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Novel SCN3A Variants Associated with Focal Epilepsy in Children

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Abstract

Voltage-gated sodium (Na_v) channels are essential for initiating and propagating action potentials in the brain. More than 800 mutations in genes encoding neuronal Na_v channels including *SCN1A* and *SCN2A* have been associated with human epilepsy. Only one epilepsy-associated mutation has been identified in *SCN3A* encoding the Na_v1.3 neuronal sodium channel. We performed a genetic screen of pediatric patients with focal epilepsy of unknown cause and identified four novel *SCN3A* missense variants: R357Q, D766N, E1111K and M1323V. We determined the functional consequences of these variants along with the previously reported K354Q mutation using heterologously expressed human Na_v1.3. Functional defects were heterogeneous among the variants. The most severely affected was R357Q, which had significantly smaller current density and slower activation than the wild-type (WT) channel as well as depolarized voltage dependences of activation and inactivation. Also notable was E1111K, which evoked a significantly greater level of persistent sodium current than WT channels. Interestingly, a common feature shared by all variant channels was increased current activation in response to depolarizing voltage ramps revealing a functional property consistent with conferring neuronal hyper-excitability. Discovery of a common biophysical defect among variants identified in unrelated pediatric epilepsy patients suggests that *SCN3A* may contribute to neuronal hyperexcitability and epilepsy.

Keywords

SCN3A; sodium channel; epilepsy; persistent current; ramp currents

INTRODUCTION

Voltage-gated sodium (Na_v) channels are essential for initiating and propagating action potentials in the brain. The channels exist in a native protein complex composed of a pore-forming α subunit and two smaller accessory β subunits (Catterall, 1988; Isom et al., 1994).

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More than 800 sodium channel mutations, mostly in *SCN1A* and a few in *SCN2A*, have been associated with epilepsy (Lossin, 2009). *SCN3A* is clustered with *SCN1A* and *SCN2A* on human chromosome 2q24, but only one mutation in *SCN3A*, encoding Na_v1.3, is known to be associated with epilepsy (Holland et al., 2008). The involvement of Na_v1.3 in epilepsy is supported by studies showing that *SCN3A* mRNA is expressed at higher levels in human CA4 hilar cells in the epileptic hippocampus (Whitaker et al., 2001) and in rat neurons of CA1–CA3 and in the dentate granule cell layer after the induction of status epilepticus (Bartolomei et al., 1997; Aronica et al., 2001).

Na_v1.3 channels possess several properties that may cause neuronal hyper-responsiveness. For example, Na_v1.3 channels recover from inactivation rapidly and sustain high-frequency firing (Cummins et al., 2001), activate during slow ramp depolarizations, and produce persistent sodium current (Chen et al., 2000; Cummins and Waxman, 1997; Sun et al., 2007; Estacion et al., 2010). The Na_v1.3 epilepsy-associated mutation K354Q was previously demonstrated to enhance persistent current and ramp current (Holland et al., 2008; Estacion et al., 2010). Several studies indicate that Na_v persistent current participates in spontaneous neuronal firing in a variety of cell types including hippocampal neurons (Agrawal et al., 2001; Kearney et al., 2001) and subicular neurons isolated from patients with temporal lobe epilepsy (Vreugdenhil et al., 2004). Moreover, several epilepsy-associated Na_v1.1 mutations are known to enhance persistent current (Lossin et al., 2002; Rhodes et al., 2004; Spanpanato et al., 2004; Kahlig et al., 2008).

We performed a genetic screen of pediatric patients with cryptogenic focal epilepsy and identified four novel *SCN3A* missense variants: R357Q, D766N, E1111K and M1323V. Electrophysiological studies revealed a diversity of functional defects, but interestingly, all mutant channels exhibited abnormal current activation during a slow depolarizing voltage ramp. This common defect among the novel alleles could explain neuronal hyperexcitability.

MATERIALS AND METHODS

Study subjects

Subjects were ascertained from the St. Louis Children's Hospital Pediatric Epilepsy Center and the Cincinnati Children's Hospital Comprehensive Epilepsy Center. Diagnoses were classified as focal epilepsy of unknown cause according to the International League Against Epilepsy guidelines (Berg et al., 2010). Additional inclusion criteria used at the Cincinnati Children's Hospital Comprehensive Epilepsy Center were: i) treatment with carbamazepine or oxcarbazepine; and ii) have classifiable treatment response (as defined in Holland et al., 2007). Research was approved by the local institutional review boards and informed consent was obtained from the parent or guardian of each individual. Additional clinical details for the four subjects with novel *SCN3A* variants are provided below.

Individual 1 presented with complex focal seizures with secondary generalization at age 5. Seizures were all <2 minutes in duration and were well controlled on carbamazepine with only a few breakthrough seizures. At age 10 he had been seizure free for 2 years and was successfully weaned off carbamazepine. He also has mild speech delay and attention deficit hyperactivity disorder (ADHD), but was otherwise healthy. He has a cousin with febrile seizures and no additional family history of epilepsy.

