Characterization of SEMA3A-Encoded Semaphorin as a Naturally Occurring Kv4.3 Protein Inhibitor and its Contribution to Brugada Syndrome


Rationale: Semaphorin 3A (SEMA3A)-encoded semaphorin is a chemorepellent that disrupts neural patterning in the nervous and cardiac systems. In addition, SEMA3A has an amino acid motif that is analogous to hanatoxin, an inhibitor of voltage-gated K+ channels. SEMA3A-knockout mice exhibit an abnormal ECG pattern and are prone to ventricular arrhythmias and sudden cardiac death.

Objective: Our aim was to determine whether SEMA3A is a naturally occurring protein inhibitor of Kv4.3 (Ito) channels and its potential contribution to Brugada syndrome.

Methods and Results: Kv4.3, Na1.5, Ca1.2, or K4.2 were coexpressed or perfused with SEMA3A in HEK293 cells, and electrophysiological properties were examined via whole-cell patch clamp technique. SEMA3A selectively altered K4.3 by significantly reducing peak current density without perturbing K4.3 cell surface protein expression. SEMA3A also reduced Ito current density in cardiomyocytes derived from human-induced pluripotent stem cells. Disruption of a putative toxin binding domain on K4.3 was used to assess physical interactions between SEMA3A and K4.3. These findings in combination with coimmunoprecipitations of SEMA3A and K4.3 revealed a potential direct binding interaction between these proteins. Comprehensive mutational analysis of SEMA3A was performed on 198 unrelated SCN5A genotype–negative patients with Brugada syndrome, and 2 rare SEMA3A missense mutations were identified. The SEMA3A mutations disrupted SEMA3A’s ability to inhibit K4.3 channels, resulting in a significant gain of K4.3 current compared with wild-type SEMA3A.

Conclusions: This study is the first to demonstrate SEMA3A as a naturally occurring protein that selectively inhibits K4.3 and SEMA3A as a possible Brugada syndrome susceptibility gene through a Kv4.3 gain-of-function mechanism. (Circ Res. 2014;115:460–469.)

Key Words: Brugada syndrome ▪ genetics, medical ▪ ion channels ▪ potassium channels ▪ semaphorin-3A

Semaphorins are extracellular and membrane-associated proteins involved in many different cellular processes and are well known for their role in nervous system development through neuronal migration and axon guidance.1 Semaphorin 3A (SEMA3A) was the first molecularly characterized neural chemorepellent, which when inactivated disrupts neural patterning and projections.2 Although initial studies focused on SEMA3A’s role in neurodevelopment, SEMA3A is also involved in cardiac innervation patterning.3,4

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In developing murine hearts, SEMA3A is expressed at abundant levels, with expression pattern gradients opposite of sympathetic innervation, emphasizing its role as a chemorepellent essential to neuronal migration.4 Dysregulation of cardiac innervation is associated with an increased risk for ventricular arrhythmias and sudden cardiac death (SCD).5 SEMA3A-knockout mice display decreased basal sympathetic activity, SCD during the...
first postnatal week, and electrocardiographic features of sinus bradycardia and mild ST-segment elevation, whereas transgenic SEMA3A-overexpressing mice have reduced sympathetic innervation, reduced I_{to} density, prolonged action potential duration, spontaneous ventricular arrhythmias, and premature SCD.9–11 Sympathetic and parasympathetic patterning may determine patient subtype, disease often presenting in the fourth decade of life, characterized by a small proportion of Brugada syndrome (BrS), a male-predominated disease, where BrS is defined by the presence of a rightward deviation of the ST segment and a prolonged QT interval (>440 ms) during rest or stress.12–14

The patterning of murine SEMA3A is in a gradient, with greater expression in the endocardium and lesser expression in the epicardium. This differential gradient is opposite to the expression pattern gradient of the transient outward repolarizing current (epicardium:endocardium; I_{to}, K_{4.3}).4 Interestingly, a portion of the SEMA3A protein is analogous to a tarantula toxin, hanatoxin.6 Hanatoxin and closely related Heteropoda venatoria toxin (HpTx2) block Kv2.1 and Kv4.3 channels, respectively, by modifying energetics of activation via voltage sensor binding.7,8 Because of its sequence homology with toxins, we hypothesized that SEMA3A may act as a naturally occurring K_{4.3} (I_{to}) ion channel blocker, and the disruption of this interaction would lead to a pathological increase in I_{to} current density.

Increase in I_{to} current density is the pathogenic basis for a proportion of Brugada syndrome (BrS), a male-predominated disease often presenting in the fourth decade of life, characterized by cardiac conduction abnormalities, ST-segment elevation, and an increased risk for ventricular arrhythmias and SCD.9–11 Sympathetic and parasympathetic patterning may also play an important role in the pathogenesis of BrS,12 and arrhythmias in BrS are exacerbated by vagal stimulation.13 Here, we demonstrate that SEMA3A is a naturally occurring protein inhibitor of K_{4.3} (I_{to}) channels and that SEMA3A is a possible BrS susceptibility gene.

