

## Idiopathic Epilepsies with Seizures Precipitated by Fever and *SCN1A* Abnormalities

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**Summary:** *Purpose:* *SCN1A* is the most clinically relevant epilepsy gene, most mutations lead to severe myoclonic epilepsy of infancy (SMEI) and generalized epilepsy with febrile seizures plus (GEFS+). We studied 132 patients with epilepsy syndromes with seizures precipitated by fever, and performed phenotype–genotype correlations with *SCN1A* alterations.

*Methods:* We included patients with SMEI including borderline SMEI (SMEB), GEFS+, febrile seizures (FS), or other seizure types precipitated by fever. We performed a clinical and genetic study focusing on *SCN1A*, using dHPLC, gene sequencing, and MLPA to detect genomic deletions/duplications on SMEI/SMEB patients.

*Results:* We classified patients as: SMEI/SMEB = 55; GEFS+ = 26; and other phenotypes = 51. *SCN1A* analysis by dHPLC/sequencing revealed 40 mutations in 37 SMEI/SMEB (67%) and 3 GEFS+ (11.5%) probands. MLPA showed genomic deletions in 2 of 18 SMEI/SMEB. Most mutations were de novo

(82%). SMEB patients carrying mutations (8) were more likely to have missense mutations (62.5%), conversely SMEI patients (31) had more truncating, splice site or genomic alterations (64.5%). SMEI/SMEB with truncating, splice site or genomic alterations had a significantly earlier age of onset of FS compared to those with missense mutations and without mutations ( $p = 0.00007$ , ANOVA test). None of the remaining patients with seizures precipitated by fever carried *SCN1A* mutations.

*Conclusion:* We obtained a frequency of 71% *SCN1A* abnormalities in SMEI/SMEB and of 11.5% in GEFS+ probands. MLPA complements DNA sequencing of *SCN1A* increasing the mutation detection rate. SMEI/SMEB with truncating, splice site or genomic alterations had a significantly earlier age of onset of FS. This study confirms the high sensitivity of *SCN1A* for SMEI/SMEB phenotypes. **Key Words:** SMEI—GEFS+—*SCN1A*—Fever-provoked seizures—MLPA.

Mutations in the gene coding for the  $\alpha 1$  subunit of the neuronal sodium channel (*SCN1A*) have been associated with various types of epilepsy. *SCN1A* abnormalities have been found in about 5–10 % of generalized

epilepsy with febrile seizures plus (GEFS+) families, but the overwhelming majority of known *SCN1A* mutations lead to severe myoclonic epilepsy of infancy (SMEI) including borderline SMEI (SMEB). From 35% to 100% of SMEI/SMEB patients carry *SCN1A* mutations that are de novo in most (Mulley et al., 2005).

Clinical analysis of the phenotypes shows that the first clinical manifestations of mutations in *SCN1A* are recurrent, often prolonged, seizures provoked by fever in infancy. This association suggests that, in clinical prac-

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tice, mutations in *SCN1A* should be suspected in every child with new onset, recurrent seizures precipitated by fever.

With the aim of better defining the spectrum of phenotypes that might be associated with *SCN1A* mutations, we performed a clinical and genetic study focusing on *SCN1A* screening in patients in whom fever was a precipitant factor for seizures. We studied three subgroups of patients with: (1) SMEI/SMEB, (2) GEFS+, and (3) a group of patients in whom febrile seizures (FS) or seizures precipitated by fever had occurred although they were not characteristic of their epilepsy syndrome.

To increase the power of detecting *SCN1A* abnormalities we applied a new technique named multiplex ligation-dependent probe amplification (MPLA), searching for genomic abnormalities that are not identifiable with DNA sequencing.

## METHODS

### Patients recruitment

We recruited subjects with a clinical diagnosis of SMEI/SMEB, GEFS+ and either focal or generalized idiopathic epilepsies whose seizures were increased during febrile episodes as well as patients with classical FS. Patients were collected in an international collaborative study. Informed consent was obtained from parents or legal guardians. The study was approved by the Ethical committee of the IRCCS Stella Maris Foundation.

