Arrhythmia/Electrophysiology

Striking In Vivo Phenotype of a Disease-Associated Human SCN5A Mutation Producing Minimal Changes in Vitro

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Background—The D1275N SCN5A mutation has been associated with a range of unusual phenotypes, including conduction disease and dilated cardiomyopathy, as well as atrial and ventricular tachyarrhythmias. However, when D1275N is studied in heterologous expression systems, most studies show near-normal sodium channel function. Thus, the relationship of the variant to the clinical phenotypes remains uncertain.

Methods and Results—We identified D1275N in a patient with atrial flutter, atrial standstill, conduction disease, and sinus node dysfunction. There was no major difference in biophysical properties between wild-type and D1275N channels expressed in Chinese hamster ovary cells or tsA201 cells in the absence or presence of β1 subunits. To determine D1275N function in vivo, the Scn5a locus was modified to knock out the mouse gene, and the full-length wild-type (H) or D1275N (DN) human SCN5A cDNAs were then inserted at the modified locus by recombinase mediated cassette exchange. Mice carrying the DN allele displayed slow conduction, heart block, atrial fibrillation, ventricular tachycardia, and a dilated cardiomyopathy phenotype, with no significant fibrosis or myocyte disarray on histological examination. The DN allele conferred gene-dose–dependent increases in SCN5A mRNA abundance but reduced sodium channel protein abundance and peak sodium current amplitudes (H/H, 41.0±2.9 pA/pF at −30 mV; DN/H, 19.2±3.1 pA/pF, P<0.001 vs H/H; DN/DN, 9.3±1.1 pA/pF, P<0.001 versus H/H).

Conclusions—Although D1275N produces near-normal currents in multiple heterologous expression experiments, our data establish this variant as a pathological mutation that generates conduction slowing, arrhythmias, and a dilated cardiomyopathy phenotype by reducing cardiac sodium current. (Circulation. 2011;124:1001-1011.)

Key Words: cardiomyopathy ■ electrophysiology ■ genetics ■ ion channels

Voltage-gated sodium channels play a critical role in the generation and propagation of the cardiac action potential, and mutations in SCN5A, the gene encoding the major pore-forming sodium channel α subunit in the heart (Nav1.5), cause multiple inherited cardiac arrhythmia syndromes, including long-QT syndrome, the Brugada syndrome, isolated cardiac conduction disease, sinus node dysfunction, and atrial fibrillation.1–7 More recently, SCN5A mutations have been associated with dilated cardiomyopathy (DCM), and such DCM mutations have been associated with a similar range of arrhythmias.8–15

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Clinical Perspective on p 1011

The D1275N SCN5A mutation was initially reported in a Dutch family affected by atrial standstill, mild conduction disease, and atrial enlargement but no ventricular structural abnormality; only subjects who carried a variant in the connexin 40 promoter displayed the clinical phenotype.16 Subsequently, D1275N was implicated in a large family affected by DCM and various arrhythmias such as sinus node dysfunction, atrial and ventricular tachyarrhythmias, and conduction disease.8,9,17 Most recently, the mutation was reported in a family with atrial tachyarrhythmias, conduction disease, and ventricular enlargement without impaired contractility.18 Heterologous expression systems are conventionally used to assess function of ion channel mutations.7,19 Most studies (including our own data described below) that have compared wild-type and D1275N channels in heterologous expression systems have not shown major differences in the biophysical
properties of the variant channel. Thus, although the mutation has been reported as a cause of unusual phenotypes in a number of kindreds, its relationship to the clinical phenotypes remains uncertain.

To address this discrepancy, we have used recombinase-mediated cassette exchange \(^{21}\) to engineer mice expressing the mutant human channel (here called DN); we compared the functional properties of these animals with those expressing wild-type human alleles (H) that we previously generated in an identical fashion. \(^{21}\) The data demonstrate that D1275N causes a severe defect in sodium channel function in vivo, consistent with the reported clinical phenotypes.

**Methods**

**Study Subjects**

The proband and family members were screened for mutations in SCN5A by polymerase chain reaction amplification of coding regions and flanking intronic sequences, followed by direct sequencing of amplicons on an ABI PRISM 3730 DNA Sequence Detection System (Applied Biosystems, Foster City, CA). Informed consent was obtained for presentation of the kindred.

