Identification of an Na\textsubscript{v}1.1 sodium channel (SCN1A) loss-of-function mutation associated with familial simple febrile seizures


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Febrile seizures (FS) affect 5–12% of infants and children up to 6 years of age. There is now epidemiological evidence that FS are associated with subsequent afebrile and unprovoked seizures in ~7% of patients, which is 10 times more than in the general population. Extensive genetic studies have demonstrated that various loci are responsible for familial FS, and the FEB3 autosomal-dominant locus has been identified on chromosome 2q23–24, where the SCN1A gene is mapped. However, gene mutations causing simple FS have not been found yet. Here we show that the M145T mutation of a well conserved amino acid in the first transmembrane segment of domain I of the human Na\textsubscript{v}1.1 channel \alpha-subunit cosegregates in all 12 individuals of a large Italian family affected by simple FS. Functional studies in mammalian cells demonstrate that the mutation causes a 60% reduction of current density and a 10-mV positive shift of the activation curve. Thus, M145T is a loss-of-function mutant. These results show that monogenic FS should also be considered a channelopathy.

channelopathy | FEB3 locus | convulsions | epilepsy | neuronal excitability

It has long been known that there is a major genetic component in the etiology of febrile seizures (FS), and an autosomal-dominant (AD) inheritance with incomplete penetrance has been proposed in large pedigrees or groups of families with FS (1). Six loci for familial FS have been reported, but no genes were identified; they have been mapped at chromosomes 8q13–21 (FEB1) (2), 19q (FEB2) (3), 2q23–24 (FEB3) (4), 5q14–15 (FEB4) (5), 6q22–24 (FEB5) (6), and 18p11.2 (FEB6) (7). FEB5 has been described as a pure FS because most of the members with FS had a long follow-up period, and the development of afebrile seizures was excluded, but pathogenic mutations have not been identified. Although it has been hypothesized that FEB2 is responsible for complex FS and later development of afebrile seizure, also FEB1, FEB3, and FEB4 are characterized by families in which many individuals developed afebrile seizures (1). For example, the locus 2q24, in which generalized epilepsy with FS plus (GEFS\textsuperscript{*}) mutations of various families have been mapped, harbors at least four voltage-dependent Na channels (Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, Na\textsubscript{v}1.3, and Na\textsubscript{v}1.7). Thus, despite these findings, specific genetic defects causing FS have yet to be identified.

In this article, we report a mutation of SCN1A cosegregating in a family whose members expressed a homogenous phenotype of simple FS that always ceased by the age of 6 years. FS segregated as an AD trait with high penetrance. We studied the functional effects of a highly conserved amino acid change that resulted a loss-of-function mutant.

Methods

Patients. The family that we studied (Fig. L4) originated in the Calabria region of Italy. Written informed consent was obtained from the patients or responsible adults as necessary, and the study was approved by the ethical committees of the research institutions. Peripheral blood samples were obtained for DNA extraction from 23 members of the family, including 12 individuals with FS. One affected member that was shown on the pedigree did not undergo venipuncture.

Linkage Analysis. Linkage analysis was performed by using the following polymorphic DNA markers to exclude reported loci for FS: D8S553, D8S1840, and D8S530 for FEB1 (2); D19S65, D19S216, and D19S951 for FEB2 (3); D2S156, D2S382, D2S330, and D2S2345 for FEB3 (4, 8–10); D5S652, D5S644, and D5S2079 for FEB4 (5); D6S1620, D6S1572, D6S262, D6S1656, and D6S474 for FEB5 (6); and D18S1153, D18S1158, and D18S53 for FEB6 (7). Two-point linkage analysis was carried out by using the MLINK program of the LINKAGE package (11). The FS phenotype was analyzed by an AD trait with high (90%) penetrance, assuming equal allele frequencies and with a disease gene frequency of 0.001. Logarithm of odds (LOD) score values were negative for FEB1, FEB2, FEB4, FEB5, and FEB6 but showed strong evidence in favor of linkage to the FEB3 locus.

