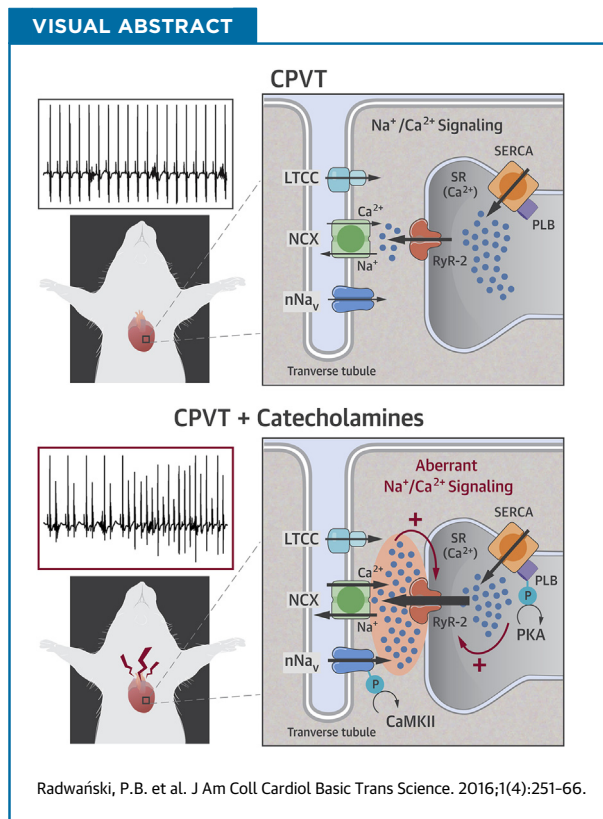


PRE-CLINICAL RESEARCH

# Neuronal Na<sup>+</sup> Channels Are Integral Components of Pro-Arrhythmic Na<sup>+</sup>/Ca<sup>2+</sup> Signaling Nanodomain That Promotes Cardiac Arrhythmias During β-Adrenergic Stimulation



Przemysław B. Radwański, PHARM D, PhD,<sup>a,b,c</sup> Hsiang-Ting Ho, PhD,<sup>a,b</sup> Rengasayee Veeraraghavan, PhD,<sup>d</sup> Lucia Brunello, PhD,<sup>a,b</sup> Bin Liu, PhD,<sup>a,b</sup> Andriy E. Belevych, PhD,<sup>a,b</sup> Sathya D. Unudurthi, PhD,<sup>a,e</sup> Michael A. Makara, PhD,<sup>a,b</sup> Silvia G. Priori, MD, PhD,<sup>f</sup> Pompeo Volpe, PhD,<sup>g</sup> Antonis A. Armoundas, PhD,<sup>h</sup> Wolfgang H. Dillmann, MD,<sup>i</sup> Bjorn C. Knollmann, MD, PhD,<sup>j</sup> Peter J. Mohler, PhD,<sup>a,b</sup> Thomas J. Hund, PhD,<sup>a,d</sup> Sándor Györke, PhD<sup>a,b</sup>



**HIGHLIGHTS**

- CPVT is often caused by increased levels of circulating catecholamines; however, the mechanistic link between β-AR stimulation and the subcellular/molecular arrhythmogenic trigger(s) is unclear.
- In both CPVT and wild type mice, a subpopulation of Na<sup>+</sup> channels (nNa<sub>v</sub>) colocalize with RyR2 and NCX.
- Augmented Na<sup>+</sup> entry via nNa<sub>v</sub> and enhanced SR Ca<sup>2+</sup>-ATPase (SERCA)-mediated SR Ca<sup>2+</sup> refill are both essential and necessary for CPVT.
- Augmentation of Na<sup>+</sup> entry involves β-AR-mediated activation of Ca<sup>2+</sup>/CAMKII.
- Selective pharmacological blockade as well as silencing of Na<sub>v</sub>1.6 inhibit myocyte arrhythmic potential and prevent arrhythmias in vivo.

From the <sup>a</sup>Dorothy M. Davis Heart and Lung Research Institute, College of Medicine, The Ohio State University Wexner Medical Center, Columbus, Ohio; <sup>b</sup>Department of Physiology and Cell Biology, College of Medicine, The Ohio State University, Columbus, Ohio; <sup>c</sup>Division of Pharmacy Practice and Sciences, College of Pharmacy, The Ohio State University, Columbus, Ohio; <sup>d</sup>Center for Heart and Regenerative Medicine Research, Virginia Tech Carilion Research Institute, Virginia Polytechnic University, Roanoke, Virginia; <sup>e</sup>Department of Biomedical Engineering, College of Engineering, The Ohio State University, Columbus, Ohio; <sup>f</sup>Division of

## ABBREVIATIONS AND ACRONYMS

**β-PMTX** = β-pompilidotoxin

**β-AR** = β-adrenergic receptor

**CaMKII** = Ca<sup>2+</sup>/calmodulin-dependent protein kinase II

**CASQ2** = calsequestrin

**CPVT** = catecholaminergic polymorphic ventricular tachycardia

**DCR** = diastolic Ca<sup>2+</sup> release

**I<sub>Na</sub>** = Na<sup>+</sup> current

**ISO** = isoproterenol

**nNa<sub>v</sub>** = neuronal Na<sup>+</sup> channels

**NCX** = Na<sup>+</sup>/Ca<sup>2+</sup> exchange

**PLB** = phospholamban

**Ril** = riluzole

**RyR2** = ryanodine receptor 2

**SR** = sarcoplasmic reticulum

**SERCA2a** = sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase 2a

**TTX** = tetrodotoxin

**VT** = ventricular tachycardia

**WT** = wild type

## SUMMARY

Although triggered arrhythmias including catecholaminergic polymorphic ventricular tachycardia (CPVT) are often caused by increased levels of circulating catecholamines, the mechanistic link between β-adrenergic receptor (AR) stimulation and the subcellular/molecular arrhythmogenic trigger(s) is unclear. Here, we systematically investigated the subcellular and molecular consequences of β-AR stimulation in the promotion of catecholamine-induced cardiac arrhythmias. Using mouse models of cardiac calsequestrin-associated CPVT, we demonstrate that a subpopulation of Na<sup>+</sup> channels, mainly the neuronal Na<sup>+</sup> channels (nNa<sub>v</sub>), colocalize with ryanodine receptor 2 (RyR2) and Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX) and are a part of the β-AR-mediated arrhythmogenic process. Specifically, augmented Na<sup>+</sup> entry via nNa<sub>v</sub> in the settings of genetic defects within the RyR2 complex and enhanced sarcoplasmic reticulum (SR) Ca<sup>2+</sup>-ATPase (SERCA)-mediated SR Ca<sup>2+</sup> refill is both an essential and a necessary factor for arrhythmogenesis. Furthermore, we show that augmentation of Na<sup>+</sup> entry involves β-AR-mediated activation of CAMKII, subsequently leading to nNa<sub>v</sub> augmentation. Importantly, selective pharmacological inhibition as well as silencing of Na<sub>v</sub>1.6 inhibit myocyte arrhythmic potential and prevent arrhythmias in vivo. Taken together, these data suggest that the arrhythmogenic alteration in Na<sup>+</sup>/Ca<sup>2+</sup> handling evidenced during β-AR stimulation results, at least in part, from enhanced Na<sup>+</sup> influx through nNa<sub>v</sub>. Therefore, selective inhibition of these channels and of Na<sub>v</sub>1.6 in particular can serve as a potential antiarrhythmic therapy. (J Am Coll Cardiol Basic Trans Science 2016;1:251-66) © 2016 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Cardiac arrhythmias are a leading cause of death in the United States (1). Arrhythmias caused by abnormal impulse generation are often associated with aberrant diastolic Ca<sup>2+</sup> release (DCR) through dysregulated ryanodine receptor 2 (RyR2) Ca<sup>2+</sup> release channels. This is especially evident when genetic defects in the RyR2 complex—either the RyR2 itself or 1 of the regulatory proteins associated with the channel (i.e., calmodulin, calsequestrin [CASQ2], triadin and/or calstabin)—facilitate aberrant DCR (2-5). In particular, recent findings demonstrate that either dysfunction or loss of cardiac calsequestrin (CASQ2), an intra-sarcoplasmic reticulum (SR) Ca<sup>2+</sup>-binding protein and a regulator of RyR2, impairs the ability of RyR2s to deactivate and become refractory following systolic Ca<sup>2+</sup> release (6-12). This compromised refractoriness of Ca<sup>2+</sup> release, in turn, permits the RyR2 channels to reopen during diastole, causing DCR to activate depolarizing membrane currents, resulting in pro-arrhythmic delayed

afterdepolarizations (10,13-15). Independent of the underlying etiology, compromised RyR2 function is a hallmark of catecholaminergic polymorphic ventricular tachycardia (CPVT).

Episodes of cardiac arrhythmias in CPVT patients are precipitated by emotional stress or exercise, which are associated with increased levels of circulating catecholamines (2,11,16). In accordance with the clinical presentation of a vast majority of these arrhythmias, β-blocker therapy is the mainstay of treatment for cardiac rhythm disorders (17). Recent years have witnessed research endeavors that have focused on alterations in Ca<sup>2+</sup> handling and their roles in precipitating triggered arrhythmias; however, the precise mechanistic link between β-adrenergic receptor (β-AR) stimulation and arrhythmogenesis in Ca<sup>2+</sup>-mediated arrhythmias remains elusive. Several targets for phosphorylation, including Ca<sub>v</sub>1.2, phospholamban (PLB), and RyR2, may be involved in the arrhythmogenic effects of β-AR stimulation (15). For instance, protein kinase A phosphorylation of PLB will

Cardiology and Molecular Cardiology, Maugeri Foundation-University of Pavia, Pavia, Italy; <sup>6</sup>Department of Biomedical Sciences, University of Padova, Padova, Italy; <sup>7</sup>Cardiovascular Research Center, Massachusetts General Hospital, Charlestown, Massachusetts; <sup>8</sup>Department of Medicine, University of California San Diego, La Jolla, California; and the <sup>9</sup>Division of Clinical Pharmacology, Vanderbilt University Medical School, Nashville, Tennessee. This work was supported by National Institutes of Health grants R01-HL074045 and R01-HL063043 (to Dr. Györke); K99-HL127299 (to Dr. Radwański); R01-HL084583, R01-HL083422, and R01-HL075649 (to Dr. Mohler); and R01-HL114893 (to Dr. Hund). Additionally funding was provided by the Russian Science Foundation (N15-15-20008), which was used to support the image analysis. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

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accelerate SR  $\text{Ca}^{2+}$ -ATPase 2a (SERCA2a)-mediated  $\text{Ca}^{2+}$  refilling of the SR, thereby providing adequate substrate for aberrant DCR (18). However, it is unclear whether phosphorylation of RyR2 by  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) plays a role in the pathogenesis of cardiac arrhythmias (19). Surprisingly, considering the  $\text{Ca}^{2+}$ -dependent nature of CPVT, these patients often respond to treatment with  $\text{Na}^+$ -channel blockers such as flecainide (20-22). It has been proposed that flecainide may exert its antiarrhythmic effect through a direct effect on RyR2 (23); however, this would not explain the effect of other  $\text{Na}^+$ -channel blockers on aberrant  $\text{Ca}^{2+}$  handling (24,25). Recently, we suggested that a subset of  $\text{Na}^+$  channels, mainly the neuronal  $\text{Na}^+$  channels ( $\text{nNa}_v$ ), are present in the transverse (T)-tubule, near  $\text{Ca}^{2+}$  handling machinery (26). These channels were initially described in neurons (hence their eponym) and are characterized by a high sensitivity to tetrodotoxin (TTX) (24,27-29). However, little is known about the pro-arrhythmic interaction between  $\text{nNa}_v$  and aberrant  $\text{Ca}^{2+}$  handling during  $\beta$ -AR stimulation as well as the effects flecainide may have on this cross talk. Furthermore, because there are multiple  $\text{nNa}_v$  isoforms expressed in cardiac myocytes (30-32) their specific roles need to be characterized.