Methods

Gene Constructs and Site-Directed Mutagenesis

SEMA3A complementary DNA in the pCR-BluntII-TOPO vector (Open Biosystems, Pittsburgh, PA) was subcloned into the pIREs2 dsRED2 vector (Clontech, Mountain View, CA). The plreGFP plasmid encoding wild-type (WT) human K_{4.3} (KCND3) and green fluorescent protein represented I_{to} current for electrophysiological studies. The K_{4.3}-L274A and K_{4.3}-V275A mutations were engineered into plreGFP-KCND3-WT, and the SEMA3A-R552C and SEMA3A-R734W mutations were engineered into pIREs2 dsREDSEMA3A using primers containing each point mutation (available on request) in combination with the Quikchange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Constructs for Na_{1.5}, Ca_{1.2}, and K_{4.2} are included in the Online Data Supplement. The integrity of all constructs was verified by DNA sequencing. 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.

HEK293 Cell Culture and Transfection

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. All cells were plated in T25 flasks and stored in a 5% CO_{2} incubator at 37°C for 24 hours. Heterologous expression of K_{4.3} and SEMA3A was accomplished by cotransfecting 0.5 μg of pREGFP-KCND3W with 1.0 μg pIREs2 dsRED2-SEMA3A_WT or pIREs2 dsRED2-SEMA3A_HIpc (0.5 μg of SEMA3A-WT plus 0.5 μg of mutant SEMA3A were used for heterozygote coexpression studies) using 5 μL of Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA) in Gibco OPTI-MEM media (Invitrogen). Cells fluorescing 48 hours post-transfection were selected for electrophysiological experiments. HEK293 cell culture and transfection procedures for Na_{1.5}, Ca_{1.2}, and K_{4.2} are included in the Online Data Supplement.

Electrophysiological Measurements and Data Analysis for K_{4.3}

Standard whole-cell patch clamp technique using an Axopatch 200B amplifier, Digidata 1440A, and pClamp version 10.2 software (Axon Instruments, Foster City, CA) was used to measure electrophysiological properties at room temperature (22–24°C). The extracellular (bath) solution contained (mmol/L): 140 NaCl, 4 KCl, 2 CaCl_{2}, 1 MgCl_{2}, and 10 HEPES, pH adjusted to 7.4 with NaOH. The pipette solution contained (mmol/L): 110 KOH, 10 EDTA, 5.17 CaCl_{2}, 1.42 MgCl_{2}, 4 MgATP, and 10 HEPES, pH adjusted to 7.2 with KOH following established protocols.8,14 Microelectrodes were pulled on a P-97 puller (Sutter Instruments, Novato, CA) and fire-polished to a final resistance of 2 to 3 MΩ. Series resistance was compensated by 80% to 85%. Currents were filtered at 5 kHz and digitized at 10 kHz. The voltage dependence of activation and inactivation was determined using voltage clamp protocols described in the figure legend. Data were analyzed using Clampfit (Axon Instruments), Excel (Microsoft, Redmond, WA), and fitted with Origin 8 (OriginLab Corporation, Northampton, MA).

Voltage-dependent inactivation curve was fitted with a Boltzmann function: \[ I_{to} = \frac{I_{to,\max}}{1 + \exp\left(\frac{V - V_{1/2}}{k}\right)} \], where \( V_{1/2} \) and \( k \) are the half-maximal voltage of inactivation and the slope factor, respectively. Inactivation time constants for each voltage step were determined by fitting a monoexponential function to current decay.

Electrophysiological measurements and data analysis for Na_{1.5}, Ca_{1.2}, and K_{4.2} are included in the Online Data Supplement.

K_{4.3} Total Cell and Cell Surface Western Blot Analysis

Cells were transfected in 25-cm^{2} flasks using 4 μg lipofectamine mixed with 4 μg total plasmids (pBK-CMV encoding KCND3 with td-Tomato coexpressed with or without pBK-CMV encoding KChIP2 or pIREs2 dsRED2-SEMA3A). Plasmids were mixed in equal ratios and kept constant at 4 μg by the addition of empty (pBK-CMV) vector. Using previously described methods,15,16 Western blot analyses were performed on total protein lysates prepared from transfected HEK293 cells. The primary antibody was anti-K_{4.3} (UC Davis/NIH NeuroMab facility, Davis, CA). The mouse monoclonal anti-Transferrin receptor antibody (Invitrogen) was used as a loading control. The rabbit antimouse horseradish peroxidase-conjugated secondary antibody (Bethyl Laboratories, Montgomery, TX) followed by SuperSignal West Dura Extended Duration substrate (Pierce, Rockford, IL) was used for signal detection, captured using a Molecular Imager Chemidoc XRS system running Quantity One software version 4.6 (Bio-Rad Laboratories, Hercules, CA). The intensities of the K_{4.3} protein bands were determined using volume analysis from the Quantity One software and were normalized to Transferrin receptor in the same lane on the same blot. Results are expressed as means±SEM from 4 to 6 experiments.

Expanded protocols for the immunoblots of SEMA3A in mouse brain and human heart, as well as the coimmunoprecipitations of SEMA3A and K_{4.3}, are available in the Online Data Supplement.

Cell Culture, Electrophysiological Measurements, and Data Analysis for Human-Induced Pluripotent Stem Cell–Derived Cardiomyocytes

Control human-induced pluripotent stem cell (hiPSC)–derived cardiomyocytes were a kind gift from Dr Timothy Nelson (Mayo

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<th>Nonstandard Abbreviations and Acronyms</th>
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Clinic, Rochester, MN). Cardiomyocyte aggregate cultures were maintained in B27/RPMI media (Gibco Invitrogen). At differentiation days 25 to 30, the enriched hiPSC-derived cardiomyocytes were subjected to enzymatic dissociation using 0.25% Trypsin/EDTA plus 5% FBS to obtain single-cell suspensions of cardiomyocytes. These cells were added to 0.1% gelatin-coated glass coverslips maintained in B27/RPMI media and stored in a 5% CO₂ incubator at 37°C before use.

