α1-Syntrophin Mutations Identified in Sudden Infant Death Syndrome Cause an Increase in Late Cardiac Sodium Current

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**Background**—Sudden infant death syndrome (SIDS) is a leading cause of death during the first 6 months after birth. About 5% to 10% of SIDS may stem from cardiac channelopathies such as long-QT syndrome. We recently implicated mutations in α1-syntrophin (SNTA1) as a novel cause of long-QT syndrome, whereby mutant SNTA1 released inhibition of associated neuronal nitric oxide synthase by the plasma membrane Ca-ATPase PMCA4b, causing increased peak and late sodium current ($I_{Na}$) via S-nitrosylation of the cardiac sodium channel. This study determined the prevalence and functional properties of SIDS-associated SNTA1 mutations.

**Methods and Results**—Using polymerase chain reaction, denaturing high-performance liquid chromatography, and DNA sequencing of SNTA1’s open reading frame, 6 rare (absent in 800 reference alleles) missense mutations (G54R, P56S, T262P, S287R, T372M, and G460S) were identified in 8 (3%) of 292 SIDS cases. These mutations were engineered using polymerase chain reaction–based overlap extension and were coexpressed heterologously with SCN5A, neuronal nitric oxide synthase, and PMCA4b in HEK293 cells. $I_{Na}$ was recorded using the whole-cell method. A significant 1.4- to 1.5-fold increase in peak $I_{Na}$ and 2.3- to 2.7-fold increase in late $I_{Na}$ compared with controls was evident for S287R-, T372M-, and G460S-SNTA1 and was reversed by a neuronal nitric oxide synthase inhibitor. These 3 mutations also caused a significant depolarizing shift in channel inactivation, thereby increasing the overlap of the activation and inactivation curves to increase window current.

**Conclusions**—Abnormal biophysical phenotypes implicate mutations in SNTA1 as a novel pathogenic mechanism for the subset of channelopathic SIDS. Functional studies are essential to distinguish pathogenic perturbations in channel interacting proteins such as α1-syntrophin from similarly rare but innocuous ones. (Circ Arrhythm Electrophysiol. 2009;2:667-676.)

Key Words: death ■ sudden ■ long-QT syndrome ■ genetics ■ ion channels ■ nitric oxide synthase

**Clinical Perspective on p 676**

More than a decade ago, an impressive clinical investigation based on 34,442 Italian newborn infants indicated that prolongation of the QTc interval (>440 ms) in the first week of life was strongly associated with SIDS. Soon after, molecular evidence provided definitive evidence to link SIDS and type 1 long-QT syndrome (LQTS). Recent postmortem molecular analyses have established a pathogenic basis for stem abnormalities, SIDS remains poorly understood with largely unknown etiology.2–6
channelopathic SIDS with the identification and functional characterization of mutations in LQTS and short-QT syndrome susceptibility genes (KCNQ1, KCNH2, SCN5A, KCNE2, CAV3, and SCN4B) in SIDS victims. Notably, these molecular studies suggest that LQTS mutations are responsible for approximately 5% to 10% of SIDS, with approximately 50% of this subset of channelopathic SIDS stemming from mutations occurring in either the SCN5A-encoded pore-forming α-subunit of the Nav1.5 cardiac sodium channel (SCN5A) or channel interacting proteins (ChIPs) of the SCN5A macromolecular complex.

SCN5A gain-of-function mutations resulting in persistent late sodium current (I\(_{Na}\)) provide the molecular substrate for approximately 5% to 10% of congenital LQTS known as LQT3, in which patients most often present with potentially lethal arrhythmias predominantly at rest or while sleeping.

α1-Syntrophin (SNTA1), a dystrophin-associated protein, is the dominant syntrophin isoform in cardiac muscle. As a scaffolding adapter with several protein interaction motifs, SNTA1 binds to neuronal nitric oxide synthase (nNOS) and the cardiac isoform of the plasma membrane Ca2+-ATPase (PMCA4b) to form a complex in which PMCA4b inhibits nNOS-mediated nitric oxide (NO) synthesis. Through a PDZ domain, SNTA1 interacts with the C-terminus of SCN5A. Recently, we discovered that SNTA1 connects the pore-forming cardiac sodium channel α-subunit to the nNOS-PMCA4b complex in cardiomyocytes and implicated SNTA1 as a novel LQTS-susceptibility gene (LQT12), where the LQTS-associated mutation, A390V-SNTA1, disrupted binding with PMCA4b, released inhibition of nNOS, and accentuated both peak and late I\(_{Na}\) via S-nitrosylation of the cardiac sodium channel. We also recently reported on a different LQTS-associated mutation, A257G-SNTA1, which also demonstrated altered channel kinetics in HEK293 cells and cardiomyocytes.