In this present study, we have systematically investigated the subcellular and molecular consequences of  $\beta$ -AR stimulation in the promotion of catecholamine-induced cardiac arrhythmias. Because, in certain variants of human CPVT, CASQ2 may be virtually absent or may exist at very low levels due to missense or other mutations, knocking out or mutating CASQ2 in a mouse realistically mimics the phenotype of human disease (2,33). Therefore, to investigate the role of  $\text{Na}^+/\text{Ca}^{2+}$  signaling, we used well-established murine models of CVPT in which arrhythmogenic oscillation of intracellular  $\text{Ca}^{2+}$  and membrane potential are caused by depletion or dysfunction in CASQ2 (CASQ2 null and R33Q, respectively) (6,14,26). We report that, in the setting of dysregulated RyR2 channels, catecholamines promote aberrant DCR by facilitating SR  $\text{Ca}^{2+}$  refilling while enhancing  $\text{nNa}_v$ -mediated persistent  $\text{Na}^+$  current ( $I_{\text{Na}}$ ), respectively, forming the functional basis for catecholamine-induced polymorphic ventricular tachycardia (CPVT).

## METHODS

All animal procedures were approved by The Ohio State University Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S.

National Institutes of Health (NIH Publication No. 85-23, revised 2011).

**GENETICALLY-ENGINEERED MOUSE MODELS.** All genetically-engineered mice used in our study were homozygous for their respective mutations and/or deletions. Cardiac calsequestrin (CASQ2) null mice (on mixed background) (34) were crossbred with: 1) mice conditionally overexpressing SERCA2a in a doxycycline-dependent manner (on FVB/N background) (35); or 2) RyR2 S2814A mice (on C57BL/6 background; generous gift from Dr. Xander Wehrens) (36). The genotypes of the crossbred mice were confirmed by polymerase chain reactions (PCR) (for CASQ2, reverse tetracycline transactivator driven by the cardiac specific  $\alpha$ -myosin heavy chain promoter [35], tetracycline response element-SERCA2a (35), and RyR2 S2814A mutation) using tail deoxyribonucleic acid. To induce the overexpression of SERCA2a, animals received doxycycline diet (Harlan TD 09295 1000 ppm Doxycycline Diet 2018, Harlan, Indianapolis, Indiana) for 14 to 21 days. We also used cardiac CASQ2-R33Q as well as wild type (WT) mice (both on C57BL/6 background) to examine the role of  $\text{Na}_v1.6$  and  $\text{Na}^+/\text{Ca}^{2+}$  exchange (NCX) in aberrant  $\text{Na}^+/\text{Ca}^{2+}$  signaling (26).

## MYOCYTE ISOLATION, CONFOCAL $\text{Ca}^{2+}$ IMAGING, AND $\text{Na}^+$ CURRENT RECORDINGS.

Ventricular myocytes were obtained by enzymatic isolation from 3- to 9-month-old mice of both sexes. Mice were anesthetized with isoflurane, and after a deep level of anesthesia was reached, the heart was rapidly removed and perfused via a Langendorff as previously described (14,26). Peak  $I_{\text{Na}}$  was recorded using an internal solution that contained (in mmol/l): 10 NaCl, 20 tetraethylammonium chloride, 123 CsCl, 1  $\text{MgCl}_2$ , 0.1 Tris guanosine-5'-triphosphate, 5 Mg adenosine triphosphate, 10 HEPES, and 10 BAPTA (pH 7.2, CsOH). For persistent  $I_{\text{Na}}$  recordings, we substituted BAPTA with 1 mmol/l EGTA and maintained free  $\text{Ca}^{2+}$  100 nmol/l with  $\text{CaCl}_2$ . The extracellular bathing solution for peak  $I_{\text{Na}}$  contained (in mmol/l): 10 NaCl, 130 tetraethylammonium chloride, 4 CsCl, 0.4  $\text{CaCl}_2$ , 2  $\text{MgCl}_2$ , 0.05  $\text{CdCl}_2$ , 10 HEPES, and 10 glucose. The extracellular bathing solution for persistent  $I_{\text{Na}}$  recordings contained (in mmol/l): 140 NaCl, 4 CsCl, 1  $\text{CaCl}_2$ , 2  $\text{MgCl}_2$ , 0.05  $\text{CdCl}_2$ , 10 HEPES, 10 glucose, 0.03 niflumic acid, 0.004 strophanthidin, and 0.2  $\text{NiCl}_2$ . The pH was maintained at 7.4 with CsOH for both types of solutions. Whole-cell capacitance and series resistance compensation ( $\geq 60\%$ ) was applied along with leak subtraction. Signals were filtered with 10 kHz Bessel filter, and  $I_{\text{Na}}$  was then normalized to membrane capacitance. Late  $I_{\text{Na}}$  was estimated by integrating  $I_{\text{Na}}$  between 50 and 450 ms.

Electrical field stimulation experiments were performed using the following external solution (in mmol/l): 140 NaCl, 5.4 KCl, 1.0  $\text{CaCl}_2$ , 0.5  $\text{MgCl}_2$ , 10 HEPES, and 5.6 glucose (pH 7.4, NaOH). To assess the SR  $\text{Ca}^{2+}$  load, 20 mmol/l caffeine was applied at the end of the experiments. Intracellular  $\text{Ca}^{2+}$  cycling was monitored by a Nikon A1 laser scanning confocal microscope (Nikon Instruments Inc., Melville, New York). For intact myocytes, we used the cytosolic  $\text{Ca}^{2+}$ -sensitive indicators Fluo-3 AM. For more reliable measurements of SR  $\text{Ca}^{2+}$  release from inside the SR in control and isoproterenol-treated CPVT cardiomyocytes, we performed experiments in **Figure 1** using a low-affinity  $\text{Ca}^{2+}$  indicator Fluo-4FF-AM. The fluorescent probes were excited with the 488-nm line of an argon laser, and emission was collected at 500 to 600 nm. Fluo-3/Fluo-4FF fluorescence was recorded in the line scan mode of the confocal microscope. For  $\text{Ca}^{2+}$  wave recordings, myocytes were paced at 0.3 Hz using extracellular platinum electrodes to obtain DCR frequency. Any DCR event (i.e., wave, wavelet) that increased cell-wide fluorescence intensity above 10% of the signal generated by the preceding stimulated  $\text{Ca}^{2+}$  transient was included in the analysis. The fluorescence emitted was expressed as  $F/F_0$ , where  $F$  is the fluorescence at time ( $t$ ), and  $F_0$  represents the background signal. All experiments were performed at room temperature (26°C).

**CONFOCAL MICROSCOPY OF IMMUNOLABELED MYOCYTES.** Isolated ventricular myocytes were prepared for immunofluorescence as well as proximity ligation assay (PLA) as described previously (26). PLA is a histochemical/cytochemical and confocal microscopy technique for determining when specific proteins are colocalized within <40 nm (37). Briefly, cells were plated on laminin-coated glass coverslips, fixed with 4% paraformaldehyde (5 min), permeabilized with 0.1% Triton X-100, and washed with PBS. Endogenous immunoglobulin was blocked using a mouse-on-mouse blocking reagent (M.O.M. kit, Vector Laboratories, Burlingame, California) for 1 h at room temperature and subsequently incubated with primary antibodies ( $\text{Na}_v1.1$ , 1.3, 1.6, 1.5: 1:32, 1:32, 1:50, and 1:50, respectively, for  $\text{nNa}_v$ s, Alomone, Jerusalem, Israel;  $\text{Na}_v1.5$  was a generous gift from Dr. Peter Mohler; and RyR2 1:100 and NCX 1:50 were from Pierce Antibodies, Rockford, Illinois) overnight at 4°C. After washing for immunofluorescence, goat secondary antibodies (antimouse and antirabbit) conjugated to Alexa Fluor (Life Technologies, Grand Island, New York) were added for 1 h, whereas the PLA reactions were carried out using appropriate Duolink secondary antibodies (Sigma, St. Louis, Missouri) according to the manufacturer's instructions. The sensitivity of PLA