Standard whole-cell patch clamp technique, as described above, was used to measure Iₜ currents in hiPSC-derived cardiomyocytes at room temperature (22–24°C). Currents were filtered at 1 kHz and digitized at 5 kHz. Data were analyzed as described above.

**Study Subjects and SEMA3A Mutational Analysis**

Expanded methods regarding the BrS study subjects and SEMA3A mutational analysis are available in the Online Data Supplement.

**Statistical Analysis**

All data are expressed as mean±SEM. One-way ANOVA was performed to determine statistical significance among multiple groups, and paired t test was used to compare statistical significance before and after SEMA3A perfusion. *P<0.05 was considered to be significant.

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**Results**

**Kv4.3 Current Inhibition by SEMA3A in Heterologous Expression System**

Figure 1A shows the representative tracings of Kv4.3-WT, coexpression with SEMA3A-WT, and with paracrine expression of SEMA3A-WT in HEK293 cells. The paracrine expression of SEMA3A-WT represent cells themselves that are not expressing SEMA3A; however, they are in the same media as cells expressing SEMA3A (as confirmed by fluorescence). Analysis of the current–voltage relationship indicated that both SEMA3A-WT coexpression and paracrine expression significantly inhibited Kv4.3 current density from −20 to +40 mV (n=10 for each group; P<0.05 versus Kv4.3-WT; Figure 1B). Kv4.3 peak current density at +40 mV (154.7±24.3 pA/pF; n=10) was significantly reduced by 66.3% with SEMA3A-WT coexpression (52.2±12.1 pA/pF; n=10; P<0.05) and 62.2% with paracrine expression of SEMA3A-WT (58.5±14.5 pA/pF; n=10; P<0.05), indicating that SEMA3A-WT is working on the extracellular surface to block Kv4.3 current. We also coexpressed Kv4.3 with KChIP2, a Kv4.3 chaperone, and SEMA3A had a similar marked inhibitory effect as described above (Online Figure 1).

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**Figure 1. SEMA3A-WT inhibits Kv4.3-WT current in heterologous system but does not affect total or cell surface Kv4.3 protein expression.**

A, Representative whole-cell tracings of Kv4.3-WT, coexpression with SEMA3A-WT, and paracrine expression of SEMA3A-WT elicited by step depolarization of 500 ms duration from a holding potential of −80 to +40 mV in 10 mV increments. B, Current–voltage relationship for Kv4.3-WT (n=10), coexpression with SEMA3A-WT (n=10), and paracrine expression of SEMA3A-WT (n=10). *P<0.05 versus Kv4.3-WT. Representative Western blots of fractionated total (C) and cell surface (E) proteins prepared from HEK293 cells transiently transfected with complementary DNA constructs encoding td-Tomato and Kv4.3 in the absence (−) and the presence (+) of KChIP2 or SEMA3A. The intensities of the total and cell surface Kv4.3 protein bands were measured and normalized to the expression of endogenous transferrin receptor (TransR) in the same lane and subsequently to the total or surface Kv4.3 in the ±SEMA3A or KChIP2 in the same blot. Mean±SEM (n=4 blots) total (D) and cell surface (F) Kv4.3 protein expression levels in cells expressing Kv4.3 in the presence and absence of KChIP2 or SEMA3A are plotted. As reported previously,15,16 coexpression of Kv4 α-subunits with KChIP2 markedly increased total and cell surface Kv4 protein expression.
SEMA3A’s Inhibitory Effect on Ito Is Independent of Kv 4.3 Expression

To better understand how SEMA3A may be altering the properties of Kv4.3, we first examined the effects of SEMA3A on K v4.3 protein expression. The overall loss of K v4.3 current density when coexpressed with SEMA3A is independent of the expression levels of K v4.3. Specifically, total cell and cell surface K v4.3 expression is unaffected by SEMA3A in the presence and absence of KChIP2 (Figure 1C–1F).

SEMA3A Alters the Kinetic Properties of Kv 4.3

Like SEMA3A coexpression, 100 nmol/L hSEMA3A protein perfusion significantly inhibited K v4.3 current density from −10 to +40 mV (n=15; P<0.05 versus before hSEMA3A perfusion; Online Figure II). To further determine if hSEMA3A protein could alter Kv4.3-WT current kinetics, we analyzed Kv4.3-WT inactivation time constants and steady-state inactivation parameters before and after perfusion with 100 nmol/L hSEMA3A. About 100 nmol/L hSEMA3A protein perfusion significantly decreased K v4.3 decay time from 0 to 40 mV (n=15; P<0.05). At +40 mV, 100 nmol/L hSEMA3A decreased inactivation time constant by 37.2% from 67.4±3.2 to 42.3±4.2 ms (n=15; P<0.05 versus before hSEMA3A; Figure 2A). Steady-state inactivation was assessed by a standard 2-pulse voltage clamp protocol (Figure 2B), and steady-state inactivation curves were fit using a Boltzmann function.10 About 100 nmol/L hSEMA3A protein significantly shifted V1/2 of inactivation from −38.9±1.5 mV (before hSEMA3A; n=12) to −51.5±4.7 mV (after hSEMA3A; n=12; P<0.05; Figure 2B). However, recovery from inactivation remained unchanged after perfusion of 100 nmol/L hSEMA3A (Online Figure III).