Considering that perturbations in the Nav1.5 sodium channel complex may account for the majority of channelopathic SIDS and our recent identification of mutations in another sodium channel interacting protein, α1-syntrophin, as a novel cause of LQTS, we hypothesized that mutations in SNTA1 may increase the risk for a malignant ventricular arrhythmia during infancy and account for some cases of SIDS. In this study, we aimed to determine the spectrum, prevalence, and functional properties of SNTA1 mutations in SIDS.

Methods

Population-Based Cohort of SIDS

Two-hundred ninety-two SIDS cases derived from population-based cohorts of unexplained infant deaths (114 girls, 177 boys, 1 unknown; 203 white, 76 black, 10 Hispanic, 2 Asian, 1 unknown; average age, 2.9±1.9 months; range, 6 hours to 12 months) were submitted for postmortem genetic testing. To be rendered SIDS, the death of the infant under age 1 year had to be sudden, unexpected, and unexplained after a comprehensive medico-legal autopsy. Infants whose death was due to asphyxia or specific disease were excluded. This study was approved by Mayo Clinic Institutional Review Board as an anonymous study. As such, only limited medical information was available, including sex, ethnicity, and age at death. Time of day, medication use, and position at death were not available. By definition, the infant’s medical history and family history were negative.

SNTA1 Mutational Analysis

Genomic DNA was extracted from frozen necropsy tissue with the Qiagen DNeasy Tissue Kit (Qiagen, Inc, Valencia, Calif) or from autopsy blood with the Puregene DNA Isolation Kit (Gentra, Minneapolis, Minn). Using polymerase chain reaction (PCR), denaturing high-performance liquid chromatography, and direct DNA sequencing, open reading frame/slice site mutational analysis on SNTA1 (chrosome 20q11.2, 8 exons) was performed as previously described. Primer sequences, PCR conditions, and denaturing high-performance liquid chromatography conditions are available on request.

Plasmid Constructions of Mammalian Expression Vectors

The cDNA of wild-type (WT) human SNTA1 gene (Genbank accession No. NM_003098) was subcloned into pRES2EGFP plasmid vector (Clontech Laboratories, Palo Alto, Calif). The G54R, P565, T262P, S287R, T372M, and G460S-SNTA1 missense mutations were incorporated into WT SNTA1 using the PCR-based overlap-extension method as previously reported. The cDNAs of nNOS (Genbank accession No. NM_052799) and PMCA4b (Genbank accession No. AY560895) were a generous gift from Solomon H. Snyder (Johns Hopkins University) and Emanuel E. Strehler (Mayo Clinic), respectively. All clones were sequenced to confirm integrity and to ensure the presence of the introduced mutations and the absence of other substitutions caused by PCR.

Chemical Reagent

The NOS inhibitor NG-nitro-L-arginine (L-NMMA) was obtained from Cayman Chemical (Ann Arbor, Mich). The L-NMMA was diluted in PBS buffer (pH 7.2) 10 minutes before use.

Mammalian Cell Transfection

The WT or mutant SNTA1 in pRES2EGFP vector was transiently cotransfected with expression vectors containing SCN5A (hNav1.5, Genbank accession No. AB158469), nNOS, and PMCA4b at a ratio of 1:4:4:4, respectively, into HEK293 cells with FuGENE6 reagent (Roche Diagnostics, Indianapolis, Ind) according to manufacturer’s instructions.

Electrophysiological Measurements

Macroscopic voltage-gated I\(_{Na}\) was measured 48 hours after transfection with the standard whole-cell patch clamp method at 21°C to 23°C in the HEK293 cells. The extracellular (bath) solution contained the following (in mM): CsF 120, CsCl2 20, EGTA 2, NaCl 5, and CaCl2 1.8, MgCl2 0.75, and HEPES 5 and was adjusted to pH 7.4 with NaOH. The intracellular (pipette) solution contained the following (in mM): CsF 120, CsCl2 20, EGTA 2, NaCl 5, and HEPES 5 and was adjusted to pH 7.4 with CsOH. Microelectrodes were manufactured from borosilicate glass using a puller (P-87, Sutter Instrument Co, Novato, Calif) and were heat polished with a microforge (MF-83, Narishige, Tokyo, Japan). The resistances of microelectrodes ranged from 1.0 to 2.0 MΩ. Voltage-clamp data were generated with pClamp software 10.2 and an Axopatch 200B amplifier (Axon Instruments, Foster City, Calif) with series-resistance compensation. Membrane current data were digitalized at 100 kHz, low-pass filtered at 5 kHz, and then normalized to membrane capacitance.

