SUPPLEMENTAL MATERIAL

DETAILED METHODS

1.1 Heterologous Expression of WT-K_v4.2 and -K_v4.3 Channels (Online Figure I)

All reagents were from Sigma Chemical Company (St. Louis, MO) unless noted otherwise.
HEK293 cells, obtained from the American Tissue Culture Collection (ATCC: Manassas, VA), were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco/Invitrogen, Carlsbad, CA) supplemented with 5% horse serum (Gibco), 5% heat-inactivated fetal calf serum (Gibco) and 1 unit/ml penicillin-streptomycin (Gibco). Cells were passaged at confluence every 3-4 days by brief trypsinization.[1] For transient transfections, plasmids (pBK-CMV) encoding human K_v4.3 (**Online Figure I**) or K_v4.2 and td-Tomato in the absence and presence of (pBK-CMV) plasmids encoding human KChiP2 or SEMA3A were mixed with lipofectamine 2000 (Life Technologies, Inc., Gaithersburg, MD) in Opti-MEM (Gibco) and incubated at room temperature for ~30 minutes prior to addition to the cultures. For electrophysiology, cells were transfected in 35 mm tissue culture dishes using 1 µg of lipofectamine mixed with 0.5 µg of total plasmids. Plasmids were mixed in equal ratios, and the total amount of plasmid was kept constant (at 0.5 µg for electrophysiological) by the addition of empty (pBK-CMV) vector.

Approximately 8 hours after addition, the plasmid-containing medium was removed and replaced with the normal HEK293 cell culture medium (above). Electrophysiological experiments were performed 24-36 hours after transfections.

1.2 Electrophysiological Measurements and Data Analysis for WT-K_v4.2 and -K_v4.3 (Online Figure I)

Whole-cell voltage-clamp recordings were obtained at room temperature (22-24°C) within 36 hour of transfections using an Axopatch-1D amplifier (Axon Instruments, Sunnyvale, CA) interfaced to a Dell (model Precision 340) personal computer using a Digidata 1322A A/D converter (Axon Instruments). Voltage-clamp paradigms were controlled and data were collected using Clampex 9.2 (pClamp 9, Axon Instruments). Data were acquired at 100 kHz, and current signals were filtered on-line at 5 kHz prior to digitization and storage. Recording pipettes contained (in mM): KCl 115, KOH 20, EGTA 10, HEPES 10 and glucose 5 (pH 7.2; 300-310 mOsm). Pipette resistances were 1.5-3.0 MΩ when filled with the recording solution. The bath solution contained (in mM): NaCl 140, KCl 4, $MgCl_2$ 2, $CaCl_2$ 1, HEPES 10 and glucose 5 (pH 7.4; 300-310 mOsm).

After establishing the whole-cell configuration, brief (20 ms) ± 10 mV steps from a holding potential of -70 mV were applied to allow measurements of whole-cell membrane capacitances and input resistances. Whole-cell membrane capacitances and series resistances were routinely compensated (≥ 85%) electronically. Only data obtained from cells with input resistances ≥ 300 MΩ were analyzed. Leak currents were always < 200 pA and were not corrected. Voltage-gated Kv4-encoded K^+ currents were evoked in response to 450 ms depolarizing voltage steps to potentials between -60 and + 40 mV from a holding potential of -70 mV; voltage steps were presented in 10 mV increments at 10 sec intervals.

Electrophysiological data were analyzed using Clampfit 9.2 (Axon). Whole-cell membrane capacitances were calculated by integrating the capacitive transients (evoked during ± 10 mV voltage steps from -70 mV). Kv4.2-encoded currents (at each test potential) were measured as the difference between the maximal outward current amplitudes and the currents remaining at 450 ms.

1.3 Statistical Analysis

Electrophysiological data are presented as means ± SEM, as indicated in the text and figure legends. The statistical significance of observed differences between groups was evaluated using a one-way Student's t-test; a $p<0.05$ was considered significant.