**Animal Model**

All studies using animals were approved by the institutional animal care and use committees at Vanderbilt University and performed in accordance with National Institutes of Health guidelines. We have previously modified the Scn5a locus in mouse embryonic stem cells to enable the technique of recombinase mediated cassette exchange. \(^{21}\) In our initial studies, we inserted the full-length human SCN5A cDNA into the targeted locus. \(^{21}\) Mice homozygous for the exchanged allele (called H/H) expressed only the human allele and had normal ECGs and ventricular sodium current, supporting the hypothesis that expression of the exchanged allele was under control of endogenous Scn5a regulatory mechanisms.

For the present study, we used the same technique to generate DN mice in which the exchanged construct was identical to that previously used for the H/H mice with the exception of a c.3823G→A mutation resulting in p.D1275N and insertion of a FLAG epitope between residues 153 and 154 of the extracellular linker S1-S2 in domain I; the FLAG insertion into S1-S2 linker has previously been found to have no effect on channel gating or cell surface expression. \(^{22}\) We also generated FG mice bearing the wild-type SCN5A allele with the FLAG tag. Initial matings between mice heterozygous for engineered alleles resulted in H/H, DN/H, and FG/H mice, and these mice were then bred into the 129/Sv background. H/H, DN/H, and DN/DN mice were generated from DN/H×DN/H matings, and H/H littermates were used as controls for all experiments. To genotype mice, genomic DNA was isolated from mouse tails, and the target SCN5A polymerase chain reaction amplicon (c.3688 to c.4082) was incubated with Taq1 (New England Biolabs, Ipswich, MA) and then electrophoresed in agarose gels.

**Echocardiogram**

Transsthoracic echocardiograms were performed on resting conscious mice and analyzed by a sonographer blinded to the genotype. Signals were acquired with a 15-MHz transducer (Sonos 5500 system, Agilent, Santa Clara, CA) at the Murine Cardiovascular Core at Vanderbilt University as previously described. \(^{29}\)

**Histology**

Hearts were fixed overnight in 10% formalin, paraffin embedded, sectioned at 5 μm, and stained with Masson trichrome.

**mRNA Quantification**

Real-time polymerase chain reaction was conducted with a 7900HT Real-Time Instrument (Applied Biosystems). mRNA was isolated from the left ventricles, and cDNA was synthesized from 2 μg of the RNA by use of the Transcripter First Strand cDNA Synthesis Kit with random hexamer primers (Roche Applied Science, Indianapolis, IL) and used as a template. To generate a standard curve for absolute quantification, genes of interest were subcloned into the pGEM-T vector (Clontech, Mountain View, CA). cDNA and 5 different dilutions of the vector with target DNA were prepared with predeigned 6-carboxyfluorescein–labeled fluorescent TaqMan probe and primers (Applied Biosystems) for SCN5A (Hs01165693 m1) or β-actin (Hm00650774 s1) in triplicate in the same 96-well plate for real-time polymerase chain reaction amplification. Data were collected with instrument spectral compensation and analyzed by use of absolute quantification and a standard curve with SDS 2.2 software (Applied Biosystems). Each value was normalized to that for β-actin.

**Western Blotting**

Protein was extracted from flash-frozen hearts that were pulverized into powder and homogenized in a Dounce apparatus with 1× radiimmunoprecipitation assay buffer. Lysates were centrifuged at 10 000g for 5 minutes, and protein content was analyzed with a bicinchoninic acid assay (Pierce Biochemicals, Rockford, IL). Protein (40 to 100 μg) from each cardiac sample was separated by running the sample on a NuPage 8% Tris-acetate gel (Invitrogen, Carlsbad, CA). The protein was transferred to 0.2 μm nitrocellulose membranes (Amersham Biosciences, Sweden), which were blocked overnight in 0.05% Tween-20 Tris-buffered saline (TBBS) plus 5% nonfat dry milk at 4°C and then incubated with antibodies targeting anti–Nav1.5 (polyclonal antibody, 1:200; Alomone Labs, Israel) or anti-calnexin (polyclonal antibody, 1:1000, Stressgen Bioreagents, Belgium) at room temperature for 2 hours. Membranes were washed 3 times with TTBS for 10 minutes each and incubated with secondary anti-mouse and anti-rabbit horseradish peroxidase-linked antibodies (Amersham Biosciences) in TTBS at room temperature for 1 hour. The blots were then washed 4 times for 10 minutes each in TTBS. We visualized antibody interactions with the ECL system (Amersham Biosciences).