### Clinical assessment

Clinical history, genealogical information and family trees, EEG recordings, and MRI were collected from the parents and/or other relatives for patients seen at the Epilepsy, Neurophysiology and Neurogenetic Unit, Stella Maris Foundation, Pisa, Italy. For patients referred from other centers, medical records were collected and reviewed.

Epileptic seizures and epilepsy syndrome diagnoses were performed, prior to the molecular genetic study, according to the clinical criteria established by the International League Against Epilepsy (ILAE, 1981; 1989; Engel, 2001). Classical SMEI was diagnosed following the criteria described by Dravet et al. (2002). The term SMEB was used for cases without a number of the key features of SMEI (Oguni et al., 2001; Dravet et al., 2002; Fukuma et al., 2004).

GEFS+ phenotypes were diagnosed following the clinical criteria described by Scheffer and Berkovic 1997 (Scheffer and Berkovic, 1997; Singh et al., 1999). By definition, a diagnosis of GEFS+ requires a positive family history. However, patients with FS extending beyond 6 years and/or with afebrile seizures (FS+) without a family history were also included as *de novo* mutations are known to occur in familial epilepsies (Phillips et al.,

2000). Myoclonic astatic epilepsy (MAE) was considered as sporadic if no other family members had seizures or it was included in the GEFS+ spectrum when occurring in families. Patients in whom, despite clinical information, a diagnosis could not be made, were designated as “unclassified epilepsy.” Outside SMEI and GEFS+ syndromes, fever was considered as an important provoking factor when the patients’ habitual seizures increased by at least 50% during febrile episodes.

### Molecular analysis

The coding regions of the *SCN1A* gene were analyzed by denaturing high performance liquid chromatography (dHPLC) (Transgenomic Inc., Cheshire, U.K.). Abnormal profiles observed on dHPLC screening were subsequently sequenced (ABI3100*avant*; Applied Biosystems, Foster City CA, U.S.A.).

### Multiplex ligation-dependent probe amplification assay (MPLA)

SMEI/SMEB patients without *SCN1A* mutations upon dHPLC or sequencing were analyzed with MLPA assay, aiming to uncover genomic deletions/duplications. The MLPA *SCN1A* kit (SALSA P137, MRC-Holland, Amsterdam, The Netherlands) contains 25 paired-probes from the *SCN1A* region (covering 25/26 exons) and 14 control probes specific for other chromosomes. Screening was performed according to the manufacturer’s protocol. MLPA products were separated on an automated sequencer (Applied Biosystems, Foster City CA, U.S.A.) and sized using ROX-500 (Applied Biosystems, Foster City CA, U.S.A.). Peaks corresponding to *SCN1A* exons and control probes were identified according to their size. Peak areas were exported into an Excel Spreadsheet and normalized as previously reported (Hogervorst et al., 2003). Values of 1.7–2.3 indicate normal results whereas, values <1.7 or >2.3, indicate a deletion or a duplication. Alterations were confirmed with a second MLPA reaction.

### Genotype–phenotype correlations

Based on epilepsy syndromes, the 132 patients were divided into: (1) SMEI/SMEB, (2) GEFS+, and (3) other phenotypes. A clinical analysis focused on seizure types and age of seizures onset, with particular attention to seizures precipitated by fever, was performed in the three groups. Based on previous studies of genotype–phenotype correlations (Ohmori et al., 2003; Ceulemans et al., 2004; Fukuma et al., 2004) we further subdivided SMEI/SMEB probands in three subgroups according to *SCN1A* analysis: (a) truncating, splicing alterations, and genomic alterations, (b) missense mutations, and (c) no genomic alterations. ANOVA test was used for statistical analysis to evaluate differences of age of seizure onset between the three groups.

To evaluate the existence of hotspot regions, where mutations of *SCN1A* are more likely to fall, we compared the number of the identified mutations in each exon (including 10 nucleotides up and down stream the exon) to the number of expected mutations using the Fisher's exact test. We performed the same test to evaluate the location of *SCN1A* mutations according to their coding protein domain: N-terminal + DI; Loop 1; DII; Loop 2; DIII; Loop 3 and DIV + C-term.

## RESULTS

A total of 132 probands, 74 females and 58 males were studied. The mean age at the time of the study was 9 years (median 6.5 ranging from 1 to 38). Epilepsy phenotypes of the 132 probands are shown on Table 1.