2.1 Heterologous Expression of WT-SCN5A Channels

HEK293 cells were cultured in minimum essential medium supplemented with 1% nonessential amino acid solution, 10% horse serum, 1% sodium pyruvate solution, and 1.4% penicillin/streptomycin solution in a 5% CO_2 incubator at 37°C. 1 µg *SCN5A* wild type (H558/Q1077del, Genbank accession no.AY148488) human cardiac voltage-dependent Na^+ channel α-subunit in the pcDNA3 vector (Invitrogen, Carlsbad, CA) was co-transfected with 0.25 µg Green Fluorescence Protein (GFP) cDNA

(kindly provided by Dr. Gianrico Farrugia, Mayo Clinic, Rochester, MN) with the use of 3μl Lipofectamine (Invitrogen, Carlsbad, CA). The integrity of the constructs was verified by direct DNA sequencing. Transfected HEK293 cells were cultured in OPTI-MEM (Gibco, Carlsbad, CA) and incubated for 24 hours. Cells exhibiting green fluorescence were selected for electrophysiological experiments. For perfusion based experiments, human SEMA3A protein (hSEMA3A; R&D Systems, Minneapolis, MN) was dissolved in PBS at a concentration of 1 mM and diluted to work concentrations before experiments.

2.2 Electrophysiological Measurements and Data Analysis for $Na_v1.5$

Standard whole-cell patch clamp technique was used to measure SCN5A wild type currents before and after 100 nM human SEMA3A protein perfusion at room temperature (22-24°C) with the use of an Axopatch 200B amplifier, Digidata 1440A and pclamp 10 software (Axon Instruments, Sunnyvale, CA). The extracellular (bath) solution contained (mmol/L): 140NaCl, 4 KCl, 1.8 $CaCl_2$, 0.75 $MgCl_2$ and 5 HEPES, pH adjusted to 7.4 with NaOH. The pipette solution contained (mmol/L): 120 CsF, 20 CsCl, 2 EGTA, and 5 HEPES, pH adjusted to 7.4 with CsOH.[2, 3] Microelectrodes were pulled on a P-97 puller (Sutter Instruments, Novato, CA) and fire polished to a final resistance of 2-3 MΩ. Series resistance was compensated by 80-85%. Currents were filtered at 5 kHz and digitized at 10 kHz. The voltage-dependence of activation was determined from a holding potential of -100 mV to testing potential of +90 mV in 10 mV increments with 24 ms duration. Data were analyzed using Clampfit (Axon Instruments, Sunnyvale, CA), Excel (Microsoft, Redmond, WA), and plotted with Origin 8 (OriginLab Corporation, Northampton, MA) software.

2.3 Statistical Analysis

Results are expressed as mean ± SEM. Paired t test was performed to examine before and after 100 nM human SEMA3A protein comparison. A $p<0.05$ was considered significant.

3.1 Heterologous Expression of WT- $Ca_v1.2$ Channels

The human wild-type (WT) *CACNA1C* cDNA with an N-terminal enhanced yellow fluorescence protein (EYFP) tag [(EYFP) Nα1c, 77] in the pcDNA vector was a gift from Dr. Charles Antzelevitch. cDNA of *CACNA2D1* gene was cloned in pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) and was also a gift from Dr. Charles Antzelevitch. The cDNA of the *CACNB2b* gene was subcloned into the bicistronic pIRES2-dsRED2 vector (Clontech, Mountain View, CA). The integrity of the constructs was verified by direct DNA sequencing. HEK293 cells were cultured in minimum essential medium supplemented with 1% nonessential amino acid solution, 10% horse serum, 1% sodium pyruvate solution, and 1.4% penicillin/streptomycin solution in a 5% CO_2 incubator at 37°C. Heterologous expression of $Ca_v1.2$ was accomplished by co-transfecting 1 μg *CACNA1C* cDNA with 1 μg *CACNB2b*, 1 μg *CACNA2D1* and 0.25 μg Green Fluorescence Protein (GFP) cDNA with the use of 9μl Lipofectamine 2000. The media was replaced with OPTI-MEM after 4-6 hours. Transfected HEK293 cells were cultured in OPTI-MEM and incubated for 48 hours before electrophysiological experiments. For perfusion based experiments, human SEMA3A protein (hSEMA3A; R&D Systems, Minneapolis, MN) was dissolved in PBS at a concentration of 1 mM and diluted to work concentrations before experiments.