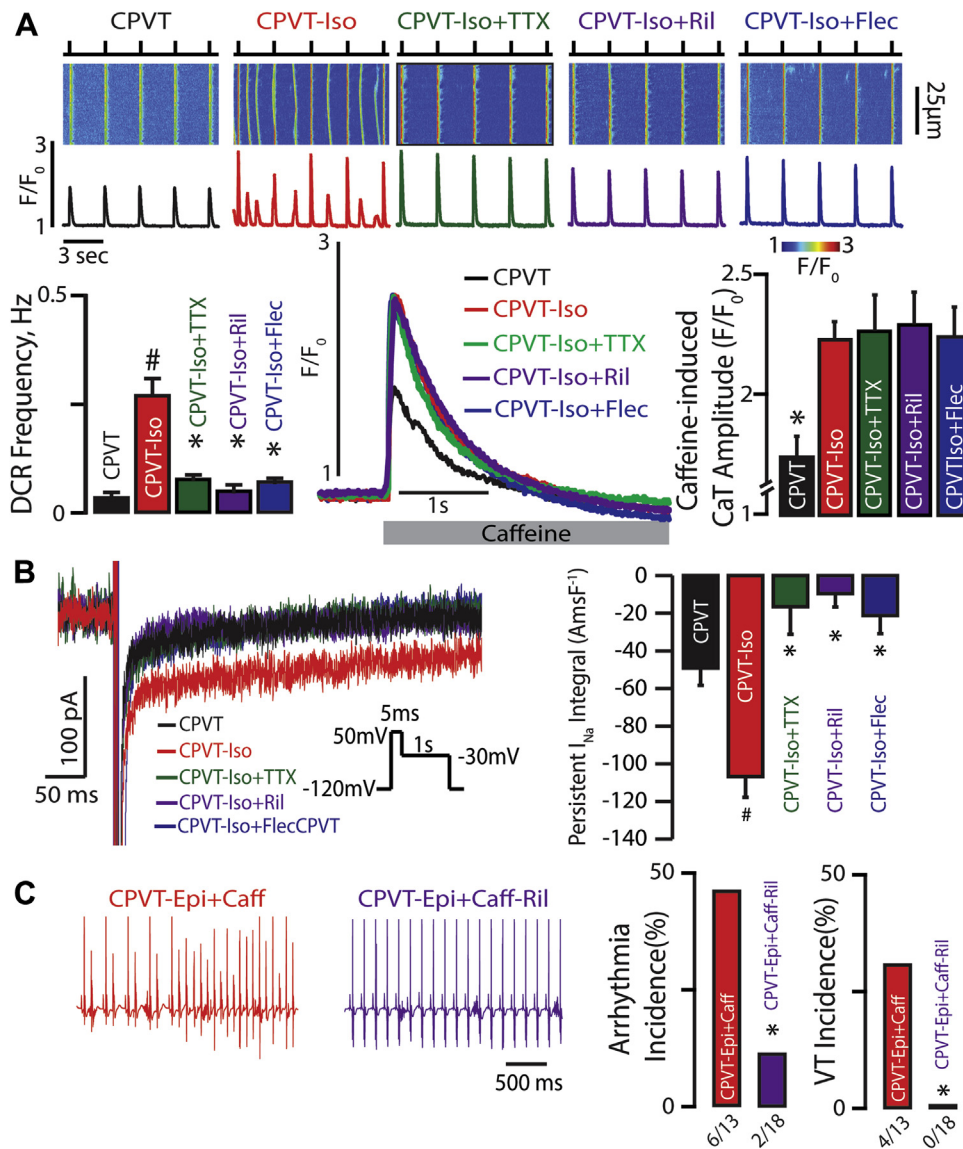
was assessed by staining for  $\text{Na}_v1.5$  (1:50, a generous gift from Dr. Peter Mohler) and Connexin 43 (1:100, Millipore, Billerica, Massachusetts) (**Supplemental Figure 1**), which were previously demonstrated to colocalize at the intercalated disc (37).

**SILENCING RIBONUCLEIC ACID.** Targeting silencing ribonucleic acid (siRNA) was purchased from Santa Cruz (Santa Cruz Biotechnology, Inc., Dallas, Texas). We used a previously validated approach of intraperitoneal injection (1.5 mg/kg) mixed with an equal volume of siPORT amine (Ambion, Thermo Fisher Scientific, Waltham, Massachusetts,) in the live animal (38). We administered the siRNA every 24 h for 2 days. Silencing efficacy was evaluated 72 h after initiation of therapy by quantitative real-time (qRT) PCR as well as protein analysis.

**QUANTITATIVE REAL-TIME PCR.** Hearts were collected 72 h after initiations of siRNA therapy ( $n = 3$  per each group). Total ribonucleic acid (RNA) was prepared from cells using an RNA Purification Kit (Norgen Biotek, Thorold, Ontario, Canada) in accordance with the manufacturer's instructions. Total RNA was subjected to qRT PCR. RNA levels were analyzed using the TaqMan Gene Expression Assays in accordance with the manufacturer's instructions (scn1a: Mm00450580\_m1, scn3a: Mm00658167\_m1, scn5a: Mm01342518\_m1, and scn8a: Mm00488110\_m1, Life Technologies). RNA concentrations were determined with a NanoDrop 20000 (Thermo Fisher Scientific, Waltham, Massachusetts). Samples were normalized to OAZ1 for mRNAs (Life Technologies). Gene expression levels were quantified using the ABI Prism 7900HT Sequence detection system (Life Technologies). Comparative real-time PCR was performed in triplicate. Relative expression was calculated using the comparative Ct method.

**IMMUNOBLOTS.** Heart tissue lysates, following quantitation by the bicinchoninic acid (BCA) assay (Pierce), were loaded into 4% to 15% pre-cast TGX gels (Bio-Rad) and transferred to nitrocellulose membranes. Membranes were blocked for >1 h at room temperature in 3% bovine serum albumin and incubated in primary antibody overnight at 4°C. Primary antibodies included:  $\text{Na}_v1.6$  (1:500, Alomone) and glyceraldehyde 3-phosphate dehydrogenase (1:5,000, Fitzgerald, Acton, Massachusetts). Secondary antibodies used were donkey antimouse-horseradish peroxidase (HRP) and donkey antirabbit-HRP (Jackson Laboratory, Farmington, Connecticut). Densitometric analysis was performed using Image Lab software (Bio-Rad Laboratories, Hercules, California), and all data was normalized to glyceraldehyde 3-phosphate dehydrogenase.

**FIGURE 1**  $\beta$ -AR Stimulation Increases Propensity for CPVT by Augmenting TTX-Sensitive  $\text{nNa}_v$ -Mediated Late  $\text{I}_{\text{Na}}$



**(A)** Effect of  $\beta$ -adrenergic receptor ( $\beta$ -AR) stimulation on neuronal  $\text{Na}^+$  channel ( $\text{nNa}_v$ ) blockade and  $\text{Ca}^{2+}$  handling. **(Top)** Representative examples of the line-scan images and corresponding  $\text{Ca}^{2+}$  transients (CaT) recorded in catecholaminergic polymorphic ventricular tachycardia (CPVT) ventricular cardiomyocytes loaded with  $\text{Ca}^{2+}$  indicator, Fluo-4FF AM, and paced at 0.3 Hz. Cells were treated with isoproterenol (Iso) (100 nmol/l) and tetrodotoxin (TTX) (100 nmol/l), riluzole (Ril) (10  $\mu\text{mol/l}$ ), or flecainide (Flec) (2.5  $\mu\text{mol/l}$ ).  $\beta$ -AR stimulation with Iso promotes diastolic  $\text{Ca}^{2+}$  release (DCR) events in the form of  $\text{Ca}^{2+}$  waves relative to untreated CPVT cardiomyocytes ( $n = 166$  and  $n = 34$  cells, respectively;  $\#p < 0.001$  Wilcoxon rank sum test). TTX, Ril, and Flec significantly decreased DCR frequency in CPVT cardiomyocytes exposed to Iso ( $n = 109$ , 48, and 66 cells, respectively;  $p < 0.001$  Kruskal-Wallis test;  $*p = 0.003$ ,  $*p < 0.001$ , and  $*p = 0.032$  Wilcoxon rank sum test for TTX, Ril, and Flec vs. ISO, respectively). **(Bottom)** Representative caffeine (Caff)-induced (20 mmol/l) CaT. ISO significantly increased caffeine-induced CaT relative to untreated CPVT cardiomyocytes ( $n = 13$  and  $n = 11$  cells, respectively;  $*p = 0.005$  Wilcoxon rank sum test). This elevation in caffeine-induced CaT persisted despite concomitant treatment with TTX, Ril, and Flec ( $n = 11$ , 13, and 10 cells, respectively;  $p = 0.99$  Kruskal-Wallis test). **(B)** Effect of  $\beta$ -AR stimulation and subsequent  $\text{nNa}_v$  blockade of persistent  $\text{Na}^+$  current ( $\text{I}_{\text{Na}}$ ). Representative traces of persistent  $\text{I}_{\text{Na}}$  elicited using the protocol are shown in the inset. Iso enhanced persistent  $\text{I}_{\text{Na}}$  in CPVT cardiomyocytes ( $n = 18$  and  $n = 21$  cells, respectively;  $\#p = 0.004$  Wilcoxon rank sum test). This response to Iso was completely abolished upon addition of TTX, Ril, or Flec ( $n = 9$ , 7, and 9 cells, respectively;  $p < 0.001$  Kruskal-Wallis test;  $*p < 0.001$  Wilcoxon rank sum test for each treatment group vs. ISO). Summary data presented as persistent  $\text{I}_{\text{Na}}$  integral amp-ms/F (AmsF<sup>-1</sup>). **(C)** Effect of  $\beta$ -AR stimulation on  $\text{nNa}_v$ -mediated ventricular arrhythmias in vivo. Representative electrocardiography (ECG) recordings of CPVT mice after catecholamine challenge with intraperitoneal injection of epinephrine (1.5 mg/kg) and caffeine (120 mg/kg; red ECGs). A subset of mice was pre-treated with Ril (15 mg/kg; purple ECGs). Arrhythmia and ventricular tachycardia (VT) incidence (%) in CPVT mice exposed to catecholamine challenge during  $\text{Na}^+$ -channel blockade with riluzole ( $n = 13$  vs.  $n = 18$  CPVT-Epi+Caff vs. CPVT-Epi+Caff-Ril treated mice.  $*p = 0.043$  and  $*p = 0.023$  Fisher exact test for arrhythmia and VT incidence, respectively).

**ELECTROCARDIOGRAPHIC RECORDINGS.** Continuous electrocardiographic (ECG) recordings (PL3504 PowerLab 4/35, ADInstruments, Sydney, Australia) were obtained from mice anesthetized with isoflurane (1.0% to 1.5%) as previously described (26). Briefly, after baseline recording (5 min), a subset of animals received either riluzole (15 mg/kg) or β-pompilidotoxin (β-PMTX) (30 mg/kg). After 5 min, those animals that were pre-treated with β-PMTX received vehicle, riluzole, or flecainide (20 mg/kg). After an additional 5 to 10 min, animals were exposed to an intraperitoneal epinephrine (1.5 mg/kg) and caffeine (120 mg/kg) challenge, and ECG recording continued for 10 min. We also obtained continuous ECG recordings from CPVT-SERCA mice pre- and post-doxycycline induction. After baseline recording (5 min), each CPVT-SERCA mouse received only β-PMTX (30 mg/kg) intraperitoneally, and ECG recording continued for 10 min. ECG recordings were analyzed using the LabChart 7.3 program (ADInstruments). Arrhythmia was defined as bigeminy or frequent ectopic ventricular activity, whereas ventricular tachycardia (VT) was defined as 3 or more premature ectopies.

**REAGENTS.** Unless otherwise stated, all chemicals were purchased from Sigma, Torcis (Bristol, United Kingdom), Focus Biomolecules (Plymouth Meeting, Pennsylvania), Cusabio (Wuhan, China), Medchem-express LLC (Monmouth Junction, New Jersey), Millipore, or Alomone. Fluorescent dyes were purchased from Molecular Probes (Eugene, Oregon).

