Supplemental Material

Methods

Clinical Analysis and Participants

The clinical diagnosis of Brugada Syndrome (BrS) and early repolarization syndrome (ERS) was based on criteria provided in the 2005 Consensus Conference document (1) in the case of BrS and criteria suggested in our recent review of the J wave syndromes in the case of ERS. (2) Briefly, a diagnosis of BrS was assigned to an individual presenting with a diagnostic Type 1 Brugada ECG pattern (coved-type ST-segment elevation in the right precordial leads) either spontaneously, during fever, and/or after intravenous injection of a sodium channel blocking agent (ajmaline, flecainide, or procainamide), and either a personal or family history of arrhythmic syncope, cardiac arrest, or sudden cardiac death (SCD). ERS is divided into three subtypes: Type 1, displaying an early repolarization (ER) pattern predominantly in the lateral precordial leads, is prevalent among healthy male athletes and rarely seen in ventricular fibrillation (VF) survivors; ERS Type 2, displaying an ER pattern predominantly in the inferior or infero-lateral ECG leads, is associated with a higher level of risk; whereas ERS Type 3, displaying an ER pattern globally in the inferior, lateral and right precordial leads, is associated with the highest level of risk for development of malignant arrhythmias and is often associated with VF storms. It is noteworthy that, when the ER pattern present in lateral and right precordial leads, it was considered BrS. (3)

Informed consent was obtained from all patients upon referral to the Masonic Medical Research Laboratory for genetic testing and patients were tracked anonymously. This study was approved by the regional institutional ethics review board of Faxton St. Luke's Healthcare, Utica, New York and conducted according to Declaration of Helsinki principles.

For each patient, we collected age at time of diagnosis, gender, clinical presentation (SCD, aborted cardiac arrest, syncope, chest pain, *et al*.), family history, and therapy (implantable cardioverter-defibrillator (ICD), medication). 12-lead ECGs obtained were recorded at paper speeds of 25 or 50 mm/s. The PR, QRS, and QT intervals were measured manually and the QTc interval was calculated according to Bazett's formula. The presence of arrhythmias was evaluated on the basis of 12-lead ECGs, 24-h Holter recordings, or interrogation of ICDs. Coronary artery disease and/or spasm were excluded before the diagnosis of BrS/ERS was reached.

Genetic Screening and Analysis

Blood was collected from probands and family members after obtaining informed consent. Genomic DNA was extracted from peripheral blood leukocytes using a commercial kit (Gentra Puregene, Quiagen Sciences, Germantown, Maryland) and amplified by polymerase chain reaction (PCR) on GeneAmp® PCR System 9700 (Applied Biosystems, Life Technologies, Grand Island, New York). All exons and intron borders of the *SCN5A*, *GPD1L*, *CACNA1C, CACNB2B*, *SCN1B*/*SCN1Bb*, *KCNE3*, *SCN3B*, *KCNJ8*, *CACNA2D1*, *KCND3*, *MOG1*, *SLAMP*, and *SCN10A* genes were amplified and analyzed by direct sequencing. PCR products were purified with a commercial reagent (ExoSAP- IT, USB, Affymetrix, Santa Clara, California) and directly sequenced from both directions using an ABI PRISM 3730 Automatic DNA Analyzer (Applied Biosystems, Life Technologies), as previously described. (4) The primer sequences for *SCN10A* are shown in **Table S1** (Reference Sequence: NM_006514). More than 200 ethnically matched healthy controls (400 reference alleles), plus all available online databases for allele frequency (including the 1000 Human Genome Project database and Exome Sequencing Project), conservation score (including Genomic Evolutionary Rate Profiling and PhastCons), and *in silico* pathogenic prediction tool (including Sorting Intolerant From Tolerant

and Polyphen), were probed for prediction of pathogenicity of the variants found. Mutations are defined as rare, case-only (absent in the 200+ healthy controls) variants that are possibly pathogenic. If the minor allele frequency is <0.5%, these variants will be termed uncommon/rare polymorphisms; otherwise, termed as common polymorphisms. (5)