3.2 Electrophysiological Measurements and Data Analysis for $Ca_v1.2$

Standard whole-cell patch clamp technique was used to measure $Ca_v1.2$ currents without or with100 nM human SEMA3A protein incubation at room temperature (22-24°C) with the use of an Axopatch 200B amplifier, Digidata 1440A and pclamp version 10.2 software (Axon Instruments, Sunnyvale, CA). The extracellular (bath) solution contained (mmol/L): 130 NMDG, 5 KCl, 15 $CaCl_2$, 1 $MgCl_2$, 5 mM TEA-Cl and 10 HEPES, pH adjusted to 7.35 with HCl. The pipette solution contained (mmol/L): 120 CsCl, 2 $MgCl_2$, 10 EGTA, 2 MgATP, 5 $CaCl_2$ and 10 HEPES, pH adjusted to 7.25 with CsOH.[4-6] Microelectrodes were pulled on a P-97 puller (Sutter Instruments, Novato, CA) and fire polished to a final resistance of 2-3 MΩ. Series resistance was compensated by 80-85%. Currents were filtered at 1 kHz and digitized at 5 kHz with an eight-pole Bessel filter. The voltage dependence of activation was determined from a holding potential of -90 mV to testing potential of +70 mV in 10 mV increments with 500 ms duration. Data were

analyzed using Clampfit (Axon Instruments, Sunnyvale, CA), Excel (Microsoft, Redmond, WA), and plotted with Origin 8 (OriginLab Corporation, Northampton, MA) software.

3.3 Statistical Analysis

Results are expressed as mean ± SEM. Student t test was performed to examine $Ca_v1.2$ without or with 100 nM human SEMA3A protein comparison. A $p<0.05$ was considered significant.

4.1 Immunoblots of SEMA3A in Mouse Brain and Human Heart

Adult mouse brains were homogenized in ice-cold lysis buffer containing: 20 mM HEPES, pH 7.4, 150 mM NaCl, 0.5% CHAPS, complete mini EDTA-free protease inhibitor mixture tablet (Roche, Switzerland), and 1X Halt phosphatase inhibitor mixture (Pierce, Rockford, IL). Proteins were extracted on ice for 1 hour, centrifuged at 12,000 x g to remove debris, and protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). For isolation of mouse brain and human ventricular myocyte membranes, flash frozen adult mouse brain or human ventricular tissue samples were homogenized in ice-cold buffer containing (in mM): 10 HEPES, 320 sucrose, 3 $MgCl_2$, 25 sodium phosphate, 5 EGTA, 20 NaF, 2 sodium orthovanadate, complete mini EDTA-free protease inhibitor mixture tablet (Roche, Switzerland), and 1X Halt phosphatase inhibitor mixture (Pierce, Rockford, IL). Protein lysates were centrifuged at 10,000 x g to remove large tissue debris, and membranes were pelleted by centrifugation at 100,000 x g for 1 hour at 4°C. Isolated membranes were first washed with ice-cold lysis buffer without detergent and then solubilized in ice-cold lysis buffer containing 0.5% CHAPS. For Western blot analysis, proteins separated on 4-12% gradient SDS-PAGE gels (Invitrogen, Carlsbad, CA) were transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA), blocked with 5% skim milk in wash buffer (0.1% Tween 20/phosphate buffered saline) and incubated with primary antibodies overnight at 4°C. For comparison, recombinant human SEMA3A (R&D Systems, Minneapolis, MN) was run in parallel with all protein lysates. PVDF membranes were washed several times and incubated with either rabbit anti-mouse (Bethyl, Montgomery, TX) or donkey anti-rabbit (GE Healthcare, United Kingdom) horseradish peroxidase conjugated secondary antibodies and SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL). Signals were detected using a ChemiDoc MP imaging system and Image Lab Software (Bio-Rad Laboratories, Hercules, CA).