Individual 2 presented at 22 months of age with complex focal seizures characterized by staring an unresponsiveness lasting less than 1 minute. He had rare seizures but became seizure-free on carbamazepine, which discontinued at 10 years of age after a 3-year period of seizure freedom. His initial EEG demonstrated midline occipital epileptiform discharges and subsequent EEGs were abnormal because of mild slowing of the background activity.

He was diagnosed with a non-verbal learning disability after testing demonstrated a borderline full scale IQ (78) with impairment in non-verbal skills but normal verbal skills.

Individual 3 is an 8 year old girl whose seizures began at 2 days of age. Seizures consisted of clonic movements involving the left arm that corresponded to rhythmic right central discharges on the EEG. She was treated with phenobarbital and had excellent seizure control until age 3 months when she had a single breakthrough seizure also involving the left arm with unresponsiveness. At the time, her phenobarbital level was 15, and she responded to an increase in medication with no further seizure activity. At age 2 years, the EEG was repeated and was normal and she was successfully weaned off phenobarbital. Birth history was unremarkable and development was normal. She has a paternal aunt who had seizures that began at 1 month of age and lasted until 11 months.

Individual 4 had two simple febrile seizures at 16 months of age. One month later the individual had an unprovoked generalized tonic clonic seizure lasting 45 minutes which was followed by postictal eye deviation to the left and tremulousness of the right arm. At that time oxcarbazepine was initiated. Over the following months the individual had several seizures characterized by loss of awareness and right sided jerking and one with left sided jerking. The dose of oxcarbazepine was progressively increased over a 3 month period until the individual became seizure-free and oxcarbazepine was stopped at 5 years of age. Multiple EEGs were normal; however no EEGs were done prior to the initiation for treatment. The individual's mother had surgery for intractable epilepsy (pathology unknown) and the paternal grandfather also had epilepsy (details unknown).

SCN3A Screening

Genomic DNA was extracted from peripheral blood and the 26 coding exons of *SCN3A* were amplified by PCR using previously described primers (Weiss et al., 2003). Exons were screened for variants by sequencing as previously described (Holland et al., 2008) or by heteroduplex analysis using conformation-sensitive gel electrophoresis followed by sequencing as previously described (Escayg et al., 2000). Nucleotide and amino acid residue numeration correspond to the major *SCN3A* isoform that includes the exon 5 adult (5A) and exon 12v1 (646 bp) splice variants (Genbank NM_001081676.1 (Thimmapaya et al., 2005). NINDS Neurologically Normal Control Panels (Coriell NDPT006 and NDPT009; $n = 184$) were screened for variants R357Q, D766N, M1323V and E1111K to determine population allele frequencies. For some variants, we screened an additional panel of 111 subjects who were >60 years of age without personal or family history of neurological disease (Rainier et al., 2006). Screening of this same control panel was previously reported for variant K354Q (Holland et al., 2008). DNA from family members of study subjects was not available for screening.

Cell Culture

All electrophysiology experiments were conducted using tsA201 cells (HEK-293 cells stably transfected with SV40 large T antigen) grown at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (ATLANTA Biologicals, Norcross, GA, USA), 2 mM L-glutamine, and penicillin (50 units/ml)-streptomycin (50 µg/ml). Only cells from passage number <13 were used. Unless otherwise stated, all tissue culture media was obtained from Life Technologies, Inc. (Grand Island, NY, USA).

Plasmids and Cell Transfection

A plasmid encoding the major splice isoform of human Na_v1.3, with the exon 5 adult (5A) and exon 12v1 (646 bp) splice variants (GenBank NM_001081676.1), was previously

described (Wang et al., 2010). Full-length Nav1.3 was propagated in STBL2 cells at 30°C (Invitrogen) and the open reading frame of all plasmid preparations was fully sequenced prior to transfection. Variants were introduced by site-directed mutagenesis. Plasmids encoding the human Nav channel accessory subunits $\beta 1$ or $\beta 2$ in vectors containing the marker genes CD8 (pCD8-IRES- $\beta 1$) or GFP (pGFP-IRES- $\beta 2$) were also used. Expression of Nav1.3, $\beta 1$ and $\beta 2$ subunits was achieved by transient transfection (2 μ g of total cDNA: SCN3A, $\beta 1$, $\beta 2$ mass ratio was 10:1:1) using Superfect Transfection Reagent (QIAGEN, Valencia, CA, USA). Cells were incubated for 48 hr as described above after transfection before their use in electrophysiology experiments. Transfected cells were dissociated by brief exposure to trypsin/EDTA, resuspended in supplemented DMEM medium, plated on glass coverslips and allowed to recover for ~2 hrs at 37°C in 5% CO₂. Polystyrene microbeads precoated with anti-CD8 antibody (Dynabeads M-450 CD 8, Dynal, Great Neck, NY, USA) were added and only cells positive for both CD8 antigen (i.e., $\beta 1$ expression) and GFP fluorescence (i.e., $\beta 2$ expression) were studied.