Activation was measured by clamp steps of −120 to 60 mV in 10 mV increments from a holding potential of −140 mV. The midpoint of activation was obtained using a Boltzmann function, where \(G_{Na} = G_{Na0} \times \exp \left( \frac{V_{1/2} - V}{k} \right) \), where \(V_{1/2}\) and \(k\) are the midpoint and slope factor, respectively. \(G_{Na} = \frac{G_{Na0} - G_{m}}{G_{Na0} - G_{m}} \), where \(G_{m}\) is the reversal potential and \(V\) is the membrane potential. Steady-state inactivation was measured in response to a test depolarization to 0 mV for 24 ms from a holding potential of −140 mV, following a 1-second conditioning pulse from −150 mV to 0 mV in
Table 1. Demographic Information and Functional Consequences for SNTA1 Mutation-Positive SIDS

<table>
<thead>
<tr>
<th>Age, mo</th>
<th>Sex</th>
<th>Ethnicity</th>
<th>Nucleotide Change</th>
<th>Amino Acid Change</th>
<th>Protein Location</th>
<th>Functional Consequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>H</td>
<td>160 G&gt;C</td>
<td>G54R</td>
<td>PH1</td>
<td>WT</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>B</td>
<td>166 C&gt;T</td>
<td>P56S</td>
<td>PH1</td>
<td>WT</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>B</td>
<td>166 C&gt;T</td>
<td>P56S</td>
<td>PH1</td>
<td>WT</td>
</tr>
<tr>
<td>0</td>
<td>F</td>
<td>B</td>
<td>166 C&gt;T</td>
<td>P56S</td>
<td>PH1</td>
<td>WT</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>W</td>
<td>784 A&gt;C</td>
<td>T262P</td>
<td>PH1</td>
<td>↑ Peak I(_{\text{Na}}) + Shift inact</td>
</tr>
<tr>
<td>0.75</td>
<td>F</td>
<td>W</td>
<td>861 C&gt;G</td>
<td>S287R</td>
<td>Linker</td>
<td>↑ Peak I(_{\text{Na}}) + Shift inact</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>W</td>
<td>1115 C&gt;T</td>
<td>T372M</td>
<td>PH2</td>
<td>↑ Late I(_{\text{Na}}) + Shift inact</td>
</tr>
<tr>
<td>1</td>
<td>F</td>
<td>W</td>
<td>1378 G&gt;A</td>
<td>G460S</td>
<td>SU</td>
<td>↑ Peak I(_{\text{Na}}) + Shift inact</td>
</tr>
</tbody>
</table>

F indicates female; M, male; H, Hispanic; B, black; W, white; Inact, inactivation.

Results

SNTA1 Mutational Analysis in SIDS

Overall, 6 distinct, rare SNTA1 missense mutations (G54R, P56S, T262P, S287R, T372M, and G460S) were detected in 8 of the 292 SIDS cases (2.7%; 7 girls, average age 1.7 months; range, just after birth to 4 months; Table 1 and Figure 1A). SNTA1 mutations were identified in 7 of 114 (6.1%) female infants compared with only 1 of 177 (0.6%) male infants (P<0.01). P56S was found in 3 cases, all black infants. Demographic data for all 1 SNTA1 mutation-positive SIDS is shown in Table 1. No other putative cardiac channelopathic gene mutations had been previously identified in these 8 SIDS victims.9,13,15,16,26,27 One of the P56S-SNTA1 infants also hosted the previously described SCN5A late I\(_{\text{Na}}\)-producing T78M-CAV3 rare polymorphism.13 In addition, 4 cases and 3 control subjects were heterozygous for the combined variants P74L and A257G. Given the ~1% frequency for both cases and control subjects, P74L/A257G was excluded from further studies due to its status as a common polymorphism. Because of the anonymized nature of the study, determination of genetic variants as transmitted or de novo was not feasible.

All 6 mutations were absent in 800 reference alleles and involved residues with various degrees of conservation across species, with the G54R, S287R, T372M, and G460S being most highly conserved (Figure 1B). Two of the mutations (G54R and P56S) localized to the first pleckstrin homology 1 (PH1) domain (amino acids 1 to 80), 1 mutation (T262P) localized to the second PH1 domain (aa 161 to 263), 2 mutations (S287R and T372M) localized either in or very near the pleckstrin homology 2 (PH2) domain (aa 292 to 399), and 1 mutation (G460S) localized to the syntrophin unique (SU) domain (aa 447 to 503) of SNTA1 (Figure 1C and 1D).

SNTA1 Mutations in SIDS Increase Peak and Late I\(_{\text{Na}}\) in HEK293 Cells

Functional characterization of SNTA1 mutations was performed in HEK293 cells, which transiently expressed SCN5A, nNOS, PMCA4b, and either the wild-type SNTA1 (WT-SNTA1) or the mutant SNTA1. Compared with WT-SNTA1, 4 of the 6 SNTA1-encoded missense mutations; T262P-, S287R-., T372M-, and G460S-SNTA1, had significantly larger peak I\(_{\text{Na}}\) amplitudes, whereas G54R- and P56S-SNTA1 were similar to WT-SNTA1 (Figure 2A and 2B and Table 2).