4.2 Immunoprecipitations of SEMA3A and $K_v4.3$

For immunoprecipitation (IP) of $K_v4.3$ channel complexes, anti-$K_v4.3$ antibody (clone K75/41, a kind gift of Dr. James Trimmer, UC Davis) or an isotype matched non-specific control antibody (anti-beta-galactosidase, clone 40-1A, Developmental Studies Hybridoma Bank) was cross-linked to Dynabeads Protein G beads (Invitrogen, Carlsbad, CA) using dimethyl pimelimidate (Pierce, Rockford IL). Mouse brain membrane lysates were first pre-cleared using protein G agarose beads (Invitrogen, Carlsbad, CA) and then mixed with anti-$K_v4.3$ conjugated beads for 3 hr at 4°C. Following mixing, beads were washed several times with ice-cold lysis buffer containing 0.05% CHAPS, and immunoprecipitated proteins were eluted using sample reducing buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% Glycerol, 2% Betamercaptoethanol, 0.01% Bromophenol Blue) for Western blot analysis.

5.1 Study Subjects

The study population consisted of 198 unrelated patients with clinically diagnosed but *SCN5A* mutation negative Brugada syndrome (BrS; **Table 1**) who were referred to the Windland Smith Rice Sudden Death Genomics Laboratory at Mayo Clinic, Rochester, Minnesota (n=11), the Molecular Cardiology Laboratory, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy (n=95), or the Cardio-Genetic Clinic Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands (n=92) for genetic testing. This study was approved by the Mayo Foundation Institutional Review Board, the Medical Ethical Committee of Fondazione IRCCS Policlinico San Matteo, and the Medical Ethical Committee of the Academic Medical Center. Informed consent was obtained for all patients.

5.2 SEMA3A Mutational Analysis

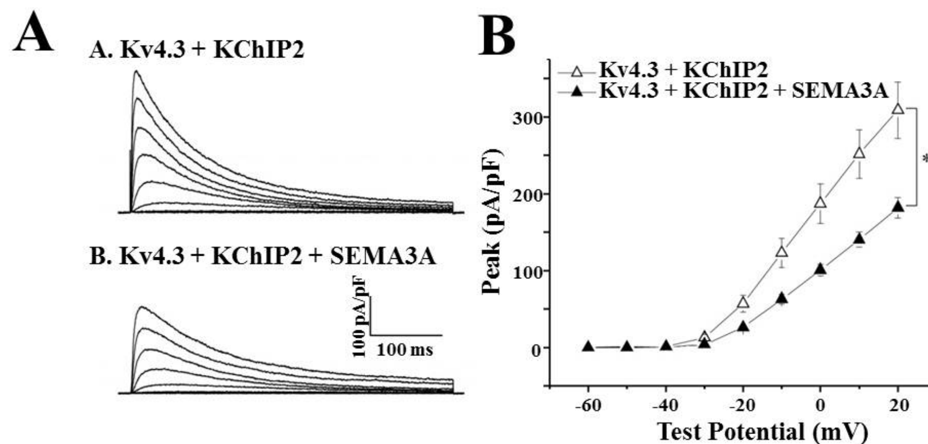
Comprehensive open reading frame and splice junction mutational analysis of the entire *SEMA3A* coding region (Genbank accession number NM_006080.2) was performed on genomic DNA from these 198 BrS

patients using PCR, denaturing high performance liquid chromatography (DHPLC; WAVE DNA Fragment Analysis System, Transgenomic Inc., Omaha, NE), and direct DNA sequencing (ABI Prism 377, Applied Biosystems Inc., Foster City, CA). Primer sequences, PCR conditions, and DHPLC conditions are available upon request.