Electrophysiology

Coverslips were placed into a recording chamber on the stage of an inverted microscope with epifluorescence capability and allowed to equilibrate for 10 min in bath solution before starting the experiments. Bath solution contained (in mM): 145 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 HEPES (*N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulphonic acid), pH 7.35, 310 mOsm/kg. The composition of the pipette solution was (in mM): 10 NaF, 110 CsF, 20 CsCl, 2 EGTA (ethyleneglycol-bis-(β -aminoethylether), 10 HEPES, pH 7.35, 310 mOsm/kg. Osmolarity and pH values were adjusted with sucrose and NaOH, respectively. As a reference electrode, a 2% agar-bridge with composition similar to the bath solution was utilized. Junction potentials were zeroed with the filled pipette in the bath solution. Unless otherwise stated, all chemicals were obtained from (Sigma-Aldrich, St. Louis, MO, USA).

Patch pipettes were pulled from thin-wall borosilicate glass (World Precision Instruments, Inc., Sarasota, FL, USA) with a multistage P-97 Flaming-Brown micropipette puller (Sutter Instruments Co., San Rafael, CA, USA) and fire-polished with a Micro Forge MF 830 (Narashige, Japan). After heat polishing, the resistance of the patch pipettes was 1–3 M Ω in standard bath and pipette solutions. Once whole-cell recording mode was achieved the access resistance averaged 2.5 ± 0.1 M Ω . Whole-cell sodium currents were recorded as previously described (Lossin et al., 2002; Vanoye et al., 2006) at room temperature (20–23°C) in the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) using an Axopatch 200B series amplifier (Molecular Devices Corp. Sunnyvale, CA, USA). Cells were allowed to equilibrate for 10 min after establishment of the whole-cell configuration before currents were recorded. Pulse generation was done with Clampex 8.1 (Molecular Devices Corp., Sunnyvale, CA) and whole-cell currents acquired at 20 kHz and filtered at 5 kHz. Linear leak and residual capacitance artifacts were subtracted out using the P/4 method. To reduce voltage errors, 80–90% series resistance and prediction compensation were applied. Cells were excluded from analysis if the predicted voltage error exceeded 3 mV or the current at the holding potential (–120 mV) was >5% of the peak current.

Peak currents were measured using 20 ms pulses between –80 and +50 mV every 5 s from a holding potential of –120 mV. The peak current was normalized for cell capacitance and plotted against voltage to generate peak current density–voltage relationships. Whole-cell conductance (G) was calculated from the peak current amplitude using the formula: $G = I / (V - E_{rev})$ where G is the conductance, I is the peak inward current, V is the membrane potential step, and E_{rev} is the estimated Na⁺ reversal potential. Conductance data were normalized by maximum conductance recorded between –80 and +20 mV. The normalized

G-V curves were fit with the Boltzmann function: $G = 1 / (1 + \exp[(V - V_{1/2}) / k])$ to determine the voltage for half-maximal channel activation ($V_{1/2}$) and slope factor (k). The voltage-dependence of channel availability was assessed following a 100 msec pre-pulse to various potentials followed by 20 ms pulse to -10 mV. The normalized current was plotted against the voltage and the steady-state channel availability curves were fit with Boltzmann functions ($I/I_{\max} = 1 / (1 + \exp[(V - V_{1/2}) / k])$) to determine the voltage for half-maximal channel inactivation ($V_{1/2}$) and slope factor (k). Recovery from fast inactivation was determined using a two-pulse protocol. A 100 ms pre-pulse to -10 mV was followed by a variable amount of time for channel recovery at -120 mV and a 10 ms test pulse to -10 mV. The peak current from the test pulse was normalized to the peak current from the pre-pulse and plotted against the recovery period. Data were fit with the two exponential function, $I/I_{\max} = A_f \times [1 - \exp(-t/\tau_f)] + A_s \times [1 - \exp(-t/\tau_s)]$, where τ_f and τ_s denote time constants (fast and slow components, respectively), and A_f and A_s represent the fast and slow fractional amplitudes.

Persistent current was evaluated by 200 msec depolarizations to -10 mV first in the absence and then in the presence of 500 nM tetrodotoxin (TTX; Sigma, St. Louis, MO). The average steady-state persistent current for each cell was measured during the final 10 ms of five 200 ms voltage steps to -10 mV. The voltage steps were elicited from a holding potential of -120 mV with an inter-pulse duration of 10 s. Percent late current was normalized to peak TTX-sensitive Na^+ channel current and plotted as % persistent current.

Ramp current was assessed by changing the membrane potential from -120 mV to $+40$ mV over 800 ms (0.2 mV/ms). For each cell the average of 5 ramp pulses with an inter-pulse duration of 10 s were recorded before and after TTX (500 nM) addition. The TTX-sensitive ramp currents were then obtained by digital subtraction of the average sodium currents recorded before and after TTX. The inward charge (pC) for each cell was calculated between the voltage at which the Na_v currents activated and the voltage where the currents reversed polarity, and normalized to the peak current measured during a pulse test to -10 mV.