Co-Expression of $Na_V1.5$ and $Na_V1.8$ for Co-Immunoprecipitation (Co-IP) Analysis. Site-directed mutagenesis was performed on full-length human wild-type (WT) and mutant *SCN10A*-3XFLAG cDNA cloned in pCMV2 vector, the WT *SCN3B* cloned in pCMV6-XL6 vector, and the WT *SCN5A* cloned in pcDNA3.1. The mutated plasmid was sequenced to ensure the presence of the mutation without spurious substitutions. HEK293 cells were used to express Nav1.8 sodium channels. (6-8) Three genes were co-expressed in cells using FuGENE6. Since there was no data available concerning the level of expression of *SCN10A* in affected individuals, we choose to empirically us equal amount of *SCN10A* and *SCN5A* cDNA. A total of 2.25µg of DNA was transfected at a ratio of 5:5:1 for *SCN5A*:*SCN10A*:*SCN3B*. A plasmid encoding enhanced green fluorescent protein (eGFP) was used to control for DNA quantity. Total protein was isolated 24 hours after transfection with Lysis buffer (150m NaCl, 10m Tris-HCl, 5mM EDTA, 1% Triton X-100, pH 7.4) supplemented with protease inhibitors. Scraped lysates were centrifuged at 16,000g for 30min at 4°C. For total protein lanes, soluble proteins were quantitated and equal amounts were diluted with Laemmli sample buffer containing 5% β-mercaptoethanol (Input lanes). For the Co-IP lanes, equal amounts of soluble protein were diluted to 500uL in Lysis buffer and incubated overnight at 4°C with the anti-FLAG M2 antibody (1:1000, Sigma F-1804, Sigma-Aldrich, St. Louis, Missouri). Protein A/G beads (50uL, Pierce 88802, Thermo Fisher Scientific Inc., Rockford, Illinois) were then added and incubated for 2 hours at 4°C. The beads were washed three times with TBS (50mM Tris-HCl, 150m NaCl, pH 7.4) supplemented with 2M NaCl, 0.05% Tween-20. Bound proteins were eluted using

Laemmli sample buffer containing 5% β-mercaptoethanol and analyzed using SDS-PAGE. Separated proteins were transferred into polyvinylidene difluoride membranes and blocked with 5% non-fat dry milk (Lab Scientific, Highlands, New Jersey) in Tris-Buffered Saline supplemented with 1% tween-20. The membranes were then probed with either anti-FLAG M2 (for $Na_V1.8$, 1:10,000, Sigma F-1804, Sigma-Aldrich), anti-$Na_V1.5$ (1:2000, ASC-013, Alomone Labs, Jerusalem, Israel) or anti-transferrin (1:20,000, Sigma 13-6800, Sigma-Aldrich) followed by an appropriate horseradish peroxidase conjugated secondary antibody (Anti-mouse 1:30,000 Abcam ab97023 or Anti-rabbit 1:30,000 Abcam ab97051). All antibody dilutions were made in Tris-Buffered Saline and Tween 20 supplemented with 5% non-fat dry milk. Immunoreactive bands were visualized using Immune-Star WesternC and Bio-Max film (Life Science, Hercules, California). The endogenous protein transferrin was probed as a loading control.

Co-Expression of $Na_V1.5$ and $Na_V1.8$ for Electrophysiological Investigations

TSA201 cells transfected with *SCN5A*, *SCN10A* and *SCN3B* plasmids were also used for patch-clamp study. A plasmid encoding eGFP was used to identify transfected cells. Briefly, transient transfection using fugene6 (Roche Diagnostics, Indianapolis, Indiana), was carried out with *SCN10A* (WT or mutant), *SCN5A* and SCN3B with a molar ratio of 5:5:1. The cells were grown in GIBCO DMEM medium (No. 10566, Gibcowith FBS (No. 16000) and antibiotics (No. 15140) Life Technologies) on polylysine coated 35 mm culture dishes (Cell+, Sarstedt INC., Newton, North Carolina). Cells were placed in a 5% CO2 incubator at 37°C for 24 to 48 hours prior to patch clamp study.

Membrane currents were measured using whole-cell patch-clamp techniques. All recordings were obtained at room temperature (20 - 22°C) using an Axopatch 200B amplifier equipped with a CV-201A head stage (Axon Instruments Inc./Molecular Devices, Union City,

California). Currents were filtered with a four pole Bessel filter at 5 kHz and digitized at 50 kHz. Series resistance was compensated at around 80% to assure that the command potential was reached within microseconds with a voltage error <2 mV. Cells were allowed to stabilize for 10 min after establishment of the whole-cell configuration before current was measured. Macroscopic whole cell Na^+ current was recorded by using bath solution perfusion containing (in mmol/L) 140 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 Dextrose (pH 7.35 with NaOH). Osmolarity adjusted to 310 mmol/kg with sucrose. Patch pipettes were fabricated from 1.5 mm OD borosilicate glass capillaries (Fisher Scientific Inc.). They were pulled using a gravity puller (Model PP-830, Narishige International USA, Inc., East Meadow, New York) to obtain resistances between 0.8 - 2.2 MΩ when filled with a pipette solution containing (in mmol/L) 10 NaF, 105 CsF, 20 CsCl, 2 EGTA, and 10 HEPES with a pH of 7.35 adjusted with CsOH and an osmolarity of 300 mmol/kg with sucrose.