### *SCN1A* analysis

#### (a) dHPLC/sequencing

dHPLC analysis and sequencing of *SCN1A* in the 132 patients revealed 40 mutations: 19 missense, 5 nonsense, 10 frameshift, 4 splice site mutations (one identified twice) and two silent nucleotide substitutions that in silico seemed to modify the mRNA splicing process (Fig. 1A and B). Molecular analysis of the 68 available parents showed that 28 mutations were de novo and 6 were inherited (two truncating and four missense mutations) (Tables 2 and 3, supplementary material). The position of the missense mutations within the *SCN1A* protein is shown in Fig. 2.

**TABLE 1.** Epilepsy phenotypes of the 132 patients screened for *SCN1A*

Syndromic phenotypes	No. of patients	<i>SCN1A</i> alterations
1) SMEI/SMEB	55	39 (71%)
2) GEFS+ spectrum	26	3 (11.5%)
3) Other phenotypes		
Sporadic MAE	3	0
FS	10	0
CAE	7	0
CAE > JME	2	0
JAE	2	0
JME	1	0
IGE-TCS	2	0
Benign infantile convulsions	2	0
Atypical BRE	1	0
Idiopathic fever-sensitive focal epilepsy	3	0
Uncl epilepsy with fever-sensitive sz	18	0
Total	132	42 (32%)

BRE, benign rolandic epilepsy; CAE, childhood absence epilepsy; FS, febrile seizures; IGE-TCS, idiopathic generalized epilepsy with tonic-clonic seizures; GEFS+, generalized epilepsy with febrile seizures plus; JME, juvenile myoclonic epilepsy; JAE, juvenile absence epilepsy; MAE, myoclonic astatic epilepsy; SMEI, severe myoclonic epilepsy of infancy; SMEB, borderline severe myoclonic epilepsy of infancy; sz, seizures; Uncl, unclassified.

#### (b) Multiplex ligation-dependent probe amplification (MLPA)

MLPA analysis revealed genomic deletions of *SCN1A* in 2 of 18 patients with SMEI/SMEB (11%) without *SCN1A* mutations on dHPLC and/or sequencing. The first patient carried a deletion of the entire *SCN1A* gene, in the second patient exons 12 to 14 were deleted (Table 2, supplementary material). Both patients had classical SMEI.

### Distribution of the *SCN1A* alterations among the epilepsy syndromes

#### (1) Severe myoclonic epilepsy of infancy (SMEI/SMEB)

We studied 55 patients (27 females and 28 males) with a clinical diagnosis of SMEI (40 patients) and SMEB (15 patients). Mean age at the time of the study was 9.5 years (median 8.6  $\pm$  5.1, ranging from 2.5 to 20.8). Among the 55 patients with SMEI/SMEB, 39 (71%) had *SCN1A* abnormalities.