Data Analysis

Data were collected for each experimental condition from at least 3 transient transfections and analyzed and plotted using a combination of Clampfit 8.1 (Molecular Devices Corp.), Excel 2002 (Microsoft, Seattle, WA, USA), SigmaPlot 10 (Systat Software, Inc., San Jose, CA, USA) and OriginPro 7.0 (OriginLab, Northampton, MA, USA) software. Results are represented as means $\pm$ SEM, and number of cells used for each experimental condition is given in the Figure legends or Table 2. Current values were normalized to membrane capacitance. In some figures, the SE bars are smaller than the data symbols.

Differences between the wild-type channel and each mutant channel were assessed by Student's t test using SigmaStat 2.03 (Systat Software, Inc.) with $p < 0.05$ considered statistically significant.

RESULTS

We identified four novel *SCN3A* missense variants (R357Q, D766N, E1111K, M1323V) in a screen of 179 pediatric patients with focal epilepsy who were *SCN1A*-mutation negative. None of the variants were detected in up to 590 chromosomes from control subjects (Table 1). Three of the variants (R357Q, D766N, M1323V) were not detected by the NHLBI Exome Sequencing Project (Exome Variant Server, 2013) in 4300 EuropeanAmerican or 2203 AfricanAmerican subjects. One heterozygous E1111K carrier was reported in this database with a minor allele frequency = 0.0001 (EuropeanAmerican). The observed

frequency of singleton missense variants in our clinical population of (4/179) was 3-fold greater than observed in the general population (46/6503) (Exome Variant Server, 2013). In a concurrent screen of 75 pediatric patients with generalized epilepsy we did not identify any singleton missense variants in *SCN3A*. The clinical features associated with these *SCN3A* variants are summarized in Table 1.

The locations of the variants in the $\text{Na}_v1.3$ protein and the evolutionary conservation of the variant residues are illustrated in Fig. 1. Amino acids K354 and R357 are located in the pore region of domain 1 (Fig. 1A). Both of these residues are invariant in other vertebrate $\text{Na}_v1.3$ orthologs, and are highly conserved in other human sodium channel genes (Fig. 1B). D766 lies at the intracellular face of a transmembrane alpha helix and exhibits a high degree of evolutionary conservation (Fig. 1B). E1111 is located in the domain II/III cytoplasmic linker and is evolutionarily invariant in other vertebrate $\text{Na}_v1.3$ orthologs and in the other human brain sodium channel genes (Fig. 1B). M1323 is located in the extracellular linker between transmembrane segment 5 and the pore region of domain 3. M1323 is evolutionarily invariant through birds and reptiles, while fish have conservative substitutions. Among the other human sodium channel genes, a methionine is present at this position in the most closely related *SCN2A* gene, while this position is not conserved in more distantly related sodium channel genes. We utilized two bioinformatic algorithms, Provean and SIFT, to predict the consequences of these $\text{Na}_v1.3$ variants (Kumar et al, 2009; Choi et al 2012). Both programs predict R354Q, R357Q, D766N and E1111K to have deleterious effects on protein function, while M1323V is predicted to be benign (Table S1). To experimentally determine whether the novel *SCN3A* variants cause channel dysfunction, we performed electrophysiological studies of heterologously expressed $\text{Na}_v1.3$ channels.

Biophysical properties of wild-type human $\text{Na}_v1.3$ channels

We first examined the functional properties of wild-type (WT) $\text{Na}_v1.3$ to obtain a control activity profile under our expression and recording conditions. Figure 2 illustrates the biophysical properties of WT human $\text{Na}_v1.3$ co-expressed with both β_1 and β_2 accessory subunits in tsA201 cells. Rapidly activating and inactivating voltage-dependent inward whole-cell currents were observed in response to depolarizing test pulses from a holding potential of -120 mV (Fig. 2A). Inward currents observed at potentials more depolarized than -50 mV peaked between -10 and 0 mV (Fig. 2B). Wild-type $\text{Na}_v1.3$ channels are fast activating as illustrated by the voltage dependence of time-to-peak current plotted in Fig. 2C. The channels inactivated rapidly with a bi-exponential time course. Inactivation time constants and corresponding fractional amplitudes for the fast and slow components are shown in Figs. 2D and 2E. Voltage dependence of activation and fast inactivation (Fig. 2F), and recovery from fast inactivation were also examined (Fig. 2G, Table 2). $\text{Na}_v1.3$ recovery from fast inactivation following a 100 ms inactivating pulse to -10 mV was distributed into two populations, with the majority of channels ($A_f = 95 \pm 3\%$) recovering within a few milliseconds ($\tau_f = 3.5 \pm 0.2$ ms) and a minor population ($A_s = 5 \pm 1\%$) recovering more slowly ($\tau_s = 196.5 \pm 42.0$ ms, $n = 11$; Fig. 2G, Table 2).

