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Infant sudden death: Mutations responsible for impaired Nav1.5 channel trafficking and function

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Abstract

Background: Two genetic variants in *SCN5A*, encoding the Nav1.5 Na⁺ channel α -subunit, were found in a 5-month-old girl who died suddenly in her sleep. The first variant is a missense mutation, resulting in an amino acid change (Q1832E), which has been described (but not characterized) in a patient with Brugada syndrome. The second is a nonsense mutation that produces a premature stop codon and a C-terminal truncation (R1944).

Methods and Results: To investigate their functional relevance with patch clamp experiments in transfected HEK-293 cells. The Q1832E mutation drastically reduced Nav1.5 current density. The R1944 C-terminal truncation had negligible effects on Nav1.5 current density. Neither of the mutations affected the voltage dependence of steady activation and inactivation or influenced the late Na⁺ current or the recovery from inactivation. Biochemical and immunofluorescent approaches demonstrated that the Q1832E mutation caused severe trafficking defects. Polymerase chain reaction cloning and sequencing the victim's genomic DNA allowed us to determine that the two variants were in *trans*. We investigated the functional consequences by coexpressing Nav1.5(Q1832E) and Nav1.5(R1944), which led to a significantly reduced current amplitude relative to wild-type.

Conclusions: These sudden infant death syndrome (SIDS)-related variants caused a severely dysfunctional Nav1.5 channel, which was mainly due to trafficking defects caused by the Q1832E mutation. The decreased current density is likely to be a major contributing factor to arrhythmogenesis in Brugada syndrome and the sudden death of this SIDS victim.

Keywords

Brugada syndrome; channelopathies; Na⁺ Channels; sudden infant death syndrome

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1 INTRODUCTION

Infant mortality, defined as the death of an individual before his or her first birthday, is a major national concern. In the United States, the infant mortality rate has decreased from ~45 deaths per 1,000 live births in the 1960s to approximately six deaths per 1,000 live births in 2011.¹⁻³ Despite substantial progress over the last 50 years, further reduction of preventable infant deaths is a major challenge. Sudden infant death syndrome (SIDS) is the leading cause of mortality in apparently normal infants and most commonly occurs during sleep.³ The American Academy of Pediatrics has issued specific recommendations in an effort to curb the persistently high incidence of SIDS, which involves improved sleeping habits and positioning; breastfeeding; routine immunizations; using a pacifier; and avoidance of overheating and exposure to tobacco smoke, alcohol, and illicit drugs. The physiological basis of SIDS involves respiratory or cardiac abnormalities, but mechanistic insight remains elusive. SIDS is likely multifactorial in nature and several different mechanisms may participate, including metabolic and genetic disorders.⁴ Mutations in cardiac ion channel genes (cardiac channelopathies) may lead to electrophysiological defects and lethal arrhythmias in a structurally normal heart, leaving no evidence to be found during an autopsy.

Ion channels are essential membrane proteins in cardiac myocytes. Their orchestrated activities are responsible for the exact shape of the action potential, for Ca^{2+} influx/efflux with each heart beat and for determining cardiac contractility. Even minor disruptions of ion channel function can upset the delicate balance between inward and outward ionic currents and may cause abnormal action of potential shortening or lengthening, disruption of ionic homeostasis, and the onset of life-threatening arrhythmias. The dysfunction of ion channels, or channelopathies, is responsible for many human diseases. In the heart, channelopathies cause syndromes such as the long QT syndrome (LQTS), catecholaminergic polymorphic ventricular tachycardia, and Brugada syndrome (BrS) and sudden death, particularly in the young.⁵⁻⁷ The genetic etiology of SIDS is multifactorial, heterogeneous, and most likely involves several different genetically controlled pathways. A clear role for channelopathies has emerged.⁸ For example, in a study of 34,442 infants over an 18-year period, with a 1-year follow-up on 33,034 infants, QT interval prolongation of the electrocardiogram was found to be a major risk factor in SIDS.⁹ This finding is in keeping with an earlier report, which found a prolonged QT interval of at least one parent in a set of 26% of the parents studied who had an infant with SIDS.¹⁰ In a large study of infant death in Norway, genetic screening of seven ion channel genes (KCNQ1, KCNH2, SCN5A, KCNE1, KCNE2, KCNJ2, CAV3) demonstrated mutations and rare variants in 26 of 201 confirmed SIDS cases.¹¹ Other studies also suggested a role for ion channel gene mutations in SIDS, including SCN5A¹²⁻¹⁷ (and its β subunit),¹⁸ KCNH2,¹⁵ KCND3,^{16,19} KCNJ8,²⁰ connexin-43,²¹ and KCNQ1.^{9,22} In a recently study, mutations in KCNQ1 and KCNH2 were found in ~3% of cases of intrauterine fetal death (stillbirth), which accounts for 50% of all perinatal deaths, consistent with potentially proarrhythmic phenotypes that may have contributed to premature fetal death.²³