#### (a) *SCN1A* truncating mutations, splicing alterations, or genomic alterations.

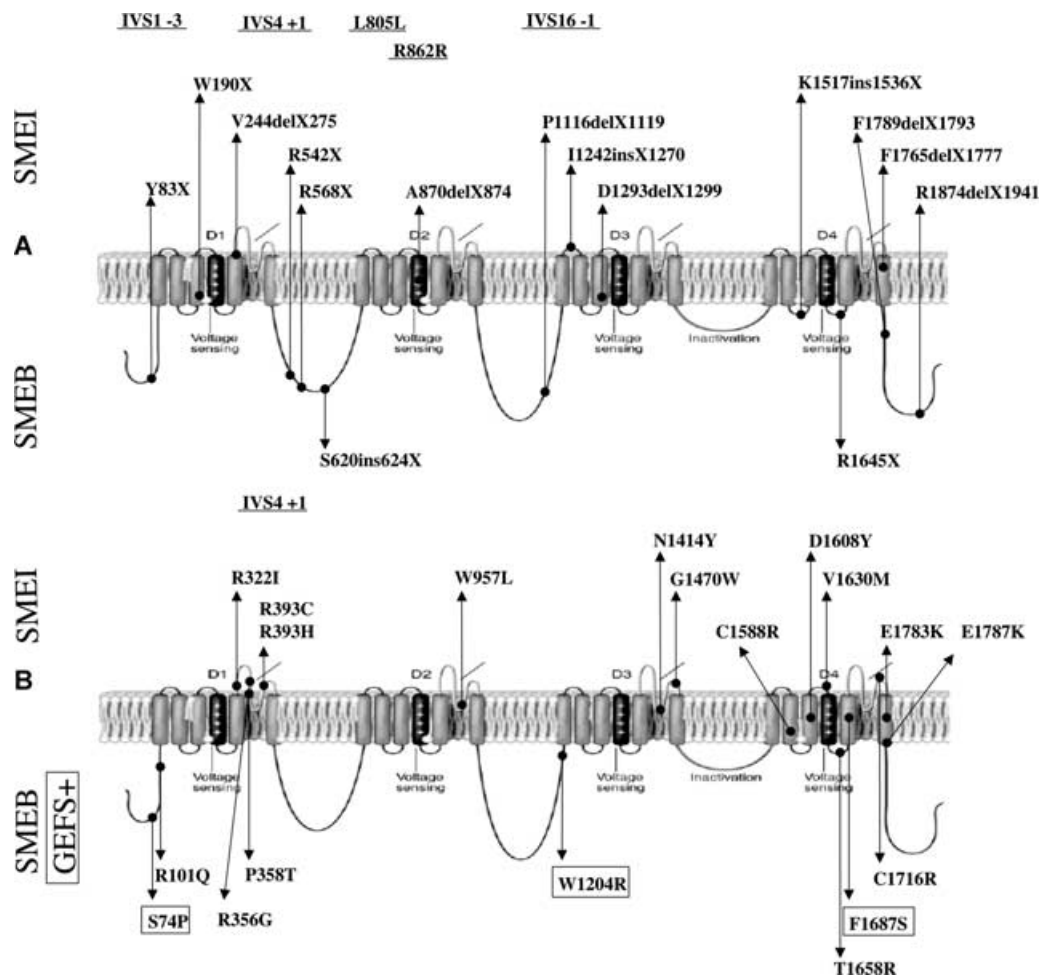
*SCN1A* truncating, splice site or genomic alterations were identified in 23 patients (59% of patients with mutations) of whom 20 had classical SMEI and 3 had SMEB (Fig. 1A). All patients had recurrent FS and in most of them FS was the first clinical expression occurring between the fourth to fifth months of life (mean 4.6 months; median 4; range 2–9). All patients had mild-to-severe cognitive impairment. A family history for seizures, including FS, was present in 11 (48%) probands and in one of them there was a bilineal occurrence of seizures. The sister of a patient with SMEI had a clinical history also consistent with SMEI. Their father had FS from infancy to childhood. (Patients' clinical details are reported on Table 2, supplementary material).

#### (b) *SCN1A* missense mutations

Missense mutations were found in 16 patients (43% of patients with mutations) (Fig. 1B). A diagnosis of SMEI was made in 11 probands, whereas five had SMEB. Recurrent, often prolonged, FS were seen in all patients, with a mean age at onset of 6.7 months (median 7; range 3–12). All patients had mild-to-severe cognitive impairment. A family history of seizures, including FS, was present in 9 of 16 (56%) patients, and in 2 of 9 there was a bilineal occurrence of seizures. (Patients' clinical details are reported on Table 3, supplementary material)

#### (c) Without *SCN1A* mutations

Molecular analysis did not reveal *SCN1A* mutations in 16 patients with SMEB (7) and SMEI (9). FS beginning around age of 10.7 months (median 9.5; range 4–24 months) occurred in all patients. Borderline cognitive functions were seen in five patients, and the remaining 11 had mild-to-severe cognitive impairment. A family history



**FIG. 1.** (A) Graphic representation of the SCN1A protein showing the location of the truncating and splice site mutations. In the upper part of the figure are reported mutations leading to truncated SCN1A protein and to mRNA splicing alteration (underlined), associated with severe myoclonic epilepsy of infancy; in the lower part are reported the truncating mutations associated with borderline severe myoclonic epilepsy of infancy (SMEB). (B) Graphic representation of the SCN1A protein showing the location of the missense mutations. In the upper part of the figure are reported missense mutations associated with severe myoclonic epilepsy of infancy (SMEI); in the lower part are reported the missense mutations associated with borderline severe myoclonic epilepsy of infancy (SMEB) and with generalized epilepsy with febrile seizures plus (GEFS+).

of seizures, including FS, was present in 10 (62.5%) probands.