**DATA ANALYSIS.** I<sub>Na</sub> analysis was performed using pCLAMP9 software (Molecular Devices, Sunnyvale, California). Line scanning images of Ca<sup>2+</sup> were normalized for baseline fluorescence (14). The Ca<sup>2+</sup> imaging data were processed using ImageJ (NIH, Bethesda, Maryland) and Origin 7.0 software Origin-Lab Corporation, Northampton, Massachusetts. Confocal micrographs of PLA signal were low pass filtered (Gaussian) and thresholded to generate a black and white mask of the whole myocyte. This was used to calculate myocyte area. The unfiltered image was then thresholded using Otsu's method, followed by nearest-neighbor cluster detection to segment the PLA punctae. The punctae within the whole cell mask areas were counted to determine the density of PLA punctae within the cell (per μm<sup>2</sup>). Statistical analysis of the data was performed using a Wilcoxon signed rank test and Wilcoxon rank sum test for paired and nonpaired continuous data, respectively, or a Kruskal-Wallis test. The Šidák correction was applied to adjust for multiple comparisons. Fisher exact or McNemar tests were used to test differences in VT

incidence. On the basis of our previous observations of mice with high incidence of VT (≥70%) (26), 4 CASQ2-R33Q or other high-VT incidence mice/group were required to have an 80% chance of detecting, as significant at the 5% level, a decrease in the VT incidence from 70% in the control group to 0% in the treatment group. However, due to a lower VT incidence in the CASQ null mice (39), a total sample size of 30 mice in that group was needed. All statistical analyses were performed using Origin 7.0 or R (R Foundation for Statistical Computing, Vienna, Austria). All values are reported as mean ± SEM unless otherwise noted. A p value <0.05 was considered statistically significant.

## RESULTS

**β-AR STIMULATION IS NECESSARY FOR ABERRANT DCR.** In this study, we used mouse models of cardiac calsequestrin-associated CPVT. Consistent with the dependence of arrhythmia in CPVT patients on β-AR stimulation, CPVT murine myocytes presented only a few incidents of aberrant DCR in the absence of isoproterenol (ISO) (Sigma) (Figure 1A, black trace and bars). Addition of ISO (100 nmol/l) markedly increased the frequency of arrhythmogenic DCRs (Figure 1A, red traces and bars). This effect of ISO was accompanied by a significant increase in the SR Ca<sup>2+</sup> content (Figure 1A, red trace and bar). The ISO-dependent increase in the frequency of arrhythmogenic DCRs could, therefore, be attributed to: 1) increase in the SR Ca<sup>2+</sup> content (via phosphorylation of PLB and/or Ca<sub>v</sub>1.2); 2) altered RyR2 function (via phosphorylation of RyR2 at S2814); or 3) augmented nNa<sub>v</sub>-dependent local Na<sup>+</sup>/Ca<sup>2+</sup> signaling (26).

**β-AR SIMULATION INCREASES PROPENSITY FOR CPVT BY AUGMENTING TTX-SENSITIVE nNa<sub>v</sub>-MEDIATED PERSISTENT I<sub>Na</sub>.** In addition to the predominant TTX-resistant cardiac Na<sup>+</sup> channels (Na<sub>v</sub>1.5) localized predominantly at the intercalated disc and lateral membrane (26,37), cardiac myocytes express several types of TTX-sensitive nNa<sub>v</sub> localized in the cardiac T-tubule (26,30,31). The nNa<sub>v</sub> blockade with 100 nmol TTX (Tocris Bioscience, Avonmouth, United Kingdom) significantly decreased the frequency of ISO-promoted DCRs (Figure 1A, green traces and bars). Recently, Na<sup>+</sup> channel inhibitors flecainide and riluzole emerged as effective therapies in CPVT models (23,26). Interestingly, riluzole (10 μmol/l, Sigma) and flecainide (2.5 μmol/l, Sigma) both also reduced DCR frequency (Figure 1A, purple and blue traces and bars). Notably, consistent with previous reports

(25,26), none of the aforementioned interventions (i.e., TTX, riluzole, or flecainide) was associated with alterations in the SR  $\text{Ca}^{2+}$  content (Figure 1A). Taken together, these findings suggest that, in the setting of dysregulated RyR2 function, increased  $\text{Na}^+$  influx through  $\text{nNa}_v$  during the post-systolic phase may contribute to the arrhythmogenesis evidenced in this model upon  $\beta$ -AR stimulation. To examine the possibility of increased  $\text{Na}^+$  flux through  $\text{nNa}_v$  during  $\beta$ -AR stimulation, we assessed persistent  $I_{\text{Na}}$  in CPVT and WT cardiomyocytes. Exposure to ISO (100 nmol/l) elicited persistent  $I_{\text{Na}}$  both in CPVT and WT myocytes (Figure 1B, Supplemental Figure 2, respectively, red traces and bars). Notably, this current was sensitive to 100 nmol/l TTX (Figure 1B, Supplemental Figure 2, green traces and bars), riluzole, as well as flecainide (Figure 1B, purple and blue traces and bars, respectively), despite the 2 former agents exhibiting only a fraction of flecainide's total peak  $I_{\text{Na}}$  blocking potential (Supplemental Figure 3).

Next, we examined the effect of  $\text{nNa}_v$ -mediated persistent  $I_{\text{Na}}$  on CPVT in vivo. A catecholamine challenge composed of caffeine (Sigma) and epinephrine (Sigma) induced frequent ventricular arrhythmias, which degenerated into polymorphic VT (Figure 1C, red ECG and bars). Consistent with the notion of  $\beta$ -AR-mediated TTX-sensitive persistent  $I_{\text{Na}}$  contributing to pro-arrhythmic DCR, the vast majority of CPVT animals tested remained in sinus rhythm when pre-treated with riluzole (Figure 1C, purple ECG and bars). Therefore, augmentation of  $\text{Na}^+$  influx through  $\text{nNa}_v$  by catecholamines appears to be necessary for the pro-arrhythmic aberrant  $\text{Na}^+/\text{Ca}^{2+}$  signaling in CPVT.

**TTX-SENSITIVE  $\text{nNa}_v$ -MEDIATED PERSISTENT  $I_{\text{Na}}$  AUGMENTATION AND INCREASED SR  $\text{Ca}^{2+}$  LOAD ARE NECESSARY AND SUFFICIENT FOR ARRHYTHMIAS IN CPVT.** To determine whether augmentation of  $\text{nNa}_v$ -mediated  $\text{Na}^+$  influx alone (independent of  $\beta$ -AR stimulation) is sufficient for inducing arrhythmogenic DCR, we induced persistent  $I_{\text{Na}}$  via  $\text{nNa}_v$  augmentation with  $\beta$ -PMTX (40) in CPVT cardiomyocytes (Figure 2A). Persistent  $I_{\text{Na}}$  induced by 40  $\mu\text{mol/l}$   $\beta$ -PMTX (Alomone) was completely reversed by TTX (100 nmol/l), riluzole (10  $\mu\text{mol/l}$ ) as well as flecainide (2.5  $\mu\text{mol/l}$ ) (Figure 2A). Stimulation of  $\text{nNa}_v$  channels by  $\beta$ -PMTX (30 mg/kg intraperitoneally) in the absence of catecholamine challenge, however, failed to induce VT in vivo (Figure 2D).

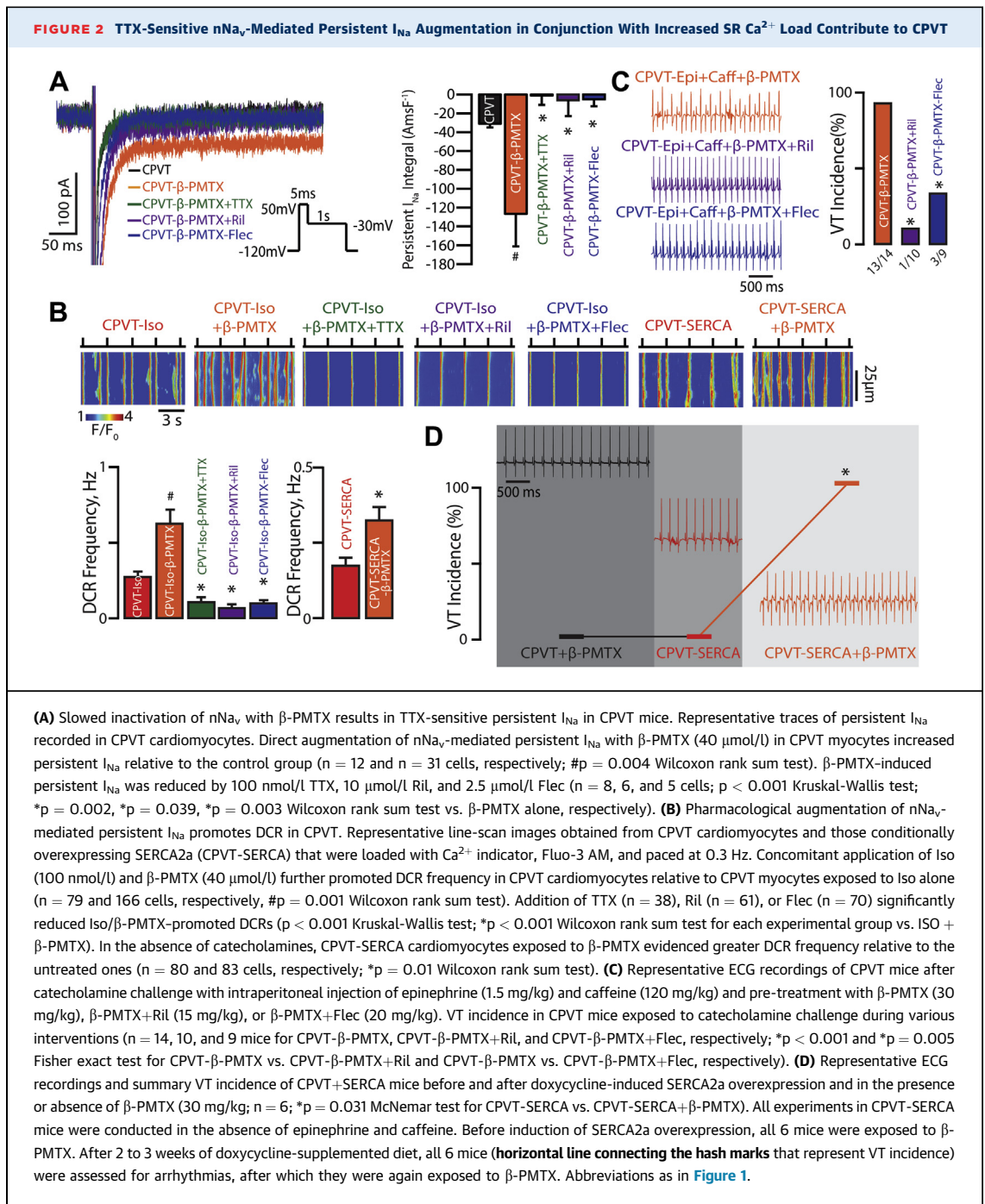
Of note,  $\beta$ -PMTX further promoted DCR in the presence of ISO on the cellular level (Figure 2B). This resulted in over 90% VT incidence in the CPVT mice undergoing concomitant  $\beta$ -PMTX treatment and

catecholamine challenge (Figure 2C, orange ECG and bar). Confirming the involvement of  $\text{nNa}_v$  in this pro-arrhythmic process,  $\text{Na}^+$ -channel blockade—both selective and nonselective—significantly reduced DCR and VT incidence in  $\beta$ -PMTX exposed, catecholamine challenged myocytes and animals, respectively, which was independent of changes in SR  $\text{Ca}^{2+}$  load (Figures 2B and 2C, Supplemental Figure 4A, green, purple and blue bars, and ECGs). Thus, stimulation of  $\text{nNa}_v$  alone, although necessary, is not sufficient to reproduce the proarrhythmic action of catecholamines in CPVT.