To be considered a putative pathogenic BrS-causing variant, identified SEMA3A variants had to be i) non-synonymous, ii) involve highly conserved amino acids, iii) and absent in 500 ostensibly healthy controls obtained from the European collection of Cell Cultures (HPA Culture Collections, UK), absent in 300 Italian donor blood samples, and absent in the publically available databases including the 1000 Genomes Project[7] (n=1094), the NHLBI Go Exome Sequencing Project[8] (n=6503), and the 12000 Exome Chip[9] (n=12000).

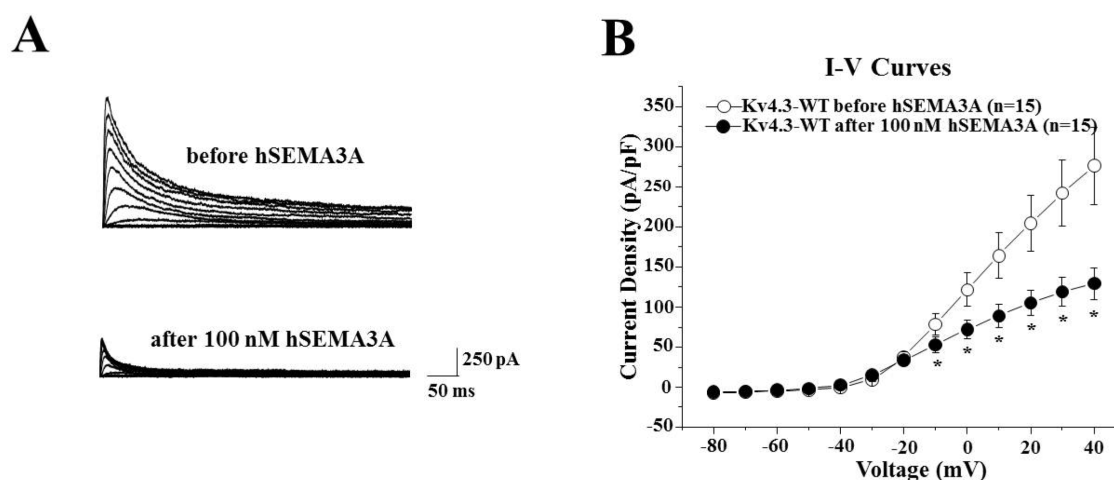
SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

Online Figure 1



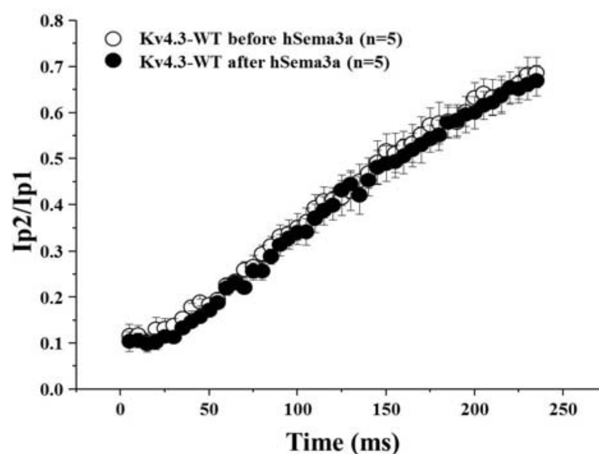
Online Figure I. Co-Expression of SEMA3A Attenuates K_v4.3 + KChIP2-Encoded K_v Currents. Whole-cell voltage-gated outward K^+ currents were recorded from HEK293 cells transiently transfected with cDNA constructs encoding tdTomato plus K_v4.3 in the absence and in the presence of KChIP2 and/or SEMA3A in response to depolarizing voltage steps between -60 and +40 mV (in 10 mV increments) from a holding potential of -70 mV. Representative K_v4.3 + KChIP2-encoded K_v currents, recorded from HEK293 cells transfected in the absence (**A upper**) or the presence (**A lower**) of SEMA3A, are shown. (**B**) Mean ± SEM (n = 5-8 cells) peak K_v4.3 current densities in the absence and presence of KChIP2 and/or SEMA3A are plotted. Co-expression of SEMA3A significantly ($*P < 0.01$) attenuated K_v4.3 + KChIP2 current densities.