(2) Generalized epilepsy with febrile seizure plus (GEFS+) or FS+.

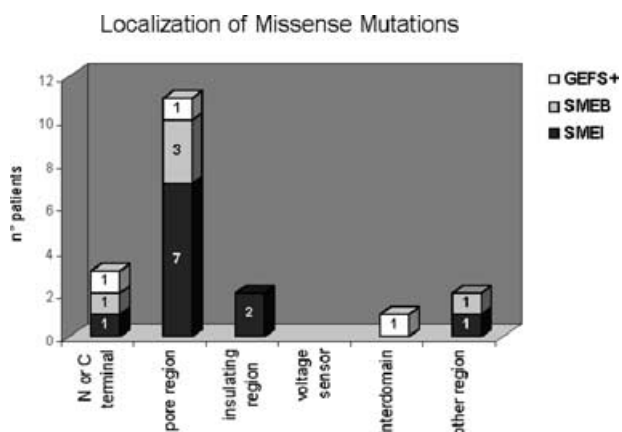
A diagnosis of GEFS+ was made in 26 patients (15 females and 11 males) aged from 1 to 26 years (mean 8; median 6.5). FS occurred in all patients with a mean age at onset of 17 months (median 12, range 5–60). Afebrile tonic-clonic seizures occurred in 19 patients with mean age at onset of 43 months (median 19; range 3–108 months). Other seizure types included: myoclonic (7 patients), absences (2 patients), myoclonic-astatic (6 patients), and partial seizures (9 patients). A family history for seizures, including FS, was present in 25.

Of the 26 patients with GEFS+ spectrum, 3 (11.5%) harbored SCN1A mutations (Fig. 1B). The available fam-

ily history of these three patients was limited to the parents-proband triad. In the first proband with FS+ and a left malformed hippocampus, the SCN1A mutation was inherited from the father who had classical FS. In the third proband with MAE the mutation was transmitted from the mother with FS+. The second proband harbored a de novo mutation, no family history of seizures was detected (Clinical details of the 3 patients are reported on Table 4, supplementary material).

(3) Other phenotypes

Classical febrile seizures (Table 1) were diagnosed in 10 patients; mean age of FS onset was 15 months (median 12.5; range 8–31). One patient had right hippocampal sclerosis (HS) on MRI and a mild cognitive deficit. A family history of epilepsy was detected in nine probands. None had SCN1A mutations.



**FIG. 2.** Graphic representation showing the location of the missense mutation according to protein domains: N or C terminal; pore region; insulating region; voltage sensor; interdomain and other regions. Within protein domains, mutations have also been subdivided according to the phenotype: white for GEFS+, gray for SMEB, and black for SMEI.

*Idiopathic generalized epilepsies* (Table 1) with antecedents of FS were documented in 14 patients, none carrying *SCN1A* mutations. A family history of epilepsy, including FS, was detected in 11 of 14 probands. Three patients had *sporadic myoclonic atstatic epilepsy* and *SCN1A* screening was negative.

The remaining 24 patients had *various epilepsy phenotypes* (Table 1) and most (18), despite clinical and EEG information, could not be classified. These patients had either focal or generalized seizures, or both, which exhibited increase in frequency (>50%) during febrile episodes. Mean age at seizure onset was 20 months (median 17, range: neonatal period: 84 months). A family history of epilepsy was noticed in six probands, in five patients genealogical information was not available. None had *SCN1A* mutations.

### Genotype–phenotype correlations

Age at onset of FS showed a highly significant difference ( $p = 0.00007$ ; ANOVA test) between the three groups of SMEI/SMEB patients with: (a) *SCN1A* truncating mutations, splicing alterations, or genomic alterations, (b) *SCN1A* missense mutations, and (c) no *SCN1A* abnormalities. Patients with truncating mutations had an earlier onset, around the fifth month of age, those with missense mutations had a later onset of FS, around the seventh month, and finally, individuals without *SCN1A* mutations had an age of onset around the tenth month of life. For the remaining seizure types—febrile and afebrile status, afebrile tonic–clonic or hemiclonic, myoclonic, absence and partial seizures—there were no significant differences in age at seizure onset between the three groups.