To test whether increased SR  $\text{Ca}^{2+}$  content is another necessary condition for arrhythmogenesis in CPVT, we performed experiments in CPVT mice that conditionally overexpress SERCA2a (CPVT-SERCA) (35). Even without  $\beta$ -AR stimulation, CPVT-SERCA myocytes evidenced comparable SR  $\text{Ca}^{2+}$  load to ISO-exposed CPVT myocytes (Supplemental Figure 4A) and significantly more arrhythmic DCR events relative to ISO-naive CPVT myocytes (Figures 1A and 2B). However, this was insufficient to promote VT in vivo (Figure 2D, red ECG and bar). Importantly, augmentation of  $\text{Na}^+$  flux through  $\text{nNa}_v$  with  $\beta$ -PMTX in ISO-naive CPVT-SERCA myocytes was sufficient to significantly increase aberrant DCR on the cellular level, relative to untreated CPVT-SERCA myocytes (Figure 2B) This, in turn, precipitated VT in all the CPVT-SERCA mice exposed to  $\beta$ -PMTX (Figure 2D, orange ECG and bar). Of note, in 2 instances when SERCA2a overexpression was reversed in CPVT-SERCA mice by stopping the doxycycline-rich diet for 14 days, exposure to  $\beta$ -PMTX failed to induce VT. Taken together, these results suggest that  $\text{nNa}_v$ -mediated persistent  $I_{\text{Na}}$  combined with genetically impaired RyR2 function and enhanced SR  $\text{Ca}^{2+}$  refill are necessary and sufficient for the arrhythmogenic phenotype responsible for CPVT.

**PROARRHYTHMIC EFFECT OF  $\beta$ -AR STIMULATION ON TTX-SENSITIVE PERSISTENT  $I_{\text{Na}}$  AUGMENTATION INVOLVES CAMKII PHOSPHORYLATION OF  $\text{nNa}_v$  AND IS INDEPENDENT OF RyR2 PHOSPHORYLATION.**

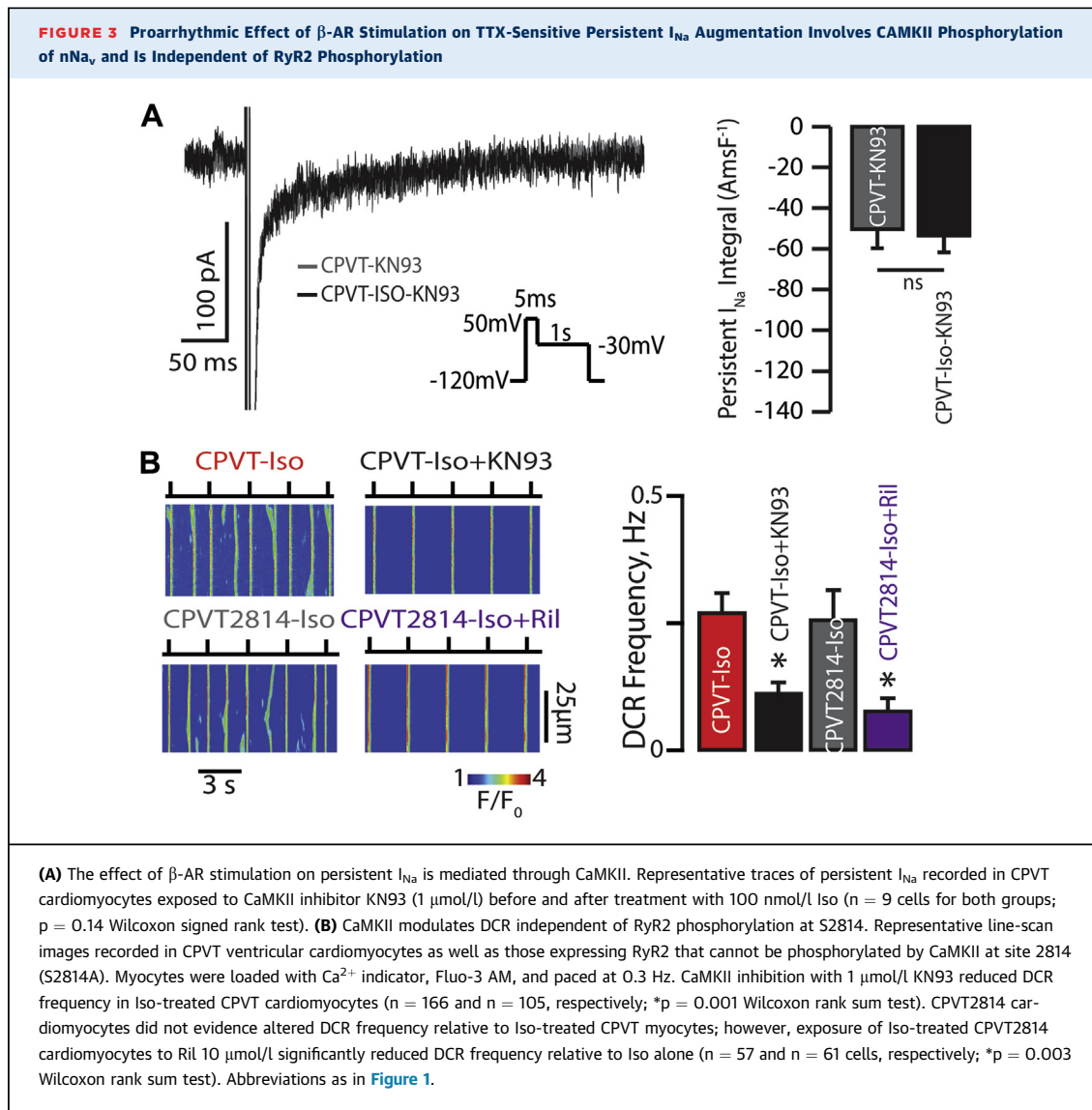
The aforementioned finding that  $\beta$ -AR stimulation promotes  $\text{Na}^+$  influx through  $\text{nNa}_v$  suggests that catecholamines may modulate  $\text{nNa}_v$  function through phosphorylation. Recently,  $\text{Na}^+$  channels have been shown to be subject to phosphorylation by CaMKII (41,42). To investigate the role of CaMKII-mediated modulation of  $\text{Na}^+/\text{Ca}^{2+}$  signaling in CPVT, we pharmacologically or genetically perturbed CaMKII signaling in CPVT cardiomyocytes. First, we observed that pharmacological blockade of CaMKII with KN93 (1  $\mu\text{mol/l}$ , Sigma) prevented ISO-induced persistent



$\text{I}_{\text{Na}}$  (Figure 3A). Second, KN93 significantly reduced ISO-promoted DCR in CPVT myocytes (Figure 3B, black and red bars, respectively). These results suggested that CaMKII promotes aberrant  $\text{Na}^+/\text{Ca}^{2+}$  signaling by augmenting  $\text{Na}^+$  influx through  $\text{nNa}_v$ . To examine the potential direct effects of CaMKII phosphorylation on RyR2 function in CPVT, we used CPVT-S2814A mice in which RyR2 is rendered nonphosphorylatable by CaMKII at S2814 (36).

Cardiomyocytes isolated from CPVT-S2814A mice evidenced similar frequency of ISO-promoted DCR relative to those isolated from CPVT mice (Figure 3B, red and gray bars, respectively). Furthermore, the frequency of these aberrant DCRs was significantly reduced by  $\text{Na}^+$  blockade with riluzole (Figure 3B, purple bar). Notably, none of the aforementioned interventions affected SR  $\text{Ca}^{2+}$  load (Supplemental Figure 4B). Thus CaMKII-mediated  $\text{Na}^+$  influx