**Immunostaining/Confocal Microscopy**

Unfixed hearts were frozen in Tissue Tek and sectioned at 6 μm. Sections were washed in 1× Dulbecco phosphate-buffered saline and then incubated in 1× Dulbecco phosphate-buffered saline containing 0.3% fish gelatin and 0.1% Triton (block) for 1 hour at 4°C. Sections were immunostained with antibodies targeting anti–Nav1.5 (polyclonal antibody 1:50, Alomone Labs) diluted in block solution overnight. Samples were then washed 3 times and incubated with Alexa 488–conjugated goat anti-mouse IgG (1:400, Invitrogen) secondary antibody for 1 hour at room temperature. Then sections
were washed and coverslips were applied with Vectashield (Vector Labs, Burlingame, CA). Images were collected with a Zeiss LSM510 Meta confocal imaging system with 20×1.3 NA lens (pinhole equals 1 airy disk) with 2× zoom and analyzed with LSM 4.0 software.

### Sodium Current Recordings

Sodium current was recorded with the whole-cell voltage-clamp technique in single ventricular myocytes isolated by a modified collagenase/protease method or in Chinese hamster ovary (CHO) cells transiently expressing wild-type or D1275N SCN5A.21,30,31 The SCN5A DNA (NM 198056) was subcloned into the pBKC-MV vector (Stratagene, La Jolla, CA), and the mutation was prepared with the QuickChange II XL site-directed mutagenesis kit (Stratagene), followed by verification by resequencing. SCN5A DNA (1 μg) was transfected with the plasmid encoding the enhanced green fluorescent protein (pEGFP-IRES, Clontech) by use of Fugene6 (Roche Applied Science, Indianapolis, IN) in CHO cells. Cells were grown for 48 hours after transfection before study. Similar methods were used to study the biophysical properties of wild-type and D1275N sodium channels transfected with the sodium channel β1 subunit in human embryonic kidney cells (tsA201). Late sodium current was measured at the end of 200-ms test pulses to −20 mV from a holding potential of −120 mV (interpulse duration, 5 seconds).

The extracellular bath solution contained (in mmol/L) 145 NaCl, 4.0 KCl, 1.0 MgCl2, 1.8 CaCl2, 10 glucose, and 10 HEPES, pH 7.4 (NaOH). Patch pipettes (in mmol/L) NaCl 140, KCl 5.4, CaCl2 1.8, MgCl2 1, HEPES 5, and glucose 10, pH 7.4 (adjusted by NaOH). Microelectrodes contained (in mmol/L) NaCl 140, KCl 5, CaCl2 1.8, MgCl2 1, HEPES 5, and glucose 10, pH 7.4 (adjusted by NaOH). Patch pipettes contained (in mmol/L) KCl 110, K2-ATP 5, MgCl2 1, BAPTA 0.1, and HEPES 10, pH 7.2 (adjusted by KOH). Microelectrodes of 3 to 5 mol/LQ were used. Data acquisition was carried out with an Axopatch 200B patch-clamp amplifier and pCLAMP. The action potential durations at 50% and 90% repolarization and the action potential amplitude were measured.

### Data Analysis

Results are presented as mean±SEM. The unpaired t test was used for comparisons of electrophysiological characteristics between D1275N and wild-type channels expressed in heterologous expression systems. We used ANOVA followed by a post hoc analysis with Bonferroni correction for all of comparisons among the genotypes of mice, except for the linear mixed-effects models with Bonferroni correction for comparisons of in vitro electrophysiological characteristics of mice. All statistical analyses were performed with SPSS, version 12.0 (SPSS Inc, Chicago, IL). A 2-tailed value of P<0.05 was considered statistically significant.

### Results

#### Clinical Case Presentation

A 19-year-old white man (II-1) presented with recurrent exertional syncope (Figure 1A, arrow). Physical examination and echocardiography were normal, and his ECG demonstrated unusually slow atrial flutter that was conducted 1:1 to the ventricles with hypotension during exertion (Figure 1B). After catheter ablation of the cavo-tricuspid isthmus for atrial flutter, he had atrial standstill, prolonged QRS duration, sinus node dysfunction, high-degree atrioventricular block, and normal QT interval. A cardioverter-defibrillator was implanted. He has been...
asymptomatic for 10 years, and his echocardiography has been normal. We identified a missense mutation in SCN5A, c.3823G→A in exon 21 (Figure 1C), resulting in p.D1275N within a transmembrane domain of the protein (segment 3, domain III); the variant connexin 40 associated with atrial standstill in the reported Dutch kindred was absent.16 Both his mother (I-2) and 1 son (III-2) share the mutation but have no clinical findings.