Specific voltage-clamp protocols assessing channel activation and fast inactivation were used as depicted in figure insets. Cardiac sodium channel current (I_{Na}) was elicited by depolarizing pulses ranging from -90 mV to +40 mV in 5 mV increments with a holding potential of -120 mV. Peak currents were measured and I_{Na} densities (pA/pF) were obtained by dividing the peak I_{Na} by the cell capacitance obtained. Activation properties were determined from I/V relationships by normalizing peak I_{Na} to driving force and maximal I_{Na}, and plotting normalized conductance *vs.* Vm. Voltage-dependence of steady-state inactivation was obtained by plotting the normalized peak current (40-ms test pulse to –0 mV after a 1000-ms conditioning pulse from -140 mV to -10 mV with the holding potential of -120 mV, see inserted protocol of Fig. 5F) *vs.* Vm. The steady-state channel availability and inactivation curves were fitted to the Boltzmann equation, $I/I_{max} = 1/(1+exp((V-V_{1/2})/k))$ to determine the membrane potential for half-maximal activation/inactivation ($V_{1/2}$) and the slope factor (k). Pulses for recovery from

inactivation were of 100 ms duration for P1 and 50 ms for P2. Peak current elicited during the second pulse was normalized to the value obtained during the initial test pulse. It was analyzed by fitting data to a double exponential function: $I_{(t)}/I_{max}= A_f \cdot(1-exp(-t/\tau_f))+A_s \cdot(1-exp(-t/\tau_s))$, where A_f and A_s are the fractions of fast and slow inactivating components, respectively, and τ_f and τ_s are their time constants.

All data acquisition and analysis were performed using pCLAMP V9.2 (Molecular Devices), EXCEL (Microsoft, Redmond, Washington) and ORIGIN 7.5 (MicroCal Software, GE Healthcare, Pittsburgh, Pennsylvania).

Statistical Analysis

Human data were presented as mean±SD, experimental data were presented as mean±SEM, and unless otherwise noted. For statistical analysis, two-tailed Student's t-test and ANOVA coupled with Student-Newman-Keuls test, were used for compare of two groups and more than three groups of continuous variables separately; Chi-square test were used for compare of categorical variables (SigmaStat, Systat Scientific Inc., San Jose, California). Differences were considered statistically significant at a value of $P<0.05$.

References

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Table S1. Sequences of Primers in *SCN10A*.