We measured the level of persistent/late I\(_{\text{Na}}\) as a percentage of peak I\(_{\text{Na}}\) elicited by prolonged depolarization and leak subtraction. Compared with WT-SNTA1, S287R-, T372M-, and G460S-SNTA1 caused a significant 2.3- to 2.7-fold increase in late I\(_{\text{Na}}\), whereas the other 3 missense mutations (G54R-, P56S-, and T262P-SNTA1) were comparable to WT-SNTA1 (Figure 2C and 2D and Table 2).
The Sodium Channel Gain of Function Caused by the 3 SIDS-Associated SNTA1 Mutations Is nNOS-SNTA1-PMCA4b Complex Dependent

To observe the effect of NOS inhibitor on the PMCA4b-nNOS-SNTA1-SCN5A complex, L-NMMA (100 μmol/L) was introduced into the HEK293 cell culture medium 12 hours before testing. The marked accentuation in late $I_{Na}$ precipitated by S287R-, T372M-, and G460S-SNTA1 was abolished by L-NMMA, and the corresponding peak $I_{Na}$ were also reversed (Table 2). These results indicated that akin to the original LQT12-associated mutation, A390V, NO was the key factor by which SNTA1 affected function. To determine whether these SNTA1 mutations cause abnormal $I_{Na}$ through modulation of its interaction with SCN5A, we performed functional characterization of SIDS-associated SNTA1 mutations using HEK293 cells coexpressing only SCN5A and either the WT-SNTA1 or the SNTA1 mutants. None of the 6 SNTA1 mutations coexpressed with SCN5A alone showed a significant difference in peak $I_{Na}$, late $I_{Na}$, or channel kinetics compared with WT-SNTA1 (supplemental Table 1).

To clarify whether PMCA4b (ie, the full nNOS-PMCA4b-SNTA1 complex) is required for SNTA1 mutation-mediated effects on SCN5A function, we tested HEK293 cells only coexpressing SCN5A, nNOS, and either the WT-SNTA1 or the SNTA1 mutants, without PMCA4b expression. Again, none of the mutations showed a significant difference in peak $I_{Na}$, late $I_{Na}$, or channel kinetics compared with WT-SNTA1 (supplemental Table 2). These data suggest that the sodium channel gain of function caused by the 3 SIDS-associated SNTA1 mutants...
is mediated by the entire nNOS-PMCA4b-SNTA1 complex.

**SNTA1 Mutations Changed Sodium Channel Gating Properties Through an nNOS-Dependent Mechanism**

We analyzed the kinetic parameters of activation and inactivation of all 6 SNTA1 mutations and compared these data with the WT-SNTA1. Although none of the SNTA1 mutations showed a significant difference in activation parameters compared with WT-SNTA1 (Figure 3A and Table 2), the T262P-, S287R-, T372M-, and G460S-SNTA1 mutations caused a statistically significant depolarizing shift in inactivation (Figure 3B and Table 2). For the mutants S287R-, T372M-, and G460S-SNTA1, the increase in overlap of the activation and inactivation curves resulted in the increase of late $I_{Na}$.

**Table 2. Electrophysiological Properties of Sodium Channels in HEK293 Cells Coexpressing SCN5A, PMCA4b, nNOS, and Either WT or Mutant SNTA1**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Peak $I_{Na}$</th>
<th>Activation</th>
<th>Inactivation</th>
<th>Late $I_{Na}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$pA/pF$</td>
<td>V1/2, mV</td>
<td>K</td>
<td>n</td>
</tr>
<tr>
<td>WT-SNTA1</td>
<td>−185±18</td>
<td>−40.7±0.9</td>
<td>4.0</td>
<td>30</td>
</tr>
<tr>
<td>G460S-SNTA1</td>
<td>−254±23*</td>
<td>−39.8±1.1</td>
<td>4.0</td>
<td>12</td>
</tr>
<tr>
<td>T372M-SNTA1</td>
<td>−271±28*</td>
<td>−40.8±1.0</td>
<td>4.1</td>
<td>12</td>
</tr>
<tr>
<td>S287R-SNTA1</td>
<td>−284±35*</td>
<td>−42.5±2.3</td>
<td>4.2</td>
<td>9</td>
</tr>
<tr>
<td>T262P-SNTA1</td>
<td>−335±51*</td>
<td>−40.3±0.6</td>
<td>4.1</td>
<td>9</td>
</tr>
<tr>
<td>P56S-SNTA1</td>
<td>−221±30</td>
<td>−42.8±1.4</td>
<td>4.3</td>
<td>14</td>
</tr>
<tr>
<td>G54R-SNTA1</td>
<td>−240±40</td>
<td>−39.9±1.0</td>
<td>4.0</td>
<td>9</td>
</tr>
<tr>
<td>G460S-SNTA1+L-NMMA</td>
<td>−231±39</td>
<td>−43.0±1.7</td>
<td>4.2</td>
<td>5</td>
</tr>
<tr>
<td>T372M-SNTA1+L-NMMA</td>
<td>−228±34</td>
<td>−41.7±2.1</td>
<td>4.3</td>
<td>5</td>
</tr>
<tr>
<td>S287R-SNTA1+L-NMMA</td>
<td>−258±41</td>
<td>−41.4±1.4</td>
<td>4.0</td>
<td>5</td>
</tr>
</tbody>
</table>

