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## 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 $\beta$ -adrenergic Stimulation

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P.B.R. and S.G. designed research; P.B.R., L.B., H-T.H., A.E.B., B.L., S.D.U., M.A.M. and R.V. performed research; A.A.A., W.H.D., B.C.K., P.V. and S.G.P. contributed murine models; R.V., T.J.H. and P.M.J. contributed analytic tools; P.B.R., M.A.M., H-T.H. and R.V. analyzed data; P.B.R., and S.G. wrote the paper.

### Conflict of Interest

None

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## Abstract

**Background**—Cardiac arrhythmias are a leading cause of death in the US. Vast majority of these arrhythmias including catecholaminergic polymorphic ventricular tachycardia (CPVT) are associated with increased levels of circulating catecholamines and involve abnormal impulse formation secondary to aberrant  $\text{Ca}^{2+}$  and  $\text{Na}^{+}$  handling. However, the mechanistic link between  $\beta$ -AR stimulation and the subcellular/molecular arrhythmogenic trigger(s) remains elusive.

**Methods and Results**—We performed functional and structural studies to assess  $\text{Ca}^{2+}$  and  $\text{Na}^{+}$  signaling in ventricular myocyte as well as surface electrocardiograms in mouse models of cardiac calsequestrin (CASQ2)-associated CPVT. We demonstrate that a subpopulation of  $\text{Na}^{+}$  channels (neuronal  $\text{Na}^{+}$  channels;  $\text{nNa}_v$ ) that colocalize with RyR2 and  $\text{Na}^{+}/\text{Ca}^{2+}$  exchanger (NCX) are a part of the  $\beta$ -AR-mediated arrhythmogenic process. Specifically, augmented  $\text{Na}^{+}$  entry via  $\text{nNa}_v$  in the settings of genetic defects within the RyR2 complex and enhanced sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$ -ATPase (SERCA)-mediated SR  $\text{Ca}^{2+}$  refill is both an essential and a necessary factor for the arrhythmogenesis. Furthermore, we show that augmentation of  $\text{Na}^{+}$  entry involves  $\beta$ -AR-mediated activation of CAMKII subsequently leading to  $\text{nNa}_v$  augmentation. Importantly, selective pharmacological inhibition as well as silencing of  $\text{Na}_v1.6$  inhibit myocyte arrhythmic potential and prevent arrhythmias *in vivo*.

**Conclusion**—These data suggest that the arrhythmogenic alteration in  $\text{Na}^{+}/\text{Ca}^{2+}$  handling evidenced during  $\beta$ -AR stimulation results, at least in part, from enhanced  $\text{Na}^{+}$  influx through  $\text{nNa}_v$ . Therefore, selective inhibition of these channels and  $\text{Na}_v1.6$  in particular can serve as a potential antiarrhythmic therapy.

## Keywords

$\beta$ -adrenergic receptor; Ventricular arrhythmias; Neuronal  $\text{Na}^{+}$  channels; Diastolic  $\text{Ca}^{2+}$  release

## Introduction

Cardiac arrhythmias are a leading cause of death in the US.<sup>1</sup> Arrhythmias caused by abnormal impulse generation are often associated with aberrant diastolic  $\text{Ca}^{2+}$  release (DCR) through dysregulated ryanodine receptor 2 (RyR2)  $\text{Ca}^{2+}$  release channels. This is especially evident when genetic defects in the RyR2 complex – either the RyR2 itself or one of the regulatory proteins associated with the channel (i.e., calmodulin, CASQ2, triadin and/or calstabin) – facilitate aberrant DCR.<sup>2–5</sup> In particular, recent findings demonstrate that either dysfunction or loss of cardiac calsequestrin (CASQ2), an intra-sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$ -binding protein and a regulator of RyR2, impairs the ability of RyR2s to deactivate and become refractory following systolic  $\text{Ca}^{2+}$  release.<sup>6–12</sup> This compromised refractoriness of  $\text{Ca}^{2+}$  release, in turn, permits the RyR2 channels to reopen during diastole, causing DCR to activate depolarizing membrane currents, resulting in pro-arrhythmic delayed afterdepolarizations (DADs).<sup>10,13–15</sup> 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.<sup>2,11,16</sup> In