SEMA3A Inhibits Kv4.3 Peak Current in a Dose-Dependent Manner

To explore whether hSEMA3A pharmacologically inhibits K v4.3 current in a dose-dependent manner, K v4.3-expressing cells were perfused with 0.1, 1, 10, and 100 nmol/L hSEMA3A protein for 5 to 10 minutes. hSEMA3A protein dose-dependently inhibited K v4.3 current with an IC50 of 4.4±1.3 nmol/L (n=5; Figure 2C and 2D).

SEMA3A May Be a Kv4.3 Channel–Specific Blocker

To determine the specificity of SEMA3A’s effects, we incubated hSEMA3A with other cardiac ion channels related to BrS, including the sodium channel (SCN5A, I Na), the l-type calcium channel (CACNA1C, I Ca,L, Cav1.2), and the Kv4.3 highly homologous voltage-gated potassium channel, K v4.3 (KCND2).

Figure 2. SEMA3A perfusion alters kinetic properties and inhibits K v4.3 in a dose-dependent manner. A, Inactivation time constants (τ) for K v4.3-WT as a function of voltage before and after 100 nmol/L human SEMA3A (hSEMA3A) perfusion. Inactivation time constants for each voltage step were determined by fitting a monoexponential function to current decay. *P<0.05 vs before 100 nmol/L hSEMA3A perfusion. B, Steady-state inactivation curves of K v4.3-WT before and after 100 nmol/L hSEMA3A perfusion determined from a holding potential of −80 mV to prepulse of +20 mV in 5 mV increments with 0.5-s duration followed by a test pulse of +20 mV with 0.5-s duration and fitted with a Boltzmann function.10 C, Representative whole-cell K v4.3-WT tracings with 0.1, 1, and 10 nmol/L hSEMA3A protein perfusion. D, Dose-dependent curve with an IC50 of 4.4±1.3 nmol/L (n=5). All values shown represent mean±SEM.
We found that Na\textsubscript{1.5} (Figure 4A and 4B), Ca\textsubscript{2.1} (Figure 4C and 4D), and K\textsubscript{4.2} (Figure 4E and 4F) current densities were not measurably affected by SEMA3A, compared with SEMA3A’s inhibitory effect on K\textsubscript{4.3} with or without KCHIP2 coexpression.

**Heart Expression of SEMA3A and Coimmunoprecipitation With K\textsubscript{4.3}**

Because of the effects of SEMA3A on K\textsubscript{4.3}, we wanted to confirm whether SEMA3A is expressed in human cardiac tissue. As illustrated in Figure 5, the polyclonal anti-SEMA3A antibody reliably detects native SEMA3A protein in (human) heart and (mouse) brain lysates. Western blot analysis of human ventricular brain tissue. We found that SEMA3A coimmunoprecipitates with K\textsubscript{4.3} (Figure 5A). SEMA3A expression in adult mouse brain was particularly robust in the membrane fraction (Figure 5B); therefore, to determine if there was a binding interaction between SEMA3A and K\textsubscript{4.3}, we did a coinmunoprecipitation in mouse brain tissue. We found that SEMA3A coinmunoprecipitates with K\textsubscript{4.3} (Figure 5C). The coinmunoprecipitation of SEMA3A with the anti-K\textsubscript{4.3} antibody was specific, as evidenced by the absence of signal in the control coinmunoprecipitation (Figure 5C). These data suggest a direct binding interaction between these 2 proteins.

**SEMA3A’s Inhibitory Effect on K\textsubscript{4.3} Is Related to its Hanatoxin-Like Domain**

A portion of SEMA3A’s amino acid sequence is analogous to hanatoxin\textsuperscript{6} (Figure 6A), which is closely related to HpTx2. HpTx2 selectively inhibits K\textsubscript{4.3} through K\textsubscript{4.3}’s voltage sensor, mediated by interactions with 2 K\textsubscript{4.3} amino acids (L275 and V276, rat isoform).\textsuperscript{8} Therefore, to determine whether SEMA3A may be binding to K\textsubscript{4.3} in a similar location to HpTx2, we mutated the homologous amino acid residues in human K\textsubscript{4.3}, L274, and V275 to alanine. The L274A-K\textsubscript{4.3} (266.50 pA/pF) and V275A-K\textsubscript{4.3} (257.02 pA/pF) channels electrophysiologically behave like K\textsubscript{4.3}-WT (291.85 pA/pF) at +40 mV voltage (Online Figure V). Although SEMA3A reduces the current density of K\textsubscript{4.3}-WT (176.94 pA/pF; 38.44%), L274A-K\textsubscript{4.3} (215.14 pA/pF; 18.83%), and V275A-K\textsubscript{4.3} (185.52 pA/pF; 27.93%) channels (Figure 6B; Online Figure V), the overall effect of SEMA3A on peak current density is reduced for L274A. SEMA3A leads to a 38% reduction in K\textsubscript{4.3}-WT peak current density; however, SEMA3A leads to only an 18% reduction in L274A-K\textsubscript{4.3} current density (P<0.05; Figure 6C). SEMA3A has a larger effect on V275A-K\textsubscript{4.3} peak current density, with a 29% reduction (Figure 6C).

Because the effects of SEMA3A are attenuated by L274A and V275A K\textsubscript{4.3} substitutions, it may be possible that SEMA3A binds to the voltage sensor region on K\textsubscript{4.3}, and these mutations are disrupting this interaction.