Online Figure II



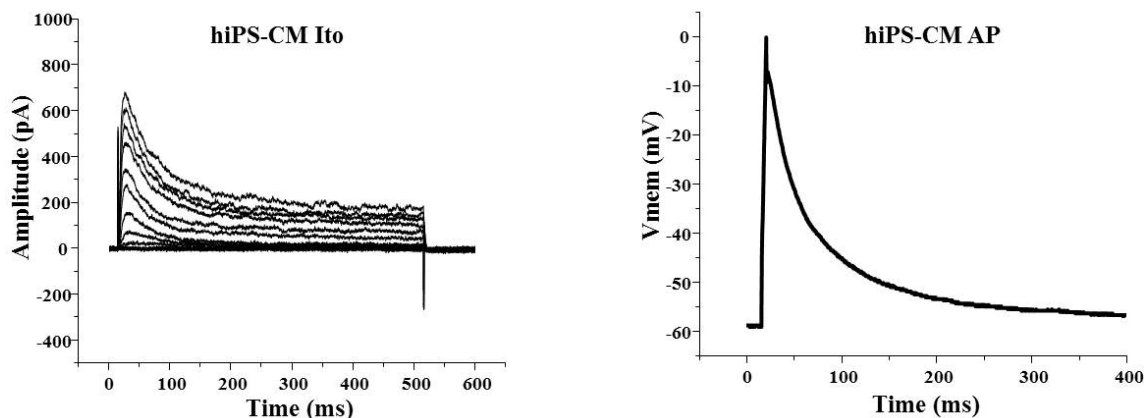
Online Figure II. Perfusion of 100 nM SEMA3A Decreases Current Density of $K_v4.3$.
(A) Representative whole-cell $K_v4.3$-WT tracings before and after 100 nM human SEMA3A protein perfusion. **(B)** The current voltage relationship for $K_v4.3$-WT before and after 100 nM hSEMA3A perfusion. All values represent mean±SEM. * $p<0.05$ vs. $K_v4.3$-WT before hSEMA3A perfusion.