DN Mice Are Viable and Display Gene-Dose-Dependent Conduction Slowing and Arrhythmias

The distribution of pups from DN/H×DN/H matings was in Hardy-Weinberg equilibrium (52 H/H, 107 DN/H, 54 DN/DN). During a follow-up of 12 weeks, 1 DN/DN mouse died suddenly, but no DN/H or H/H mice died. ECG recordings revealed that the DN allele caused abnormal phenotypes in a gene-dose–dependent fashion at 3 weeks (Figure 2A and 2B, and Table 1). The DN allele was associated with slow heart rate and slow cardiac conduction (prolongation of the P-wave duration, PR interval, and QRS duration) at 3 weeks, and similar changes were observed at 12 weeks. In mice with ECGs recorded at both 3 and 12 weeks, the prolongation of the P-wave duration, PR interval, and QRS duration associated with the DN allele was progressive with age. In addition, spontaneous monomorphic and polymorphic ventricular tachycardia was observed in 7 of 9 DN/DN mice during 15-minute recording periods under light anesthesia at 12 weeks, but no arrhythmia was observed in 18 DN/H or 10 H/H littermates studied under the same conditions (Figure 2C). Sinus node dysfunction (n=3), atrioventricular block (second degree or higher; n=4), and atrial fibrillation/tachycardia (n=5) also occurred only in DN/DN mice, not in DN/H or H/H littermates.

Reduced Contractile Function in DN Mice

There was consistent and statistically significant end-diastolic and end-systolic left ventricular dilatation and calculated left ventricular fractional shortening reduction in a gene–dose-dependent fashion (Figure 3A and Table 2). Histological examination of mouse hearts revealed that the DN allele was associated with ventricular dilatation but was not associated with significant fibrosis or myocyte disarray (Figure 3B). One possibility is that the FLAG tag incorporated into the DN allele contributes to the phenotypes in the DN animals. However, we found no difference in ECG and echocardiographic phenotypes between H/H and FG/FG animals, indicating that the FLAG tag does not
Table 1. ECG Phenotype

<table>
<thead>
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<th>H/H</th>
<th>DN/H</th>
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<tr>
<td><strong>At 3 weeks</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>11</td>
<td>20</td>
<td>9</td>
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<tr>
<td>Heart rate, bpm</td>
<td>388±8</td>
<td>354±8*</td>
<td>335±15*</td>
</tr>
<tr>
<td>P-wave duration, ms</td>
<td>13.0±0.6</td>
<td>17.0±0.3*</td>
<td>19.4±0.5†</td>
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<tr>
<td>PR interval, ms</td>
<td>33.7±0.7</td>
<td>35.6±0.7</td>
<td>44.1±0.9†</td>
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<tr>
<td>QRS duration, ms</td>
<td>9.8±0.2</td>
<td>11.8±0.3*</td>
<td>22.3±2.2†</td>
</tr>
<tr>
<td>QT interval, ms</td>
<td>48.5±1.9</td>
<td>50.2±1.1</td>
<td>67.6±4.2†</td>
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<tr>
<td>QTc interval, ms</td>
<td>38.9±1.6</td>
<td>38.5±0.8</td>
<td>50.2±2.5†</td>
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**At 12 weeks**

<table>
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<th>18</th>
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<td>Heart rate, bpm</td>
<td>387±8</td>
<td>368±11</td>
<td>317±18*</td>
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<tr>
<td>P-wave duration, ms</td>
<td>14±0.5</td>
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<td>PR interval, ms</td>
<td>37.8±0.8</td>
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<tr>
<td>QRS duration, ms</td>
<td>10.7±0.4</td>
<td>12.9±0.3</td>
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<tr>
<td>QT interval, ms</td>
<td>51.2±0.6</td>
<td>54.8±0.8</td>
<td>77.9±3.7†</td>
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<td>QTc interval, ms</td>
<td>41.1±0.6</td>
<td>42.8±0.9</td>
<td>55.9±1.8†</td>
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</table>