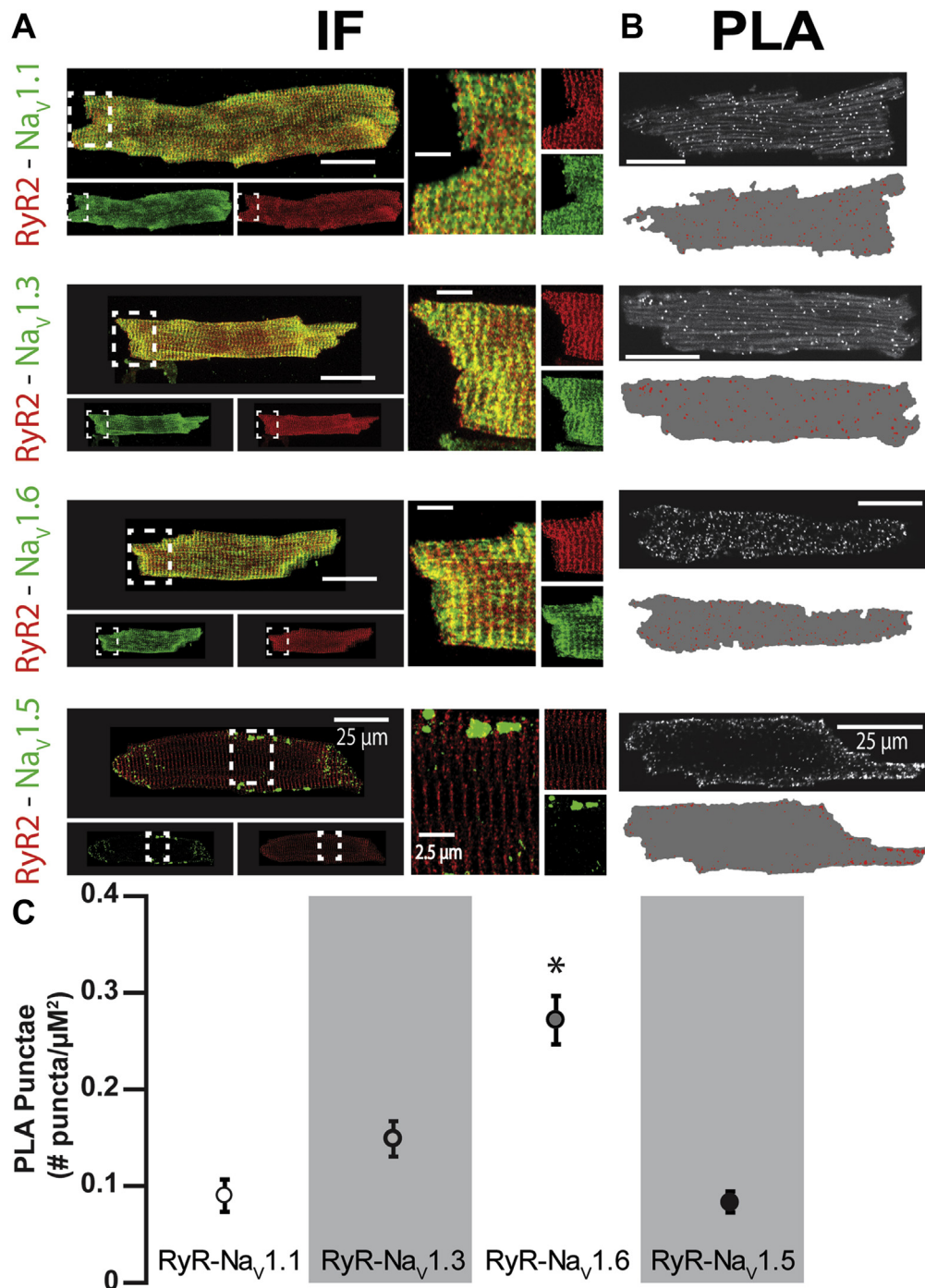




through nNa<sub>v</sub> can modulate DCR independently of RyR2 phosphorylation.

**ARRHYTHMOGENESIS IN CPVT DEPENDS ON Na<sub>v</sub>1.6-MEDIATED PERSISTENT I<sub>Na</sub>.** As we have previously demonstrated (26), cardiac myocytes contain several types of Na<sup>+</sup> channels, including TTX-sensitive nNa<sub>v</sub> (Na<sub>v</sub>1.1, Na<sub>v</sub>1.3, and Na<sub>v</sub>1.6) as well as the TTX-resistant Na<sub>v</sub>1.5. The former are located in the vicinity of RyR2 in the junctional microdomain, and the latter, in the lateral membrane and the intercalated discs (Figure 4A). To more precisely examine the localization of these channels with respect to RyR2 in CPVT, we performed a PLA (37). We found that all Na<sup>+</sup> channel isoforms were closely colocalized (within <40 nm [37]) with RyR2 (Figure 4B); however, Na<sub>v</sub>1.5 appeared to be primarily colocalizing with RyR2 in

the cell periphery, whereas the nNa<sub>v</sub>s exhibited a more diffuse pattern of colocalization. Specifically, Na<sub>v</sub>1.6 evidenced the highest degree of colocalization with RyR2 relative to the other nNa<sub>v</sub> isoforms (Figure 4C). The pattern and degree of colocalization of Na<sub>v</sub>1.6 with RyR2 were similar between myocytes isolated from WT and CPVT hearts, whereas this was not the case for Na<sub>v</sub>1.1 and 1.3 (Supplemental Figure 5). These data, in the context of recent work suggesting a role for Na<sub>v</sub>1.6 in progression of demyelinating disease (43), led us to hypothesize a mechanistic role for Na<sub>v</sub>1.6 in CPVT. Further, the nNa<sub>v</sub> inhibitor riluzole may exert a therapeutic effect in amyotrophic lateral sclerosis, a demyelinating disorder, through the blockade of Na<sub>v</sub>1.6 (44). We therefore examined the functional role of Na<sub>v</sub>1.6 in CPVT.

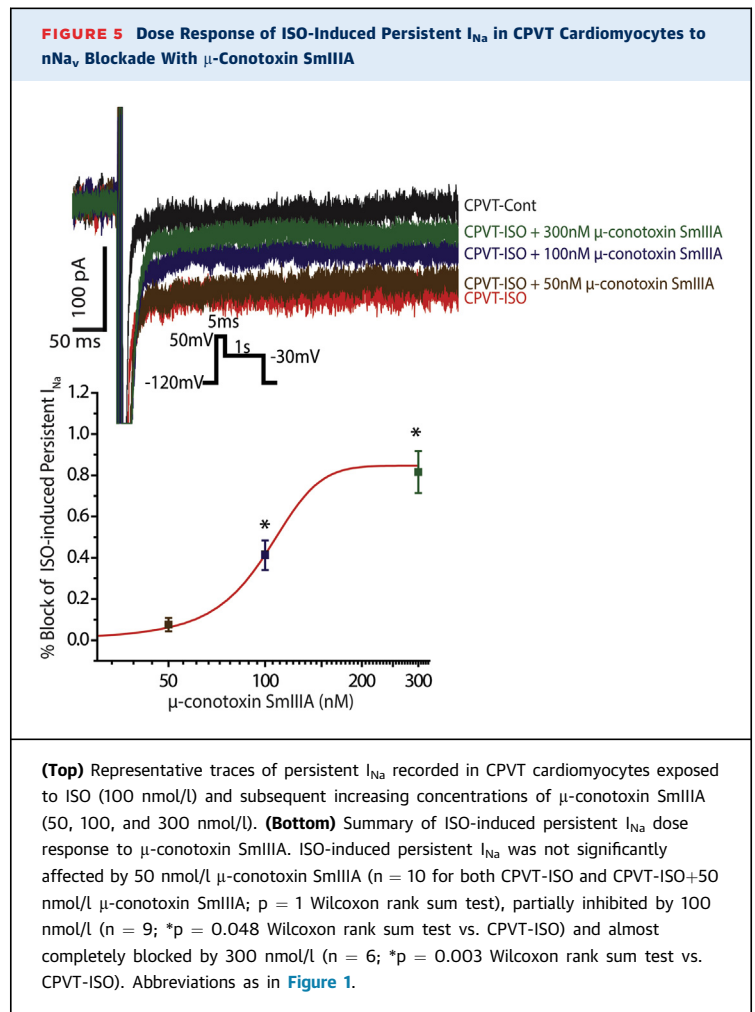
**FIGURE 4** Neuronal  $\text{Na}^+$  Channels and RyR2 Colocalize to the Same Discrete Subcellular Regions

(A) Representative confocal micrographs of isolated CPVT ventricular myocytes labeled for RyR2 (red) with various  $\text{Na}_V$  isoforms ( $\text{Na}_V1.x$ , green) often resulted in an overlap between the immunofluorescent (IF) signals (yellow) when overlaid. (Right) Close-up views of regions highlighted by dashed white boxes. (B) Representative confocal micrographs of ventricular myocytes isolated from CPVT mice showing fluorescent proximity ligation assay (PLA) signal for RyR2 with different  $\text{Na}_V$  isoforms ( $\text{Na}_V1.x$ ). Below each image are the results of digital segmentation, with the cell mask in gray and PLA signal in red. (C) Plot of average number of PLA punctae/ $\mu\text{M}^2$  ( $p < 0.001$  Kruskal-Wallis test; \* $p = 0.002$ , \* $p = 0.019$ , \* $p < 0.001$  Wilcoxon rank sum test for  $\text{Na}_V1.6$  vs.  $\text{Na}_V1.1$ , 1.3, and 1.5, respectively;  $n = 1,231, 1,223, 1,291$ , and  $2,848$  punctae from 7, 6, 12, and 7 cells for  $\text{Na}_V1.1, 1.3, 1.5$ , and 1.6, respectively). Abbreviations as in Figure 1.

To test this, first we conducted a dose response experiment with  $\mu$ -conotoxin SmIIIa, which can discriminate between TTX-sensitive  $\text{Na}^+$  channel isoforms (45). Specifically, at very low nmol/l concentrations,  $\mu$ -conotoxin SmIIIa inhibits  $\text{Na}_v1.1$  and  $\text{Na}_v1.3$  (45). Despite the putative inhibition of  $\text{Na}_v1.1$  and  $\text{Na}_v1.3$ , 50 nmol/l  $\mu$ -conotoxin SmIIIa (Cusabio, Wuhan, China) did not significantly alter ISO-induced persistent  $I_{\text{Na}}$  in CPVT myocytes (Figure 5). However, a concentration of  $\mu$ -conotoxin SmIIIa near the  $\text{IC}_{50}$  for  $\text{Na}_v1.6$  (100 nmol/l) (45) partially reduced ISO-induced persistent  $I_{\text{Na}}$ , whereas 300 nmol/l  $\mu$ -conotoxin SmIIIa virtually abolished this ISO-induced phenomenon (Figure 5). These data suggest that  $\text{Na}_v1.6$  can potentially contribute to the ISO-induced persistent  $I_{\text{Na}}$  and arrhythmias in CPVT. To examine this possibility further in both cardiac myocytes and in vivo we used a selective  $\text{Na}_v1.6$  inhibitor, 4,9-anhydro-TTX (Focus Biomolecules) (46,47). Notably, ISO-induced persistent  $I_{\text{Na}}$  in CPVT cardiomyocytes was sensitive to 300 nmol/l 4,9-anhydro-TTX (Figure 6A), suggesting that this ISO-promoted persistent  $I_{\text{Na}}$  is for the most part carried by  $\text{Na}_v1.6$ .

Furthermore, the addition of 300 nmol/l 4,9-anhydro-TTX to CPVT myocytes reduced the frequency of ISO-promoted DCRs (Figure 6B). Similarly, pre-treatment of CPVT mice with 4,9-anhydro-TTX (750  $\mu\text{g}/\text{kg}$ ) markedly reduced VT vulnerability during catecholamine challenge (Figure 6C, blue ECG and bar). Notably, this intervention had no significant effect on SR  $\text{Ca}^{2+}$  load (Supplemental Figure 6). We further addressed the role of  $\text{Na}_v1.6$  in CPVT by an siRNA approach to selectively target  $\text{Na}_v1.6$  (Supplemental Figure 7). CPVT mice injected with siRNA against  $\text{Na}_v1.6$  showed a marked decrease in arrhythmia episodes during catecholamine challenge (Figure 6C, purple ECG and bar). Taken together, these results suggest that  $\text{Na}_v1.6$  may be in part involved in CPVT-related arrhythmogenesis, which likely involves NCX.