Genetic evaluation (or a “molecular autopsy”) is becoming an indispensable step following sudden cardiac death in infants and in the young.²⁴ Since 2008, the New York City (NYC)

Office of Chief Medical Examiner has routinely integrated molecular testing of cardiac channelopathy genes in cases of sudden unexplained death (SUD). They recently described genetic testing of six major arrhythmia-associated genes in 274 autopsy-negative cases of SUD during 2008–2012.²⁵ A total of 141 of these cases were infants below 1 year of age, with the majority (92.9%) being younger than 6 months of age at the time of death.²⁵ In this infant cohort, eight previously classified channelopathy-associated variants and 13 novel putative channelopathy-associated variants were identified, which included mutations in *SCN5A*, *KCNQ1*, *KCNE2*, *KCNH2*, and *RYR2*. Knowledge of these variants by itself, however, is not diagnostic and functional studies are needed to match these SIDS-associated genetic variations with possible effects on channel function. The focus of this study was a case of SIDS of a 5-month-old girl who died suddenly in her sleep (Case ID: AN11B/H; tables 2 and 3 of Wang et al.).²⁵ Genetic screening revealed two point mutations in *SCN5A* (NM_198056.2:c.5504C>G and c.5830C>T), which respectively results in the substitution of glutamine residue for a glutamate residue at amino acid residue 1832 and the introduction of a premature stop codon at amino acid position 1944, resulting in a C-terminal truncation (Nav1.5-R1944). The Q1832E mutation has previously been associated with BrS,²⁶ but has not been characterized functionally. The purpose of this study was therefore to determine if these mutations affect Na⁺ current function and to assess whether these variants may have had potential role(s) in cardiac arrhythmogenesis and SUD.

2 METHODS

2.1 Site-directed mutagenesis

The human Nav1.5 cDNA in pcDNA3.1 (a gift from Nicole Schmitt, Ph.D., University of Copenhagen) was subjected to site-directed mutagenesis (Quickchange II XL, Aligent Technologies, Santa Clara, CA, USA) using primer 5'-GCCAAGCCCAACGAGATAAGCCTC-3' to generate Nav1.5(Q1832E) and 5'-AGGATGCCCCTGAGTGAGAGGGCCTC-3' to generate Nav1.5(R1944), which has an early truncation at position 1944. The cDNA mutations were verified by sequencing (Macrogen USA, Rockville, MD, USA).

2.2 Cell culture

Human Embryonic Kidney (HEK) 293 cells were cultured in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with heat-inactivated 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA), and penicillin-streptomycin (Mediatech, Manassas, VA, USA). Cells were grown in 35-mm culture plates until 70-80% confluence was reached. Cells were transfected using the Lipofectamine 2000 reagent (Life Technologies, Carlsbad, CA, USA) with 2 μ g of total DNA: 1.8 μ g of Nav1.5 and 0.2 μ g of a GFP plasmid, to allow visualization of successfully transfected cells for patch clamping. Cells were used for patch clamping or biochemistry 48 hours after transfection.

2.3 Patch clamping

Whole-cell currents were recorded at room temperature (Axopatch 200B; Molecular Devices, Sunnyvale, CA, USA), low-pass filtered with an 8-pole Bessel filter (−3 dB @ 1