**SEMA3A Mutations May Contribute to the Pathogenesis of BrS**

Overall, 4 SEMA3A missense mutations were identified in 10 patients (N153S, 2 cases; V435I, 6 cases; R552C, 1 case; R734W, 1 case) within our BrS cohort (Online Table I). However, 2 missense mutations, R552C and R734W (Figure 6A), in 2 of 198 (1%) unrelated BrS patients (Table), were absent in 500 European white controls, 300 Italian controls, and 1094 subjects from the 1000 Genomes Project,\textsuperscript{17} 6503 subjects from the NHLBI GO Exome Sequencing Project,\textsuperscript{18} and the 12000 Exome Chip\textsuperscript{19} and were, therefore, considered as potentially pathogenic mutations and investigated functionally.

R552C-SEMA3A was identified in a 45-year-old man with a history of palpitations at rest, ST-segment elevation was observed in leads V1 and V2 on a Holter ECG, especially after large meals. Flecainide testing revealed a type 1 Brugada ECG pattern (Online Figure V1A and V1B). Despite a reported negative family history of cardiac events, the patient’s only living family member, a daughter, was clinically evaluated. She had a negative flecainide challenge and was R552C-SEMA3A mutation–negative.

R734W-SEMA3A was identified in an asymptomatic 44-year-old man with no family history. An ECG performed
for chest pain identified a type 2 Brugada ECG pattern, and a subsequent flecainide test induced a positive type 1 Brugada pattern (Online Figure VIC and VID). Ventricular fibrillation was noted during the diagnostic electrophysiology study, and an implantable cardioverter defibrillator was implanted subsequently. The patient’s son had a negative flecainide test and was R734W-SEMA3A mutation–negative.

Both R552C- and R734W-SEMA3A mutations when coexpressed with K\textsubscript{4.3}-WT resulted in an increased I\textsubscript{to} current density from −20 to +40 mV (R552C n=23; R734W n=20; P<0.05) compared with K\textsubscript{4.3}-WT plus SEMA3A (n=20; Figure 7A and 7B). SEMA3A mutant coexpression significantly increased K\textsubscript{4.3} peak current density at +40 mV by 333.5% from 69.3±8.1 pA/pF (WT; n=20) to 300.4±55 pA/pF (R552C; n=23; P<0.05) and by 137.4% to 164.5±31.1 pA/pF (R734W; n=20; P<0.05; Figure 7C). Additionally, R552C and R734W both significantly increased the I\textsubscript{to} total charge from −10 to +40 mV (P<0.05 versus K\textsubscript{4.3}-WT plus SEMA3A; Figure 7D). However, neither SEMA3A mutations resulted in significant changes in decay time (Figure 7E) or steady-state inactivation (Figure 7F) when compared with K\textsubscript{4.3} plus SEMA3A-WT coexpression.

In addition, electrophysiological analysis was completed in a heterozygous state, with K\textsubscript{4.3}-WT coexpression with SEMA3A-WT and SEMA3A-WT plus SEMA3A-R552C or SEMA3A-WT plus SEMA3A-R734W (Online Figure VII). With these mutant SEMA3A-WT coexpressions, SEMA3A-WT plus SEMA3A-R552C still precipitated a marked increase in K\textsubscript{4.3} current density from −30 to +40 mV (WT plus R552C n=15; P<0.05) compared with K\textsubscript{4.3}-WT plus SEMA3A-WT (n=15; Online Figure VIIC and VIIID). SEMA3A-WT plus

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**Figure 4. Channel specificity of SEMA3A.** A. Representative whole-cell voltage-gated inward Na\textsuperscript{+} currents were recorded from HEK293 cells transiently transfected with complementary DNA (cDNA) constructs encoding Na\textsubscript{1.5}-WT before and after perfusion with 100 nM SEMA3A. B. Current–voltage (I–V) relationship for Na\textsubscript{1.5} (n=10), perfused with SEMA3A (n=10). C. Representative whole-cell voltage-gated inward Ca\textsuperscript{2+} currents were recorded from HEK293 cells transiently transfected with cDNA constructs encoding Ca\textsubscript{1.2}-WT (a, b) before and after exposure to 100 nM SEMA3A. Tracings were recorded from holding potential of −90 mV to testing potential of +70 mV in 10 mV increments. D. Current–voltage relationship for Ca\textsubscript{1.2} (n=10), exposed to SEMA3A (n=10). SEMA3A was detected in the control IP. A

**Figure 3.** Western blot analysis with human recombinant SEMA3A. A. Western blot analysis of SEMA3A in mouse brain lysates, compared directly with human recombinant SEMA3A. B, Western blot analysis of SEMA3A in mouse brain lysates, compared directly with human recombinant SEMA3A. C, Western blot demonstrating robust expression of SEMA3A in the membrane fraction from mouse brain and communoprecipitation with K\textsubscript{4.3} (K\textsubscript{4.3} IP); no SEMA3A was detected in the control IP. A

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SEMA3A-R552C significantly increased Kv4.3 plus SEMA3A-WT peak current density at +40 mV by 220% from 70.9±10.6 pA/pF (SEMA3A-WT; n=15) to 226.9±44.5 pA/pF (SEMA3A-WT plus R552C; n=15; P<0.05; Online Figure VIID). In contrast, SEMA3A-WT plus SEMA3A-R734W increased the Kv4.3 current density, by only 25.2% compared with SEMA3A-WT (peak current density 88.8±19.9 pA/pF; SEMA3A-WT plus R734W; n=14; Online Figure VIIC and VIID).

