The clinical and electrophysiological spectrum of cardiac sodium channel mutations
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CHAPTER 2.2

CARDIAC CONDUCTION DISEASE CAUSED BY A REDUCTION OF MEMBRANE SODIUM CHANNEL EXPRESSION

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to be submitted
Cardiac conduction disease by a reduction of membrane sodium channel expression

ABSTRACT

Background: Autosomal-dominant mutations in the SCN5A gene are responsible for several cardiac electrical disorders, including the Long-QT syndrome type 3, Brugada syndrome, and inherited cardiac conduction disease (ICCD). Patients suffering from these disorders are at risk of cardiac arrhythmias leading to syncope and sudden death. Here, we report two novel ICCD SCN5A mutations. One led to an insertion of an isoleucine at amino acid position 1569-1570 (I1569-1570ins) and the other was a missense mutation resulting in the substitution of an arginine at position 367 by a cysteine (R367C). We report on the clinical, genetic, biophysical characteristics and protein expression of these two new SCN5A mutations.

Methods and Results: Analysis of the biophysical properties of these mutations in HEK-293 cells showed a dramatic reduction in sodium current. Measurement of whole cell sodium currents using the patch-clamp technique revealed peak sodium current amplitudes of $1.7 \pm 0.3 \text{nA (n}=15\text{)}$ for I1569-1570ins vs. $7.4 \pm 1.5 \text{nA ((n}=13\text{)}$ (P<0.0005) for wild-type control channels in the presence of the β-subunit at -20 mV. In case of R367C, there was a complete absence of expressed sodium current. The reduction in current magnitude for I1569-1570ins could not be explained by changes in channel kinetics. The R367C current could not be rescued by using another cell expression system (tsA201), decreasing the incubation temperature or incubation of transfected cells with sodium channel blocking drugs. Assessing the surface expression of both mutants and wild-type channels by GFP tagging showed that surface expression of the mutant channels was reduced.

Conclusions: The reduced/absent sodium current of these mutant channels is very likely a consequence of a reduction in membrane channel expression. The conduction disease in carriers of these mutations is explained by these findings.
INTRODUCTION
The SCN5A gene encodes the α-subunit of the voltage gated cardiac Na⁺ channel, which exerts an essential role in the generation and propagation of the cardiac impulse.1-3 Autosomal-dominant mutations in the SCN5A gene are responsible for several cardiac electrical disorders, including the long QT syndrome type 3 (LQT3),1-5 Brugada syndrome (BS)2-4,6-8, idiopathic ventricular fibrillation (IVF)9 and inherited cardiac conduction disease (ICCD).4,10-17 Distinct electrocardiographic (ECG) phenotypes and risks characterize these syndromes. In none of these diseases underlying structural abnormalities of the heart are involved.

The LQT3 syndrome is characterized by a prolonged QT-interval on the ECG, as evidence of delayed ventricular repolarization, due to gain-of-function SCN5A mutations.3-5 These patients are at risk for developing ventricular tachyarrhythmias, specifically torsades de pointes, and ventricular fibrillation.5

The Brugada syndrome, on the other hand, is in approximately 15-30% of cases associated with loss-of-function SCN5A mutations.18,19 The syndrome is characterized by J-point elevation in leads V1-V3 appearing as (incomplete) right bundle branch block ((i)RBBB).2-4,6-8 Brugada syndrome is associated with a high mortality resulting from (often nocturnal) ventricular fibrillation.2,8

ICCD is an inherited cardiac arrhythmia disorder characterized by prolongation of the conduction parameter in the His-Purkinje conduction system. Like the Brugada syndrome, ICCD is associated with loss-of-function SCN5A mutations.4,10-17 Patients suffering from ICCD are at risk of developing complete atrioventricular block leading to syncope and sudden cardiac death (SCD).4,10-17

In both BS and ICCD, the mechanism of the reduction in sodium current (I_{Na}) can be either a reduction in the expression and trafficking of the channels, or a gating defect.2-4 When specific gating defects are involved, it is often possible to explain the phenotype of the disease.11 Sometimes, however, the phenotype is less readily explained. A number of SCN5A mutations are even causally involved in both Brugada syndrome and ICCD.3,4,12,13 In carriers of the G1406R SCN5A mutation for example, 4 of 6 male carriers presented with a BS phenotype, while all 6 female mutation carriers developed an ICCD phenotype.12 The identification, and further characterization of ICCD and Brugada syndrome associated SCN5A mutations, and genetic and environmental modifying factors, may be helpful in increasing our understanding of these diseases and the phenotypical differences.
In this paper, we report clinical, genetic, and biophysical characterizations of two new SCN5A mutations causing ICCD in two non-related families.