Online Figure III



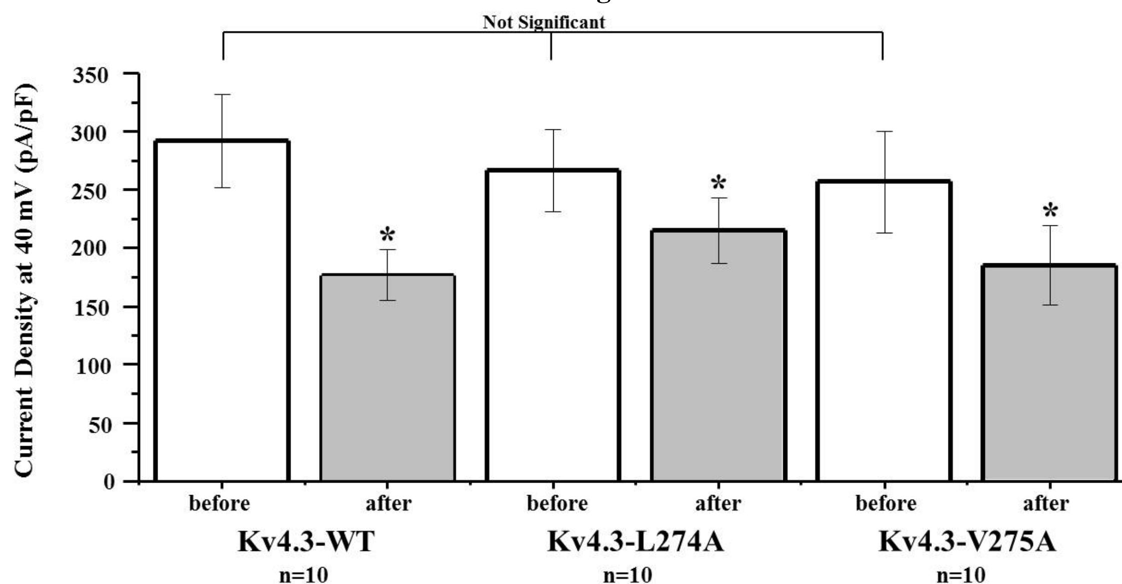
Online Figure III. Perfusion of 100 nM SEMA3A Does Not Affect Recovery from Inactivation.
Recovery from inactivation of $K_v4.3$-WT and $K_v4.3$-WT + hSEMA3A perfusion determined from a holding potential of −80 mV to pre-pulse of +20 mV with 0.5s duration, with increased recovery interval, followed by a test pulse of +20 mV with 500 ms duration and fitted with a one-exponential function. All values shown represent mean ±SEM.

Online Figure IV



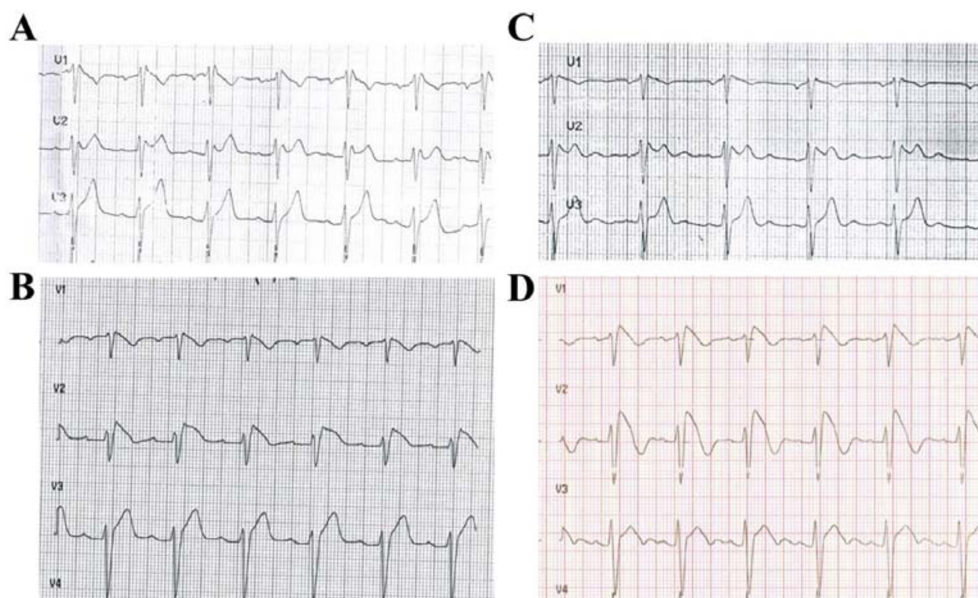
Online Figure IV. Ito and Action Potential (AP) Recordings in Control hiPSC-CMs. A representative hiPSC-CM showing Ito recording (left) and AP recording with clear phase 1 notch (right) in the same cell using current clamp mode at a constant rate of 1 Hz through 5 ms depolarizing current injections of 400-500 pA.