*P<0.05 vs WT-SNTA1.
the “window current” (Figure 3C, 3D, and 3E). Time constants ($\tau_f, \tau_s$) were obtained from 2-exponential fits of decay phase of macroscopic $I_{Na}$ measured at various test potentials. Compared with WT-SNTA1, the S287R-, T372M-, and G460S-SNTA1 mutations showed significantly larger $\tau_f$ values across a wide range of test potentials (Figure 4), indicating that fast inactivation was impaired and sodium current decay was slower. There was no difference in time constant $\tau_s$ or fractional amplitudes for the 2 time constants observed. Notably, the inactivation parameters (Table 2) and time

Figure 3. Voltage-dependent gating for SCN5A coexpressed with PMCA4b, nNOS, and either WT or mutant SNTA1. A, None of the 6 SNTA1 mutations altered steady-state activation parameters significantly. B, G460S-, T372M-, S287R-, and T262P-SNTA1 caused a statistically significant depolarizing shift in inactivation by 2.5 to 4.6 mV. The peak current activation data are replotted as a conductance ($G$) curve with steady-state inactivation relationships to show C, G460S-; D, T372M-; and E, S287R-SNTA1 increase the overlap of these relationships. Lines represent fits to Boltzmann equations with parameters of the fit and $n$ numbers in Table 2. Triangles represent inactivation curves for WT (filled) and mutant (open), whereas the filled boxes and open circles represent activation curves for WT and mutant, respectively. The window area of each mutant (right-slanted line area under curves) was significantly enhanced beyond WT (left-slanted line area under curves).

Figure 4. Decay of macroscopic current and voltage dependence of inactivation fast time constants. A, Representative normalized whole-cell current traces at $-20$ mV showing slower decay in G460S-, T372M, and S287R-SNTA1 compared with WT-SNTA1. B, Compared with WT-SNTA1, G460S-, T372M-, and S287R-SNTA1 showed significantly larger fast component ($\tau_f$) values across a wide range of test potentials from $-20$ mV to $10$ mV except T372M, where deviations from WT were from $-10$ to $10$ mV. *$P<0.05$ versus WT-SNTA1.
constants $\tau_1$ of S287R-, T372M-, and G460S-SNTA1 (data not shown) returned to normal levels after the application of L-NMMA, suggesting the alteration of channel gating properties caused by these mutants was mediated by an NO-dependent mechanism akin to the NO-dependent effect of these 3 particular SNTA1 missense mutations on both peak and persistent sodium current. Last, there were no significant differences between WT and mutants in recovery from inactivation (data not shown).

**Discussion**

**SNTA1: A Novel Susceptibility Gene for SIDS**

Cardiac channelopathies, especially LQTS, have been shown to account for up to 10% of SIDS. So far, mutations in 8 cardiac channelopathy-susceptibility genes have been implicated in the pathogenesis of SIDS. Four of these genes encode cardiac ion channel $\alpha$-subunits ($SCN5A$, $KCNJ1$, $KCNH2$, and $RYR2$), 3 encode ion channel $\beta$-subunits ($KCNE2$, $SCN3B$, and $SCN4B$), and 2 encode other channel-interacting proteins ($CAV3$, $GPD1L$). Most recently, the SNTA1-encoded sodium ChIP, $\alpha$1-syntrophin, a key component of the PMCA4b-NOS-SNTA1-SCN5A macromolecular complex, was implicated as a new LQTS-susceptibility gene by our study group.

In the present study, we provide molecular and functional evidence implicating SNTA1 as a novel susceptibility gene for SIDS. In total, 6 SNTA1 missense mutations (G54R, P56S, T262P, S287R, T372M, and G460S) were identified in 8 SIDS cases, with 1 particular mutation, P56S, identified in 3 unrelated cases. Interestingly, 7 of the 8 mutations were found in girls and 1 in boys, minimizing any potential sex effect on risk of sudden death in SNTA1 mutation-positive individuals.