**MATERIALS AND METHODS**

*Genetic analysis*

Genetic studies were performed in accordance with the recommendations of the medical ethics committee of the involved hospitals and by the agreement of the patients and their family members. Genomic DNA was prepared from peripheral blood lymphocytes by standard methods. Haplotype analysis was done by genotyping of microsatellite markers around the SCN5A gene and by genotyping of intragenic SCN5A polymorphisms. Mutation analysis was done by SSCP analysis followed by direct sequencing (ABI 377 automated sequencer) of the aberrant conformers. Genotyping of paraffin embedded sections from deceased individuals was done with the DNA isolated using the QIAamp DNA blood kit (Qiagen) by allele-specific polymerase chain reaction using an allele specific oligonucleotide primer.

*Clinical analysis*

All consenting family members were evaluated by medical history and 12-lead electrocardiograms (ECG). Of the 12-lead ECG’s, the following parameters were determined: heart rate, P wave duration (leads II and V1), PQ-, QRS- and QTc-intervals. Because in family A (I1569-1570ins sodium channel mutation) initially the diagnosis Brugada syndrome was considered based on the occurrence of sudden death (SD) and baseline ECG’s abnormalities, 11 family members (AIII-1, AIII-2, AIII-3, AIII-7, AIII-8, AIII-9, AIII-10, AIII-11, AIII-13, AIII-14, AIII-17) were tested for this disease using the class I drugs flecainide (6 patients), procainamide (4 patients) and propafenone (1 patient). With this test, patients suspected of the BS can be identified. BS patients may have spontaneous J-point elevation in leads V1 and V2 of their ECG. Three ST-segment shapes, type 1 (coved), type 2 and type 3 (saddle) are recognized. While a type 1 ECG is considered diagnostic for the Brugada syndrome, a type 2 or 3 ECG is not necessarily so. In patients with a normal baseline ECG, but who are suspected of Brugada syndrome, ST-segment elevations can sometimes be induced using this test.
The brothers and sisters (AIII-11, AIII-13, AIII-14, AIII-17) of the two index patients of family A and the index patient of family B (BII-3) underwent electrophysiological study (EPS), to evaluate their risk for developing malignant ventricular arrhythmias.

Generation of expression vectors
Mutant sodium (Na\(^+\)) channel cDNAs were prepared by mutagenesis on the pSP64T-hH1 plasmid (Makita et al.), using the QuikChange™ (Stratagene) site-directed mutagenesis kit and the following oligonucleotides:

5'-TGTGGCCATCATCTTCACAGGCGAG-3' (sense) and
5'-CTCGCCTGTGAAGATGATGGCCACA-3' (antisense) for the 11569-1570ins mutant;
5'-TGCACTCTTCTGCCTGATGACGCAG-3' (sense) and
5'-CTGCGTCATCAGGCAGAAGAGTGA-3' (antisense) for the R367C mutant. The 11569-1570ins and R367C cDNAs were then subcloned into the HindIII-XbaI sites of the expression vector pCGI (kindly provided by David Johns, Johns Hopkins University, Baltimore MD) for bicistronic expression of the channel protein and GFP reporter in a Human Embryonic Kidney cell line (HEK-293). These constructs were used for biophysical studies.

GFP-tagged wild-type (WT) SCN5A (kindly provided by Dr. Sophie Demolombe, INSERM U533, Nantes) was inserted in frame into a pEGFP-N3 plasmid (Clontech) for N-terminus tagging of the channel protein. HindIII- BstE II fragment from hH1/R367C was swapped with the same enzyme digested fragment from GFP tagged wild SCN5A in pEGFP-N3. For the construction of GFP tagged 11569-1570ins, Bst EII- BsaAI fragment from the hH1/11569-1570ins was exchanged with the same enzyme digested wild type in pEGFP-N3.