Online Figure V



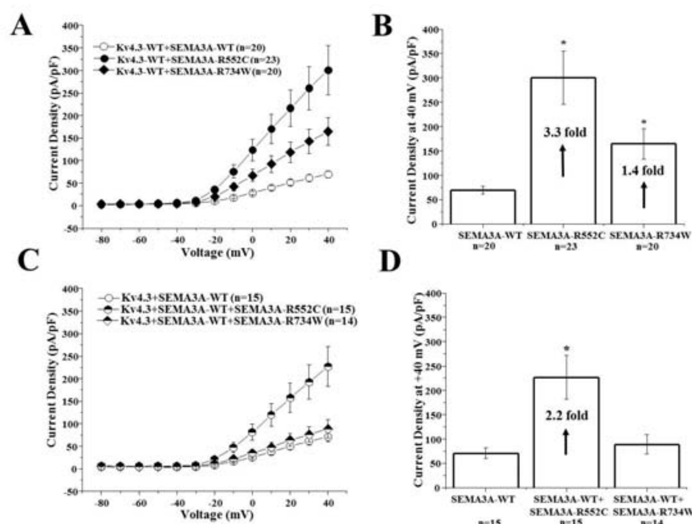
Online Figure V. Targeted Disruption of Toxin Binding Domain on K_v4.3 with SEMA3A Perfusion. Bar graph showing peak current density at +40 mV for K_v4.3-WT (n=10), K_v4.3-L274A (n=10), and K_v4.3-V275A (n=10) before SEMA3A, which is not statistically significant, and after 100nM perfusion of SEMA3A. * $p<0.05$ vs. K_v4.3 before, all values shown represent mean ±SEM.

Online Figure VI



Online Figure VI. Representative ECG Traces from BrS Patients. Representative basal **(A)** and Flecanide **(B)** ECG traces from patient harboring R552C-SEMA3A. Representative basal **(C)** and Flecanide **(D)** ECG traces from the patient harboring R734W-SEMA3A mutation.

Online Figure VII



Online Figure VII. Heterozygote Co-Expression of Mutant and WT-SEMA3A. **(A)** The current voltage relationship representing a homozygous state for K_v4.3-WT co-expressed with SEMA3A-WT (n=20), SEMA3A-R552C (n=23), or SEMA3A-R734W (n=20) (as shown in Figure 6). **(B)** Bar graph showing peak current density at +40 mV for K_v4.3-WT co-expressed with SEMA3A-WT (n=20), SEMA3A-R552C (n=23), or SEMA3A-R734W (n=20). * $p<0.05$ vs. K_v4.3-WT + SEMA3A-WT. **(C)** The current voltage relationship representing a heterozygous state for K_v4.3-WT co-expressed with SEMA3A-WT (n=15), SEMA3A-WT+SEMA3A-R552C (n=15), or SEMA3A-WT+SEMA3A-R734W (n=14) **(D)** Bar graph showing peak current density at +40 mV for K_v4.3-WT co-expressed with SEMA3A-WT (n=15), SEMA3A-WT+SEMA3A-R552C (n=15), or SEMA3A-WT+SEMA3A-R734W (n=15). * $p<0.05$ vs. K_v4.3-WT + SEMA3A-WT.

SUPPLEMENTAL TABLES

Online Table I. Spectrum and Prevalence of SEMA3A Nonsynonymous Mutations in our BrS Cohort

cDNA Position	Protein Position	Prevalence in BrS Cohort	Frequency in BrS Cohort (%)	Prevalence in NHLBI ESP	Frequency in NHLBI ESP (%)
c.458A>G	p.(N153S)	AG=2	1.01	AA=6458/ AG=44/GG=1	0.69
c.1303G>A	p.(V435I)	GA=6	3.03	GG=6368/GA=134/AA=1	2.08
c.1654C>T	p.(R552C)	CT=1	0.51	--	--
c.2200C>T	p.(R734W)	CT=1	0.51	--	--

SUPPLEMENTAL REFERENCES

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