Ratio of week 12 to week 3, %‡

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<tbody>
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<td>Heart rate, bpm</td>
<td>101±2</td>
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<td>119±18</td>
</tr>
<tr>
<td>P-wave duration, ms</td>
<td>104±4</td>
<td>112±5</td>
<td>143±11†</td>
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<tr>
<td>PR interval, ms</td>
<td>113±3</td>
<td>115±2</td>
<td>125±5†</td>
</tr>
<tr>
<td>QRS duration, ms</td>
<td>111±4</td>
<td>116±4</td>
<td>154±14†</td>
</tr>
<tr>
<td>QT interval, ms</td>
<td>106±5</td>
<td>114±3</td>
<td>117±11</td>
</tr>
<tr>
<td>QTc interval, ms</td>
<td>106±5</td>
<td>115±3</td>
<td>123±6</td>
</tr>
</tbody>
</table>

*QTc=QT/(RR/100)1/2 (mouse-specific).

*P<0.05 vs H/H; †P<0.05 vs DN/H.

‡For animals with measurements at both time points.

Table 2. Echocardiographic Phenotype at 12 Weeks

<table>
<thead>
<tr>
<th></th>
<th>H/H (n=9)</th>
<th>DN/H (n=19)</th>
<th>DN/DN (n=12)</th>
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<tr>
<td><strong>Septal wall, mm</strong></td>
<td>0.75±0.02</td>
<td>0.72±0.02</td>
<td>0.69±0.03</td>
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<tr>
<td><strong>Posterior wall, mm</strong></td>
<td>0.51±0.03</td>
<td>0.47±0.01</td>
<td>0.51±0.04</td>
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<td><strong>Left ventricle, mm</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>End diastole</strong></td>
<td>3.01±0.08</td>
<td>3.09±0.08</td>
<td>3.33±0.07*</td>
</tr>
<tr>
<td><strong>End systole</strong></td>
<td>1.49±0.09</td>
<td>1.76±0.05*</td>
<td>2.01±0.05†</td>
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<tr>
<td><strong>Fractional shortening, %</strong></td>
<td>52.0±1.6</td>
<td>43.1±0.7*</td>
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*P<0.05 vs H/H; †P<0.05 vs DN/H.

Sodium Current Is Reduced in DN Myocytes

The manifest conduction slowing in DN mice is consistent with loss of sodium channel function. However, sodium current amplitudes and gating observed with heterologous expression of wild-type and D1275N channels in CHO cells were nearly indistinguishable (Figure 4A through 4C and Table 3). In CHO cells, there was also no difference in the voltage dependence of activation or in the time course of inactivation. Similarly, only minor differences were observed between wild-type and D1275N channels coexpressed with β1 subunits in tsA201 cells; current amplitudes were nearly identical, but there was a slight shift in the voltage dependence of activation and an increase in late sodium current (percent to peak current: wild-type, 0.22±0.05%; n=7; D1275N, 1.34±0.11%, n=8; P<0.001; Figure 4D through 4F and Table 3).

In contrast, in ventricular cardiomyocytes, peak sodium current amplitude was markedly reduced in DN/H and DN/DN mice compared with H/H littermates (Figure 5A and 5B and Table 3). In addition, late sodium current was increased in DN/DN mice compared with DN/H and H/H littermates (Figure 5C). We also found that sodium current in

DN/DN myocytes displayed consistent changes in gating. The voltage dependence of inactivation was positively shifted in DN/DN mice compared with DN/H and H/H mice (Figure 5D). The time course of inactivation was slower in DN/DN mice compared with DN/H and H/H littermates (time constant at −30 mV: DN/DN, 5.5±0.2 milliseconds; DN/H, 2.8±0.1 milliseconds; H/H, 2.7±0.2 milliseconds; Figure 5E and 5F). There was no difference in the voltage dependence of activation. The DN allele was associated with decreased action potential amplitude, consistent with the decrease in peak sodium current, and with prolonged action potential duration, consistent with the increase in late current (Figure 6).

Sodium Channel Protein Abundance Is Reduced in DN Myocytes

Western blotting showed a reduction in sodium channel protein abundance associated with the DN allele, and the changes were much more dramatic in DN/DN compared with DN/H hearts (Figure 7A and 7B). The abundance of

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*P<0.05 vs H/H; †P<0.05 vs DN/H.