Transfections of HEK-293 cell line for confocal laser microscopy
HEK-293 cells were seeded in a six well plate (Nunc, Nalge Nunc International, Denmark) at a density of 10\(^6\) cells/well and cultured for 15-18h prior to transfection. Cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco-BRL, Rockville, MD,USA) supplemented with 10% fetal calf serum (FCS) (Gibco-BRL). Lipofectamine (Gibco-BRL) was used for transfection according to the manufacturer’s instructions. 200 ng of DNA was used to transfect the cells in each well, and the cells were grown for a further 15h at 37°C in a 5% CO\(_2\) incubator. Then the transfected cells were trypsinized and were seeded on a glass slide and incubated in DMEM/10%FCS for a further 12h. A HEK-293 cell line constitutively expressing β1-subunit (SCN1b) (courteous contribution of Dr. Antoinette Groenewegen, University of Utrecht, the Netherlands) was also transfected in the same way.
**Electrophysiology**

I569-1570ins, R367C or WT sodium channel α-subunit cDNA (1 μg) was transfected into HEK-293 cells with and without 1 μg hβ1-subunit using lipofectamine. Cells displaying green fluorescence 24-48 hours after transfection were used for electrophysiological experiments. Sodium currents were measured in the whole-cell configuration of the patch-clamp technique using an Axopatch 200B amplifier (Axon Instruments). Patch electrodes were pulled from borosilicate glass. When filled with solution the pipettes had a tip resistance of 2-3 MΩ. 80% of the series resistance was compensated. Whole-cell sodium currents were filtered at 5Hz and digitized at 30kHz.

All experiments were performed at room temperature (21°C). The bath (external) solution contained (in mmol/l): NaCl 140, KCl 4.7, CaCl2 1.8, MgCl2 2.0, NaHCO3 4.3, Na2HPO4 1.4, glucose 11.0, HEPES 16.8, pH adjusted to 7.4 (NaOH). The pipette (internal) solution contained (in mmol/l): CsF 100, CsCl 40, EGTA 10, NaCl 10, MgCl2 2.0, HEPES 10, pH adjusted to 7.3 (NaOH).

**Voltage protocols and data analysis**

The voltage dependence of activation, steady state inactivation and recovery from inactivation were determined by using the voltage clamp protocols provided as insets with the relevant figures. For all protocols, the pulse cycle time was 5 seconds and the holding potential was -120mV. The steady-state activation and inactivation curves were fitted using the Boltzmann equation: \( I/I_{\text{max}} = A/[1.0+\exp((V_{1/2}-V)/k)] \) to determine the membrane potential for the half maximal (in)activation \((V_{1/2})\) and the slope factor \(k\).

The time course of inactivation was determined by fitting current decay with a two-exponential function: \( I/I_{\text{max}} = A_f \exp(-t/\tau_f) + A_s \exp(-t/\tau_s) \), where \(A_f\) and \(A_s\) are fractions of fast and slow inactivation components and \(\tau_f\) and \(\tau_s\) are the time constants of the fast and slow inactivating components, respectively.

Recovery from inactivation was analyzed by fitting the data with the function: \( I/I_{\text{max}} = A[1-\exp(-t/\tau)] \) where \(t\) is the recovery time interval and \(\tau\) is the time constant of recovery from inactivation.

**Statistical analysis**

The results are expressed as mean±SEM and statistical comparisons were made using an unpaired student's t-Test with \(P<0.05\) indicating statistical significance.
**Confocal laser microscopy**

Cells adherent to the thin glass cover slips were rinsed with PBS, and the coverslip was then mounted upside down on a slide glass with a drop of vectashield mounting medium (Vector Laboratories, Inc. CA 94010). The localisation of GFP-tagged proteins was analysed by Confocal Laser Scanning Microscope (Bio-Rad MRC-1024) with a Krypton-argon laser beam and Zeiss Axioplan HBO 100 microscope. Confocal images of GFP induced fluorescence were collected at a magnification of 252 using a 488 nm excitation light from the argon/krypton laser and a 515-540 nm band pass filter. Digitalized image data obtained from the experiment were prepared by using Adobe Photoshop.

