses data from a retrospective cohort of 1018 individuals with *SCN1A*-related epilepsies, exploring how seizure characteristics, variant type, position, and in silico scores relate to the epilepsy phenotype. We hope that further elucidating the relationships between these features will aid clinicians in improved prediction of phenotype in patients with *SCN1A*-related epilepsies.

2 | MATERIALS AND METHODS

2.1 | Phenotype and clinical data

This was a retrospective cohort study of 1018 *SCN1A*-positive patients from the United Kingdom ($n=276$), Australia ($n=203$),[6,26] France ($n=201$), Italy ($n=126$), the Netherlands ($n=109$), Belgium ($n=72$), and Denmark ($n=31$). Cohort details have recently been published as part of the development and validation of a prediction model for early diagnosis of *SCN1A*-related epilepsies.[27] The cohorts included cases that came via research referrals and from consecutive referrals for clinical genetic testing across various centers in their respective countries.

Epilepsy phenotype was classified by clinicians with expertise in epilepsy diagnosis. The criteria for classification of phenotypes were as follows. DS was defined as generalized or hemiclonic seizures frequently triggered by fever and often prolonged, typically followed by other seizure types including myoclonic, focal impaired awareness, and absence seizures; and normal cognitive and psychomotor development prior to seizure onset, with subsequent slowing, including plateauing or regression of skills in the second year of life.[28] Cases were classified as GEFS+ if phenotypes were concordant with the GEFS+ spectrum and of normal intellect regardless of family history.[26]

Onset was recorded as age at first seizure in months. Status epilepticus as the initial seizure type was classified as a first seizure lasting longer than 30 min.[29]

2.2 | Variant identification

We report on patients whose variants were previously identified and validated as part of published cohorts.[27] A summary of the methodology used to identify variants in those cohorts is as follows. For identification of single nucleotide variants, all 26 exons of *SCN1A* were examined by Sanger or next generation sequencing. Large-scale gene rearrangements were identified by multiplex ligation-dependent probe amplification. Missense variants and PTVs including premature stop codons, frameshifts that resulted in premature stop codons, large deletions, and whole gene deletions were included. Intronic variants predicated to disrupting splicing were omitted due to the complexity in predicting their functional effects.

2.3 | Variant stratification

Missense variants were grouped into one of 43 regions according to their amino acid protein position. The region boundaries were determined by the protein topology provided by the SCN1A protein annotation on UniProt.[30] Missense variants at the same amino acid position with matching amino acid substitutions were classified as identical variants.

2.4 | Control of repeated family data

If identical variants originated from the same family, data were only counted once per family for analyses that could be affected by bias imparted by repeated measurement. For those variables where data may have differed between family members, for example, age at seizure onset and variant scores, the median value was used so as to best reflect a random sample. An exception was made for families with intrafamilial variable phenotypes, for example, including both DS and GEFS+ affected individuals. Within these families, data could neither be consolidated to one representative data point nor included without introducing repeated measurement of familial data, hence they were excluded from those analyses. This applied to six families with the following variants: p.G163R (Dutch), p.A201V (Dutch), p.V406A (Dutch), p.S570I (Italian), p.T875K (Australian), and p.V982A (Dutch).

For analyses specific to identical variants, family members were examined as separate individuals.

2.5 | Variant scoring

A number of tools designed to assess the deleteriousness or pathogenicity of a specific variant were used. All variants were given an *SCN1A* genetic score, which estimates a variant's pathogenicity based on paralogue conservation and physicochemical difference. The higher the *SCN1A* genetic score, the greater the predicted severity (range = 0 [similar]—207 [dissimilar]).[27] For variants with available chromosomal position data, we obtained a Combined Annotation Dependent Depletion (CADD) score: a derivation of genomic data including sequence context, gene models, evolutionary constraint,

TABLE 1 Phenotypic differences in variant scores.

Variant score	DS mean (SD)	GEFS+ mean (SD)	Mean difference	*p*
Grantham	93.7 (51.0)	74.2 (46.1)	19.5	<.001
SCN1A genetic score	133.1 (79.2)	51.6 (55.4)	81.5	<.001
CADD	31.0 (5.3)	27.2 (3.9)	3.8	<.001
REVEL	.91 (.11)	.85 (.16)	.06	<.001

Note: Mean values for variant scores are based on phenotype. Results of independent two-sample *t*-tests compare differences in value means between DS and GEFS+.

Abbreviations: CADD, Combined Annotation Dependent Depletion; DS, Dravet syndrome; GEFS+, genetic epilepsy with febrile seizures plus; REVEL, Rare Exome Variant Ensemble Learner.

epigenetic influence, and functional prediction. The higher the CADD score (range = 1–99, log derived), the greater the likelihood of pathogenicity.[19] Additionally, for all missense variants, the Rare Exome Variant Ensemble Learner (REVEL) score and Grantham score were calculated.[20,21] REVEL scores (range = 0–1) combine the output of 13 individual tools covering amino acid attributes, conservation, and biochemical features, with a higher score corresponding to pathogenicity. Grantham scores (range = 5–215) estimate the degree of physicochemical difference between amino acids, with higher scores indicative of greater dissimilarity.