Biophysical properties of mutant $\text{Na}_v1.3$ channels associated with pediatric epilepsy

We determined the functional properties of the four novel epilepsy-associated $\text{Na}_v1.3$ variants, and included the previously identified K354Q mutation (Holland et al., 2008; Estacion et al., 2010) in our analyses for comparison. Figure 3 shows the average whole-cell currents obtained from tsA201 cells transiently expressing K354Q, R357Q, D766N, E1111K or M1323V channels. Figure 3F illustrates the average current density-voltage relationships measured from cells transiently expressing the variant channels with the average WT- $\text{Na}_v1.3$ currents illustrated as a gray line. Expression of R357Q yielded sodium current

density smaller than the wild type channel (Table 2), but all other Na_v1.3 variants exhibited current densities that were not significantly different from WT channels.

All variant channels exhibited fast activating and inactivating kinetics in response to depolarizing test pulses (Tables S2 and S3). Only R357Q affected Na_v1.3 activation kinetics with time-to-peak values ~10% longer than WT (Table S2). Entry into fast inactivation was slowed by both R357Q and M1323V, but by different mechanisms. Whereas R357Q increased the fast time constant (τ_F) for inactivation at certain voltages, M1323V increased both the proportion of the slow component of inactivation and increased the fast time constant at various test potentials (Table S3). Both R357Q and M1323V exhibited depolarized voltage-dependence of activation and inactivation when compared to the WT channel (Table 2). However, the voltage sensitivity (slope factor, k) for both activation and inactivation were not different from WT-Na_v1.3. The K354Q, D766N and E1111K variants did not affect the voltage-dependence of activation or inactivation (Table 2). Recovery from inactivation was faster for M1323V but the other variants were similar to WT channels (Table 2).

We also determined channel availability of WT and mutant Na_v1.3 channels during repetitive stimulation (use-dependence) with trains of 100 depolarizing voltage steps to -10 mV at various frequencies. Only K354Q and E1111K channels exhibited altered channel availability, an effect only observed at the highest stimulation frequency (100 Hz, Fig. S1). These mutant channels had lower channel availability than WT Na_v1.3 (WT = 70.2 ± 1.3 %, K354Q = 62.8 ± 2.8 %, E1111K = 64.4 ± 2.6 %).

Persistent currents in WT and variant Na_v1.3 channels

Many epilepsy-associated mutant Na_v channels exhibit impaired inactivation resulting in increased persistent sodium current (Lossin et al., 2002; Rhodes et al., 2004; Spanpanato et al., 2004; Kahlig et al., 2008; Holland et al., 2008). Because a larger persistent current could accentuate subthreshold depolarizations and facilitate action potential generation leading to neuronal hyperexcitability, we compared this property between WT and variant Na_v1.3 channels. Figure 4A illustrates TTX-sensitive whole-cell currents (normalized to peak transient current) recorded from cells transiently expressing WT or variant Na_v1.3 channels. Only E1111K (red traces) displayed a persistent current larger than WT channels (Fig. 4B). Persistent current generated by E1111K was more than 2-fold greater than WT channels measured as a percentage of peak current (E1111K = 1.2 ± 0.2 %, $n = 5$; WT = 0.5 ± 0.1 %, $n = 8$; $p = 0.009$) and absolute current density (E1111K = 4.9 ± 2.1 pA/pF, $n = 5$; WT = 1.9 ± 0.5 pA/pF, $n = 8$; $p = 0.024$).

Ramp currents in WT and variant Na_v1.3 channels

Because aberrant activation of inward currents during ramp depolarizations can aid in neuronal hyperexcitability, we examined the effects of depolarizing voltage ramps from -120 mV to +40 mV over 800 ms (0.2 mV/ms) on the *SCN3A* variants. Figure 4C illustrates the average TTX-sensitive ramp currents expressed as percent of peak current recorded from cells expressing WT or variant Na_v1.3 channels. Ramp current was quantified as the total conducted charge (pC) for each cell by integrating the TTX-sensitive inward current between the voltage at which the Na₊ currents first activated and the voltage where the currents reversed polarity. Total charge was normalized to the peak current measured during a pulse test to -10 mV to correct for variation in channel expression levels between cells. Figure 4D demonstrates that all mutant channels conduct significantly more inward charge than WT channels.

DISCUSSION

Among the myriad epilepsy-associated Na_V mutations described to date, only one *SCN3A* mutation has been reported previously in a patient with focal epilepsy (Holland et al., 2008; Lossin, 2009). In this study we report four new *SCN3A* variants that were identified in pediatric focal epilepsy. We further demonstrated that all four novel *SCN3A* variants confer significant functional defects to the encoded $\text{Na}_V1.3$ sodium channel suggesting these as putative epilepsy-associated mutations. Two functional properties of the variant $\text{Na}_V1.3$ channels deserve in-depth discussion – persistent current and ramp current.