**RESULTS**

**Genetic studies**

In family A (Figure 1, upper panel), affected individuals AII-1, AII-6, AIII-1, AIII-3, AIII-5, AIII-7, AIII-9, AIII-12, AIII-13, AIII-14, AIII-15, AIII-17, AIV-1, AIV-2 and AIV-4 were carriers of a mutated SCN5A allele containing an in-frame insertion of 3 nucleotides leading to insertion of an isoleucine (I1569-1570ins) in the second transmembrane segment of domain IV of the sodium channel protein (Figure 1, lower panel). The deceased brothers (AIII-12 and AIII-15) were also carriers of this mutation, which was proven in stored heart specimens.
Cardiac conduction disease by a reduction of membrane sodium channel expression

![Pedigree diagram]

**Figure 1.** Upper panel: Pedigree of family A. Open symbols depict unaffected members, filled symbols depict the carriers of the ICCD phenotype (circles indicate females, squares indicate males). Bottom panel: Sequence analysis of exon 27 of SCN5A in mutation carriers and non-mutation carriers, showing the insertion of ATC resulting in the addition of an isoleucine between position 1569-1570 (11569-1570ins) of the SCN5A protein (arrow).

In family B (Figure 3, upper panel), an abnormal conformer was identified in exon 9 of SCN5A in two affected sisters (BII-1 and BII-3). DNA sequence analysis revealed a C>T (1098th nucleotide) substitution in exon 9 leading to the substitution of the uncharged polar arginine at position 367 by the charged polar cysteine (R367C) at domain DI-DII linker, of the channel (Figure 3, lower panel).

Both these mutations were absent in 150 control individuals (300 chromosomes) of Dutch descent.
Clinical data family A

Family A came to attention because of the sudden death (SD) of two males at the age of 21 and 28 years (pedigree AIII-12 and AIII-15). In both cases SD occurred immediately after stressful conditions. In neither of the indexes post mortem examination revealed signs of structural, macroscopic or microscopic, heart disease. Their previous clinical history was unremarkable. No ECG is available of either patient. Clinical screening of the family members revealed a high incidence of cardiac conduction abnormalities characterised by prolonged P-, PQ- and QRS-intervals on the 12 lead ECG of mutation carriers (Table 1, Figure 2A). Carriers of the I1569-1570ins mutation had broadened P-waves, $114 \pm 6$ ms (n=13) vs. $87 \pm 6$ ms (n=10) (p=0.002), compared to family members without the mutation. PQ-intervals in mutation carriers were found to be significantly longer, $192 \pm 6$ ms (n=13) in mutation carriers vs. $164 \pm 8$ ms (n=10, p=0.02) in non-affected family members. The QRS-intervals in mutation carriers were also longer than in non-mutation carriers, $112 \pm 9$ ms (n=13) vs. $102 \pm 6$ ms (n=10) respectively. However, this difference did not reach statistical significance (p=0.07). In 1 non-carrier the P wave was too low in amplitude for accurate measurement.

Family member AIV-4 is a 3 year old girl with recurrent fainting episodes at the age of 3 years, that resulted from broad complex tachycardia. The tachycardias were both atrial fibrillation (AF) with aberrant ventricular conduction and ventricular tachycardia (VT), occurring at different times. When in sinus rhythm she had prolonged PQ- and QRS-intervals and sinus pauses. At present she is the only living family member with symptoms other than those of conduction delay.

Because of a family history with SD, the presence of an SCN5A mutation and the fact that mutation carriers AIII-14 and AIII-17 displayed J-point elevation on their baseline ECG, the diagnosis Brugada syndrome was considered. To further investigate this, eight mutation carriers: AII-1, AIII-1, AIII-3, AIII-7, AIII-9, AIII-13, A-III-14 and AIII-17 and three family members without the mutation: AIII-2, AIII-10, AIII-11 were tested for this disease using sodium channel blockers. During this test the spontaneous saddle back shaped ST-segment elevation in the two mutation carriers (AIII-14 and AIII-17) changed into coved shape ST-segment elevation. Also one family member without the mutation developed some ST-segment elevation (<1mm) of a coved type during flecainide administration (AIII-11). This is however not sufficient according to the present criteria to be diagnostic for a Brugada syndrome. The others did not develop ST-segment elevations. During drug testing the PQ-interval prolonged, both in mutation carriers ($19 \pm 9$ %) and in non-affected family members.
Cardiac conduction disease by a reduction of membrane sodium channel expression