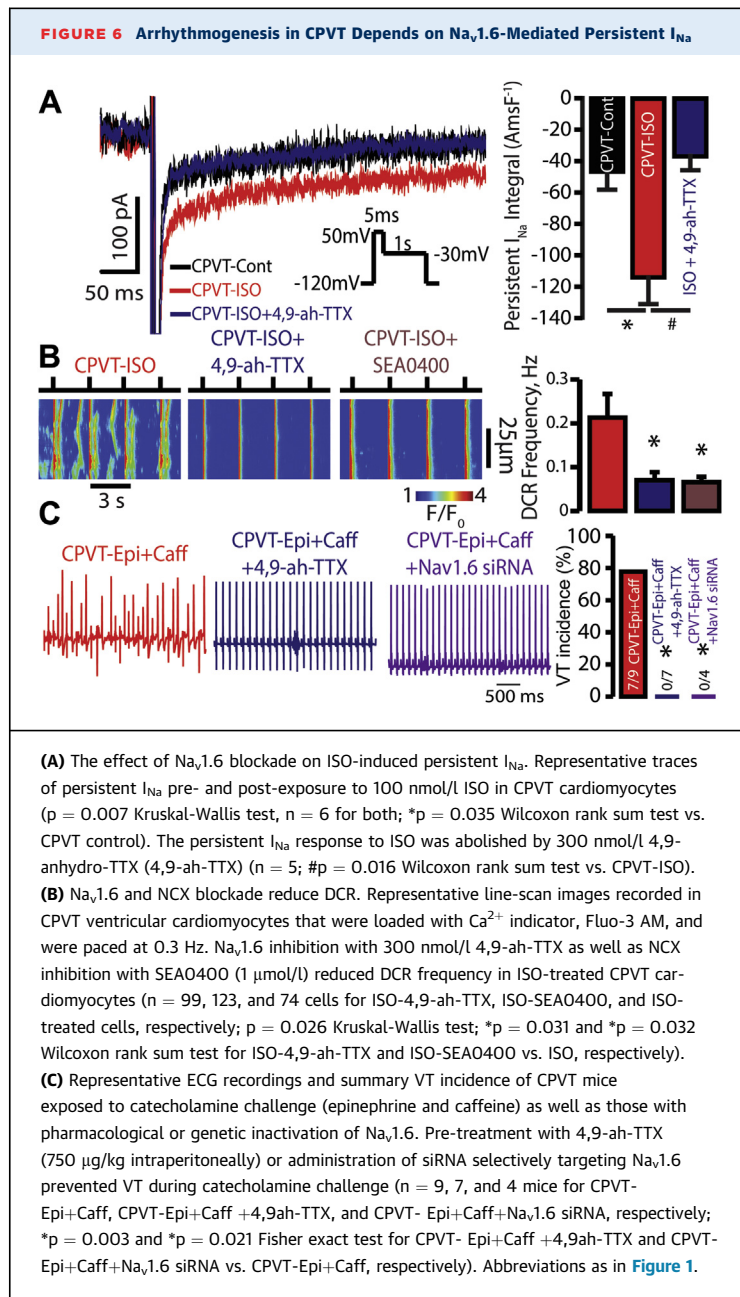
Last, to assess the potential role of NCX in the  $\text{Na}^+/\text{Ca}^{2+}$  signaling, we examined the structural correlation between NCX and  $\text{nNa}_v$ s as well as the functional effect of NCX inhibition on aberrant DCR. We found with the aid of PLA that NCX colocalizes with the TTX-sensitive  $\text{nNa}_v$  isoforms (Supplemental Figure 8). Furthermore, NCX inhibition with SEA0400 (48) (1  $\mu\text{mol}/\text{l}$ , MedChem Express, Monmouth Junction, New Jersey) had a similar effect on DCR relative to that observed with 4,9-anhydro-TTX (Figure 6B). Therefore, these data suggest that NCX may be a component of the pro-arrhythmic



interaction between  $\text{nNa}_v$ s and RyR2 that, in part, may be responsible for CPVT.

## DISCUSSION

Cardiac arrhythmias are often precipitated by catecholamine release during physical or emotional stress. The role of  $\beta$ -AR stimulation is particularly evident in inherited forms of cardiac arrhythmia such as CPVT, where genetic defects in the RyR2 complex (i.e., RyR2, CaM, CASQ2, TRD, and/or calstabin) alter RyR2 channel function and facilitate arrhythmogenic, aberrant DCR (2-5). Specifically, in the normal heart after each systolic  $\text{Ca}^{2+}$  release, RyR2s become refractory via a process that involves a decrease in the SR luminal  $\text{Ca}^{2+}$  (10). An intra-SR  $\text{Ca}^{2+}$  buffering protein, CASQ2, has been implicated in this process, acting as a  $\text{Ca}^{2+}$  buffer and a luminal  $\text{Ca}^{2+}$  sensor that regulates RyR2 gating (7-9,11,12,15). Therefore, CPVT-associated mutations in CASQ2 impair the ability of



the RyR2 channel to deactivate during the diastolic phase, thereby making RyR2s prone to premature activation that result in DCR (10,13–15). This defective RyR2 gating and the resulting DCR, which are evidenced in CPVT, are enhanced by β-AR stimulation. Despite the critical role of β-AR stimulation as an arrhythmia trigger, the precise mechanisms that link β-AR signaling to arrhythmogenesis remain elusive. Here, we demonstrate that augmented Na<sup>+</sup> entry via nNa<sub>v</sub> in the settings of the genetically compromised RyR2s and enhanced SR Ca<sup>2+</sup> refill are essential and necessary for the arrhythmogenesis during β-AR

stimulation in CPVT. Furthermore, we show that augmentation of Na<sup>+</sup> entry involves β-AR-mediated activation of CAMKII, subsequently leading to nNa<sub>v</sub> augmentation. Importantly, selective inhibition of Na<sub>v</sub>1.6 effectively prevents arrhythmia in vivo, thus potentially presenting a clinically useful antiarrhythmic approach.

Recently we and others have suggested that nNa<sub>v</sub> may facilitate excitation-contraction coupling and contribute to aberrant local Na<sup>+</sup>/Ca<sup>2+</sup> signaling, that, in part, may contribute to cardiac arrhythmias (24,26,49–52). On the basis of these studies, we set out to determine whether β-AR stimulation augments nNa<sub>v</sub>-mediated Na<sup>+</sup> entry and thereby facilitates Ca<sup>2+</sup> influx via the NCX that, in turn, may stimulate arrhythmogenic DCR through RyR2s. Here, we show that Na<sup>+</sup> influx via nNa<sub>v</sub> is not merely a compounding factor, but rather that augmentation of this Na<sup>+</sup> influx plays a key role in mediating the proarrhythmic effect of β-AR stimulation in CPVT. Specifically, our findings highlight a distinct nanodomain where nNa<sub>v</sub> are in close proximity (<40 nm) to RyR2s (Figure 4) and NCX (Supplemental Figure 8), and where β-AR-augmented Na<sup>+</sup> entry enhances aberrant Na<sup>+</sup>/Ca<sup>2+</sup> signaling, including DCR, thus resulting in CPVT. Of note, the amplitude of the nNa<sub>v</sub>-mediated persistent I<sub>Na</sub> was similar between WT and CPVT myocytes both at baseline and in the presence of ISO (Figures 1 and 6, Supplemental Figure 2). Thus, putative “physiological” β-AR augmentation of nNa<sub>v</sub> activity can become arrhythmogenic in a setting of genetically compromised RyR2 in CPVT.

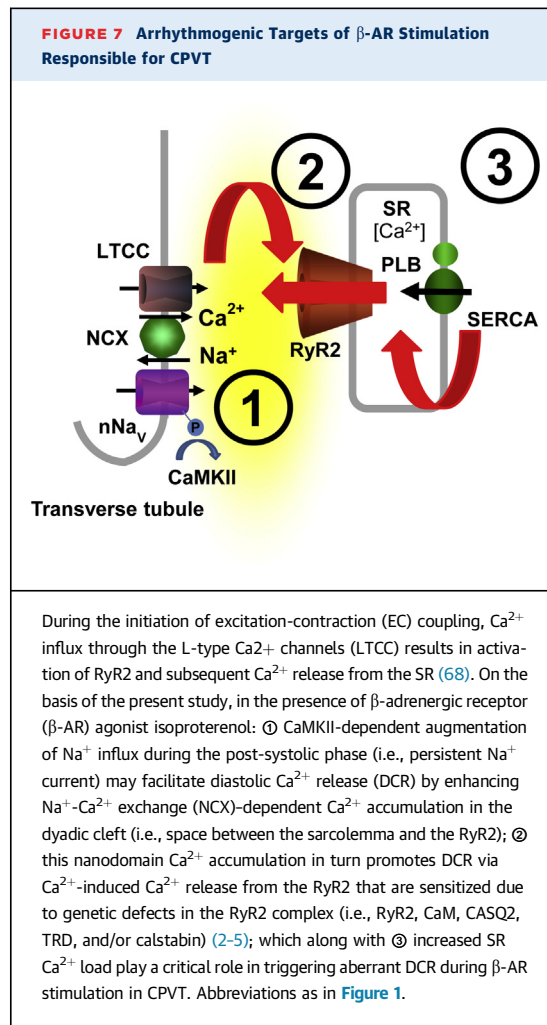
Stimulation of β-AR has been previously shown to affect intracellular Na<sup>+</sup> influx both early and late after a depolarizing stimulus (53,54). In the case of peak I<sub>Na</sub>, Yarbrough et al. (53) suggested that this phenomenon is coordinated by caveolin-3. Recently, caveolin-3 has been demonstrated to coordinate local nanodomain β<sub>2</sub>-AR-mediated regulation of L-type Ca<sup>2+</sup> channels in the T-tubules (55,56). However, future studies will need to address the role of caveolin-3 compartmentation on regulation of β-AR-mediated signaling of subpopulations of Na<sup>+</sup> channels in various cellular compartments.

Our structural and functional studies make a very compelling case for the involvement of nNa<sub>v</sub> in the arrhythmogenic process. However, this does not preclude the cardiac isoform of the Na<sup>+</sup> channels (Na<sub>v</sub>1.5) from contributing to arrhythmogenesis. In fact, early reports have described late I<sub>Na</sub> as a component of the cardiac I<sub>Na</sub> that can be inhibited by ranolazine (54). This late I<sub>Na</sub> was presumably carried by Na<sub>v</sub>1.5 and is a reflection of cell-wide sarcolemmal Na<sub>v</sub>1.5 activity. Here, we show that Na<sub>v</sub>1.5 is present

in the core-compartment of cardiomyocytes, presumably in the T-tubules, albeit its presence in that compartment is very limited (Figure 4). However,  $n\text{Na}_v$ , which include  $\text{Na}_v1.6$ , are the predominant isoforms present within these distinct nanodomains (Figure 4) and are responsible for the persistent  $I_{\text{Na}}$  phenotype during  $\beta$ -AR stimulation (Figures 1 and 6, Supplemental Figure 2). In this vein, nonselective ( $\text{Na}_v1.5$  and  $n\text{Na}_v$ ) inhibition with flecainide (57), despite having similar effect on persistent  $I_{\text{Na}}$ , more profoundly affected peak  $I_{\text{Na}}$  relative to 100 nmol/l TTX (Supplemental Figure 3), a concentration that completely blocks  $n\text{Na}_v$  although mostly sparing  $\text{Na}_v1.5$  (24,27-29). These data would further suggest that because 10  $\mu\text{mol/l}$  riluzole inhibits both peak and persistent  $I_{\text{Na}}$  to a similar extent as 100 nmol/l TTX, it may perhaps elicit its DCR-stabilizing effect through blockade of  $n\text{Na}_v$ . However, future studies will need to determine the specific  $\text{Na}^+$  channel isoforms blocked by this agent.