accordance with the clinical presentation of a vast majority of these arrhythmias,  $\beta$ -blocker therapy is the mainstay of treatment for cardiac rhythm disorders.<sup>17</sup> Recent years have witnessed research endeavors that have focused on alterations in  $\text{Ca}^{2+}$  handling and their roles in precipitating triggered arrhythmias; however, the precise mechanistic link between  $\beta$ -adrenergic receptor ( $\beta$ -AR) stimulation and arrhythmogenesis in  $\text{Ca}^{2+}$ -mediated arrhythmias remains elusive. Several targets for phosphorylation, including  $\text{Ca}_v1.2$ , phospholamban (PLB) and RyR2, may be involved in the arrhythmogenic effects of  $\beta$ -AR stimulation.<sup>15</sup> For instance protein kinase A (PKA) phosphorylation of PLB will accelerate sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$ -ATPase 2a (SERCA2a)-mediated  $\text{Ca}^{2+}$  refilling of the SR thereby providing adequate substrate for aberrant DCR.<sup>18</sup> On the other hand, 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.<sup>19</sup>

Surprisingly, considering the  $\text{Ca}^{2+}$ -dependent nature of CPVT, these patients often respond to treatment with  $\text{Na}^+$  channel blockers such as flecainide.<sup>20–22</sup> It has been proposed that flecainide may exert its antiarrhythmic effect through a direct effect on RyR2,<sup>23</sup> however, this would not explain the effect of other  $\text{Na}^+$  channel blockers on aberrant  $\text{Ca}^{2+}$  handling.<sup>24,25</sup> 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.<sup>26</sup> These channels were initially described in neurons (hence their eponym) and are characterized by a high sensitivity to tetrodotoxin (TTX).<sup>24,27–29</sup> 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 crosstalk. Furthermore, since there are multiple  $\text{nNa}_v$  isoforms expressed in cardiac myocytes<sup>30–32</sup> 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. Since, 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.<sup>2,33</sup> Therefore, to investigate the role  $\text{Na}^+/\text{Ca}^{2+}$  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).<sup>6,14,26</sup> 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).

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

## Genetically-engineered mouse models

All the genetically-engineered mice used in our study were homozygous for their respective mutations and/or deletions. Cardiac calsequestrin (CASQ2) null mice (on mixed background)<sup>34</sup> were crossbred with a) mice conditionally overexpressing SERCA2a in a doxycycline dependent manner (on FVB/N background),<sup>35</sup> or b) with RyR2 S2814A mice (on C57BL/6 background; generous gift from Dr. Xander Wehrens).<sup>36</sup> 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 (MHC-rtTA),<sup>35</sup> tetracycline response element (Tet-RE)-SERCA2a,<sup>35</sup> and RyR2 S2814A mutation) using tail DNA. To induce the overexpression of SERCA2a, animals received doxycycline diet (Harlan TD 09295 1000 ppm Doxycycline Diet 2018) for 14-21 days. We also used cardiac CASQ2-R33Q as well as wild type (WT) mice (both on C57BL/6 background) to examine the role of Na<sub>v</sub>1.6 and NCX in aberrant Na<sup>+</sup>/Ca<sup>2+</sup> signaling.<sup>26</sup>

## Myocyte isolation, confocal Ca<sup>2+</sup> imaging, Na<sup>+</sup> current recordings

Ventricular myocytes were obtained by enzymatic isolation from 3-9 month old mice of both genders. Mice were anaesthetized with isoflurane and once a deep level of anaesthesia was reached the heart was rapidly removed and perfused via a Langendorff as previously described.<sup>14,26</sup> Peak sodium currents ( $I_{Na}$ ) were recorded using internal solution containing: 10 NaCl, 20 TEACl, 123 CsCl, 1 MgCl<sub>2</sub>, 0.1 Tris GTP, 5 MgATP, 10 HEPES, 10 BAPTA (pH 7.2, CsOH). For persistent  $I_{Na}$  recordings we substituted BAPTA with 1 EGTA and maintained free Ca<sup>2+</sup> 100 nmol/L with CaCl<sub>2</sub>. The extracellular bathing solution for peak  $I_{Na}$  contained: 10 NaCl, 130 TEACl, 4 CsCl, 0.4 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 0.05 CdCl<sub>2</sub>, 10 HEPES and 10 glucose. The extracellular bathing solution for persistent  $I_{Na}$  recordings contained: 140 NaCl, 4 CsCl, 1 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 0.05 CdCl<sub>2</sub>, 10 HEPES, 10 glucose, 0.03 niflumic acid, 0.004 strophanthidin and 0.2 NiCl<sub>2</sub>. pH was maintained at 7.4 with CsOH for both types of solutions. Whole-cell capacitance and series resistance compensation (60%) was applied along with leak subtraction. Signals were filtered with 2.9 kHz Bessel filter and  $I_{Na}$  was then normalized to membrane capacitance. Late  $I_{Na}$  was estimated by integrating  $I_{Na}$  between 50 and 450 ms.