(12 ± 3 %) (ns). The QRS- interval, however, prolonged significantly more in mutation carriers (30 ± 6 %) than in non-affected family members (0.1 ± 5.5 %) (p=0.008).

Patients AIII-11, AIII-13, AIII-14 and AIII-17 underwent EPS. Interestingly patient AIII-11, who does not carry the 11569-1570ins mutation, but developed some ST-segment elevation during flecainide challenge, developed ventricular fibrillation (VF) after 2 premature stimuli. Patients AIII-13 and AIII-14, both carriers of the 11569-1570ins mutation, developed ventricular flutter (Figure 3B) and monomorphic ventricular tachycardia respectively, both after 3 premature stimuli. In patient AIII-17 no arrhythmias could be induced.

<table>
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<th>age (yrs)</th>
<th>gender (m:f)</th>
<th>n</th>
<th>HR beats/min</th>
<th>P width lead II (ms)</th>
<th>PO (ms)</th>
<th>QRS (ms)</th>
<th>QTc (ms)</th>
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<td>13</td>
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<td>114±6</td>
<td>192±6</td>
<td>112±9</td>
<td>435±18</td>
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<td>7:3</td>
<td>10</td>
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<td>87±6</td>
<td>164±8</td>
<td>102±6</td>
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<td>0.015</td>
<td>ns</td>
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</tr>
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Table 1. Averaged ECG parameters of 11569-1570ins mutation carriers compared to family members who did not carry the mutation (controls).

Clinical data family B

In family B (Figure 3) the proband (BII-3) came to attention because of recurrent syncope. Similar symptoms were found in her sister (BII-1). Both of them were found to be carriers of the R367C mutation. The index patient was previously reported to have a Brugada syndrome because of a prolonged QRS interval of 160 ms, with a right bundle branch block (RBBB) morphology and type I (coved type) ST segment elevation in leads V1 and V2 (Figure 4A). However after re-evaluation of the ECG the QRS-interval was considered to be 200 ms in duration, followed by a negative T-wave which would argue for ICCD rather than BS (Figure 4A). The conduction abnormalities were not limited to the right precordial leads but were present in all ECG leads. She had documented polymorphic and monomorphic VT, which could be terminated by administration of procainamide, after which the QRS-complex widened even more (Figure 4B). The monomorphic VT could be reproducibly initiated during EPS.
Figure 2. Electrocardiograms recorded from individuals from family A. A: Baseline 12 lead ECG recording of patient AIII-13 carrying the I1569-1570ins mutation. Note the prolonged PQ interval (240ms) and widened (100-120ms) QRS interval. The paper-speed was 25mm/sec. B: Registration of patient AIII-13, carrying the I1569-1570insl mutation, during electrophysiological study. Spontaneously terminating monomorphic ventricular tachycardia, in this case ventricular flutter, developed after 3 premature stimuli.
Cardiac conduction disease by a reduction of membrane sodium channel expression

Figure 3. Upper panel: Pedigree of family B. Open symbols depict unaffected members, filled symbols depict the carriers of the ICCD phenotype (circles indicate females, squares indicate males). Bottom panel: Sequence analysis of exon 9 of SCN5A in mutation carriers and non-mutation carriers, showing the change of C nucleotide for a T at 1098th nucleotide position resulting in the substitution of arginine to cysteine (R367C) of the SCN5A protein (arrow).