Mutational Analysis. Flanking intron primers were used to amplify and sequence 26 exons of the Na\textsubscript{v}1.1 Na channel gene by using a 3100 automated DNA sequencer (Applied Biosystems).

Mutagenesis. The plasmid pCDN5-human Na\textsubscript{v}1.1 (hNav1.1) containing the cDNA for human Na\textsubscript{v}1.1 Na channel was generously donated by J. J. Clare (GlaicoSmithKline, Stevenage, Herts, U.K.). We subcloned the hNav1.1 cDNA into the plasmid pCDM8 (Invitrogen), thus obtaining the plasmid pCDM8-hNav1.1; we propagated it in MCI0161-P3 or Top10-P3 Escherichia coli (Invitrogen). Because hNav1.1 has a high spontaneous rearrangement rate when propagated in bacteria, the cells were grown at 30°C for 48 h to decrease the rearrangements, and the entire coding sequence was resequenced after each propagation. The mutation M145T was introduced into pCDM8-hNav1.1 by using the QuikChange II XL site-directed mutagenesis kit (Stratagene) and MCI0161-P3 or Top10-P3 E. coli, with the following primers: 5’-CTGTGTGTTTACCAGAATGATTTAA (forward) and 5’-TTACTCATTGTTCGTAAGCAGTGCT (reverse). Thus, we obtained the plasmid pCDM8-hNav1.1-

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Abbreviations: FS, febrile seizure(s); AD, autosomal-dominant; MTLE, mesial temporal lobe epilepsy; MTS, mesial temporal sclerosis; hNav1.1, human Na\textsubscript{v}1.1; D1S1, 51 segment of domain I; AP, action potential.

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M145T. The entire coding sequence of the mutated hNav1.1 cDNA was sequenced to confirm the presence of the introduced mutation and the absence of spurious mutations. The human clone of Na channel $\beta_1$ accessory subunit (a generous gift from Al George, Department of Medicine, Vanderbilt University, Nashville, TN), was subcloned into the bicistronic plasmid pIRES-YFP (Clontech), which expresses both the protein of interest and yellow fluorescent protein (YFP) as reporter, obtaining the plasmid pIRES-YFP-h$\beta_1$.

**Cell Culture and Transfection.** TsA-201 human embryonic kidney cells were cultured in DMEM and a mixture of Ham’s medium and F12, supplemented with 10% FBS, as described in ref. 12. The cells were transiently cotransfected with pCDM8-hNav1.1 or pCDM8-hNav1.1-M145T and pIRES-YFP-h$\beta_1$ by using Lipofectamine 2000 (Invitrogen).

**Solutions and Drugs.** The standard extracellular solution contained 140 mM NaCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$, and 10 mM Hepes-NaOH (pH 7.40). The standard pipette solution contained 130 mM N-methyl-d-glucamine (NMDG), 10 mM NaCl, 4 mM MgCl$_2$, 5 mM EGTA-KOH, and 10 mM Hepes-KOH (pH 7.30 with $\text{H}_3\text{PO}_4$). HNav1.1 generates a variable amount of persistent Na$^+$ current in different transfected cells, as reported (12). Cells that showed incomplete inactivation (a residual current of >4% of the peak Na$^+$ current after 250 ms from the beginning of the voltage step) were disregarded from the analysis. Tetrodotoxin (Sigma) that was dissolved in the extracellular solution was used at 100 nM to block Nav1.1 currents, and the resulting traces were subtracted from the control traces to obtain the tetrodotoxin-sensitive currents. The extracellular solutions were delivered through a remote-controlled nine-hole (0.6-mm) linear positioner that was placed near the cell under study and that had an average response time of 2 or 3 s.