Electrical field stimulation experiments were performed using the following external solution (in mM): 140 NaCl, 5.4 KCl, 1.0 CaCl<sub>2</sub>, 0.5 MgCl<sub>2</sub>, 10 HEPES, and 5.6 glucose (pH 7.4, NaOH). To assess the SR Ca<sup>2+</sup> load, 20 mM caffeine was applied at the end of the experiments. Intracellular Ca<sup>2+</sup> cycling was monitored by a Nikon A1 laser scanning confocal microscope. For intact myocytes, we used the cytosolic Ca<sup>2+</sup>-sensitive indicators Fluo-3 AM. To more reliable measurements of SR Ca<sup>2+</sup> release from inside the SR in control and isoproterenol-treated CPVT cardiomyocytes, we performed experiments in Fig. 1 using a low-affinity Ca<sup>2+</sup> indicator Fluo-4FF-AM. The fluorescent probes were excited with the 488 nm line of an argon laser and emission was collected at 500–600 nm. Fluo-3/Fluo-4FF fluorescence was recorded in the line scan mode of the confocal microscope. For Ca<sup>2+</sup> wave recordings myocytes were paced at 0.3 Hz using extracellular platinum electrodes in order 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 Ca<sup>2+</sup> 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.<sup>26</sup> PLA is a histo/cyto-chemical and confocal microscopy technique for determining when specific proteins are co-localized within <40 nm.<sup>37</sup> 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, CA, USA) for 1 h at room temperature and subsequently incubated with primary antibodies (Na<sub>v</sub>1.1, 1.3, 1.6, 1.5: 1:32, 1:32, 1:50, and 1:50, respectively; Alomone, Jerusalem, Israel for nNa<sub>v</sub>s; while Na<sub>v</sub>1.5 was a generous gift from Dr. Peter Mohler. RyR2 1:100 and NCX 1:50 Pierce Antibodies, Rockford, IL, USA.) overnight at 4°C. After washing, for immunofluorescence goat secondary antibodies (anti-mouse and anti-rabbit) conjugated to Alexa Fluor (488, 549; Life Technologies, Grand Island, NY, USA) were added for 1 h, while the PLA reactions were carried out using appropriate Duolink secondary antibodies (Sigma, St. Louise, MO, USA) according to the manufacturer's instructions. The sensitivity of PLA was assessed by staining for Na<sub>v</sub>1.5 (1:50, generous gift from Dr. Peter Mohler) and Connexin 43 (Cx43, 1:100, Millipore; Supplemental Fig. 1), which were previously demonstrated to co-localize at the intercalated disc.<sup>37</sup>

### Silencing RNA

Targeting siRNA was purchased from Santa Cruz. We used a previously validated approach of intraperitoneal injection (1.5 mg/kg) mixed with an equal volume of siPORT amine (Ambion) in the live animal.<sup>38</sup> We administered the siRNA every 24 h for 2 days. Silencing efficacy was evaluated 72 hours after initiation of therapy by Quantitative Real-Time (qRT)-PCR as well protein analysis.

### Quantitative Real-Time-PCR

Hearts were collected 72 h after initiations of siRNA therapy (n=3 per each group). Total RNA was prepared from cells using RNA Purification Kit (Norgen Biotek) in accordance with manufacturer's instructions. Total RNA was subjected to qRT-PCR. RNA levels were analyzed using the TaqMan Gene Expression Assays, in accordance with 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, Inc.). 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 BCA assay (Pierce), were loaded into 4-15% precast TGX gels (BioRad) and transferred to nitrocellulose membranes. Membranes were blocked for >1hr at room temperature in 3% BSA and incubated in primary antibody overnight at 4°C. Primary antibodies included: Na<sub>v</sub>1.6 (1:500, Alomone) and GAPDH (1:5000, Fitzgerald). Secondary antibodies used were donkey anti-mouse-HRP and donkey-anti-rabbit-HR (Jackson Laboratories). Densitometric analysis was performed using Image Lab software and all data was normalized to GAPDH.