The statistical analysis, comparing the number of identified mutations in each exon to the number of expected mutations, did not show significant differences between exons ( $p > 0.05$ ) (Fisher's exact test). The highest number of mutations (10/40) was found in exon 26. These data however, did not reach a significant  $p$ -value because exon 26 is a large exon, therefore carrying a higher chance of being hit by mutations. Likewise, when performing the same analysis grouping the exons according to their coding protein domain (N-terminal + DI; Loop 1; DII; Loop 2; DIII; Loop 3 and DIV + C-term) there were no significant differences between domains ( $p > 0.05$ ).

## DISCUSSION

### Mutation rates

In our cohort of 132 patients with idiopathic epilepsies with seizures precipitated by fever in infancy and early childhood, classical SMEI was diagnosed in 40 (30%), SMEB in 15 (11%), and GEFS+ in 26 (19.6%). Mutations and genomic deletions of *SCN1A* were identified in 31 patients (77.5%) with classical SMEI and in 8 (53%) with SMEB. We obtained an overall frequency of 71% *SCN1A* abnormalities patients, which is intermediate between the lowest and the highest previously reported percentages for SMEI/SMEB (35–100%) (Claes et al., 2001; Ohmori et al., 2002; Fujiwara et al., 2003; Nabbout et al., 2003; Sugawara et al., 2002; Wallace et al., 2003). It is likely that some differences in detection rate can be attributed to ascertainment bias due to controversial aspects in the diagnosis of SMEI. We based our clinical classification predominantly on age of onset and on the occurrence of prolonged hemiclonic or generalized seizures occurring with fever. The results of *SCN1A* analysis supported our diagnostic criteria. The 31 SMEI patients carrying mutations, manifested FS at onset, followed by febrile or afebrile seizures evolving into status epilepticus in most, whereas myoclonic seizures were noticed only in 27 of 40 (67.5%) patients with mutations.

### Genomic deletions of *SCN1A* in SMEI/SMEB

MLPA, performed in 18 SMEI/SMEB patients, showed genomic deletions in two of them (11%). This finding indicates that in a subset of SMEI/SMEB patients without *SCN1A* molecular abnormality, MLPA increases the power to detect *SCN1A* alterations. Using FISH, MAQ or MLPA, *SCN1A* deletions, cryptic chromosome deletions encompassing the *SCN1A* locus have been recently identified in 8–27% of patients with SMEI (Madia et al., 2006; Mulley et al., 2006; Suls et al., 2006). MLPA is a rapid and cost-effective complement to DNA sequencing, increasing the overall mutation detection rate and should be considered systematically if DNA sequencing fails to demonstrate *SCN1A* mutations. The percentage

of genomic abnormalities identified with MPLA in our series of SMEI/SMEB patients is similar to previously reported studies (Madia et al., 2006; Mulley et al., 2006; Suls et al., 2006). Studies with higher number of patients are needed to determine a more accurate deletion frequency. Variability in the detection of *SCN1A* genomic abnormalities might be due to patients' ascertainment bias and to different sensitivity of the methods used to uncover genomic abnormalities. For example, the study of Madia et al. used a FISH analysis that allows the detection of deletion/duplication larger than 50 kb (Pereira et al., 2004; Madia et al., 2006). Such large genomic alterations, involve *SCN1A* and other contiguous genes producing SMEI/SMEB with additional clinical features (Pereira et al., 2004; Madia et al., 2006).

### Is SMEI/SMEB an inherited disorder?

In this study, molecular analysis of the 32 SMEI/SMEB probands in whom parents' DNA was available, revealed that 28 mutations were de novo (86%), whereas only 4 (14%) were inherited (one truncating, one splice site, and two missense mutations). The inherited truncating mutation was transmitted from a father who had a single seizure (D1293delX1299 mutation). Literature review shows only one other family in which *SCN1A* truncating mutation was inherited from a mildly affected mother (Nabbout et al., 2003). The second familial *SCN1A* splice site mutation (IVS4 + 1 G>A) was also present in the proband's sister, whose phenotype was consistent with SMEI. Both sisters' had inherited the mutation from their father who had had only FS from infancy to childhood and exhibited mosaicism (Marini et al., 2006). Mosaic germline mutations of *SCN1A* have recently been identified in five additional families with SMEI probands, suggesting a possible contribution of mosaicism to individual and familial phenotypic variability (Depienne et al., 2006; Gennaro et al., 2006; Morimoto et al., 2006).