Persistent currents in wild-type and mutant $\text{Na}_V1.3$ channels

Several studies support the notion that persistent sodium currents can drive intrinsic neuronal excitability (Alzheimer et al., 1993; Baker et al., 2003). Moreover, several epilepsy-associated $\text{Na}_V1.1$ mutations can enhance persistent current (Lossin et al., 2002; Rhodes et al., 2004; Spampinato et al., 2004; Kahlig et al., 2008; Holland et al., 2008), and transgenic mice expressing mutant $\text{Na}_V1.2$ channels generating increased persistent current have a severe epilepsy (Kearney et al., 2001). However, most of the novel *SCN3A* variants we report here exhibited levels of persistent sodium current similar to the WT channel. By contrast, E1111K channels displayed greater levels of persistent current than WT channels. The residue at position 1111 is located within the DII-DIII linker and is conserved among many species (e.g., rodent, dog, cow, zebrafish) and several isoforms ($\text{Na}_V1.1$, $\text{Na}_V1.2$, $\text{Na}_V1.5$ [D instead of E] and $\text{Na}_V1.6$). The variant occurs several amino acids (~50) C-terminal from a region identified for ankyrin-G interaction (LeMaillet et al., 2003) and CK2 phosphorylation, which regulates that interaction (Brecht et al., 2008). It is conceivable that this charge reversal variant could alter the interaction of ankyrin-G with $\text{Na}_V1.3$ and affect inactivation state stability. Interestingly, ankyrin-G co-expression has been demonstrated to reduce persistent current generated by $\text{Na}_V1.6$ (Shirahata et al., 2006).

Unlike previous studies, the K354Q mutation did not generate persistent currents larger than WT- $\text{Na}_V1.3$ under our recording conditions. The previous characterizations of the K354Q mutation used human cardiac $\text{Na}_V1.5$ or rat $\text{Na}_V1.3$ to express this variant allele (Holland et al., 2008; Estacion et al., 2010) whereas we employed the human $\text{Na}_V1.3$ channel. Therefore, the divergent effects may be due simply to the molecular context used for heterologous expression.

Importantly, we did not observe large persistent sodium currents in cells expressing WT human $\text{Na}_V1.3$ in contrast to previous studies that reported values up to 10–15% of peak current. The $\text{Na}_V1.3$ splice variant used in those studies (Chen et al., 2000; Sun et al., 2007) included exon 5N, which has a serine at position 208 (Gazina et al., 2010). Because our studies were conducted using the $\text{Na}_V1.3$ exon 5A splice variant (aspartic acid at position 208), we tested whether this single amino acid substitution accounts for the different persistent current levels. Expression of the $\text{Na}_V1.3$ 5N variant in tsA201 cells generated TTX-sensitive persistent current similar to the 5A variant under our recording conditions (5A: $0.5 \pm 0.1\%$, 1.9 ± 0.5 pA/pF, $n = 8$; 5N: $0.6 \pm 0.1\%$, 1.5 ± 0.6 pA/pF, $n = 5$). Additionally, we did not observe a significant effect of using fluoride-free intracellular solution with or without nucleotides ATP and GTP (Chen et al., 2000; Cusdin et al., 2010; Estacion et al., 2010; Sun et al., 2007) on the level of TTX-sensitive persistent current recorded from cells expressing the $\text{Na}_V1.3$ 5A variant ($1.7 \pm 0.2\%$, $n = 6$). Our results along with other previous $\text{Na}_V1.3$ studies using CHO cells as the expression system that describe negligible persistent current (Chen et al., 2000; Meadows et al., 2002) suggest that large persistent current is not an intrinsic property of the $\text{Na}_V1.3$ channel. Large persistent current may be due to modulation by proteins such as β subunits [(Cusdin et al., 2010), but see (Meadows et al., 2002)] or G-proteins (Ma et al., 1994; Mantegazza et al., 2005).

Increased ramp currents in all mutant $\text{Na}_V1.3$ channels

A common defect shared by all variant $\text{Na}_V1.3$ channels was increased ramp currents. The larger ramp currents may represent a pattern of channel dysfunction capable of enhancing the response to subthreshold depolarizing inputs, leading to reduced firing threshold (Lampert et al., 2006). Similar to previous studies using rat $\text{Na}_V1.3$ (Estacion et al., 2010) the K354Q mutation expressed in human $\text{Na}_V1.3$ splice 5A variant (our data) generated ramp currents larger than the wild-type channel suggesting that this effect, unlike persistent current, is not dependent on animal species, expression system or recording conditions. Estacion and Waxman (2013) showed that $\text{Na}_V1.3$ response to ramp stimuli includes an early component dependent on ramp rate and a second one that correlates with persistent current. Our data show that the mutant channel that generated the largest persistent current, E111K, was also the one with the largest response to ramp stimulus. However, the other mutant channels yielded larger ramp currents when compared to WT- $\text{Na}_V1.3$ in the absence of larger persistent currents. This discrepancy could be due to the presence of fluoride in our internal solution which may affect persistent current levels (Jarecki et al., 2008).