### Discussion

**SEMA3A Regulates I_{to} Current Density and Kinetics**

SEMA3A has robust expression in human heart tissue, and it has been established previously that K_{4.3} is expressed in the human heart. In addition, SEMA3A transgenic mice have a reduction of I_{to} density, reduced sympathetic innervation, and have the propensity for spontaneous ventricular arrhythmias. In combination with our illustration of SEMA3A’s effect on K_{4.3}, this data suggest that SEMA3A not only regulates cardiac innervation patterning but may also regulate I_{to} current densities to maintain a transmural repolarization gradient and prevent potentially lethal cardiac arrhythmias.

Here, we identified SEMA3A as a novel inhibitory regulator of K_{4.3} current density and kinetics because of direct binding of SEMA3A and K_{4.3} in a manner similar to toxin channel binding. SEMA3A has several similarities to toxins, which are known to physically bind and inhibit voltage-gated ion channels.
SEMA3A has a 34-amino acid stretch analogous to hanatoxin, which contains the 6 stereotypical cysteines of an inhibitor cystine knot motif commonly seen in invertebrate toxins. This hanatoxin-like sequence in SEMA3A resides within a similar plexin/semaforin/integrin domain in which the structure was described in SEMA4D (a close relative of SEMA3A). Although the function of this domain is unknown, the plexin/semaforin/integrin domain folds using 3 disulfide bonds akin to the inhibitor cystine knot motif. Therefore, SEMA3A has protein sequence characteristics of a toxin, which may support its ability to bind and inhibit ion channels.

Toxins are known to bind to the extracellular surface of ion channels. In our study, SEMA3A led to reduced current density of Kv4.3 in HEK293 cells whether coexpressed within the cell (Figure 1A and 1B), or with hSEMA3A protein perfusion (Figure 2). SEMA3A perfusion also reduced current density of Ito in cardiomyocytes derived from hiPSCs (Figure 3). To establish a binding interaction, we were able to immunoprecipitate SEMA3A with an anti-Kv4.3 antibody in mouse brain (Figure 5C). Altogether, this data support the conclusion that SEMA3A is binding to Kv4.3, potentially at the extracellular surface, congruent with SEMA3A's previously established function as a naturally secreted protein binding to cell surface receptors.

In an attempt to determine where SEMA3A may be binding to Kv4.3, we focused on what has been previously established for toxin channel interaction. There are 2 major mechanisms in which toxins can interact with voltage-gated channels on the extracellular surface, through direct targeting of the ion channel pore or through binding to the channel’s voltage sensor region, which influences the stability of closed, open, or inactivated states of the channel. Hanatoxin and HpTx2 both fall into the latter category, binding to the voltage-sensing domain of Kv2.1 and Kv4.3, respectively. When bound, these toxins shift activation to more depolarized voltages, decrease current density, and rapidly inactivate channels, all of which are seen when Kv4.3 is exposed to SEMA3A. The binding interaction between toxins and channels is regulated by the overall charge of the voltage sensor paddle region, and mutagenesis of this region can significantly alter the effects of the toxins. Similar to previous studies on K,4.3 and HpTx2, mutagenesis of K,4.3 at amino acid positions L274 and V275 attenuated SEMA3A's inhibitory effect on the
channel, suggesting a direct interaction between SEMA3A and K,4.3 voltage sensor.

SEMA3A: A Possible BrS Susceptibility Gene

The phenotype of murine SEMA3A knockout consisting of sinus bradycardia, decreased basal sympathetic activity, ST-segment elevation, and SCD prompted analysis of our BrS cohort. Here, we identified 2 ultrarare SEMA3A mutations, R552C and R734W, in 2 patients diagnosed with BrS. Interestingly, a common I334V-SEMA3A polymorphism was associated recently with a high incidence of unexplained cardiac arrest with ventricular fibrillation among pilsicainide challenge–negative Japanese individuals.24 According to Nakano et al,25 using the 1000 Genomes Project,17 I334V has a prevalence of 2.1% in East Asians, 1.35% among West Africans, 1.86% among Americans, and 0% among Europeans. This mutation was not identified in our European white cohort. Functional characterization of this SEMA3A polymorphism identified a loss of function of axon collapse and led to disrupted innervation patterning in patient tissues.24 Whether our SEMA3A mutation–positive BrS patients have an abnormal cardiac innervation pattern is currently unknown.

Coexpression of K,4.3 with either SEMA3A mutation in a homozygous fashion led to a significant increase in I, current compared with K,4,3 coexpressed with SEMA3A-WT. We speculate that each mutation may cause misfolding of SEMA3A, thereby either disrupting the hanatoxin-like sequence altering SEMA3A-K,4.3 binding or preventing SEMA3A secretion. These effects would presumably disrupt SEMA3A's normal suppressive effect on K,4.3, therefore leading to an increase in I, current. Interestingly, R552C is 3 amino acids away from the SEMA3A hanatoxin-like sequence. This region, within the plexin/semaphorin/integrin domain, is known to fold with 3 disulfide bonds between 6 cysteine residues (Figure 6A). The addition of a new cysteine as a result of the R552C mutant could lead to the formation of a novel disulfide bond, thus altering the folding structure of the plexin/semaphorin/integrin domain, directly affecting this toxin-like region, which in turn could alter binding to K,4,3. In addition, the R734W mutation within the basic SEMA3A C-terminus substitutes a basic amino acid, arginine, to a hydrophobic uncharged amino acid, tryptophan, which could affect the overall charge of this region and alter its folding structure.