In our cohort of SMEI/SMEB probands 56% had first- or second-degree relatives with seizures, including FS, favoring the hypothesis that SMEI might be an inherited disorder (Nabbout et al., 2003; Wallace et al., 2003). However, high rates of family history of epilepsy and familial occurrence of SMEI are hard to reconcile with the finding that most *SCN1A* mutations in SMEI are de novo.

### Genotype—phenotype correlations

We observed a mild predominance of truncating, splice site, or genomic alterations (59%) over missense (41%) mutations. Patients with SMEB were more likely to have missense mutations (62.5%). Conversely, patients with classical SMEI had more truncating, splice site, or genomic alterations (64.5%). Similarly to previous studies, we found that missense mutations in the pore-forming parts S5-S6 of the channel occurred in 10 of

16 SMEI/SMEB patients (62.5%) and only in 1 of 3 of GEFS+ patients (Fig. 2).

Statistical analysis of the mean age of onset of FS showed that patients with truncating mutations had the earliest onset (5 months) followed by patients with missense mutations, having an intermediate onset (7 months), and individuals without *SCN1A* mutations, showing the latest age of onset (10 months). The difference between the three groups was statistically significant ( $p = 0.0007$ ). It would be interesting studying larger series of patients to determine whether truncating mutations, and therefore earlier FS onset, carry a higher chance of developing a more severe epileptic encephalopathy.

Only 6 of 39 of the mutations identified in our series had been previously described. The highest number of mutations (10/40) was found in exon 26, which is the largest exon. However no statistically significant  $p$ -value (Fisher's exact test) indicative of a hotspot was reached.

### GEFS+ and *SCN1A* mutations

Among GEFS+ phenotypes the rate of *SCN1A* mutations was 11.5%. The W1204R mutation identified in a child with FS+ and in his father had previously been reported (Escayg et al., 2001), and Spampinato et al. showed that this mutation causes an alteration of the voltage-dependent channel gating that could result in neuronal hyperexcitability (Spampinato et al., 2003). The Ser74Pro missense mutation identified in a patient with FS+ is the first de novo mutation described in GEFS+.

### *SCN1A* and other phenotypes

None of the patients with idiopathic epilepsies with seizures precipitated by fever besides SMEI/SMEB and GEFS+ carried *SCN1A* mutations. Therefore, *SCN1A* is not likely to cause seizures provoked by fever outside the SMEI/SMEB and GEFS+ spectrum.

## CONCLUSIONS

*SCN1A* is the most relevant epilepsy gene with the largest number of epilepsy-related mutations so far identified. The high correlation between SMEI/SMEB and *SCN1A* mutations suggests a phenotypic specificity of *SCN1A*. However, SMEI/SMEB represent a very small proportion of children with recurrent febrile seizures in a population, thus this study does not address the issue of diagnostic specificity from a population perspective. We do not know whether *SCN1A* also plays a role in other common epilepsy syndromes without febrile seizures, which were not examined at all in our study.

The high rate of epilepsy in families of probands with SMEI suggests that, in addition to *SCN1A* mutations, other genetic factors might play a role in the expression of the epilepsy phenotypes. *SCN1A* mosaic mutations should

also be taken into account, at least in some cases, as a possible explanation for familial phenotypic variability. MLPA assay might be a useful tool to uncover genomic deletions/duplications and to increase the power of discovering *SCN1A* abnormalities in SMEI/SMEB. What causes SMEI when there is no detectable *SCN1A* involvement remains to be identified.

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**SUPPLEMENTARY MATERIAL**

The following supplementary material is available for this article:

**Supplementary Table 1:** clinical information of the 23 SMEI/SMEB patients with *SCN1A* truncating, splice site or genomic alterations

**Supplementary Table 2:** clinical information of the 16 SMEI/SMEB patients with *SCN1A* missense mutations

**Supplementary Table 3:** clinical details of the 3 patients with GEFS<sup>+</sup> and *SCN1A* mutations

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