**Patch-Clamp Recordings and Data Analysis.** Transfected cells were selected visually by their fluorescence. The currents were recorded at room temperature (20°C) by using the MultiClamp 700A amplifier (Axon Instruments, Union City, CA); pipette resistance was 1.3–2.1 MΩ; cell capacitance and series resistance

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**Fig. 1.** Genetic linkage and DNA sequence analysis. (A) Simplified pedigree of the family. Filled symbols indicate individuals with FS. Asterisk indicates individuals who later developed mesial temporal lobe epilepsy (MTLE). (B) Electropherogram of the sequence from a T434C heterozygous mutant patient (mu) and a control (wt).
errors were carefully (85–90%) compensated for before each run of the voltage-clamp protocol to reduce voltage errors to <5% of the protocol pulse, as described in ref. 13. The P/N leak subtraction procedure was used routinely. PClAMP 8.2 (Axon Instruments) and ORIGIN 7 (Microcal Software, Northampton, MA) software were routinely used for data acquisition and analysis. The axovac program (Axon Instruments), written by S.W. Jones, was used for generating the simulations shown in Fig. 6. The right shift of the activation curve was obtained by right shifting both the $a_m$ and $b_m$ rate constants by 10 mV. The same activation right shift can also be obtained by right shifting only the $a_m$ rate constant by 23 mV, but the observed effects are so severe as to inhibit completely the all-or-none behavior of the action potential (AP) (data not shown).

**Results**

**Patients and Pedigree.** The family that we studied (Fig. 1A) contained 35 individuals spread over four generations. A total of 13 living members had generalized tonic or tonic–clonic seizures with onset associated with fever, and none had FS beyond 6 years of age. None of the spouses had a history of seizures or familial epilepsy. The neurocognitive and psychiatric status of all 13 affected members was normal. Three individuals (III-3, IV-3, and IV-5; aged 38, 17, and 22 years, respectively) at the age of 10, 13, and 11 years, respectively, developed afebrile simple partial seizures of temporal lobe origin with vegetative or experiential phenomena. Very rare partial complex seizures or nocturnal secondary generalized tonic–clonic seizures also occurred in all of them. Patient III-8 was affected by posttraumatic epilepsy; a severe traumatic head injury at the age of 13 years accounted for afebrile seizures which started 1 year later at the age of 14 years. The electroencephalogram analysis showed interictal anteromesiotemporal epileptiform abnormalities that were seen in all three patients with afebrile temporal lobe seizures, whereas it showed right frontal sharp-waves in patient III-8 with posttraumatic epilepsy.

Electroencephalograms were normal in all other affected individuals, and they never revealed generalized epileptiform discharges. One patient (IV-3) had one typical seizure recorded with onset in the right midinferonasal temporal region. Two patients (III-3 and IV-3) with afebrile seizures had MRI evidence of unilateral mesial temporal sclerosis (MTS), which consisted of an abnormal high signal intensity detected on fluid-attenuated inversion recovery (FLAIR) and T2-weighted images ipsilateral with the electroencephalogram abnormalities. MRI study of patient III-8 revealed a posttraumatic scar in the right frontal region without any MRI evidence of MTS. MRI study of patient III-8 revealed a posttraumatic scar in the right frontal region without any MRI evidence of MTS. MRI study of patient III-8 revealed a posttraumatic scar in the right frontal region without any MRI evidence of MTS. MRI study of patient III-8 revealed a posttraumatic scar in the right frontal region without any MRI evidence of MTS. MRI study of patient III-8 revealed a posttraumatic scar in the right frontal region without any MRI evidence of MTS. MRI study of patient III-8 revealed a posttraumatic scar in the right frontal region without any MRI evidence of MTS.

Thus, the pedigree analysis supports a highly (>90%) penetrant AD trait of simple FS in this family. Among the 13 affected family members, three patients developed subsequent afebrile seizures, whose electroclinical features were consistent with mesial temporal lobe epilepsy (MTLE) This phenotype resembles the phenotype that is encountered in families with AD FS, in which some individuals may develop later MTLE associated with MTS (15), whereas it differs from that reported in generalized epilepsy with FS plus (GEFS+) families (1).