Hz), and digitized (3 kHz; DigiData 1550A, Molecular Devices) using pClamp v10.5 software (Molecular Devices). Patch electrodes were manufactured (Zeitz-Instruments, Munich, Germany) using borosilicate glass (1.5-mm OD; World Precision Instruments, Sarasota, FL, USA) and had tip resistances of 1.5–2.5 M Ω when filled with (in mmol/L): 10 KCl, 105 CsF, 10 NaCl, 10 HEPES, 10 EGTA, and 10 TEA-Cl and pH adjusted to 7.2 with 2N CsOH. We performed experiments either with physiologically relevant extracellular Na⁺ concentrations, or with an extracellular Na⁺ of 30 mM. The physiologically relevant bath solution consisted of a modified Tyrode's solution (in mmol/L): 137 NaCl, 5.4 KCl, 10 HEPES, 0.5 MgCl₂, 1.8 CaCl₂, and pH adjusted to 7.4 using 2N NaOH. The low sodium bath solution consisted of (in mmol/L): 30 NaCl, 107 CsCl, 5.4 KCl, 10 HEPES, 0.5 MgCl₂, 1.8 CaCl₂, and pH adjusted to 7.4 using 2N NaOH. Data were not corrected for the liquid junction potential, which was calculated to be 7.4 mV when using the modified Tyrode's and 2.6 mV when using the low Na⁺ bath solution. The whole-cell capacitance and series resistance were compensated to levels greater than 80%. Currents were corrected for cell size by dividing by the cell capacitance and current densities are expressed as pA/pF.

2.4 Biotinylation assay

Transfected HEK-293 cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS) and biotinylated for 30 minutes using phosphate-buffered saline (PBS) containing 0.5 mg/mL of EZ Link Sulfo-NHS-SS-Biotin (Pierce, Thermo Fisher Scientific, Waltham, MA, USA). Plates were incubated for 10 minutes with PBS+100 mM glycine (to quench unlinked biotin) and washed twice with PBS. Cells were then lysed for 1 hour with lysis buffer RIPA lysis buffer (Sigma Aldrich, St. Louis, MO, USA). After centrifugation at $14,000 \times g$ for 15 minutes, supernatants were incubated with immobilized NeutrAvidin beads (Pierce) overnight and pelleted by centrifugation $1,000 \times g$ for 1 minute. All steps were performed at 4°C. After three washes with PBS, the biotinylated proteins were eluted with the 2 $\times$ Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) with 5% β -Mercaptoethanol at room temperature for 1 hour and analyzed by western blot.

2.5 Western blotting

Total protein concentration was measured using BioRad Protein Assay Dye Reagent (Bio-Rad). Samples with equal amounts of total protein were mixed with sample buffer (Thermo Fisher Scientific) containing 5% β -Mercaptoethanol and kept at room temperature prior to resolving with a 4–15% polyacrylamide gradient gel. After transfer to polyvinylidene difluoride membranes (Bio-Rad) and blocking with 5% milk in TBS-Tween (0.05%) for 1 hour, blots were incubated overnight at 4°C with primary antibodies and 1 hour at room temperature with secondary antibodies (dissolved in blocking buffer). Detection was with chemiluminescence (SuperSignal West Dura, Thermo Fisher Scientific) and photographic film or Odyssey CLx near-infrared fluorescence imaging system (LI-COR, Biosciences, Lincoln, NE, USA). Immunoblots were analyzed and quantified using NIH image software (ImageJ).

2.6 Immunofluorescence microscopy

HEK-293 cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature and permeabilized with 0.1% Triton X-100 in DPBS for 10 minutes. Nonspecific sites were

blocked by incubation with 5% normal goat serum for 30 minutes at room temperature. Subsequently, the cells were incubated with primary overnight at 4°C and incubated with fluorescent secondary antibody for 1 hour in the dark at room temperature. Finally, coverslips were mounted using mounting medium containing DAPI (VectaShield, Burlingame, CA, USA). Images were obtained using a Leica DMIRE2 microscope (Leica Microsystems, Wetzlar, Germany) using a 40× objective (NA:1.25-0.75) and analyzed using NIH image software (ImageJ).

2.7 Antibodies

Primary antibodies used were mouse monoclonal antivoltage-gated sodium channel (1:5000; K58/35, Sigma Aldrich), mouse monoclonal anti-GAPDH (1:10000; GAPDH-71.1, Sigma Aldrich), and rabbit polyclonal anti-Biotin (1:10000; ab53494, Abcam, Cambridge, MA, USA). Secondary antibodies used were goat anti-mouse IRDye 800CW (1:10000; LI-COR, Biosciences), donkey anti-Rabbit-HRP (1:20000; Jackson ImmunoResearch, West Grove, PA, USA) for western blotting, and goat anti-mouse Cy3-conjugated (1:400; Jackson ImmunoResearch) for immunocytochemistry.