2.6 | Statistical analysis

Significance level was set at $\alpha = .05$. Median values and interquartile ranges (IQRs) compared differences in onset of two groups when data were nonnormally distributed. We used Mann–Whitney *U*-tests to assess significant differences in median onset when variables were nonnormal but similarly distributed. Independent sample *t*-tests measured significant differences in mean values of continuous variables between two groups when data were normally distributed and in exploratory and descriptive analyses. If homogeneity of variances was violated, the unequal variances *t*-test was reported. Spearman rank correlations were used to find the strength of association between onset and variant scores. Binomial tests with Clopper–Pearson 95% confidence intervals, chi-squared tests, and Fisher exact tests were used to assess the significance of differences in proportions. Levene test was used to find significant differences in variance between two groups. SD and IQR were utilized as descriptive measures of variance. A Wilcoxon signed-rank test was used to assess the difference between the medians of individuals with the identical variant and the median of their comparative phenotypic cohort. In analyses where multiple testing was conducted, Bonferroni correction method was applied to *p*-values. Tests were carried out on IBM SPSS Statistics v28 and R Stats Package v4.1.2. The figures with structural heatmaps were generated with PyMOL v2.4.1. Preparation of the variants for the heatmaps was done using custom Python scripts.

Anonymized data not published within this article will be available from the corresponding author by email on reasonable request.

2.7 | Ethical approval

Retrospective review of anonymized clinical referral data and variant findings was approved by the relevant institutional review boards (West of Scotland Research Ethics Committee, reference number 16/WS/0203).

3 | RESULTS

3.1 | Cohort characteristics

From our large international cohort of 1018 individuals, 823 (80.8%) were classified as DS and 195 (19.2%) were classified as GEFS+.[27] The DS cohort consisted of 440 (53.5%) patients with missense and 383 (46.5%) patients with PTVs (357 premature stop codons, 26 whole gene/large deletion). The GEFS+ cohort contained 185 patients with missense variants (94.9%) and 10 (5.1%) patients with PTVs (nine premature stop codons, one large deletion [ex2-ex4del]).

3.2 | Seizure onset and variant scores

DS had significantly earlier age at seizure onset (median = 5.3 months, IQR = 4–7) than GEFS+ (median = 12 months, IQR = 8–18, $p < .001$). All in silico variant scores were significantly higher in DS compared to GEFS+ (Table 1). Age at onset was negatively correlated with Grantham (−.169, $p < .001$), CADD (−.233, $p < .001$), and REVEL (−.247, $p < .001$) scores in the combined DS/GEFS+ cohort. Among DS patients only, age at seizure

onset was negatively correlated with Grantham (−.112, $p = .011$), CADD (−.084, $p = .024$), and REVEL (−.195, $p < .001$) scores.

3.3 | Variant types and distribution

Individuals with truncating variants had a significantly earlier age at seizure onset (median = 5 months, IQR = 4–7) than individuals with missense variants (median = 6 months, IQR = 4.1–9, $p < .001$). A Levene test showed significant more variability of onset in missense variants ($p < .001$). Missense variants (Figure 1A) clustered within the four functional domains. Notably, all six missense variants found in the nonconserved N-terminus were associated with GEFS+ phenotypes. Within the large D1–D2 and D2–D3 coding regions, only 16 missense variants were identified, of which seven (44%) were associated with GEFS+ phenotypes. After amino acid position 1928 at the distal end of the nonconserved C-terminus, only two missense variants were found (one GEFS+, one DS).

Truncating variants (Figure 1B) were distributed more evenly throughout the gene. An exception was noted at the extreme ends of the protein coding sequence (before amino acid position 14 and after position 1930), where only two truncating variants were found, at positions 11 and 2003. Both were associated with a milder GEFS+ phenotype. Exon 26 contained a significantly higher proportion of GEFS+-associated PTVs than exons 1–25 (8.1% vs. 1.3%, $\chi^2 = 9.96$, $df = 1$, $p = .002$).