Figure 3. Dilated cardiomyopathy phenotype. **A**, Representative echocardiograms showing prominent increased end-systolic dimensions in DN/H and DN/DN mice at 12 weeks. See Table 2 for summary results. **B**, Masson trichrome staining in mice hearts. Scale bars indicate 1 mm.

Contribute to the ventricular dysfunction or other phenotypes observed in DN animals.

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Sodium Current Is Reduced in DN Myocytes

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Sodium Channel Protein Abundance Is Reduced in DN Myocytes

Western blotting showed a reduction in sodium channel protein abundance associated with the DN allele, and the changes were much more dramatic in DN/DN compared with DN/H hearts (Figure 7A and 7B). The abundance of
the control calnexin protein was similar among H/H (reference, 100±6%), DN/H (103±4% of H/H), and DN/DN mice (100±5% of H/H) (P=NS for each). Although sodium current and sodium channel protein were reduced in DN/DN and DN/H mice compared with H/H littermates, real-time polymerase chain reaction showed that SCN5A transcript levels were elevated in mice with the DN allele (Figure 7C). Expression levels of -actin transcripts were similar among H/H (reference, 100±1%), DN/H (100±1% of H/H), and DN/DN mice (102±5% of H/H) (P=NS for each). Immunostaining experiments were conducted in heart sections at 3 weeks (Figure 8). The DN allele was associated with reduced levels of cell surface expression. Notably, staining was obvious on the lateral myocyte aspects in H/H hearts but was nearly absent in DN/DN hearts stained under identical conditions.

Discussion

The D1275N mutation has been associated with sinus node dysfunction, conduction abnormalities, tachyarrhythmias, and contractile dysfunction. However, in previous studies, the evidence implicating D1275N as the causative mutation has been weak: for example, in the large Dutch kindred, the contribution of an additional connexin variant was invoked to explain why only a minority of subjects displayed a clinical phenotype, but that variant was absent in the proband reported here. In addition, D1275N does not generate major changes in sodium channel function in heterologous expression studies. Thus, despite the previous and the present clinical case reports, the formal possibility remained that D1275N does not actually contribute to the abnormal phenotypes. To address the role of this (and other) variants in mediating sodium channel-

Table 3. Sodium Channel Gating in Heterologous Expression Systems and Ventricular Cardiomyocytes

<table>
<thead>
<tr>
<th></th>
<th>Peak Current Density at −30 mV</th>
<th>Voltage Dependence of Activation</th>
<th>Voltage Dependence of Inactivation</th>
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<tbody>
<tr>
<td></td>
<td>pA/pF n</td>
<td>V_{1/2}, mV n</td>
<td>V_{1/2}, mV n</td>
</tr>
<tr>
<td>CHO cells</td>
<td></td>
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<td>Wild type</td>
<td>−160±20 25</td>
<td>−35.4±0.6 25</td>
<td>−84.5±1.0 24</td>
</tr>
<tr>
<td>D1275N</td>
<td>−159±21 28</td>
<td>−34.7±0.6 28</td>
<td>−88.4±0.8 27</td>
</tr>
<tr>
<td>tsA201 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>−454±48 16</td>
<td>−47.7±1.1 16</td>
<td>−89.4±0.7 19</td>
</tr>
<tr>
<td>D1275N</td>
<td>−432±71 13</td>
<td>−35.7±1.1† 13</td>
<td>−88.0±1.6 18</td>
</tr>
<tr>
<td>Cardiomyocytes*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H/H</td>
<td>−40.9±2.9 10</td>
<td>−44.1±1.0 10</td>
<td>−84.1±1.0 10</td>
</tr>
<tr>
<td>DN/H</td>
<td>−19.2±3.1† 12</td>
<td>−44.3±1.4 12</td>
<td>−81.2±1.1 12</td>
</tr>
<tr>
<td>DN/DN</td>
<td>−9.3±1.1§ 12</td>
<td>−45.6±0.9 12</td>
<td>−76.5±0.88§ 12</td>
</tr>
</tbody>
</table>

CHO indicates Chinese hamster ovary; n, number of cells. Study conditions differ for heterologous expression systems and cardiomyocytes as described in Methods.

*Cardiomyocytes from 3 mice for each genotype.