After heterozygote coexpression of mutant and SEMA3A-WT together, R734W no longer accentuates K,4.3 current density significantly, at least in a HEK293 cell model system during WT plus mutant coexpression studies that attempt to mimic the heterozygous state. However, without examination of protein expression in our patient, we do not know if these 50:50 studies are truly reflective of human expression. The mutations themselves could alter the expressivity of the mutant SEMA3A, potentially leading to a more robust phenotype. In addition, growth cone collapse assays for each of the mutants have not been completed. Therefore, it is possible that either of these mutations could still contribute to a BrS-like phenotype, in a nerve growth–related manner. These SEMA3A mutations could also alter the normal expression patterning of SEMA3A, disrupting the known SEMA3A and K,4.3 expression gradients. Additional experimentation in the form of transgenic mice may help elucidate some of the potential developmental innervation changes, which may develop because of mutations within SEMA3A.

Altogether, based on our electrophysiological studies, we know that a rare R552C-SEMA3A mutation attenuates SEMA3A's ability to block K,4.3, resulting in a substantial accentuation of K,4,3 current. Previously, we have demonstrated that primary mutations in KCND3-encoded K,4,3 cause BrS through a marked gain of K,4,3 current.10 Accordingly, in a final common pathway fashion, it is possible that the SEMA3A perturbation underlies their disease. However, because the cases in which these mutations have been identified do not have sufficient pedigree information to test cosegregation, we cannot be certain that the SEMA3A mutation is solely responsible for BrS in these 2 patients.

Potential for SEMA3A as a Therapeutic I, Specific Channel Blocker

Gain of function in I, underlies a subset of BrS being first identified in patients with BrS harboring mutations in KCNE3.25 Subsequently, we identified 2 mutations within KCND3-encoded K,4,3 in patients with BrS.10 Mutations in each of these genes caused a significant gain of function in I, current. One of the current treatment strategies for patients with BrS is quinidine, which blocks a variety of channels. However, quinidine's I, blocking activity may underlie its therapeutic efficacy in patients with BrS, regardless of the primary pathogenic substrate.26–28 In principle, an I,–specific blocker might be more effective than quinidine in managing patients with symptomatic BrS. This study provides evidence that SEMA3A may have a potential as a novel therapeutic as an I,–specific blocker. First, SEMA3A has drug-like properties, with a dose-dependent response curve as shown in Figure 2C and 2D. Second, SEMA3A does not alter the current density of other inward cardiac ion channels, such as Na,,5 or Ca,1.2, or another fast transient outward current potassium channel, K,4,2 (Figure 4), lending support that SEMA3A may be a channel-specific blocker for K,4,3. Many of the known K,4,3 blockers also block other potassium channels or inward currents.29 Therefore, SEMA3A, or a synthetically derived toxin-like portion of SEMA3A, could potentially be developed into a treatment strategy for patients with symptomatic BrS.

Conclusions

We have identified a novel function for SEMA3A as a potential K,4,3–specific channel blocker. In addition, with the identification of rare functionally significant mutations, perturbations in SEMA3A may contribute to BrS. The identified effects of SEMA3A on K,4,3 may be because of a direct binding interaction in a mechanism similar to toxin channel binding, and these findings might stimulate the development of a novel I,–specific channel blocker for therapeutic intent.

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Disclosures
M.J. Ackerman is a consultant for Boston Scientific, Gilead Sciences, Medtronic, and St. Jude Medical and receives royalties from Transgenomic for FAMILION-LQTS and FAMILION-CPVT genetic tests. A.A.M. Wilde is a member of the scientific discovery board of Sorin. The other authors report no conflicts.

References
10. Giudicessi JR, Ye D, Tester DJ, Crotti L, Migione A, Nesterenko VV, Albertson AW. The function of semaphorins during nervous system patterning in the nervous and cardiac systems. In addition, SEMA3A: Novel Kv4.3 Channel Blocker Linked to BrS

Novelty and Significance
What Is Known?
- Semaphorin 3A (SEMA3A) is a chemorepellent that guides neural patterning and projections, is expressed in murine hearts, and, when disrupted through either complete knockout or transgenic overexpression, leads to sudden cardiac death in mice.
- A portion of SEMA3A is analogous to a toxin, known as Hananotix, which binds to and inhibits potassium channels.
- Gain in function of Kv4.3 potassium channels in the heart can lead to a Brugada syndrome (BrS) phenotype that may be associated with sudden cardiac death.

What New Information Does This Article Contribute?
- We have identified a novel function for SEMA3A as a Kv4.3-specific channel blocker, specifically SEMA3A reduces Kv4.3 channel current density in a dose-dependent manner, alters Kv4.3 channel kinetics, yet has no effect on other cardiac ion channels, such as Na1.5, Ca1.2, and Kv4.2.
- SEMA3A coimmunoprecipitated with Kv4.3, suggesting a direct binding interaction between these 2 proteins.

With the identification of rare SEMA3A mutations, leading to an overall gain of function in Kv4.3 current, genetic perturbations in SEMA3A may contribute to BrS.