Electrophysiological properties of the I1569-1570ins mutation

To determine the functional consequences of the I1569-1570ins sodium channel mutation, electrophysiological characteristics of mutant and WT sodium currents were studied in HEK-293 cells. Figure 5 depicts examples of current traces (Figure 5A) and the averaged current-voltage (IV) relationships (Figure 5B, left panel) of the WT and I1569-1570ins channels, clearly showing that HEK-293 cells transfected with I1569-1570ins cDNA have a lower sodium current density. The difference was independent of co-expression of hβ1 (Figure 5B, right panel). Without hβ1 co-expression, the maximum I\textsubscript{Na} was 5.0 ± 0.9 nA (n=20) for WT control and 1.6 ± 0.3 nA (n=16) (p=0.003) for I1569-1570ins channels at −25 mV (Figure 5B, left panel and Table 2.) Values obtained when the α-subunit and hβ1 were co-expressed were 7.4 ± 1.5 nA (n=13) for WT control and 1.7 ± 0.3 nA (n=15) (p=0.004) for I1569-1570ins
channels (Figure 5B, right panel and Table 2). Neither the fast ($\tau_{fast}$) nor the slow time constant of inactivation ($\tau_{slow}$) at the tested membrane potentials was different between mutant and WT sodium channels either with or without h\(b\) sub-expression (Figure 6).

**Figure 4.** A: Baseline electrocardiogram recorded from the index patient (BII-3) of family B, carrying the R367C mutation. Leads I, aVF, V1 and V6 are shown. Note the widened QRS complexes (200ms) in these leads. 

B: The same ECG leads recorded after intravenous administration of 200mg procainamide, which successfully terminated a spontaneously occurring monomorphic ventricular tachycardia. Note the further widening of the QRS-complex. In all ECG recordings the paper-speed was 25mm/sec.
Cardiac conduction disease by a reduction of membrane sodium channel expression

Figure 5. Whole-cell sodium current measurements. A: Representative whole-cell sodium current traces recorded from HEK-293 cells transfected with either WT (left) or I1569-1570ins (right) sodium channel α-subunit cDNA in the presence of hβ1 subunit cDNA. B: Average current-voltage relationship for WT and I1569-1570ins sodium channels in the absence (left) or presence (right) of β1 subunit (for values see Table 2.).

Figure 6. Time course of inactivation. Fast and slow time constants of current decay of WT and I1569-1570ins sodium channels. Values were acquired by fitting the current decay at each applied voltage with a bi-exponential function. At none of the applied potentials there was a significant difference in time constants of inactivation.

Except for a difference in the slope factor of activation, in the presence of the β1-subunit no differences were found in the properties of voltaged-dependence of activation and steady-state
inactivation and in recovery from inactivation between WT and I1569-1570ins channels, expressed with or without hβi (Figures 7 and 8, Table 2.).

**Figure 7.** Voltage dependence of activation and steady-state inactivation of WT and I1569-1570ins sodium channels in the absence (A) and presence of the β1 subunit (B). Data points were fitted with Boltzmann equations. For values see Table 2.

**Figure 8.** Recovery from inactivation of WT and I1569-1570ins sodium channels in the absence (A) and presence of the β1 subunit (B). Time constant of recovery from inactivation was obtained by fitting the data with a mono-exponential function. For values see Table 2.
Cardiac conduction disease by a reduction of membrane sodium channel expression

<table>
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<th>WT+β (n=13)</th>
<th>1569-1570insl+β (n=15)</th>
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<td>24.9±2.9</td>
<td>13.4±1.9</td>
<td>15.1±1.3</td>
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*p<0.05, †p<0.005, ‡p<0.0005

Table 2. Cellular electrophysiological properties of the I1569-1570ins sodium channel when expressed in HEK-293 cells. The peak sodium current was found to be significantly larger in WT compared to the I1569-1570ins mutant channels. The other characteristics were not found to be different.

Electrophysiological properties of the R367C mutation

In order to conduct biophysical analysis of the mutant R367C Na⁺ channels, we expressed R367C in tsA201 and HEK-293 cells. In both cell types, no R367C Na⁺ currents could be elicited (data not shown). Given that "rescue" of ER-trapped HERG channels was reported by incubation at a reduced temperature (27°C), we incubated tsA201 and HEK-293 cells transfected with R367C cDNA at 27°C. This did not result in macroscopic Na⁺ current. Similarly, incubation in the presence of Na⁺ channel blockers (lidocaine and flecainide) and butyric acid failed to "rescue" the mutant Na⁺ channels.