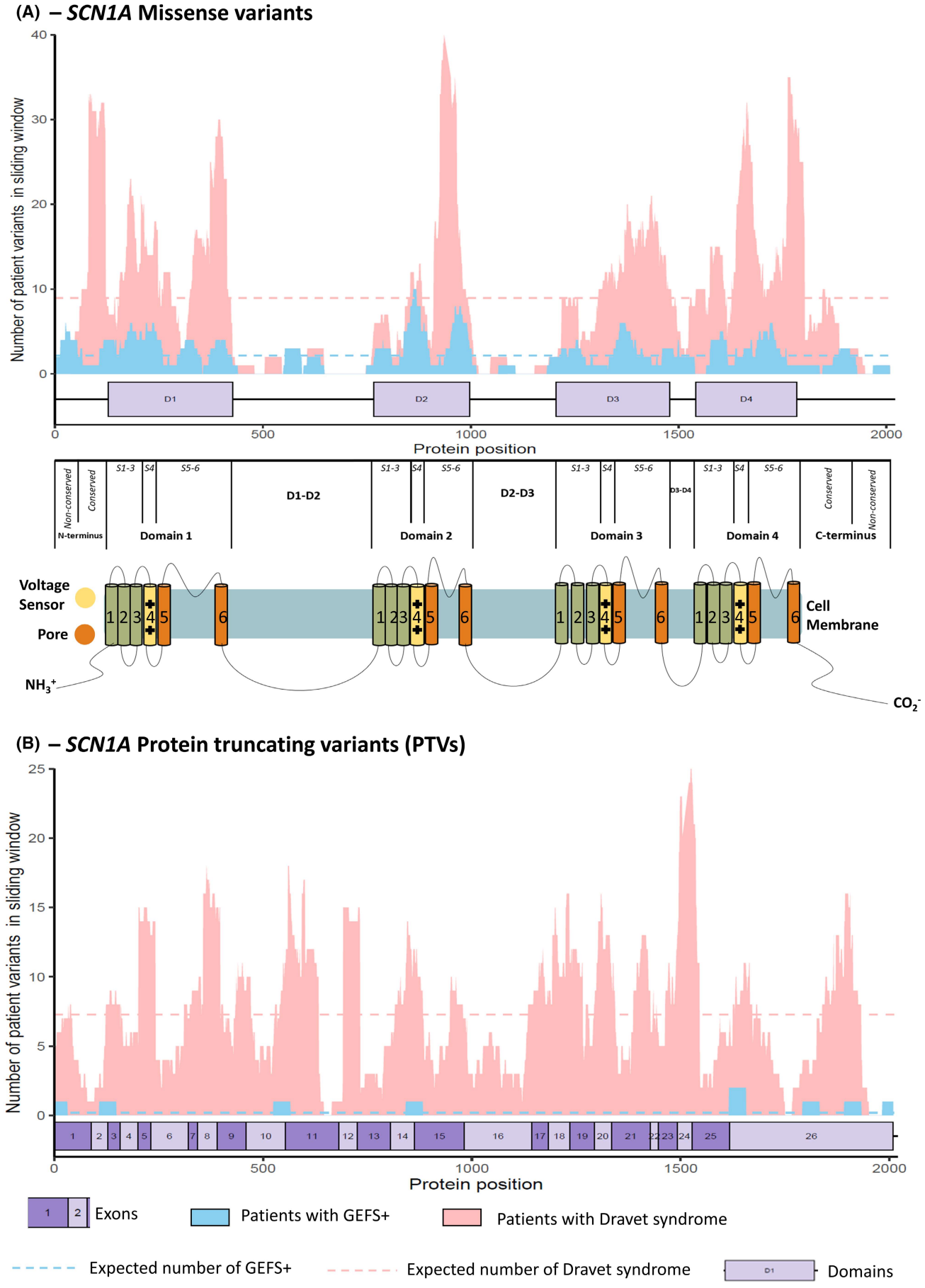
3.4 | Phenotypic differences of missense variant carriers relate to coding region

For the entire cohort, patients with missense variants located more proximally to the central pore coding region had earlier age at onset as indicated by the increased red shading centrally in Figure 2A. When focusing on the GEFS+ cohort, a similar pattern was noted. Patients with variants located further away from the central pore coding region presented with later age at seizure onset, as indicated by the increase in blue shading peripherally in Figure 2B. Overall, regardless of the phenotype (DS vs. GEFS+), patients with missense variants affecting pore forming regions (S5 + S5–S6 linker + S6) had significantly earlier seizure onset compared to patients with missense variants located outside the pore coding regions ($\text{MeanOnset}_{\text{PoreRegions}} = 7.7$ months vs. $\text{MeanOnset}_{\text{OutsidePore}} = 9.3$ months, $p_{adj} < .001$).

We then investigated how the missense variant frequency related to age at seizure onset, according to functional region. To do so, we first assessed the variant frequency across the whole protein (486 variants among 2009 amino acid residues; Table S2) and assigned this ratio a value of 1. Individual coding regions were then grouped according to their respective variant frequency ratio (Figure 3A). Regions were labeled "normal" if the ratio was valued at .5–1.5, "variant dense" if the ratio was >1.5, and "variant sparse" if the ratio was <.5. Variant dense regions included conserved N-terminus, S4, S4–S5, S5, S5–S6, and S6. Variant sparse regions included the nonconserved N-terminus, S3–S4, D1–D2, D2–D3, and nonconserved C-terminus. S1–S3, D3–D4, and the conserved C-terminus made up the remaining "normal" regions.

First, we compared differences in presentation as measured by age at seizure onset according to the coding regions of the SCN1A protein. Patients with missense variants in variant dense regions consistently presented with earlier seizure onset (all ≤7.0 months; Figure 3B). Patients with variants in variant sparse regions presented with later seizure onset, with only one of these regions being associated with median onset < 7.0 months (nonconserved C-terminus). As a whole, variant dense regions were significantly associated with earlier age at seizure onset compared with both variant sparse (6.0 vs. 10.0 months,

FIGURE 1 Distribution of *SCN1A* variants throughout the gene. (A) Excess of the *SCN1A* missense variant burden for Dravet syndrome (DS) versus milder genetic epilepsy with febrile seizures plus (GEFS+) phenotypes by protein position. X-axis displays the protein positions made up of the different domain and linker gene regions. Schematic displays the unfurled *SCN1*-alpha subunit in situ in the cell membrane scaled to the x-axis alongside with the important topological protein regions included according to the length of the protein they make up. The exact amino positions contained within each region are given in the appendix (Table S1). Four homologous functional domains (I, II, III, IV) made up of six transmembrane regions (S1, S2, S3 [green], S4 [yellow], S5, S6 [orange]) are connected by linker regions (curved black lines). The S4 regions are positively charged, voltage gating the channel. The S5 and S6 regions make up the lining of the pore. The S5–S6 linker regions critically have parts of their polypeptide chain enter into the pore region through which sodium ions pass. The protein is capped by N- and C-terminus branches, illustrated by NH_3^+ and CO_2^-, respectively. (B) Excess of the protein-truncating variant burden for DS versus GEFS+ patients by protein position. X-axis also displays the protein positions coded for the *SCN1A* exons 1–26.[31] (A, B) DS patient excess is shown in pink; GEFS+ patient excess is shown in blue. The x-axis is divided into sliding windows of 41 residues/protein positions. The horizontal lines traversing the figure represent the expected level of variants identified across the gene if spread evenly, also colored according to phenotype.