†P<0.001 versus wild type; ‡P<0.05 versus H/H; §P<0.01 versus H/H; ||P<0.01 vs DN/H.
linked clinical phenotypes, we generated a series of mouse lines in which the murine cardiac sodium channel was ablated and human alleles were substituted in the murine Scn5a locus. The technique of recombinase-mediated cassette exchange allowed us to place wild-type or mutant human sodium channel cDNAs in the murine cardiac sodium channel locus. We have previously reported that this approach eliminates expression of the murine channel and that sodium currents from unmodified wild-type murine ventricular myocytes and those expressing wild-type human \textit{SCN5A} are indistinguishable, indicating that expression of the exchanged sequence is determined by endogenous sodium channel regulatory mechanisms.21

\textbf{DN Mice Display Sodium Channel Dysfunction}

Sodium current amplitude was similar between D1275N and wild-type channels when expressed in heterologous expression systems in the present study in either the absence or presence of \textit{\beta1} subunit.16 This is in agreement with most results previously reported, although 1 group has found that D1275N channels generate significantly less current than wild-type channels in tsA201 cells; the reason for this discrepancy is unknown.20 In our mouse model, D1275N was associated with decreased levels of sodium channel protein by Western analysis of total ventricular protein, decreased expression of sodium channels at the ventricular myocyte surface, and marked reduction of sodium current. In addition, we observed increased late current and altered voltage-dependence of channel inactivation. Thus, channel dysfunction conferred by D1275N becomes evident in the myocyte environment. The major change, reduction in peak sodium current, could represent decreased cell surface expression and/or altered gating of the channel protein. One possible explanation in either case is altered interactions with sodium channel partners, present in myocytes and absent in CHO and tsA201 cells.32,33 There is precedent for such a hypothesis; the E1053K \textit{SCN5A} mutation, which is associated with a loss of sodium channel function phenotype, has no effect on current density when studied in heterologous expression systems but abolishes binding of the channel to ankyrin-G and reduces cell surface expression and sodium current in cultured cardiomyocytes.34 However, although E1053K affects channel gating under heterologous expression,34 altered channel gating by D1275N was found only in the mice, not in heterologous systems, in our study. This is clearly not a general rule because channel dysfunction observed with heterologous expression of other mutants

\textbf{Figure 5. Sodium current in male ventricular cardiomyocytes at 3 weeks showing altered sodium channel function by the DN allele. A, Representative current traces in H/H, DN/H, and DN/DN cells. See Table 3 for summary results. B, Current voltage relationships. C, Late sodium current at $-30 \text{ mV}$. Late current amplitude was normalized to peak current amplitude. D, Voltage dependence of activation and inactivation. E and F, Inactivation time constant ($\tau$) at $-30 \text{ mV}$. *$P<0.001$ vs H/H; †$P<0.001$ vs DN/H. n Indicates the number of cardiomyocytes from 3 mice.}
Association of Sodium Channel Mutations With Cardiomyopathy

In addition to arrhythmias, SCN5A mutations have been associated with cardiomyopathy. To date, 12 rare variants in SCN5A have been identified in cardiomyopathy, and all of the variants have been associated with arrhythmia phenotypes that result from loss of sodium channel function. In our mouse model, the loss of sodium channel function by D1275N is consistent with biophysical properties of other SCN5A mutations associated with DCM, and findings in clinical and experimental studies suggest that a marked reduction in sodium current is critical for the development of cardiomyopathy. In prior studies, mice with 90% reduction of Scn5a expression level developed cardiac dysfunction, whereas heterozygous Scn5a knockdown mice (Scn5a/H11001/H11002) display normal cardiac function. In our study, mice expressing D1275N, one of the initially reported SCN5A mutations in a cardiomyopathy kindred, showed a reduction in sodium current with disrupted channel gating and developed evident cardiomyopathy at 12 weeks. This is consistent with other reports describing that both R814Q occurring homozygously and the compound heterozygous occurrence of the W156X and R225W are associated with cardiomyopathy. In these settings, the cardiomyopathy phenotype is generally absent in heterozygotes.

Among 12 rare variants in SCN5A associated with cardiomyopathy, 7 are located in transmembrane domains, and 6 of them, including D1275N, are predicted to change...
the electric charge of substituted amino acids. These substitutions may lead to changes in channel structure, resulting in altered channel gating and/or reduced channel expression levels directly or by disrupted interaction with sodium channel accessory proteins.