SEMA3A-encoded semaphorin is a chemorepellent that disrupts neural patterning in the nervous and cardiac systems. In addition, SEMA3A has an amino acid motif that is analogous to hananotix, an inhibitor of voltage-gated potassium channels. Mice that are lacking SEMA3A (ie, SEMA3A-knockout mice) are prone to ventricular arrhythmias and sudden cardiac death. An increase in voltage-gated Kv4.3 potassium channel current has been recognized as a pathogenic basis for some cases of BrS. In this study, we identified a novel biological role for SEMA3A as a naturally occurring protein inhibitor of Kv4.3 potassium channels. We also showed that SEMA3A gene mutations are a potential contributor to BrS by disrupting SEMA3A’s natural ability to suppress the Kv4.3 channel, thus resulting in an increase in potassium channel current. This previous unrecognized interaction between SEMA3A and Kv4.3 could be exploited as a potential drug target, for the regulation of Kv4.3 currents, and have potential therapeutic utility for diseases like BrS.
Characterization of SEMA3A-Encoded Semaphorin as a Naturally Occurring K,4.3 Protein Inhibitor and its Contribution to Brugada Syndrome
Nicole J. Boczek, Dan Ye, Eric K. Johnson, Wei Wang, Lia Crotti, David J. Tester, Federica Dagradi, Yuka Mizusawa, Margherita Torchio, Marielle Alders, John R. Giudicessi, Arthur A.M. Wilde, Peter J. Schwartz, Jeanne M. Nerbonne and Michael J. Ackerman

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SUPPLEMENTAL MATERIAL

DETAILED METHODS

1.1 Heterologous Expression of WT-Kv4.2 and -Kv4.3 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. For transient transfections, plasmids (pBK-CMV) encoding human Kv4.3 (Online Figure I) or Kv4.2 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-Kv4.2 and -Kv4.3 (Online Figure I)

Whole-cell voltage-clamp recordings were obtained at room temperature (22-24°C) within 36 hours 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, MgCl2 2, CaCl2 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% CO2 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,1.5
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 CaCl₂, 0.75 MgCl₂ 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. 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,1.2 Channels
The human wild-type (WT) CACNA1C cDNA with an N-terminal enhanced yellow fluorescence protein (EYFP) tag [(EYFP) α1c, 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% CO₂ incubator at 37°C. Heterologous expression of Ca,1.2 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,1.2
Standard whole-cell patch clamp technique was used to measure Ca,1.2 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 CaCl₂, 1 MgCl₂, 5 mM TEA-Cl and 10 HEPES, pH adjusted to 7.35 with HCl. The pipette solution contained (mmol/L): 120 CsCl, 2 MgCl₂, 10 EGTA, 2 MgATP, 5 CaCl₂ and 10 HEPES, pH adjusted to 7.25 with CsOH. 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,1.2 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 MgCl2, 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/physiological 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 Kv4.3
For immunoprecipitation (IP) of Kv4.3 channel complexes, anti-Kv4.3 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-Kv4.3 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 Project7 (n=1094), the NHLBI Go Exome Sequencing Project8 (n=6503), and the 12000 Exome Chip⁹ (n=12000).

SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

Online Figure 1

Online Figure 1. Co-Expression of SEMA3A Attenuates K,4.3 + KChIP2-Encoded K₅ Currents. Whole-cell voltage-gated outward K⁺ currents were recorded from HEK293 cells transiently transfected with cDNA constructs encoding tdTomato plus K,4.3 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,4.3 + KChIP2-encoded K₅ 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 K₅ current densities in the absence and presence of KChIP2 and/or SEMA3A are plotted. Co-expression of SEMA3A significantly (*)P < 0.01 attenuated K,4.3 + KChIP2 current densities.
Online Figure II. Perfusion of 100 nM SEMA3A Decreases Current Density of Kv4.3.
(A) Representative whole-cell Kv4.3-WT tracings before and after 100 nM human SEMA3A protein perfusion. (B) The current voltage relationship for Kv4.3-WT before and after 100 nM hSEMA3A perfusion. All values represent mean±SEM. * p<0.05 vs. Kv4.3-WT before hSEMA3A perfusion.

Online Figure III. Perfusion of 100 nM SEMA3A Does Not Affect Recovery from Inactivation.
Recovery from inactivation of K\textsubscript{v}4.3-WT and K\textsubscript{v}4.3-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. 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. Targeted Disruption of Toxin Binding Domain on Kv4.3 with SEMA3A Perfusion. Bar graph showing peak current density at +40 mV for Kv4.3-WT (n=10), Kv4.3-L274A (n=10), and Kv4.3-V275A (n=10) before SEMA3A, which is not statistically significant, and after 100nM perfusion of SEMA3A. * p<0.05 vs. Kv4.3 before, all values shown represent mean ±SEM.
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. Heterozygote Co-Expression of Mutant and WT-SEMA3A. (A) The current voltage relationship representing a homozygous state for Kv4.3-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 Kv4.3-WT co-expressed with SEMA3A-WT (n=20), SEMA3A-R552C (n=23), or SEMA3A-R734W (n=20). * p<0.05 vs. Kv4.3-WT + SEMA3A-WT. (C) The current voltage relationship representing a heterozygous state for Kv4.3-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 Kv4.3-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. Kv4.3-WT + SEMA3A-WT.
SUPPLEMENTAL TABLES

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

<table>
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<tr>
<th>cDNA Position</th>
<th>Protein Position</th>
<th>Prevalence in BrS Cohort</th>
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<th>Prevalence in NHLBI ESP</th>
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SUPPLEMENTAL REFERENCES


2. Valdivia CR, Nagatomo T, Makielski JC. Late na currents affected by alpha subunit isoform and betal subunit co-expression in hek293 cells. *J Mol Cell Cardiol.* 2002;34:1029-1039