**Linkage and Sequence Analysis.** We undertook linkage analysis in this family by defining the disease phenotype as simple FS. A genome screen revealed linked markers to the FEB3 locus on chromosome 2q23–24 that segregated with individuals who were previously classified as affected. Sequence analysis of the entire coding region of SCN1A Na channel gene revealed a T434C substitution (GenBank accession no. AY043484) within the coding region of exon 3, which alters an highly conserved methionine (Met145Thr, Fig. 2) within the S1 segment of domain I (DIS1) of the Na$_v$1.1 a-subunit (Fig. 1B). This mutation cosegregates with the disease because it was present in 12 family members with the affected haplotype and was not detected in all normal individuals from this family, indicating a disease-specific mutation; also, it was absent in 50 ethnically matched control individuals. Human Na$_v$1.1 consists of 2,009 aa and its sequence is highly conserved, with a 98% amino acid identity with the rat clone. Fig. 2 shows the homology of the amino acid sequences in the partial DIS1 of the human neuronal tetrodotoxin-sensitive channels (Na$_v$1.1, Na$_v$1.2, Na$_v$1.3, Na$_v$1.6, and Na$_v$1.7), one tetrodotoxin-insensitive channel (Na$_v$1.8), and rat Na$_v$1.1.

**Functional Study.** To study the functional effects of this mutation, we recorded with the whole-cell configuration of the patch-clamp

![Fig. 2.](image2.png) Evolutionary conservation of the residue M145 (boxed) in SCN1A amino acid alignments of the segment DIS1. Sequence alignment of the neuronal hNav1.1, rNav1.1, hNav1.2, hNav1.3, hNav1.6, hNav1.7, and hNav1.8 channels (gene product in Homo sapiens and Rattus norvegicus) is shown.

![Fig. 3.](image3.png) Maximal Na$^+$ currents. Plot of the maximal peak current densities (mean ± SEM, pA/pf, n = 20) observed in cells expressing either the WT or the M145T mutant.

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technique the Na currents in tsA-201 human cells that were cotransfected with WT or FS mutant M145T hNav1.1 channels and hβ2 accessory subunit. The first notable difference that we systematically observed was a 60 ± 2% reduction in M145T peak current density, as shown in Fig. 3, in which WT (199.1 ± 32.6 pA/pF) and M145T (79.9 ± 10.8 pA/pF) peak current densities have been plotted. The difference was statistically significant (P = 1.1 × 10^{-4}), suggesting an evident loss-of-function of the mutant. The threshold of activation and the time course of the Na^+ currents showed considerable changes in the mutant. As shown in Fig. 4 A and B (same scales), Na^+ currents started to activate at approximately -45 mV with the WT channel and at approximately -30 mV with the M145T mutant. Although this result should suggest a shift of the voltage-dependent open probability toward positive membrane voltages, we wanted to test whether the kinetics of opening were shifted in the same direction. Indeed, superimposition of the currents elicited at -10 mV (Fig. 4C) shows that M145T currents (○) are slower than WT currents (□), but if the mutated channels are tested at 0 mV (line), they open with the same speed of the WT channels tested at -10. We studied the activation kinetics in the voltage range from -30 to +20 mV and found statistically significant differences as shown in Fig. 4D, in which the current rise time (10–90% of the peak) is plotted vs. membrane potential. We observed a clear increase in the mutant (○), with a positive shift of 4.8 ± 1.3 mV (n = 9) in the voltage-dependence of the kinetics of activation. To clarify whether this effect is due to pure changes in the kinetics of the current or instead to modifications of the voltage-dependence of activation or inactivation, we examined the normalized peak conductance—voltage relationships and the voltage-dependence of steady-state fast inactivation. To investigate how the two effects could alter the all-or-none regenerative upstroke of the AP, we simulated both effects of the M145T mutation, independently and together, on a standard Hodgkin–Huxley model. The simulation is shown in Fig. 6, in which the superimposed traces of subthreshold responses (symbols) and APs (lines) are shown in control conditions (□, WT; AP threshold, -54 mV), for the M145T modified channel (○; AP threshold, -27 mV), and with the functional biophysical effects described in Fig. 3 (○, only 40% of Na^+ current, no voltage-dependent shift; AP threshold, -48) and in Figs. 4 and 5 (○, only -10 mV right shift; AP threshold, -37 mV). It appears that the M145T channel produces an almost abortive AP, whereas the separate biophysical changes have much less severe consequences. However, these results concern a hypothetical cell with just Nav1.1 channels. Neurons express different Na^+ channel isoforms, and the final effect of the mutation on the total Na current can vary according to the relative expression of Nav1.1 in a single neuron.