2.8 Genomic PCR and sequencing

A 480 bp sequence corresponding to the amino acid region from E1810 to S1969 of Nav1.5 was polymerase chain reaction (PCR) amplified from genomic DNA of the SIDS victim using forward primer 5'-TGTAACGACGGCCAGTAGTATTCGGTCCTGT-3' and reverse 5'-TCGAGGTCGACGGTATCGATAGGAGATGGAGGAGC-3'. PCR amplification was performed with DreamTaq Green PCR 2× Master Mix (Thermo Fisher Scientific) for 25 cycles (95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds) and final extension at 72°C for 5 minutes. The PCR product was analyzed by agarose gel electrophoresis and visualized using ethidium bromide. The PCR product was subcloned into a pCR2.1 vector using TOPO TA Cloning Kit (Thermo Fisher Scientific), and transformed into DH5alpha *E. coli*. Positive clones were selected for sequencing (Macrogen USA).

2.9 Data analysis

Patch clamp data were analyzed using the pClamp 10.5 software and plotted using OriginPro 8 (OriginLab, Northampton, MA, USA). Current-voltage curves were produced by plotting the Nav1.5 peak current (normalized to the cell capacitance) against the clamp step potential. To generate activation curves, peak inward currents (I_{Na}) were converted to conductance as follows: $G_{Na} = I_{Na}/(V_m - V_{rev})$, where V_m is the clamp step voltage and V_{rev} the reversal potential, which was determined for each cell. Values of G_{Na} were normalized to the maximum conductance and plotted as a function of voltage. The voltage dependence of steady-state inactivation was determined using prepulse voltage steps from -160 mV to -20 mV (500 ms at 10 mV increments), prior to a clamping at a test voltage of -20 mV. Peak currents during the test step were normalized to the maximum current amplitude. Data points were subjected to curve fitting using a Boltzmann equation, $1/[1 + \exp[(V_m - V_{1/2})/k]]$, with V_m the prepulse potential, $V_{1/2}$ the midpoint of activation or inactivation, and k the slope factor. The inactivation process was analyzed by curve fitting of decaying currents to $[A_f * \exp(t/\tau_f)] + [A_s * \exp(t/\tau_s)]$, where A is the amplitude of the fit, t is the time, and τ is

the time constant. Recovery from inactivation was measured using a standard two-pulse protocol to -20 mV from a holding potential of -120 mV. The voltage between the pulses was -120 mV and was varied between 0.5 ms and 500 ms. Peak currents during the second step were normalized to those during the first step, plotted against the recovery time and subjected to curve fitting to $[A_f * \exp(t_f/\tau_f)] + [A_s * \exp(t_s/\tau_s)]$, with τ_f and τ_s respectively the fast and slow recovery time constants. Results are presented as mean $\pm$ standard error of the mean. Statistical comparisons were performed using Student's t -tests or an analysis of variance, as appropriate, and statistical significance was assumed when $P < 0.05$.

3 RESULTS

A *SCN5A* mutation that causes a single amino acid change in the Nav1.5 protein (Q1832E) was previously associated with BrS.²⁶ A SIDS victim was recently found to carry the same mutation, but in addition, also had another *SCN5A* missense variant that introduces a stop codon, resulting in an early Nav1.5 C-terminal truncation (R1944)²⁵ (Figure 1). We performed patch clamp experiments to investigate the functional consequences of these mutations in an *in vitro* heterologous expression system.

3.1 The Na⁺ current amplitude

HEK-293 cells were transfected with wild-type (WT) or mutant Nav1.5 cDNAs and whole cell currents were recorded using standard patch clamp techniques. Experiments were conducted in a low extracellular Na⁺ concentration to minimize current amplitudes and to facilitate voltage control during patch clamping. From a holding potential of -120 mV, the membrane was depolarized to voltages between -80 mV and 60 mV. Peak Nav1.5 currents, normalized to cell capacitance, were plotted against the test voltage to obtain current-voltage (IV) relationships (Figure 2). Compared to WT, the Nav1.5(Q1832E) current density was reduced by $\sim 50\%$, with statistical significance achieved at voltages between -50 mV and 10 mV [for example, -185.5 ± 26.5 pA/pF; $n = 12$ and -95.8 ± 15.4 pA/pF; $n = 9$; $P < 0.05$ at -20 mV, respectively, for WT and Q1832E]. We also investigated channel properties at physiologically relevant Na⁺ concentrations by conducting experiments using a standard Tyrode's solution, which produced essentially the same result. The peak current density of the Q1832E mutant was reduced by about 55% when compared to WT (Table 1). Under these conditions, we also tested effects of the other mutant on Nav1.5 current properties. Although the Nav1.5(R1944) current density appeared to be reduced (-549 ± 81.2 pA/pF; $n = 14$), this difference did not reach statistical significance.