The Disturbance in nNOS-SNTA1-PMCA4b Complex Relieved the Inhibition of nNOS by PMCA4b

Like other syntrophin isoforms ($\beta1$, $\beta2$, $\gamma1$, and $\gamma2$), SNTA1 ($\alpha1$) comprises 4 conserved domains, 2 pleckstrin homology domains (PH1 and PH2) that are involved in the recruitment of proteins to the sarcolemma, a PDZ domain that inserts within PH1 and has been shown to bind to nNOS and SCN5A, and a syntrophin unique COOH-terminal domain (SU) that binds SNTA1 to dystrophin. The fact that there are up to 4 SNTA1 binding sites in close proximity within a single dystrophin complex suggests that SNTA1 probably brings several signaling molecules together to form a large signaling complex.

In cardiomyocytes, the activity of nNOS was confirmed to be negatively regulated by PMCA4b through direct interaction mediated by a PDZ domain. When SNTA1 was introduced to the nNOS-PMCA4b complex to form the bigger complex nNOS-SNTA1-PMCA4b, the maximal inhibitory effect of PMCA4b on nNOS was observed compared with the nNOS-PMCA4b complex, suggesting that the interaction of SNTA1 and PMCA4b, as well as the formation of the entire complex were critical for PMCA4b-mediated inhibition of nNOS.

Previously, we showed the existence of the macromolecular complex SCN5A-nNOS-SNTA1-PMCA4b (Figure 1C and 1D) in cardiomyocytes and found that the LQTS-associated A390V-SNTA1 mutation disrupted binding with PMCA4b, released inhibition of nNOS, and consequently increased the peak and late $I_{\text{Na}}$ via S-nitrosylation of the cardiac sodium channel mediated by local increased NO concentration.

In this investigation, 3 of the 6 SNTA1 missense mutations (S287R, T372M, and G460S) demonstrated similarly pronounced gain-of-function effects on NaV1.5 through the nNOS-SNTA1-PMCA4b macromolecular complex. Interestingly, some structure-function observations emerge when comparing the domain localization of the 3 missense mutations with a distinct pathological phenotype to the 3 missense mutations that were essentially indistinguishable from WT-SNTA1. The functionally significant S287R, T372M, and G460S-SNTA1 mutations were located in or very close to the region between PH2 and SU domains, which was identified as the region of interaction for SNTA1 and PMCA4b. The T262P mutant with only increased peak $I_{\text{Na}}$ was near to the binding area, whereas the 2 WT-like mutations (P56S and G54R) localized outside of the specific binding area (Figure 1C).

The fact that the nNOS inhibitor L-NMMA eliminated the increased late $I_{\text{Na}}$ caused by the S287R-, T372M-, and G460S-SNTA1 mutations further supports the idea that these mutations increase late $I_{\text{Na}}$ in an nNOS-dependent manner. Moreover, the functional studies for the complex SCN5A-SNTA1 (lacking both nNOS and PMCA4b) or SCN5A-SNTA1-nNOS complex (lacking PMCA4b) suggest that the 3 SNTA1 mutations do not cause increased late $I_{\text{Na}}$ by a direct interaction between SNTA1 and SCN5A or between SNTA1 and nNOS. Based on these data, we speculate that the 3 mutations may disturb the interaction of PMCA4b and SNTA1 in the whole macromolecular complex SCN5A-nNOS-SNTA1-PMCA4b, thus re-
lieving the negative regulation of PMCA4b on nNOS and thereby resulting in an increase of local NO concentrations and a biophysical modification of the sodium channel.

Molecular Mechanism for Increased Late $I_{Na}$ Associated With NO Modulation

There is still some disagreement regarding the reported modulatory effect of NO on the sodium channel, in part due to tissue specificity and NO delivery method. Relatively high concentrations of exogenous NO reduce peak $I_{Na}$ in cardiomyocytes via a cGMP associated pathway and have no effect on activation, inactivation, or reactivation kinetics. In a different study, persistent $I_{Na}$ in rat hippocampal neurons increased by 60% to 80% through a direct action of NO on the sodium channel protein or on a closely associated regulatory protein in the plasma membrane (S-nitrosylation pathway). Still another study in nerve terminals and ventricular myocytes showed that NO reduced the inactivation of the sodium channel, increasing persistent $I_{Na}$. Further investigation confirmed the effect was independent of the cGMP pathway and was blocked by N-ethylmaleimide, suggesting the S-nitrosylation pathway. Importantly, in myocytes, persistent $I_{Na}$ was also enhanced by endogenous NO generated enzymatically by NOS, whereas NOS inhibitors abolished the increase of both NO and persistent $I_{Na}$.