Confocal Laser Microscopic Analysis of the R367C and I1569-1570ins mutants

Fluorescence confocal microscopy was used to determine the subcellular localization of the GFP tagged WT SCN5A and the ICCD causing mutants I1569-1570ins and R367C, respectively. When β₁-subunit expressing HEK-293 cells were transfected with wild-type SCN5A^{GFP} these cells exhibited a surface membrane distribution pattern consistent with the
functional electrophysiological recordings (Figure 9A). Similar type of surface membrane fluorescence was also obtained in the absence of the $\beta_1$-subunit (data not shown).

In contrast, cells transfected with I1569-1570ins SCN5A<sup>GFP</sup> and R367C SCN5A<sup>GFP</sup> revealed a predominantly perinuclear subcellular localization with a much less intense surface membrane distribution pattern both in the absence (data not shown) and presence of $\beta_1$ (Figures 9B and 9C). This is consistent with the reduction or absence of Na$^+$ current upon electrophysiological measurements.

**Figure 9.** (Sub)cellular localization of GFP-tagged WT and mutant SCN5A in HEK-293 cells constitutively expressing $\beta_1$ subunit. Confocal microscopic images of HEK-293 cells expressing WT SCN5A in a cluster of cells (A), I1569-1570ins SCN5A in a single cell (B), and R367C SCN5A also in a single cell (C).
DISCUSSION

The I1569-1570ins SCN5A mutation was found in a family with ICCD and clinical features of BS in which two male family members died suddenly. When the mutant channel was expressed in a cell expression model, its electrophysiological properties were similar to the WT channel except for a 75% reduction in peak sodium current. This is very likely due to the inability of a great proportion of the mutant channel to reach the sarcolemmal membrane, as supported by findings from GFP-tagging experiments, in which we observed a pronounced perinuclear or cytoplasmic localization of the channel protein, as compared to WT. One could speculate that this mutation results in mis-folding of the protein leading to its retention inside the cytosol. Nevertheless, since Na⁺ current could be measured, a proportion of mutant channels must escape these quality control mechanisms.

In the case of the R367C mutation, which was found in another family with ICCD and features of the BS, no Na⁺ current could be measured in a cell expression model. GFP-tagging experiments of this mutant channel displayed cytosolic or perinuclear retention suggesting that this mutation is associated with a severe trafficking defect.

Clinical phenotype of family A

SD in ICCD may result from complete heart block or ventricular tachyarrhythmias, due to functional re-entry, developing into ventricular fibrillation (VF). In the case of both the I1569-1570ins mutation indexes (AIII-12 and AIII-15), the cause of SD is unknown. Interestingly, the triggers for the events resulting in SD seem to have been stress or stress-related. This is remarkable since in both the Brugada syndrome (BS) and the long QT syndrome type 3 (LQT3), both associated with mutations in the SCN5A gene, arrhythmias typically arise at rest and SD usually occurs during sleep. When SD in carriers of the I1569-1570ins mutation indeed is stress related, during higher heart rates, this may be due to frequency dependent effects that further reduce the Na⁺ current magnitude e.g. by increased slow inactivation.

Interestingly, the only other symptomatic individual from this family (AIV-4) developed a tachyarrhythmia episode, atrial and ventricular, while having a slight fever (38°C). Fever is known to aggravate and induce symptoms in both BS and ICCD. The other family members that carried the I1569-1570ins mutation were found to have conduction abnormalities. When challenged with sodium channel blocking drugs, cardiac conduction in mutation carriers became significantly more impaired.
The clinical, cellular electrophysiological and trafficking data suggest that individuals carrying the 11569-1570ins mutation suffer from ICCD and possibly a combination of ICCD and BS.

Clinical phenotype of family B
The index patient from family B (BII-3), was evaluated for recurrent syncope preceded by palpitations and chest pain. During her hospital stay several episodes of both monomorphic and polymorphic VT were documented. The monomorphic VT, but not the polymorphic VT, could be reproducible induced during programmed electrical stimulation. Because her 12-lead ECG showed severe conduction abnormalities, including RBBB, and what appeared as coved type ST-segment elevations, the diagnosis Brugada syndrome was initially made. The finding of the R367C loss-of-function SCN5A mutation seemed to confirm this diagnosis. Additionally, the same mutation was identified in her sister who also suffered from episodes of recurrent syncope and presented with similar ECG abnormalities.