3.2 The late Na⁺ current

In separate experiments, we examined effects of the mutations on the "late" Na⁺ current by measuring the current at the end of a 175 -ms voltage pulse at -20 mV, as the difference in the currents before and after the application of 20 μ M TTX, which eliminates artifacts due to leaks. We found that the TTX-sensitive noninactivating (or persistent) component of Nav1.5 was between 0.9% and 1.7% of the peak Na⁺ current. None of the mutations significantly affected the persistent Na⁺ current (Table 1).

3.3 Nav1.5 channel activation and inactivation kinetics

We next investigated channel kinetics. Activation time appeared to be unaffected by the Q1832E mutation, since the time to peak current was not different between Nav1.5(Q1832E) and WT currents at any voltage (Figures 3A and B). The truncation mutation, Nav1.5(R1944), similarly had no effect on the time to peak current (data not shown). We also investigated inactivation kinetics by exponential curve fitting. The inactivation time course of WT Nav1.5 was best described as a sum of two exponentials (Figure 3C). The Q1832E mutant, which had the largest effect on current density, was without effect on inactivation kinetics, as illustrated when Nav1.5(Q1832E) and WT currents were normalized to their maximal amplitudes and superimposed (Figures 3A and C). Similarly, Nav1.5(R1944) had no effect on the inactivation time constants (data not shown).

3.4 Voltage dependence of the activation and inactivation kinetic variables

The voltage dependence of the steady-state activation was derived from the data in Figure 2(A) by plotting the normalized peak conductance as a function of the membrane potential (Figure 4A). The WT Nav1.5 currents activated at around -60 mV and were maximally activated at ~ 10 mV, with a voltage of half-maximal activation of -39 mV. Neither of the mutations affected the voltage of half-maximal activation (Figure 4A and Table 1).

The voltage dependence of steady-state inactivation was measured using a 500-ms conditioning pulse between -160 mV and -20 mV, followed by test pulse at -20 mV. The voltage at which steady-state inactivation was half-maximal was -93 mV for WT Nav1.5. There was a slight, but not significant, depolarizing shift in the voltage of half-maximal steady-state inactivation for Nav1.5(Q1832E) (Figure 4B) in low sodium Tyrode's bath solution. Although a small change was observed for the other mutation in the voltage of half-maximal steady-state inactivation when using standard Tyrode's solution, this change was not statistically significant (Table 1).

3.5 Recovery from inactivation

The time course of recovery from inactivation (or channel availability) was determined with a standard double-pulse voltage clamp protocol. Neither of the mutations had a significant effect on the recovery time course (Figure 5 and Table 1).

3.6 The genomic phase of the mutations

The SIDS case in which the two SCN5A variants were identified is an infant who resided in a homeless and domestic violence shelter.²⁵ Parents and other family members could not be found to perform further genetic testing in order to determine if the two variants are in the same allele or whether they were located on different alleles. Moreover, family histories could not be determined. The two variants were in the same exon and sufficiently close to each other to allow for PCR analysis of the genomic DNA of the SIDS victim. The PCR product was subcloned for clonal expansion in bacteria. Sequencing individual colonies yielded products carrying either the Q1832E or the R1944 mutation. No colony carried both of the mutations or had WT Nav1.5 sequence (Figure 6A). We conclude therefore that each of the two genomic variants were present on different alleles and that the SIDS victim had one copy of Nav1.5(Q1832E) and one copy of Nav1.5(R1944). We performed patch

clamping to examine the possibility of a dominant negative effect of Q1832E over R1944 by cotransfecting HEK-293 cells with 1 μ g of each the two constructs and comparing to the effects of each mutant individually. The current density of cells cotransfected with the mutants (Q1832E + R1944) was -403 ± 91.1 pA/pF at -20 mV ($n = 10$), which was intermediate between the effects of the individual mutations (Figure 6B). As with Nav1.5(Q1832E) alone, coexpression of the two mutants led to no observed effects in the time to peak current, fast and slow component of the time constant of inactivation, voltage dependence of steady-state activation or inactivation, or the time course of recovery from inactivation (Figures 6C and D and Table 1).