The present study showed that in the presence of an nNOS inhibitor, the marked accentuation in late $I_{Na}$ caused by S287R-, T372M, and G460S-SNTA1 decreased to WT-SNTA1 levels and that the increase in peak $I_{Na}$ was also reversed. Moreover, the mutant properties of the sodium channel (ie, positive shift of inactivation and slowing of current decay) that underlie increased late $I_{Na}$ were also reversed by an nNOS inhibitor. These findings were similar to other studies and strongly support the contention that endogenous NO generated enzymatically by NOS is the key signaling molecule by which SNTA1 mutants increase peak and late $I_{Na}$. Our group previously showed that A390V-SNTA1 released the inhibition of nNOS, thus increasing endogenous NO, which in turn caused increased direct S-nitrosylation of SCN5A compared with WT-SNTA1. With these data, we demonstrate that the direct S-nitrosylation effect of the increased endogenous NO caused by SNTA1 mutations associated with SIDS can change the characteristics of the cardiac sodium channel and modulate late $I_{Na}$ under physiological and pathophysiological conditions.

Implications of nNOS Complex in Sudden Cardiac Death

The present study demonstrates a new arrhythmic cause for approximately 1% of SIDS, characterized by increased late $I_{Na}$ originating from the disturbance of the nNOS complex, and further establishes perturbations throughout the Nav1.5 sodium channel complex as the final common pathway for the majority of channelopathic SIDS. The functional data involving 3 of the SIDS-associated SNTA1 mutations (S287R, T372M, and G460S) has provided additional evidence for implicating SNTA1 as a LQTS-susceptibility gene. Most importantly, these findings strongly suggested that nNOS plays an important role in modulating the late $I_{Na}$ underlying sodium channel-mediated LQTS and sudden unexplained cardiac death. Notably, the previously reported influences of common variation involving the neuronal nitric oxide synthase adaptor protein (NOS1AP, an nNOS regulator) on QT interval duration and most recently the observed association of NOS1AP genetic variants with sudden cardiac death as well as SIDS have confirmed the important role of nNOS in LQTS-related disorders. Thus, the deeper association of nNOS complex–related proteins (for example nNOS regulators like PMCA4b) as potential candidate genes for LQTS and sudden cardiac death deserves further study.

Study Limitations

Although we established a distinct association between SIDS and SNTA1 mutations by molecular and functional evidence, there are some limitations in the present study. First, because these mutations were detected in a “retrospective” population-based postmortem cohort, it is not possible to infer true causality but only demonstrate the association of a “proarrhythmic” genotype with certain SIDS victims. Obviously, by the nature of the study design, there are no implantable loop recordings showing an exit rhythm of ventricular fibrillation in the 3 infants who hosted one of these rare and functionally significant SNTA1 missense mutations.

Second, the electrophysiological data were generated by in vitro experiments using HEK293 cells coexpressing the macromolecular complex SCN5A-SNTA1-nNOS-PMCA4b, which is somewhat different from the physiological environment in human cardiomyocytes. Because α1-syntrophin is a scaffolding adapter with several protein interaction motifs, it may interact with other signaling molecules involved with SCN5A or other ion channel complexes and therefore we cannot exclude that these particular SIDS-associated SNTA1 mutations might exert other effects in a more native cardiomyocyte environment. However, given the demonstration of increased late $I_{Na}$ with the original LQTS-associated A390V-SNTA1 in cardiomyocytes, we expect that results for these mutations in a more native environment would demonstrate similar findings.

Last, 3 of the 8 SNTA1-positive SIDS cases also had the common SCN5A polymorphism, H558R, which has been shown to alter the disease phenotype for various SCN5A disease-associated mutations. Whether or not common channel polymorphisms affect the nitrosylation pathway represents a possible future direction for this work.

Conclusion

In conclusion, this study implicates SNTA1 as a novel SIDS-susceptibility gene, whereby mutant SNTA1 disturbs the nNOS-SNTA1-PMCA4b-SCN5A complex, releasing inhibition of associated nNOS by PMCA4b and resulting in increased peak and late $I_{Na}$ via the upregulated endogenous NO. This current study adds to the growing body of literature implicating channelopathies as causing up to 10% of SIDS, with a significant portion of channelopathic SIDS stemming...
from perturbations in the Nav1.5 cardiac sodium channel macromolecular complex.

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Disclosures
None.