At least 3 isoforms of  $n\text{Na}_v$  have been identified in the heart as follows:  $\text{Na}_v1.1$ ,  $\text{Na}_v1.3$ , and  $\text{Na}_v1.6$  (Figure 4) (26,30-32,58). To examine whether a particular  $n\text{Na}_v$  isoform is essential for both aberrant  $\text{Na}^+/\text{Ca}^{2+}$  signaling and in vivo arrhythmia in CPVT, we used structural and functional assays. PLA as well as pharmacological and silencing approaches (Figure 4B, Supplemental Figure 5, and Figures 5 and 6, respectively) pointed to the involvement of  $\text{Na}_v1.6$  in the arrhythmogenic process. Moreover, WT and CPVT myocytes exhibited a similar degree of  $\text{Na}_v1.6$  and RyR2 colocalization (Figure 4, Supplemental Figure 5), in contrast to changes in RyR2 colocalization observed with other  $n\text{Na}_v$  isoforms; thus, the bulk of ISO-promoted late  $I_{\text{Na}}$  in WT and CPVT is likely carried by  $\text{Na}_v1.6$ . Taken together, these findings are consistent with the prevalence of this  $\text{Na}^+$  channel isoform in cardiomyocytes (26,30-32,58), its substantial persistent current (59,60), and its localization in the T-tubules in the vicinity of the RyR2 (Figure 4). Furthermore, the persistent  $I_{\text{Na}}$  that was generated by  $\text{Na}_v1.6$  during application of ISO was modulated by CaMKII-dependent  $n\text{Na}_v$  augmentation (Figure 3). Although the exact CaMKII phosphorylation site(s) in  $\text{Na}_v1.6$  or the other  $n\text{Na}_v$ s are not known, there are consensus CaMKII phosphorylation sites in these channels that correspond to DI-II linker conforming to Arg/Lys-X-X-Ser/Thr (61). In particular, S571 in  $\text{Na}_v1.5$  appears to be conserved in TTX-sensitive  $n\text{Na}_v$ s, suggesting that this might be the putative CaMKII phosphorylation site; however, future studies will need to determine the particular phosphorylation site(s) responsible for catecholamine-mediated augmentation of persistent  $I_{\text{Na}}$ .

**FIGURE 7** Arrhythmogenic Targets of  $\beta$ -AR Stimulation Responsible for CPVT



During the initiation of excitation-contraction (EC) coupling,  $\text{Ca}^{2+}$  influx through the L-type  $\text{Ca}^{2+}$  channels (LTCC) results in activation of RyR2 and subsequent  $\text{Ca}^{2+}$  release from the SR (68). On the basis of the present study, in the presence of  $\beta$ -adrenergic receptor ( $\beta$ -AR) agonist isoproterenol: ① CaMKII-dependent augmentation of  $\text{Na}^+$  influx during the post-systolic phase (i.e., persistent  $\text{Na}^+$  current) may facilitate diastolic  $\text{Ca}^{2+}$  release (DCR) by enhancing  $\text{Na}^+/\text{Ca}^{2+}$  exchange (NCX)-dependent  $\text{Ca}^{2+}$  accumulation in the dyadic cleft (i.e., space between the sarcolemma and the RyR2); ② this nanodomain  $\text{Ca}^{2+}$  accumulation in turn promotes DCR via  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from the RyR2 that are sensitized due to genetic defects in the RyR2 complex (i.e., RyR2, CaM, CASQ2, TRD, and/or calstabin) (2-5); which along with ③ increased SR  $\text{Ca}^{2+}$  load play a critical role in triggering aberrant DCR during  $\beta$ -AR stimulation in CPVT. Abbreviations as in Figure 1.

What other factors, apart from  $n\text{Na}_v$  stimulation, are critical to arrhythmogenesis in CPVT? To address this question, we omitted exposure to catecholamines and selectively slowed  $n\text{Na}_v$  inactivation with  $\beta$ -PMTX (40) to mimic  $\beta$ -AR-induced  $n\text{Na}_v$  augmentation in CPVT mice with inducible SERCA2a overexpression. These studies suggested that enhanced SR  $\text{Ca}^{2+}$  refilling or phosphorylation of effector sites such as RyR2 may be necessary for arrhythmogenesis in cardiac CASQ2-associated CPVT. Further experiments where we inhibited  $n\text{Na}_v$  activity in CPVT mice exposed to  $\beta$ -AR stimulation that were deficient in RyR2 CaMKII phosphorylation (S2814A) revealed that CaMKII phosphorylation of RyR2 does not play a pivotal role in CASQ2-associated CPVT. Taken together, these data suggest a novel conceptual framework for  $\beta$ -AR-promoted arrhythmogenesis (Figure 7). Mainly, the cross talk among  $n\text{Na}_v$ , NCX, and RyR2 may play a critical role in triggering aberrant DCR during  $\beta$ -AR stimulation in CPVT.

Furthermore, it is likely that a similar mechanism may contribute to arrhythmogenesis in other genetic and acquired forms of catecholamine-dependent arrhythmias. Likewise, there is evidence to suggest that CPVT is associated with DCR in the atria as well (34,62). Further, aberrant Ca<sup>2+</sup> release events are also observed in atria of patients with various forms of atrial fibrillation (63). Thus, it is very likely that the mechanism described herein may apply to the atrium as well. However, future studies will need to address the involvement of such aberrant Na<sup>+</sup>/Ca<sup>2+</sup> signaling in atrial as well as ventricular variants of genetic and acquired forms of catecholamine-dependent arrhythmias.

Although Na<sup>+</sup>-channel blockade, with flecainide in particular, has been shown to be effective in management of CPVT (21,23), the mechanism through which it alleviates arrhythmia remains to be clarified. Initially, the antiarrhythmic effect of flecainide was attributed to the direct inhibition of the RyR2 (23). Subsequent studies have suggested that it reduces the availability of cardiac-type Na<sup>+</sup> channels (Na<sub>v</sub>1.5), thus preventing the development of triggered activity (64). Here, we propose an additional and novel antiarrhythmic mechanism for flecainide in CPVT as follows: antagonizing catecholamine-dependent augmentation of Na<sup>+</sup> influx via nNa<sub>v</sub>s in general, and Na<sub>v</sub>1.6 in particular. Considering that altered RyR2 function contributes to acquired arrhythmias of various etiologies, including ischemic and nonischemic cardiomyopathy (65), inhibition of nNa<sub>v</sub> can potentially be applied to treat these diverse conditions. Interestingly, although non-isoform-selective Na<sup>+</sup> channel inhibition initially appeared to be beneficial in the management of Ca<sup>2+</sup>-mediated arrhythmias due to myocardial infarction (20), it has proven to be proarrhythmic and enhance the risk of arrhythmic death in patients with structural heart disease, evidently due to reduced electrical excitability of the myocardium (66,67). In this context, nNa<sub>v</sub> appears to be a particularly suitable antiarrhythmic target, where the antiarrhythmic effect of selective nNa<sub>v</sub> blockade can be uncoupled from the proarrhythmic effect of reduced cellular excitability associated with Na<sub>v</sub>1.5 inhibition. Taken together, our study brings well established findings on the global plane regarding the efficacy of Na<sup>+</sup> channels as well as β-AR blockers under 1 mechanistic umbrella. Specifically, the novel catecholamine-mediated arrhythmogenic mechanism described herein relies on the maintenance of enhanced SR Ca<sup>2+</sup> load in the setting of genetically compromised RyR2 along with augmentation of nNa<sub>v</sub> activity. The combination of these factors promotes aberrant Na<sup>+</sup>/Ca<sup>2+</sup> signaling,

resulting in DCR and arrhythmias in vivo. Selective inhibition of nNa<sub>v</sub>s in general, and Na<sub>v</sub>1.6 in particular, may represent effective treatment for a wide range of arrhythmias associated with altered RyR2 function and sympathetic stimulation.

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**REPRINT REQUESTS AND CORRESPONDENCE:** Dr. Przemysław B. Radwański, Davis Heart and Lung Research Institute, The Ohio State University, 473 West 12th Avenue, Room 510, Columbus, Ohio 43210. E-mail: [Przemyslaw.Radwanski@osumc.edu](mailto:Przemyslaw.Radwanski@osumc.edu). OR Dr. Sándor Györke, Davis Heart and Lung Research Institute The Ohio State University, 473 West 12th Avenue, Room 507, Columbus, Ohio 43210. E-mail: [Sandor.Gyorke@osumc.edu](mailto:Sandor.Gyorke@osumc.edu).

## PERSPECTIVES

**COMPETENCY IN MEDICAL KNOWLEDGE:** In a mouse heart, catecholamines promote pro-arrhythmic aberrant diastolic Ca<sup>2+</sup> release (DCR) by enhancing neuronal Na<sup>+</sup> channel (nNa<sub>v</sub>)-mediated persistent Na<sup>+</sup> current (I<sub>Na</sub>) along with maintaining adequate SR Ca<sup>2+</sup> load. Thus, these form the functional basis for CPVT.

**TRANSLATIONAL OUTLOOK:** Na<sup>+</sup> channel blockade has been shown to be effective in management of CPVT. Considering that altered RyR2 function contributes to both genetic and acquired arrhythmias of various etiologies, including ischemic and nonischemic cardiomyopathy, inhibition of nNa<sub>v</sub> can potentially be applied to treat these diverse conditions. Interestingly, although non-isoform-selective Na<sup>+</sup> channel inhibition initially appeared beneficial in the management of Ca<sup>2+</sup>-mediated arrhythmias due to myocardial infarction, it has proven to be pro-arrhythmic and enhance the risk of arrhythmic death in patients with structural heart disease evidently due to reduced availability of Na<sub>v</sub>1.5 and the consequent loss of myocardial excitability. In this context, nNa<sub>v</sub> appears to be particularly suitable antiarrhythmic target, where the antiarrhythmic effect of selective nNa<sub>v</sub> blockade can be uncoupled from the proarrhythmic effect of reduced cellular excitability associated with Na<sub>v</sub>1.5 inhibition.

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- KEY WORDS**  $\beta$ -adrenergic receptor, diastolic Ca<sup>2+</sup> release, neuronal Na<sup>+</sup> channels, ventricular arrhythmias
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- APPENDIX** For supplemental figures, please see the online version of this article.