3.7 Mechanism: the Q1832E mutation resulted in trafficking defects

The patch clamp data demonstrate that the most significant effect was caused by the Q1832E mutation. Since the current density was drastically reduced without appreciable effects on kinetic variables, the possibility is raised that the Nav1.5 channel surface density is decreased. We investigated this possibility with surface biotinylation experiments. HEK-293 cells were transfected with WT Nav1.5 or Nav1.5(Q1832E), surface biotinylated, and washed with glycine to block excess biotin. Biotinylated proteins were captured from cell lysates with NeutrAvidin agarose beads, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with a Nav1.5 antibody. Consistent with the reduced Nav1.5 current density, these data revealed a 48% ($n = 3$; $P < 0.05$) decrease in the surface expression of Nav1.5(Q1832E) relative to WT Nav1.5 (Figure 7A). GAPDH was not observed as a surface protein, which confirmed that the biotinylated fraction represents specific labeling of membrane proteins. Equal loading was confirmed by similar amounts of GAPDH and Nav1.5 proteins in the cell lysates. The latter result also suggests that Nav1.5 protein stability was not greatly impaired by the Q1832E mutation. No appreciable effects on surface expression were observed for the Nav1.5(R1944) mutant channels (not shown).

The reduced surface expression of Nav1.5-Q1832E in the absence of changes in total Nav1.5 protein suggests the possibility of a trafficking defect. We therefore performed an immunofluorescence microscopy analysis to examine the channel's subcellular localization. HEK-293 cells transfected with WT-Nav1.5 exhibited diffuse staining with strong localization at the cell periphery (Figure 7B). In contrast, the Nav1.5-Q1832E protein had little evidence of surface expression, but instead was retained within large intracellular bodies (Figure 7B). Thus, these demonstrate that the Q1832E mutation led to severe trafficking defects.

4 DISCUSSION

We characterized two rare variants in the *SCN5A* gene, previously reported in a SIDS case by the NYC Chief Medical Examiner,²⁵ which both result in defects of the Nav1.5 C-terminus. The first is an amino acid change (Q1832E), which has previously been reported in a patient with BrS.²⁶ The second variant leads to a nonsense mutation that produces a premature stop codon and a C-terminal truncation (R1944). Both of the variants are computationally predicted to be potentially harmful, but neither have previously been

characterized functionally. We extensively examined the gating variables of the Nav1.5 mutant channels. None of the mutations had effects on the time course of recovery from inactivation or the voltage dependence of steady-state activation or inactivation. We also did not observe major effects on the persistent Na⁺ current properties. We conclude, therefore, that these C-terminal mutations did not have strong effects on channel gating.

Biologically, the most significant effect observed in our study was a drastic (~50%) reduction in the Nav1.5 current density by the Q1832E mutation, either when expressed by itself, or when co-expressed with Nav1.5(R1944). The latter situation recapitulates the SIDS case since our data demonstrate that the SCN5A variants occurred on different alleles, which would give rise to two different mutant channels. Although previous studies described dominant negative effects of SCN5A mutations mediated through alpha-alpha subunit interactions,²⁷ our studies show that co-expression of the two mutants led to a significant reduction of current density (~57% of WT) and an intermediate phenotype between that of Nav1.5(Q1832E) and Nav1.5(R1944). It has been demonstrated that the presence of the Nav1.5 β -subunit is needed α - α subunit interactions and for the dominant negative effect of mutations to manifest.²⁸ Since our studies were performed in the absence of exogenously expressed β -subunits, we cannot exclude the possibility of dominant negative effects that may be responsible for even larger reductions in overall Na⁺ current amplitudes, which may possibly cause cardiac rhythm defects and/or sudden death.

The current amplitude was not strongly reduced by the Nav1.5 C-terminus truncation (R1944). This was somewhat unexpected since mutations beyond position 1944 have previously been associated with LQTS, BrS, SUD, SIDS, and intrauterine fetal death.^{11,23,25,26,29-34} Moreover, the distal Nav1.5 C-terminus is known to interact (via a PDZ domain) with proteins such as syntrophin, protein tyrosine phosphatase PTPH1 and SAP97,³⁵ which regulate the Nav1.5 biophysical properties.³⁶ It was surprising, therefore, that the R1944 C-terminal truncation, which lacks these protein-protein interaction motifs, had such a mild phenotype with no appreciable effect on kinetics. In previous studies, which described the importance of the Nav1.5 C-terminus in the voltage dependence of activation, inactivation, and persistent Na⁺ current,³⁷⁻⁴⁰ truncations were made that removed some or the entire structured region of the Nav1.5 C-terminus. Smaller deletions has less of an effect. For example, a C-terminal truncation at L1921 causes a moderate reduction in peak current with no effect on gating parameters,³⁷ which is in agreement with our findings. Differences between studies may also relate to the study conditions. For example, when the last 10 amino acids of Nav1.5 are truncated, including the PDZ domain, significant effects on inactivation is observed in *Xenopus* oocytes, whereas the truncation led to currents with macroscopic biophysic properties that were not different from those of WT channels when expressed in HEK-293 cells.⁴¹