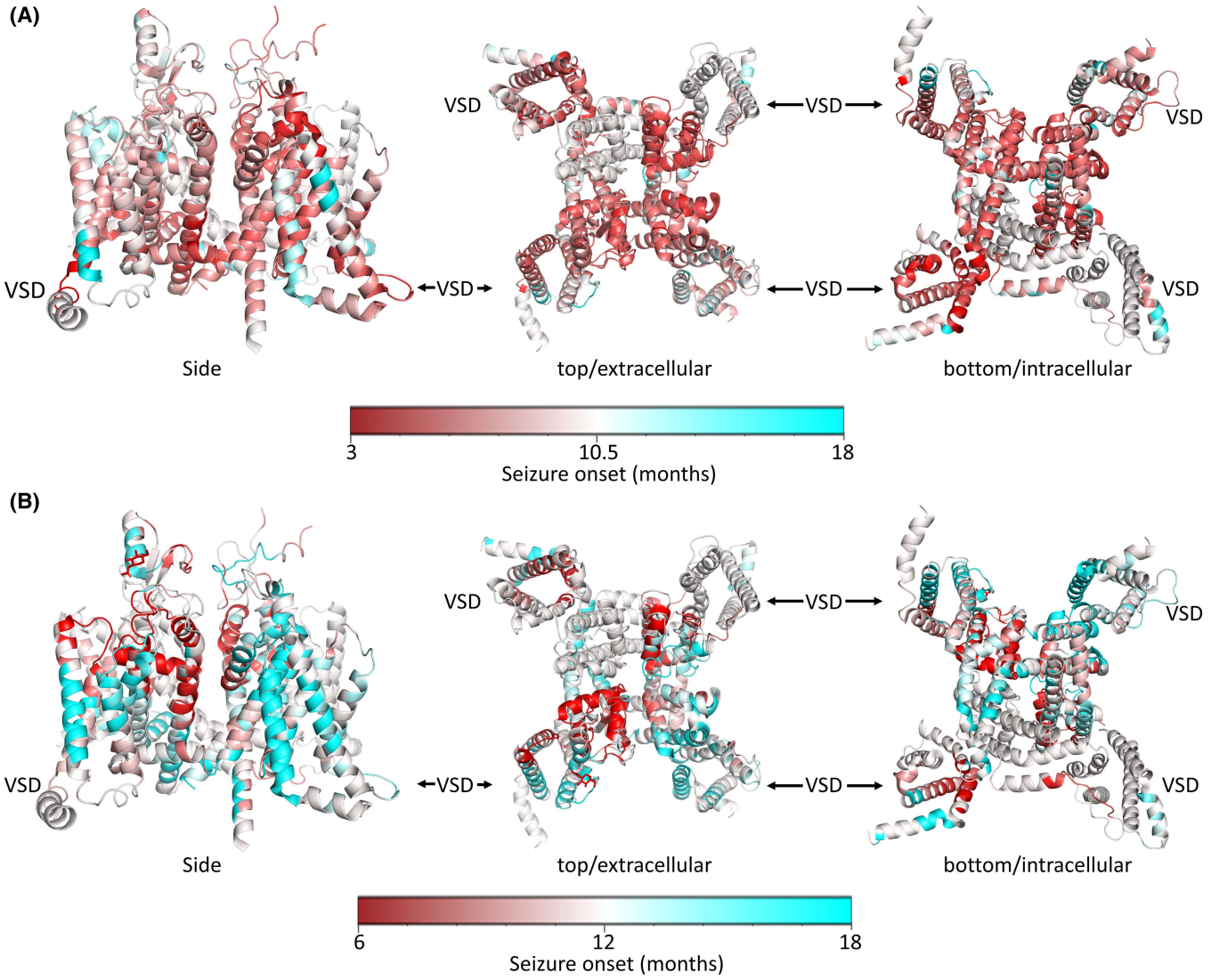


FIGURE 2 Three-dimensional schematic of the SCN1A protein shown from the side, top, and bottom angles. The central opening in the schematics represents the channel pore, and voltage-sensing domain (VSD) sites are indicated. The schematic is colored to indicate where variants are located throughout the gene, with shading representing the associated age at seizure onset of variants found within these regions. Schematic A encompasses all missense variant patients. Schematic B only displays missense variant genetic epilepsy with febrile seizures plus patients.

$p < .001$) and normal regions (6.0 vs. 7.0 months, $p = .003$). Variant sparse regions were also significantly associated with later onset seizures than normal regions (10.0 vs. 7.0 months, $p = .036$).

We then determined whether the proportion of missense variants associated with GEFS+ phenotypes could be related to this distribution pattern. The proportion of GEFS+-associated missense variants in this cohort was 21.8% (106/486, numbers consolidated as per Materials and Methods section 2.4, Control of Repeated Family Data). Variant sparse regions were associated with increased frequency of GEFS+ (15/29, 51.7%), whereas variant dense regions were mainly associated with DS (280/340, 82.3%, $\chi^2 = 19.161$, $df = 1$, $p < .001$). The exact values used to calculate these proportions are in the supplementary appendix (Table S2). Within the individual gene domains/subdomains, variant density was strongly negatively correlated with the proportion of GEFS+ patients (−.700, $p = .004$).