What appeared as ST-segment elevation in V1 on her baseline ECG, in conjunction with a widened QRS complex of 160 ms with an elevated ST-segment (Figure 4A), is, however, an extremely widened QRS complex of 200ms followed by a negative T wave. Similar QRS widening is present in all 12 ECG leads (Figure 4A, only leads I, aVF, V1 and V6 are shown). The apparent (incomplete) RBBB often described in BS patients, is actually a J-point elevation due to an increased epicardial phase 1 repolarization resulting in a voltage gradient between the epicardium and endocardium. In case of the index patient carrying the R367C mutation, it may however be a true RBBB and thus evidence of ICCD rather than BS. Theoretically a BS and/or an ICCD phenotype may result from loss-of-function SCN5A mutations. In the case of the R367C mutation the conduction disease is, however, so elaborate that these patients probably suffer from conduction disease rather than Brugada syndrome. Additionally, the occurrence of spontaneous and reproducible inducible monomorphic VT, would also argue against Brugada syndrome, in which polymorphic VT is more common. BS or an overlap syndrome of BS and ICCD rather than ICCD can, however, at present not be excluded.

Pathogenesis
Abnormal trafficking of mutant protein is increasingly recognized as a mechanism for inherited human diseases.\textsuperscript{21,24-27} Mutations in several membrane proteins also have been reported to cause defective trafficking, including the ion channels HERG,\textsuperscript{21}KCNQ1,\textsuperscript{26} and
Cardiac conduction disease by a reduction of membrane sodium channel expression

$SCN5A$. This is thought to involve misfolding or improper assembly of the protein structure, leading to its retention in the endoplasmic reticulum by the “quality control” system. Baroudi et al. first described trafficking abnormality as one of the mechanisms of Brugada syndrome in $SCN5A$ mutants, where they showed cytoplasmic accumulation of mutant sodium channel proteins. Several studies suggest that ion channel surface membrane expression can be rescued in vitro by a reduction in incubation temperature, incubation with butyric acid or incubation with specific channel blocking drugs. For a LQT3 mutation in the $SCN5A$ gene, $M1766L$, it has been shown that the peak current magnitude could be rescued by incubation of the transfected cells with mexiletine. None of the above mentioned interventions proved successful in restoring the sodium current in HEK-293 nor tsA201 cells, expressing the R367C sodium channel. The mechanisms by which the above mentioned interventions rescue mutant sodium channel expression are unclear. Possibly they act through a chaperone mechanism, whereby mutant sodium channels are able to escape from the quality control mechanism. Although these interventions have been suggested as possible therapeutic options in diseases resulting from protein trafficking defects, the applicability may be limited to only a few mutations. This is illustrated by the lack of effectiveness for rescue of the R367C mutation. Additionally it is questionable if a larger functional expression of mutant $SCN5A$ channels would be desirable. Finally, the long-term prescription of such drugs in patients with overt ICCD would be questionable. This is illustrated by the larger QRS-interval prolongation in I1569-1570ins mutation carriers when they received sodium channel blocking drugs for diagnostic purposes.

CONCLUSION

ICCD in both families is explained by a reduction in sodium current. Loss of function $SCN5A$ mutations are a commonly observed mechanism in inherited ICCD. Although fibrosis in the heart has been reported to occur in association with several loss of function $SCN5A$ mutations, the I1569-1570ins and R367C mutations illustrate that an isolated reduction in $I_{Na}$ remains an important mechanism in ICCD. The I1569-1570ins and R367C $SCN5A$ mutations would bring the number of $SCN5A$ mutations that cause ICCD as a true primary electrical disease of the heart to seven. Both mutations additionally show the diagnostic difficulties in $SCN5A$ mutation related diseases, since in neither of the two families BS or an overlap syndrome of ICCD and BS can be
excluded. Differentiation between pure BS, ICCD and overlap syndromes remains a diagnostic challenge.
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Cardiac conduction disease by a reduction of membrane sodium channel expression