References

Every year, more than 2000 infants in the United States die of sudden infant death syndrome (SIDS), a multifactorial event with environmental and genetic factors converging on the vulnerable infant during the first year of life. The QT hypothesis, first proposed in 1976, attributes a significant number of cases of SIDS to congenital cardiac channelopathies, such as long-QT syndrome (LQTS). Approximately 5% to 10% of SIDS may be precipitated by mutations in genes encoding proteins comprising the sodium channel macromolecular complex, including the sodium channel α-subunit, caveolin-3, and GPD1L. This report implicates the recently discovered LQTS-susceptibility gene SNTA1, which encodes the structural protein α1-syntrophin, as a novel potential cause of channelopathic SIDS. In vitro functional studies demonstrated that 3 of the 6 rare SNTA1 variants markedly accentuated the late sodium current consistent with an LQT3-like proarrhythmic substrate. Interestingly, this cellular phenotype is neuronal nitric oxide synthase dependent and reversible with a neuronal nitric oxide synthase inhibitor. Moreover, mutational effects were protein region–dependent, with the functionally significant, SIDS-associated mutations localizing near the syntrophin binding domain with PMCA4b, an interaction required to exert PMCA4b inhibition of neuronal nitric oxide synthase. The significance of these findings is 2-fold. First, this work contributes to the growing body of literature implicating the cardiac channelopathies and the sodium channel macromolecular complex as the pathogenic substrate for a small subset of SIDS victims, that is, channelopathic SIDS. Second, given the increasing awareness of sequence variation in the general population, this work highlights the importance of concomitant functional studies to further discern rare “deleterious” genetic variants from similarly rare yet “innocuous” ones.
α1-Syntrophin Mutations Identified in Sudden Infant Death Syndrome Cause an Increase in Late Cardiac Sodium Current

Jianding Cheng, David W. Van Norstrand, Argelia Medeiros-Domingo, Carmen Valdivia, Bi-hua Tan, Bin Ye, Stacie Kroboth, Matteo Vatta, David J. Tester, Craig T. January, Jonathan C. Makielski and Michael J. Ackerman

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**SUPPLEMENTAL MATERIAL**

**Supplemental Table 1.** Electrophysiological properties of sodium channels in HEK293 cells co-expressing SCN5A and either WT or mutant SNTA1

<table>
<thead>
<tr>
<th>Samples</th>
<th>Peak $I_{Na}$†</th>
<th>Activation</th>
<th>Inactivation</th>
<th>Late $I_{Na}$</th>
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<tr>
<td></td>
<td>$pA/pF$</td>
<td>V1/2(mV)</td>
<td>K</td>
<td>n</td>
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<tr>
<td>WT-SNTA1</td>
<td>-335 ± 35</td>
<td>-43.4 ± 1.8</td>
<td>4.0</td>
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<tr>
<td>G460S-SNTA1</td>
<td>-294 ± 73</td>
<td>-45.1 ± 2.0</td>
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<td>T372M-SNTA1</td>
<td>-315 ± 76</td>
<td>-42.8 ± 1.4</td>
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<td>S287R-SNTA1</td>
<td>-348 ± 60</td>
<td>-44.6 ± 1.5</td>
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<td>T262P-SNTA1</td>
<td>-254 ± 78</td>
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<td>P56S-SNTA1</td>
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<td>G54R-SNTA1</td>
<td>-326 ± 46</td>
<td>-44.0 ± 1.1</td>
<td>4.0</td>
<td>5</td>
</tr>
</tbody>
</table>

† $I_{Na}$ = Sodium current
**Supplemental Table 2.** Electrophysiological properties of sodium channels in HEK293 cells co-expressing SCN5A, nNOS and either WT or mutant SNTA1

<table>
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<tr>
<th>Samples</th>
<th>Peak $I_{Na}^\dagger$ (pA/pF)</th>
<th>Activation</th>
<th>Inactivation</th>
<th>Late $I_{Na}$ %</th>
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<td>p</td>
<td>V1/2 (mV)</td>
<td>K</td>
<td>n</td>
</tr>
<tr>
<td>WT-SNTA1</td>
<td>-311 ± 60</td>
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<td>-43.7 ± 2.0</td>
<td>4.0</td>
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<tr>
<td>G460S-SNTA1</td>
<td>-345 ± 52</td>
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<tr>
<td>T372M-SNTA1</td>
<td>-366 ± 63</td>
<td>5</td>
<td>-43.5 ± 1.7</td>
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<td>S287R-SNTA1</td>
<td>-302 ± 46</td>
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<td>-44.9 ± 2.1</td>
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<tr>
<td>T262P-SNTA1</td>
<td>-291 ± 44</td>
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<td>-45.2 ± 1.0</td>
<td>4.0</td>
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<tr>
<td>P56S-SNTA1</td>
<td>-338 ± 33</td>
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<td>-41.6 ± 1.0</td>
<td>4.0</td>
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<tr>
<td>G54R-SNTA1</td>
<td>-323 ± 48</td>
<td>5</td>
<td>-44.3 ± 1.4</td>
<td>4.1</td>
</tr>
</tbody>
</table>

$^\dagger I_{Na} =$ Sodium current