3.5 | PTVs in GEFS+ cohort

Ten PTVs were associated with GEFS+ (Table 2). Six of these were located within the first 100 nucleotides (nt) or regions coded by the last *SCN1A* exon, exon 26, which contains all regions including and between domain 4–S4 and the C-terminus (Figure 1B). A

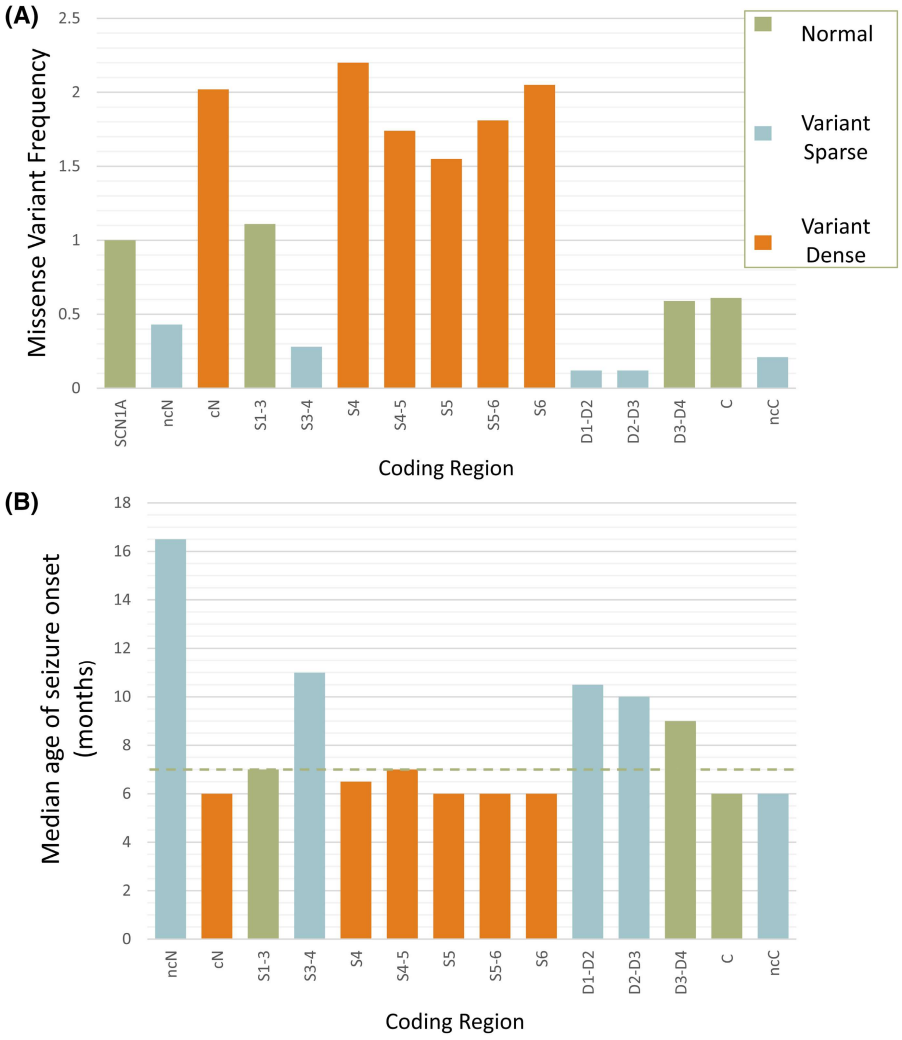


FIGURE 3 Variant density and age at seizure onset by coding region. (A) Bar chart displaying the missense variant frequency ratio associated with each coding region of the gene. The average frequency across the whole gene was defined as 1, as illustrated by the bar labeled *SCN1A* (left). (B) Bar chart displaying the median seizure onset associated with each coding region. Regions considered as having "normal" variant concentration are colored green. Variant dense regions are colored orange, and variant sparse regions are colored blue. The dotted green line in B shows the median onset of regions with "normal" variant concentration. c, conserved; C, C-terminus; N, N-terminus; nc, nonconserved.

TABLE 2 Truncating variants associated with GEFS+ phenotypes.

Variant	Phenotype	Variant type	Position	Region
p.P11TfsX18	GEFS+	PTV	11	N-Terminus
p.S128X	GEFS+	PTV	128	N-Terminus
p.Y545LfsX2	GEFS+	PTV	545	Domain 1–Domain 2
p.R862X	GEFS+	PTV	862	Domain 2–S4
p.R1636X	GEFS+	PTV	1636	Domain 4–S4
p.R1636X	GEFS+	PTV	1636	Domain 4–S4
p.W1812X	GEFS+	PTV	1812	C-Terminus
p.R1912X	GEFS+	PTV	1912	C-Terminus
D2003EfsX2	GEFS+	PTV	2003	C-Terminus
ex2-ex4del	GEFS+	PTV	N/A	N/A

Note: Summary of variant data for all truncating variants with GEFS+ phenotypes.

Abbreviations: GEFS+, genetic epilepsy with febrile seizures plus; N/A, not applicable; PTV, protein-truncating variant.

significantly larger proportion of PTVs with associated GEFS+ phenotypes were found within the <100 nt/last exon region (6/62, 9.7%, 95% confidence interval [CI] = 3.6–19.9) than the rest of the protein (3/303, 1.0%, 95% = CI .2–2.9, $p < .001$).

3.6 | Phenotypic features among patients with identical variants

The cohort contained 14 variants that were found in five or more patients, often from the same family.