**Discussion**

We have shown that a missense mutation of hNav1.1 producing loss-of-function of the channel can cause simple FS. The phenotype of the present family differs from the clinical patterns encountered in families with generalized epilepsy with FS plus (GEFS+) phenotypes, which typically include FS, FS plus, and different types of generalized epilepsies (1). Except for patient III-8, who had afebrile
seizures related to a severe traumatic head injury, three individuals had later afebrile, unprovoked seizures, whose electroclinical features were consistent with MTLE. Of these patients, two had a MRI-evidenced MTS, but only one (IV-3) showed FS lasting up to 15 min, indicating a putative example of complex FS. The association between familial AD FS and MTLE has already been proposed in large pedigrees, suggesting a common genetic basis whether MTS is present or not (2, 15). In particular, it has been shown that a family history of simple FS also can be a risk factor for MTLE (16).

To our knowledge, there is no unifying mechanism that can explain how the spectrum of the observed functional effects of epileptogenic mutations relates to the epilepsy syndromes seen in patients. Various epileptogenic mutations of SCN1A have been reported (17) that cause both loss- and gain-of-function of hNav1.1 channel. The mutation M145T that we describe here is a pathogenic mutation found in DIS1 of hNav1.1 that causes a single amino acid change; two other mutations in DIS1 (causing severe myoclonic epilepsy of infancy) have been reported (ref. 17 and supplemental material therein), but they cause the truncation of the protein and, thus, the production of a nonfunctional channel. The fact that Met-145 is conserved among both ortholog and paralog Na channels [which, thus, have both relatively low amino acid sequence identity (84%) and large distance in the phylogenetic tree (18)] suggests that the mutation is strongly pathogenetic, as described in ref. 17.

We have identified in patients affected by simple FS a mutation of Na$_{\text{v}}$.1.1 that produces a decrease in current density and a right shift in the probability of opening, thus causing a loss of function. It is not clear yet how these effects can give rise to a network hyperexcitability that is able selectively to cause FS. The development of animal models and multielectrode array experiments would probably be useful in investigating this point.

![Figure 5](image)

**Fig. 5.** Effects of the M145T mutation on conductance–voltage relationships (right $y$ axis) and steady-state fast inactivation (left $y$ axis). Boltzmann fits of the normalized peak conductance experimental data resulted in the following values: $V_{1/2}$ and slope in WT ($\circ$, $n = 8$) were $-21.7 \pm 0.7, 6.15 \pm 0.27$ and $-11.7 \pm 1.6, 7.1 \pm 0.8$ in M145T ($\square$, $n = 14$). Boltzmann fits of the normalized steady-state inactivation data resulted in the following values: $V_{1/2}$ and slope in WT ($\diamond$, $n = 8$) were $-64.9 \pm 0.8, 8.0 \pm 0.33$ and $-65.5 \pm 0.76, 9.1 \pm 0.5$ in M145T ($\blacksquare$, $n = 15$).

Moreover, because FS afflict a relatively large percentage of infants and children, it is likely that numerous other factors and genes can contribute to the generation of the heterogeneous seizure phenotypes observed in the FS spectrum.

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