Although aggravated by the 1944 C-terminal truncation when co-expressed, our data demonstrate that the reduced Nav1.5 current density was most significant with the Q1832E point mutation. Since total Nav1.5 protein in cell lysates was unaffected by the Q1832E mutant, transcriptional and translational processes appear to be unaffected. There is also unlikely to be significant effects on protein stability or degradation. In contrast, the biotinylation data demonstrate a reduction in surface protein content, consistent with a

trafficking defect. Indeed, our fluorescence microscopy demonstrate the Nav1.5(Q1832E) protein in large intracellular aggresomes, which typically occurs with misfolded proteins.⁴² Thus, a single amino acid change in the Nav1.5 C-terminus results in a severe trafficking defect. The mechanism by which Q1832E affects trafficking is not entirely clear. Apart from the distal PDZ domain, the Nav1.5 C-terminus contains several sites responsible for interaction with other proteins, including calmodulin (CaM) that binds to an IQ motif, Nedd4/Nedd4-like enzymes that bind to a PY motif, and fibroblast growth factor homologous factor family members (FHF) that interact with a proximal region of the C-terminus,⁴³ which may include the Q1832 residue.⁴⁴ Previously reported mutations (e.g., D1790G and H1849R) associated with LQT-3 and BrS, are thought to interact with FHFs.²⁶ FHFs regulate Nav1.5 function and trafficking, as demonstrated by the finding that knockdown of FHF2, the most abundant FHF in adult mouse ventricular myocytes, with shRNA leads to a reduced cell surface Nav1.5 channel levels.⁴⁵ We think it likely, therefore, that the Q1832E mutation may disrupt interactions with proteins such as FHF2, leading to defective trafficking of the channel to the cell membrane.

In conclusion, our data demonstrate that the Q1832E mutation severely impairs the ability of Nav1.5 channels to properly express on the cell surface, leading to a significant reduction in Na⁺ current, which may be partly responsible for its association with BrS and SIDS. The situation in the SIDS case is aggravated by the presence of a second mutant channel with a truncated C-terminus. A limitation of our studies is that, as in many other studies of this nature, they were performed using a heterologous expression system, which may not faithfully recapitulate the native Na⁺ current of cardiomyocytes. We think it is likely that the presence of additional regulatory subunits and associated proteins may further modify the biophysical properties of the mutant native channels.

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FIGURE 1.

(A) Genomic DNA sequencing analysis of the SCN5A gene. A heterozygous G-to-C transition at position 5494 and T-to-C transition at position 5830 of SCN5A resulted in a glutamine to glutamate and arginine and stop substitution, respectively. (B) Schematic representation showing position of each mutation on the α -subunit of the human cardiac sodium channel Nav1.5

FIGURE 2.

(A) Representative Nav1.5 current traces of WT and Q1832E Na⁺ channels, using the voltage protocol as shown in the inset. (B) The Nav1.5 current densities are plotted as a function of the test voltage. Data points represent mean $\pm$ standard error of the mean, with n = 12 in wild-type and n = 9 for the Q1832E group. *P < 0.05 versus wild-type (Student's *t*-test). These experiments were conducted in a Tyrode's bath solution containing 30 mM NaCl

FIGURE 3.

(A) Superimposed representative I_{Na} traces showing activation and inactivation phases of the WT and Q1832E currents induced by a depolarization step at -20 mV. I_{Na} were normalized for equal amplitude. (B) Time to peak, the time from the onset of depolarization to peak I_{Na} , plotted as a function of voltage. (C) Inactivation time constants plotted as a function of membrane potentials. The time constants were derived from double-exponential fit to the inactivation portions of current-traces shown in (A). Data points represent mean $\pm$ standard error of the mean, with $n = 12$ in wild-type and $n = 9$ for the Q1832E group. $*P < 0.05$ versus wild-type (Student's t -test). These experiments were conducted in a Tyrode's bath solution that contains 30 mM NaCl.