We compared the variability in age at seizure onset between those who shared the same identical variant and those with different variants. We distinguished variants associated with DS only, those associated with other GEFS+ phenotypes only (GEFS+), and those associated with both DS and other GEFS+ phenotypes (Mixed). SD of age at seizure onset for the entire DS cohort was 2.9 months ($n = 823$). The IQR of onset for the entire other GEFS+ cohort was 11 months. These were considered standard values of seizure onset variability within DS only and other GEFS+/Mixed phenotypes, respectively. All identical DS variant carrier groups exhibited SDs in seizure onset age of <2.9 (Table 3). All identical other GEFS+/Mixed variant carrier groups exhibited IQRs of <11. Identical DS only variant carriers exhibited less variability in age at onset than nonidentical DS variant carriers (1.9 vs. 2.9 months, $p = .001$). Identical other GEFS+/Mixed identical variant carriers also showed reduced variability in age at onset compared to nonidentical other GEFS+ variant carriers (8 vs. 11 months, $p = .043$).

3.7 | Status epilepticus as presenting seizure type

Data for status epilepticus as first seizure were reported in 299 patients (278 DS, 21 GEFS+). A significantly higher proportion of patients with DS presented with status epilepticus as first seizure (91/278, 32.7%) compared with patients with GEFS+ (1/21, 4.8%, $\chi^2 = 7.17$, $df = 1$, $p = .007$). The predictive and diagnostic value of status epilepticus as first seizure as an indicator for a DS phenotype in *SCN1A*-positive epilepsies was as follows: specificity = 95.2% (76.2–99.9), sensitivity = 32.7% (27.7–38.6), negative predictive value = 9.7% (6–14.5), positive predictive value = 98.9% (94.1–100).

4 | DISCUSSION

This large cohort study of patients with *SCN1A* variants provides evidence of association between gene variant features and phenotype. This includes differences in age at seizure onset associated with different variant types, identification of specific regions within *SCN1A* associated with specific presentations, and initial status epilepticus as a predictive phenotypic marker for DS. We interrogated the value of in silico variant scores as predictors of phenotype.

Age at seizure onset is both earlier and less variable for patients with truncating variants compared to missense variants. These findings are thought to be explained by the differences in disease-causing mechanisms associated with each variant type. PTVs result in nonfunctional alleles causing haploinsufficiency consistent with complete LOF.[12] Recent functional studies show *SCN1A* missense variants produce various altered states of functionality that are likely to affect severity of the phenotype. Variants associated with nonmeasurable

TABLE 3 Identical variant carriers.

Variant	Patients, *n*	Phenotype	Mean/*median* seizure onset, months	SD/*IQR*
p.R101Q	7	DS	5.4	1.7
p.R101W	13	DS	5.4	1.3
p.H127D	5	Mixed	*24*	*8*
p.R393H	7	DS	6	2.2
p.R859H	5	GEFS+	*12*	*1*
p.T875M	7	GEFS+	*12*	*10.5*
p.T875K	14	Mixed	*14.5*	*10.8*
p.R931H	6	DS	4.8	1.4
p.R946H	7	DS	5.9	2.6
p.R946C	6	Mixed	*6.5*	*6.1*
p.R1596C	7	Mixed	*6*	*7*
p.M1780T	5	DS	*8*	2.5
p.A1783V	8	DS	5.4	1.7
p.A1783T	6	DS	6.2	2.5

Note: Summary of identical variant data. Variants associated with DS only are reported with mean and SD. Variants associated with other GEFS+ and Mixed (DS and other GEFS+ phenotypes) phenotypes are reported with median and IQR.

Abbreviations: DS, Dravet syndrome; GEFS+, genetic epilepsy with febrile seizures plus; IQR, interquartile range.

sodium currents (i.e., complete LOF) are associated with severe phenotypes, whereas variants with reduced but measurable current density, and changes in polarization and activation properties (i.e., partial LOF) are associated with milder phenotypes.[13,14] The increased variance of phenotypic features associated with missense variants within this cohort may be explained by differences in the degree of LOF caused by these pathogenic variants.

Missense variants occur at higher frequencies in regions coding for important functional segments of the SCN1A protein.[11,17] We confirm that these variants broadly cluster in the four functional domains with increased clustering in regions S4–S6. S4 is highly conserved and functions as the voltage sensor, whereas the S5–S6 regions are pore-lining and are responsible for ion selectivity.[32] Our data add that collectively missense variants affecting these regions present with earlier seizure onset compared with those affecting other regions of the protein. Furthermore, these findings may also indicate a previously overlooked functional importance of the conserved N-terminus with this coding region, as this region is associated with increased pathogenic variant density, earlier age at onset, and increased proportion of DS phenotypes equivalent to regions of already well-established importance. A Japanese cohort recently demonstrated clustering within this region of the N-terminus.[17] Those missense variants occurring in functionally less important areas, the D1–D2 and D2–D3 linkers and nonconserved terminus regions, had a higher chance of being associated with GEFS+ phenotypes and later seizure onset. It is of note that the D3–D4 linker region displayed both higher variant density and lower age at onset when compared to D1–D2 and D2–D3 linkers. The D3–D4 linker region of *SCN1A* codes for part of the functionally important inactivation gate of the sodium channel and has been previously linked with gain-of-function variants in patients with familial hemiplegic migraine type 3.[33,34] This may explain why this specific linker region exhibits pathogenicity similar to that of functionally more important gene regions (S1–S3 and conserved C-terminus).