Decreased channel availability in response to repetitive stimulation indicates a loss of channel function which may nullify some of the gain-of-function properties evoked by the mutations. However, only K354Q and E111K channels exhibited a small decrease (6–5%) in channel availability when compared to WT- $\text{Na}_V1.3$, detectable only at 100 Hz. Thus, lower $\text{Na}_V1.3$ channel availability (i.e., loss of function) is not a prominent feature.

Three of the variants, R357Q, K354Q and M1323V, occur in the pore domain, specifically the S5-S6 linker (Fig. 1), which may alter channel gating by charge neutralization or allosteric effects (Estacion et al., 2010). The variant D766N is located at the intracellular end of the S2 segment in DII (Fig. 1). Segment 2 in voltage-gated channels has been implicated in voltage gating and may undergo conformational changes (Cha and Bezanilla, 1997; Ma et al., 2009; Seoh et al., 1996). The charge-neutralizing effect of this variant (D/N) could conceivably influence $\text{Na}_V1.3$ gating by a related mechanism.

Genotype-phenotype Correlations

Although all variant channels we studied exhibited increased ramp current, the associated clinical phenotypes are somewhat different. Two variants with substantial functional defects were identified in individuals with more severe phenotypes. The individual heterozygous for R357Q exhibited developmental delay, and among all $\text{Na}_V1.3$ mutant channels, R357Q channels were the most dysfunctional. Additionally, the individual who was heterozygous for E111K (largest ramp current and the only mutant expressing persistent current) had neonatal seizures that remitted. Other channel properties, variation in other genes or other cellular factors may influence the severity or timing of the disease. It is interesting to note that CK2-regulated binding of Na_V channels to ankyrin-G is required to immobilize the channels at the axonal initial segment early in development, but in mature neurons the diffusion barrier aids in immobilizing proteins that do not bind ankyrin-G (Brechet et al., 2008).

In summary, we analyzed four novel $\text{Na}_V1.3$ variants identified in childhood epilepsy patients. All variant channels studied have increased ramp currents, which may reduce neuronal firing threshold and promote hyperexcitable networks, consistent with an epilepsy phenotype. Whether increased ramp current alone or in combination with other biophysical defects correlates with clinical phenotype severity are interesting possibilities. Although defining the mechanism by which $\text{Na}_V1.3$ variants influence neuronal excitability will require additional studies, the identification of four additional *SCN3A* variants with a

common biophysical defect provides further support for a contribution of $\text{Na}_V1.3$ to childhood epilepsy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Identified four novel *SCN3A* missense variants in pediatric focal epilepsy patients.
- Variant characterization in heterologously expressed Na γ 1.3 revealed functional defects.
- All variants exhibited increased current in response to depolarizing voltage ramps.
- Increased ramp currents may be a common biophysical defect in *SCN3A*-associated epilepsy.

Figure 1. Location and evolutionary conservation of Na_v1.3 variants
(A) Predicted transmembrane topology of Na_v1.3 depicting the location of the variants (●) characterized in this study. The grey rectangle behind the depiction represents the plasma membrane. The S4 segments are illustrated as black cylinders with plus (+) signs. **(B)** Multiple alignment of human homologs and species orthologs of Na_v1.3 (Clustal Omega; Sievers et al, 2011). The variant amino acids are shaded to highlight conservation at the variant positions.

Figure 2. Biophysical properties of heterologously expressed human Nav1.3

(A) Averaged whole-cell currents recorded from cells transiently coexpressing Nav1.3 and both β_1 and β_2 subunits ($n = 29$). (B) Current-voltage relationships measured from Nav1.3-expressing cells ($n = 29$). (C) Voltage dependence of time to peak current ($n = 29$). (D) Voltage dependence of inactivation time constants (fast, $\bullet$; slow, $\circ$; $n = 25-27$). (E) Fractional amplitudes for inactivation time constants (fast, $\blacksquare$; slow, $\square$; $n = 25-27$). (F) Superimposed steady-state channel availability ($\circ$) and conductance-voltage relationship ($\bullet$) for Nav1.3 currents ($n = 29$ and 23 , respectively). Solid lines represent data fitted with Boltzmann functions. (G) Time course of recovery from fast inactivation (holding potential

was -120 mV). Line represents data fitted with a two-exponential function ($n = 10$). Biophysical fit parameters are given in Table 2.

Figure 3. Whole-cell currents recorded from heterologously expressed Na_v1.3 channels
 Average whole-cell currents recorded from tsA201 cells transiently coexpressing mutant Na_v1.3 channels plus β_1 and β_2 accessory subunits. (A) K354Q (∇), n = 28; (B) R357Q (∇), n = 14; (C) D766N ($\times$), n = 14; (D) E1111K ($\mathbf{M}$), n = 15 and (E) M1323V ($\mathbf{8}$), n = 13. (F) Current-voltage relationships measured from mutant Na_v1.3-expressing cells. The gray solid line illustrates the data from cells expressing WT channels (Fig. 1A).