Although this study and previous work strongly imply that loss of sodium channel function has a critical role for development of cardiomyopathy, the mechanisms remain controversial. The surface ECG tracings in DN mice (Figure 2) not only demonstrate gene-dose-dependent conduction slowing but also suggest altered activation sequence (with ECG complex splintering); thus, electromechanical dyssynchrony, a well-recognized cause of cardiac contractile dysfunction, may be sufficient to explain the DCM phenotype. Another possibility raised by a recent report that suggests 2 pools of sodium channel protein in heart is that the mutant channel does not target the appropriate subcellular domain to support normal cell propagation. Among causative genes for DCM, cytoskeletal proteins such as synaptotagmin and dystrophins have been associated with SCN5A channel, and disrupted interaction with such proteins may result in cardiomyopathy. Although SCN5A mutations have been associated with cardiac fibrosis, we did not observe fibrosis when the mice carrying the DN allele developed cardiac dysfunction. It has been reported that SCN5A-related DCM phenotype usually develops later (>10 years) than the onset of arrhythmia phenotypes, suggesting a possibility that the DCM phenotype is secondarily mediated by arrhythmia. In our study, however, the cardiomyopathy phenotype was evident relatively early, in the absence of sustained arrhythmia. Taking these results together, we propose that sodium channel dysfunction and electromechanical dyssynchrony represent the primary pathophysiology for DCM in this setting.

Conclusions
We found that the D1275N SCN5A mutation was associated with cardiomyopathy and multiple arrhythmias in vivo, in line with clinical findings in our and other studies. Although D1275N did not generate serious channel dysfunction when studied in heterologous expression systems, the mutation produced extensive channel dysfunction, notably marked reduction in peak current amplitude, and a cardiomyopathy phenotype in our mouse model. Further experiments along the lines outlined above are required to elucidate the precise mechanisms for channel dysfunction and how this leads to the DCM phenotype. Defining the mechanisms underlying the disconnect between the results in heterologous expression systems and those in myocytes will contribute to furthering our understanding of the variable phenotypes and penetrance of D1275N and other SCN5A mutations.

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We thank Christiana Ingram, Justine Stassum, Laura Short, and Wei Zhang at Vanderbilt University for their assistance in performing or analyzing this work. We also acknowledge the expert performance of the staff of the Vanderbilt Transgenic Mouse/Embryonic Stem Cell Shared Resource for the blastocyst microinjections. Imaging of histological and immunohistochemistry samples was performed at the VUMC Cell Imaging Shared Resource.

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Disclosures
None.

References
6. Darbar D, Kannankeril PJ, Donahue BS, Kucera G, Stubblefield T, Haines JL, George AL Jr, Roden DM. Cardiac sodium channel (SCN5A)


A conventional approach to characterize the function of ion channel mutations is to compare wild-type and variant channel function by heterologous expression in mammalian, noncardiac cells like Chinese hamster ovary or human embryonic kidney cells. The cardiac sodium channel mutation D1275N has been reported in multiple individuals and families with a range of phenotypes, including arrhythmias and dilated cardiomyopathy; however, conventional heterologous expression studies have not identified major differences between wild-type and D1275N function. Thus, it has even been uncertain whether this mutation causes the clinical phenotypes with which it has been associated. In this study, we addressed this issue by studying mice in which the cardiac sodium channel locus had been disrupted and replaced with full-length human wild-type or D1275N mutant sodium channels. We observed slowed and disordered cardiac conduction and decreased contractile function in mice bearing the mutation; mice with 2 D1275N alleles displayed worse phenotypes than those with 1 variant allele. In vitro electrophysiological studies identified reduced peak cardiac sodium current as a key defect, and this is consistent with the observed reduced conduction velocity. The major clinical implication of these findings is that heterologous expression may be insufficient to assess mutant channel function. In addition, the data lend support to the concept that sodium channel mutations are associated not only with arrhythmias but also with dilated cardiomyopathy phenotypes. The mutant mice will be an invaluable tool to dissect mechanisms underlying these findings.
Striking In Vivo Phenotype of a Disease-Associated Human SCN5A Mutation Producing Minimal Changes in Vitro

Hiroshi Watanabe, Tao Yang, Dina Myers Stroud, John S. Lowe, Louise Harris, Thomas C. Atack, Dao W. Wang, Susan B. Hipkens, Brenda Leake, Lynn Hall, Sabina Kupershmidt, Nagesh Chopra, Mark A. Magnuson, Naohito Tanabe, Björn C. Knollmann, Alfred L. George, Jr and Dan M. Roden

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