The association of milder GEFS+ phenotypes with severe truncating variants is surprising, as these are expected to lead to complete LOF and a severe DS phenotype. So far, this has only rarely been reported. Mosaic variants are thought to account for approximately 7.5% of *SCN1A* epilepsies.[35] Mosaic variants are associated with milder disease and may provide an explanation for the occurrence some of these GEFS+ phenotypes, although access to these genetic data for confirmation was not available (apart from the Dutch cohort). We observed that GEFS+-associated PTVs were nearly 10 times more likely to be found in the regions coded by exon 26 or first 100 nt. NMD, the mechanism responsible for haploinsufficiency in PTVs, has been shown to exhibit reduced efficiency within these regions.[36] NMD is least efficient for stop codon variants found within the last exon and first 100 nt of a gene. Variants in these regions may result in the production of proteins due to a faulty NMD process. These proteins may retain some functionality, which could translate into a mitigation of haploinsufficiency. Additionally, NMD efficiency decreases the further downstream from the last exon junction a stop codon variant is located. This could apply to *SCN1A*, where exon 26 is exceptionally long, and may explain why toward the extreme end of the gene and exon there appears to be a drop-off in disease-associated PTVs entirely. Only one variant within this cohort was identified after position 1930, and this was associated with a GEFS+ phenotype. The increasing frequency of milder GEFS+ phenotypes within the region in question may indicate a trend toward more benign PTV-associated phenotypes within the last exon. This drop in truncating variant frequency in the distal C-terminus may indicate the point where NMD functionality is considerably reduced. Furthermore, a similar pattern can be observed around these regions in the missense variant carrier cohort. Within the nonconserved N-terminus coding region, only four missense variants were identified, all of which were associated with GEFS+ phenotypes. Additionally, after position 1928, only two missense variants (one DS, one GEFS+) were identified, supporting the idea that variants in these functionally less important regions are rarely associated with severe phenotypes for both variant types. Further functional studies regarding truncating variants found in the last exon and <100 nt regions could provide evidence of their partially/fully rescued functional status. It is also conceivable that the real effect of the change is different from an assumed theoretical one, and could for example be associated with a nonsense-mediated altered splicing event that would restore the phase.

A lack of similarity in anecdotal clinical reports associated with identical *SCN1A* variants might suggest that *SCN1A*-related epilepsies lack strong genotype–phenotype associations. Our large sample provided us with a sufficient number of identical variants to test this hypothesis. Using age at seizure onset as our parameter of comparison, all "identical variant" groups within this cohort exhibited less variability in age at seizure onset compared with phenotypic controls (DS and GEFS+). However, we still observed phenotypic discrepancy, although reduced, among some identical variant carriers, which emphasizes the complexity of variant prediction. Recent studies have shown that some genes

exhibit disease-modifying effects on *SCN1A*-related epilepsies.[37–39] Further studies of identical variant carrier cohorts with divergent phenotypes could prove useful in the identification of modifier genes and genetic background effects.

If easily accessible in silico and biochemical variant scores determined by variant characteristics can show associations to phenotype or severity of disease, they could act as clinical tools in outcome prediction of patients presenting with *SCN1A*-related epilepsy. Current studies have proven the efficacy of tools such as CADD and REVEL in distinguishing pathogenic from benign *SCN1A* variants, but evidence linking them to severity and epilepsy type is lacking.[13] The recently developed *SCN1A* prediction model provided evidence to support its further ability to accurately predict the associated phenotype at onset.[27] However, prediction outputs complement rather than replace the clinical diagnostic workup.

SCN1A-related epilepsies can prove difficult to differentiate at initial presentation. Status epilepticus has long been associated with DS and *SCN1A*-related epilepsies.[40,41] For the subcohort of individuals where we had access to initial seizure type data, approximately one third of DS patients presented with status epilepticus compared with only 5% of GEFS+ patients. Our findings show that status epilepticus as initial presentation is a nonsensitive but highly specific and predictive feature of DS. This provides clinicians with the ability to more confidently diagnose DS phenotypes in *SCN1A*-positive individuals based on presenting seizure type.[6] Earlier identification of DS allows clinicians to make more informed treatment choices, communicate the diagnosis and prognosis with patient families/carers, and in the future, identify patients who may benefit from targeted therapies early.

The findings presented in this study must be contextualized by certain limitations. This retrospective cohort only included individuals who had been referred for genetic testing or for research purposes, which could impart bias. Our study contained a cohort with a vast number of DS patients compared to GEFS+ patients. With respect to variant types, we found missense variants were responsible for slightly more than half of DS phenotypes, with the remainder made up of truncating variants. GEFS+ phenotypes result largely from missense variants, with a small minority being truncating. These proportions were reflected by those shown in previously reported smaller cohort studies, strengthening the applicability of findings on a population scale.[11,17] It should be noted that patient data were obtained from research centers based in countries with predominantly Caucasian ethnicity and therefore may not entirely reflect differences among other ethnic groups. Additionally, due to the milder nature of disease observed in other GEFS+ phenotypes, it is likely that fewer patients will undergo molecular genetic testing. Hence, it is possible that the true population proportion of *SCN1A*-associated GEFS+ is higher than that reported in this and other similarly constructed cohorts.

5 | CONCLUSIONS

This study extends our current knowledge of genotype–phenotype associations in *SCN1A*-related epilepsies. This includes findings regarding the earlier disease onset in patients with missense variants in important functional regions, the occurrence of GEFS+ truncating variants, the similarity of identical variants, and the value of in silico variant prediction scores. The identification of highly specific early disease features such as status epilepticus aids early diagnosis and may help shape treatment choice in the early stages of disease.