Figure 4. Persistent and ramp current for Nav1.3 channels

(A) Average TTX-sensitive persistent currents normalized to the peak sodium current. Inset shows an expanded y-axis to highlight the increased persistent current of E1111K compared to WT. (B) Magnitude of persistent current as a percent of peak current for WT (n = 8), K354Q (n = 5), R357Q (n = 5), D766N (n = 6), E1111K (n = 5) and M1323V (n = 7). Asterisk indicates significance at $p < 0.01$. (C) Average TTX-sensitive ramp currents during a 0.2 mV/ms voltage ramp from a holding potential of -120 mV and expressed as a percent of peak current. (D) Average TTX-sensitive inward charge movement calculated from ramp currents and normalized to the TTX-sensitive peak current generated by a voltage step to -10 mV to account for variation in channel expression (WT, n = 5; K354Q, n = 5; R357Q, n = 6; D766N, n = 6; E1111K, n = 6 and M1323V, n = 7). * $p = 0.021$; ** $p = 0.002$; ‡ $p = 0.002$.

Table 1

Clinical features in four individuals with cryptogenic focal epilepsy.

Individual	1	2	3	4
Seizure Type(s)	Focal	Focal	Focal	Febrile seizures and Focal
Age at Onset	4 years	22 months	Neonatal	16 months
EEG	Occipital and temporal epileptiform discharges	Occipital epileptiform discharges	Normal	Normal
MRI	Normal	small focus of increased signal in left subcortical frontal white matter	Normal	Normal except for low lying cerebellar tonsils
Developmental Delay	Yes	Mild	No	No
Amino Acid Variant	R357Q	D766N	E1111K	M1323V
Location in Nav1.3 (see Fig.1)	D1 S5-S6	D2 S2	D2-D3 loop	D3 S5-S6
Family History	Yes	No	Yes	Yes
Ethnicity	European American	African American	European American	European American
Heterozygote Frequency in Neurologically Normal Controls	0/295	0/184	0/295	0/184
Heterozygote Frequency in Exome VariantServer – European American	0/4299	0/4299	1/4299	0/4299
Heterozygote Frequency in Exome VariantServer – African American	0/2203	0/2203	0/2203	0/2203

Table 2

Biophysical properties of wild-type and mutant Nav1.3 channels co-expressed with $\beta 1$ and $\beta 2$ subunits.

	Current density (-10 mV)		Voltage-dependence of activation			Voltage-dependence of fast inactivation ¹			Recovery from fast inactivation ²		
	pA/pF	n	V _{1/2} (mV)	k	n	V _{1/2} (mV)	k	n	τ_1 (ms)	τ_2 (ms)	n
WT	-374.6 ± 32.6	29	-21.0 ± 0.9	6.8 ± 0.2	29	-71.5 ± 0.4	6.6 ± 0.1	23	3.5 ± 0.2 [94.8 ± 0.8%]	196.5 ± 42.0 [5.2 ± 0.8%]	10
K354Q	-349.6 ± 28.8	28	-20.9 ± 1.2	6.8 ± 0.2	28	-73.1 ± 1.0	6.7 ± 0.2	19	4.2 ± 0.4 [95.5 ± 4.5%]	208.0 ± 61.1 [4.5 ± 1.0%]	6
R357Q	-252.8 ± 51.6 [*]	14	-17.4 ± 1.5 ^{**}	7.0 ± 0.3	14	-68.1 ± 1.0 ^{††}	6.5 ± 0.2	11	3.3 ± 0.2 [95.5 ± 1.6%]	298.3 ± 141.0 [4.4 ± 0.6%]	5
D766N	-443.0 ± 64.8	14	-19.7 ± 1.9	6.9 ± 0.3	14	-69.2 ± 1.5	6.8 ± 0.1	13	3.6 ± 0.3 [95.6 ± 0.6%]	306.4 ± 108.1 [4.4 ± 0.6%]	8
E1111K	-461.3 ± 69.3	15	-20.3 ± 1.3	6.8 ± 0.3	15	-70.8 ± 0.7	6.5 ± 0.1	14	4.0 ± 0.3 [95.5 ± 0.6%]	423.2 ± 253.8 [4.5 ± 0.6%]	6
M1323V	-312.4 ± 50.3	13	-16.9 ± 1.2 [‡]	7.3 ± 0.2	13	-68.8 ± 0.6 [‡]	6.7 ± 0.2	10	3.1 ± 0.4 [89.1 ± 2.8%] ^{†††}	52.2 ± 13.2 ^{††} [10.9 ± 2.8%] [‡]	5

¹ Steady-state inactivation determined following a 100 ms prepulse to various potentials followed by 20 ms pulse to -10 mV;

² Recovery from fast inactivation determined following a 100 ms prepulse to -10 mV;

* $P = 0.045$;

** $P = 0.036$;

†† $P < 0.001$;

‡ $P = 0.013$;

† $P = 0.002$;

††† $P = 0.028$;

†† $P = 0.041$ (mutant vs wild-type using t-test).