Exon	Sense	Antisense	PCR Size
1	5'- GAA GGG CTG TTC TGA CAA TC -3'	5'- GCC AAG GAG GAC TTT CTG G -3'	481bp
2	5'- GAG CCC TTC TTG CTC ATA AG -3'	5'- GGT ACT GGA CAC AGT AGG C -3'	296bp
3	5'- CAG CAT CAA GGT GAT CCT AG -3'	5'- CCA CAT GAG GCA TGG CAG G -3'	258bp
4	5'- CTT GGA GCA GTC AGA GCA G -3'	5'- CCT GGT ATA TTC CTG CAG TG -3'	386bp
5	5'- GAT GGC AGT GTC ACT AGA TTC -3'	5'- CCT ATA CTC CAG GCC TCA G -3'	372bp
6	5'- CCT GGA GTG TGT ACA TAC TTC -3'	5'- CCT GTC CCT ATA TGA TAC CAA -3'	379bp
7	5'- CCG AAG TCA CTG CTT AGC TC -3'	5'- CCA TCT GTG CCC ATA ATA TGC -3'	342bp
8	5'- GAC CAA GTG TCC AAG ATC ATC -3'	5'- CCT GTA CCC ATA GCA CAT GG -3'	408bp
9	5'- GCT GCA TGG CTG TGC TTT TC -3'	5'- CTG AGG CAA GAT GAT TCC TC -3'	419bp
10	5'- GGT CCT AAC CCT TCA GCT TC -3'	5'- GGT GTC TGG ATC CTT TTA GG -3'	424bp
11	5'- GGA GTA GGT AGA GGG AAG TG -3'	5'- GGA GGC ATT GGA CAG GAT G -3'	497bp
12	5'- GGC TGA ATG ATC CAA CTT TGC -3'	5'- GAC ACG AGT TAG AGA CAT TGC -3'	271bp
13	5'- GAG GAT GAC CGC AGA ATT GG -3'	5'- GTG TCC ACA TGT CTC CTG C -3'	470bp
14	5'- CCA TTC TGT CAC GTT GAC TG -3'	5'- CAC AGA CCC AAG TCT CCA G -3'	510bp
15	5'- CCA GAT CTT TGG TTT CCA GG -3'	5'- CAA CCA GAG AAG TAC AAT CTG -3'	559bp
16	5'- GCC AGC CAG CTG CTA ACC -3'	5'- CAA GGT CTC CTC TGC ATT TC -3'	625bp
17	5'- GCA GCC TGT GAT GTG CAA G -3'	5'- CGG ATT AGC CGG AGG TTG G -3'	404bp
18	5'- GGT GCA GTT GAA CCT GCA G -3'	5'- GCA AGT GGG CAC AGC TTG T -3'	321bp
19	5'- CCT AGC AGA ATG GTT TCT CTG -3'	5'- CTG CAG CTC TCC TTC TAG TG -3'	385bp
20	5'- GCT ACT GGC AAG CTG ACT AC -3'	5'- CAG ACT CCT CAC TAA ATG CTC -3'	448bp
21	5'- GAC CTC TCA TTG GAG TTC CG -3'	5'- CCA CAC CTC GTG GTG GTA G -3'	460bp
22	5'- CTC ATG AAG CAG CCT GAA TG -3'	5'- CCT CAG TTC ACA CTG AGT GC -3'	504bp
23	5'- CTG CAC AAG CCC TTT CCA AC -3'	5'- GGA CAG TGT GAG GTT GCT G -3'	303bp
24	5'- CAC TCC CTC ATT CCT GCT TC -3'	5'- CTT CCT GCA ACA GCA ATC AC -3'	438bp
25	5'- GGC AAG ACA CTC CTT GAT GC -3'	5'- CTC CAG AAG ACC AGA GTT GC -3'	448bp
26	5'- GGC AAT GAG GTA GAG TCA AC -3'	5'- GCA TGG AGA AGG CCA AGA G -3'	655bp
27a	5'- GCA GTC TAT ACC AAG CAA AGC -3'	5'- CTC TGG TGC CAT TGC TGT TG -3'	509bp
27b	5'- GCC TCT TCC AGA TTA CCA CG -3'	5'- CCT CCA TAT TTG CCT TCA GAG -3'	522bp
27c	5'- CCT GGA GAT AAG ATC CAC TG -3'	5'- CTG TAG CTG GGT GTG ATC TG -3'	583bp

Table S2. Effects of *SCN10A*, *SCN5A* and *SCN3B* Co-Expression on Equilibrium Gating Parameters

	Inactivation			Activation			Recovery		
Subunit	$V_{1/2}$ (mV)	K (mV)	n	$V_{1/2}$ (mV)	K (mV)	N	T_f (ms)	T_s (ms)	n
SCN5A/WT+	-83.83	7.94		-48.12	5.21		6.19	489.55	
SCN3B/WT+	±	±	9	±	±	9	±	±	9
SCN10A/WT	1.97	0.44		1.75	0.43		0.51	147.06	
SCN5A/WT+	-86.27	8.78		-44.37	5.98		9.29	713.89	
SCN3B/WT+	±	±	9	±	±	9	±	±	9
SCN10A/R14L	1.23	0.52		1.66*	0.38		0.58*	144.21*	
SCN5A/WT+	-91.52	8.67		-49.61	5.88		8.57	667.01	
SCN3B/WT+	±	±	8	±	±	10	±	±	8
SCN10A/R1628Q	1.56*	0.34		2.54	0.55		0.59*	91.72*	

Parameters of inactivation and activation were calculated from the Boltzmann function. $V_{1/2}$ is the voltage for half-maximal availability or activation and k is the slope factor. Parameters of recovery were fitted to a double exponential function. $^{*}P<0.05$ *vs. SCN10A*/WT + *SCN5A*/WT + *SCN3B*/WT. Data are from Figure 3 and reported as mean±SEM. WT: wild type.