FIGURE 4.

Steady-state activation (A) and steady state inactivation (B) curves for WT and Q1832E Nav1.5 channels. Solid lines represent the Boltzmann fit of data points. Data points represent mean $\pm$ standard error of the mean, with $n = 12$ in wild-type and $n = 8$ for the Q1832E group. These experiments were conducted in a Tyrode's bath solution that contains 30 mM NaCl

FIGURE 5.

(A) Representative I_{Na} traces of sodium channel elicited by standard two-pulse protocol testing recovery from inactivation. (B) Peak I_{Na} induced by second pulse were normalized to I_{Na} induced by first pulse and plotted against the time interval between pulses. Solid lines represent the fit of data points with a double exponential function. Data points represent mean $\pm$ standard error of the mean, with $n = 10$ in wild-type and $n = 8$ for the Q1832E group. These experiments were conducted in a Tyrode's bath solution that contains 30 mM NaCl

FIGURE 6.

(A) Representative DNA sequencing analysis of bacterial colonies (n = 10). (B) I_{Na} plotted as a function of voltage of Nav1.5 WT and Q1832E+ R1944 currents. Steady-state activation (C) and steady state inactivation (D) curves for WT and Q1832E+ R1944 Nav1.5 channels. Solid lines represent the Boltzmann fit of data points. Data points represent mean $\pm$ standard error of the mean, with n = 17 in wild-type and n = 10 for the Q1832E+ R1944 group. These experiments were conducted in a Tyrode's bath solution that contains 30 mM NaCl

FIGURE 7.

(A) Representative immunoblot of total and surface-biotinylated proteins from HEK-293 cells transfected with WT or Q1832E Nav1.5. Input lysates and biotinylated membrane proteins were probed with a pan-Nav antibody. GAPDH antibody and biotin antibody were used as lysate and membrane protein loading controls, respectively. GAPDH antibody was used as a negative control for membrane proteins. (B) Representative fluorescent images from HEK-293 cells cotransfected with GFP (green) and WT or Q1832E Nav1.5 constructs (red). Cell nuclei were visible by DAPI staining (blue)

TABLE 1

Biophysical and kinetic properties of wild-type (WT) and mutant Nav1.5 channels

Na _v 1.5	Peak Current (pA/pF)	n	Persistent Current(% of max peak I _{Na})	n	Steady-State Inactivation V _{1/2} (mV)	Slope (mV)	n	Steady-State Activation V _{1/2} (mV)	Slope (mV)	n	Recovery Fast Time Constants (ms)	Recovery Slow Time Constants (ms)	n
WT	-705.32 ± 78.83	17	1.4 ± 0.7	5	-79.03 ± 3.23	5.91 ± 0.45	7	-33.28 ± 1.74	5.34 ± 0.33	16	5.65 ± 0.89	34.34 ± 12.46	6
Q1832E	-312.15 ± 35.89 [*]	6	0.9 ± 0.5	5	-83.29 ± 1.94	5.51 ± 0.25	6	-35.40 ± 1.84	5.80 ± 0.55	5	5.86 ± 1.16	39.36 ± 18.80	6
R1944	-548.95 ± 82.19	14	1.7 ± 0.8	5	-77.30 ± 3.23	6.11 ± 0.35	9	-33.34 ± 1.59	5.53 ± 0.52	12	5.17 ± 0.68	56.06 ± 27.9	5
Q1832E plus R1944 [‡]	-403.18 ± 91.05 [*]	10	1.0 ± 0.4	10	-78.30 ± 1.21	5.43 ± 0.25	10	-35.76 ± 2.48	5.00 ± 0.72	10	5.11 ± 1.22	49.18 ± 17.9	6

Notes: Peak current density and persistent current were measured at -20 mV. Midpoint (V_{1/2}) of the activation, fast inactivation, and slope (k) factors were calculated from Boltzmann fit of activation and fast inactivation curves for each construct. Fast and slow time constants of recovery from fast inactivation were calculated from double exponential fit of recovery from inactivation curves. Values are given as mean ± standard error of the mean of n experiments. Experiments in Table 1 were conducted using the modified Tyrode's bath solution with a physiologically relevant extracellular Na⁺ concentration.

^{*} P < 0.05 versus WT

[‡] Q1832E plus R1944 refers to the coexpression of the two mutants.