AUTHOR CONTRIBUTIONS

Declan Gallagher: Original draft preparation (lead); writing–review & editing; data curation; formal analysis (lead); visualization; validation. **Eduardo Pérez-Palma**: Writing–review & editing; data curation; formal analysis; visualization; validation. **Tobias Bruenger**: Writing–review & editing; data curation; formal analysis; visualization; validation. **Ismael Ghanty**: Investigation; writing–review & editing. **Eva Brilstra**: Investigation; writing–review & editing. **Berten Ceulemans**: Investigation; writing–review & editing. **Nicole Chemaly**: Investigation; writing–review & editing. **Iris de Lange**: Investigation; writing–review & editing. **Christel Depienne**: Investigation; writing–review & editing. **Renzo Guerrini**: Investigation; writing–review & editing. **Davide Mei**: Investigation; writing–review & editing. **Rikke S. Møller**: Investigation; writing–review & editing. **Rima Nabbout**: Investigation; writing–review & editing. **Brigid M. Regan**: Investigation; writing–review & editing. **Amy L. Schneider**: Investigation; writing–review & editing. **Ingrid E. Scheffer**: Investigation; writing–review & editing. **An-Sofie Schoonjans**: Investigation; writing–review & editing. **Joseph D. Symonds**: Writing–review & editing. **Sarah Weckhuysen**: Investigation; writing–review & editing. **Sameer M. Zuberi**: Investigation; writing–review & editing. **Dennis Lal**: Writing–review & editing; supervision; formal analysis; visualization; validation. **Andreas Brunklaus**: Project administration (lead); conceptualization; supervision; writing–review

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CONFLICT OF INTEREST STATEMENT

A.B. has received honoraria for presenting at educational events, advisory boards, and consultancy work for Biocodex, Encoded Therapeutics, GW Pharmaceuticals, Nutricia, Stoke Therapeutics, and Zogenix. E.P.-P. has received honoraria for consultancy work for the Friends of FACES Foundation and a grant from Agencia Nacional de Investigacion y Desarrollo of Chile (grant PAI77200124) and the FamilieSCN2A foundation (2020 Action Potential Grant). B.C. has received research funding from Brabant and Zogenix, has served as a consultant for Brabant and Zogenix (patent ZX008), and with the KU Leuven University/Antwerp University Hospital may benefit financially from a royalty arrangement that is related to this research if Zogenix is successful in marketing its product, fenfluramine. N.C. has received honoraria from Nutricia and Eisai for presentations at symposia. R.G. has acted as an investigator for studies with Zogenix, Biocodex, BioMarin, UCB, Angelini, and Eisai; has been a speaker and on advisory boards for Zogenix, Biocodex, Novartis, BioMarin, GW Pharmaceuticals, and Biocodex; serves/has served on the editorial boards of *Epilepsia*, *Progress in Epileptic Disorders*, *Neuropediatrics*, *Journal of Child Neurology, Seizure*, *BMC Medical Genetics*, *Topics in Epilepsy*, and *Neurology*; and receives/has received research support from the Italian Ministry of Health, the European Community, the Tuscany Region, the Mariani Foundation, the Pisa Foundation, the Fund of Epilepsy, the GKT Special Trustees, the Italian Federation for Epilepsy, and the Italian Association for Epilepsy. R.N. has received research funding from the European Union (Seventh Framework Program), EJP-RD (European Joint Program for Rare Diseases), Shire, Zogenix, GW Pharmaceuticals, and Eisai and consultation and lecturer fees from Eisai, Zogenix, Takeda, GW Pharmaceuticals, Advicenne, Biocodex, Nutricia, Supernus, Biogen, and Novartis. I.E.S. has served on scientific advisory boards for UCB, Eisai, GlaxoSmithKline, BioMarin, Nutricia, Rogcon, Chiesi, Encoded Therapeutics, and Xenon Pharmaceuticals; has received speaker honoraria from GlaxoSmithKline, UCB, BioMarin, Biocodex, and Eisai; has received funding for travel from UCB, Biocodex, GlaxoSmithKline, BioMarin, and Eisai; has served as an investigator for Zogenix, Zynerba, Ultragenyx, GW Pharmaceuticals, UCB, Eisai, Anavex Life Sciences, Ovid Therapeutics, Epigenyx, Encoded Therapeutics, and Marinus; has consulted for Zynerba Pharmaceuticals, Atheneum Partners, Ovid Therapeutics, Care Beyond Diagnosis, Epilepsy Consortium, and UCB; may accrue future revenue on pending patent WO61/010176 (filed 2008: Therapeutic Compound); has a patent for SCN1A testing held by Bionomics and licensed to various diagnostic companies; and has a patent on a molecular diagnostic/theranostic target for benign familial infantile epilepsy ([PRRT2] 2011904493 and 2012900190 and PCT/AU2012/001321 [TECH ID:2012–009]). S.W. has received speaker and consultancy fees from UCB, Xenon, Zogenix, Lundbeck, and Biocodex. S.M.Z. has received honoraria for presenting at educational events, advisory boards, and consultancy work for GW Pharmaceuticals, Zogenix, Biocodex, Encoded Therapeutics, Stoke

Therapeutics, and Nutricia. D.L. has received honoraria for advisory board work for Encoded Therapeutics. The remaining authors have no conflicts of interest. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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