## Electrocardiographic recordings

Continuous electrocardiographic (ECG) recordings (PL3504 PowerLab 4/35, ADInstruments) were obtained from mice anesthetized with isoflurane (1-1.5%) as previously described.<sup>26</sup> Briefly, after baseline recording (5 min.), a subset of animals received either riluzole (15 mg/kg) or β-PMTX (30mg/kg). After 5 min those animals that were pre-treated with β-PMTX received either vehicle, riluzole or flecainide (20mg/kg). After additional 5-10 min animals were exposed to an intraperitoneal epinephrine (1.5 mg/kg) and caffeine (120 mg/kg) challenge and ECG recording continued for 10 mins. We also obtained continuous ECG recordings from CPVT-SERCA mice pre- and post-doxycline induction. After baseline recording (5 min.), each CPVT-SERCA mouse received only β-PMTX (30mg/kg) intraperitoneally and ECG recording continued for 10 mins. ECG recordings were analyzed using the LabChart 7.3 program (ADInstruments). Arrhythmia was defined as bigeminy or frequent ectopic ventricular activity, while ventricular tachycardia (VT) was defined as three or more premature ectopies.

## Reagents

Unless otherwise stated, all chemicals were purchased from Sigma (St Louis, MO, USA), Torcis (Bristol, UK), Focus Biomolecules (Plymouth Meeting, PA, USA), Cusabio (Wuhan, China), Medchemexpress LLC (Monmouth Junction, NJ, USA), Millipore (Billerica, MA, USA) or Alomone (Jerusalem, Israel). Fluorescent dyes were purchased from Molecular Probes (Eugene, OR, USA).

## Data analysis

I<sub>Na</sub> analysis was performed using pCLAMP9 software (Molecular Devices, Sunnyvale, CA). Line scanning images of Ca<sup>2+</sup> were normalized for baseline fluorescence.<sup>14</sup> Ca<sup>2+</sup> imaging data were processed using ImageJ and Origin software. 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 (#/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 non-paired continuous data, respectively or a Kruskal-Wallis Test. The Šidák correction was applied to adjust for multiple comparisons. A Fisher's exact or McNemar's tests were used to test differences in VT incidence. Based on our previous observations of mice with high

incidence of VT (70%),<sup>26</sup> four CASQ2-R33Q or other high-VT incidence mice per group were required to have a 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. On the other hand, due to a lower VT incidence in the CASQ null mice,<sup>39</sup> a total sample size of 30 mice in that group was needed. All statistical analysis was performed using Origin 7.0 or R. All values are reported as means  $\pm$  SEM unless otherwise noted. A  $p < 0.05$  was considered statistically significant.

## Results

### $\beta$ -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  $\beta$ -AR stimulation, CPVT murine myocytes presented only a few incidents of aberrant diastolic  $\text{Ca}^{2+}$  release (DCR) in the absence of isoproterenol (ISO; Sigma) (Fig. 1a, black trace and bars); Addition of ISO (100nM) markedly increased the frequency of arrhythmogenic DCRs (Fig. 1a, red traces and bars). This effect of ISO was accompanied by a significant increase in the SR  $\text{Ca}^{2+}$  content (Fig. 1a, red trace and bar). The ISO-dependent increase in the frequency of arrhythmogenic DCRs could therefore be attributed to either 1) increase in the SR  $\text{Ca}^{2+}$  content (via phosphorylation of PLB and/or  $\text{Ca}_v1.2$ ); 2) altered RyR2 function (via phosphorylation of RyR2 at S2814); or 3) augmented neuronal  $\text{Na}^+$  channel ( $\text{nNa}_v$ )-dependent local  $\text{Na}^+/\text{Ca}^{2+}$  signaling.<sup>26</sup>

### $\beta$ -AR stimulation increases propensity for CPVT by augmenting TTX-sensitive $\text{nNa}_v$ -mediated persistent $I_{\text{Na}}$

In addition to the predominant TTX-resistant cardiac  $\text{Na}^+$  channels ( $\text{Na}_v1.5$ ) localized predominantly at the intercalated disc and lateral membrane,<sup>26,37</sup> cardiac myocytes express several types of TTX-sensitive  $\text{nNa}_v$  localized in the cardiac T-tubule.<sup>26,30,31</sup>  $\text{nNa}_v$  blockade with 100nM TTX (Tocris) significantly decreased the frequency of ISO-promoted DCRs (Fig. 1a, green traces and bars). Recently,  $\text{Na}^+$  channel inhibitors flecainide and riluzole emerged as effective therapies in CPVT models.<sup>23,26</sup> Interestingly, riluzole (10 $\mu\text{M}$ ; Sigma) and flecainide (2.5 $\mu\text{M}$ ; Sigma) both also reduced DCR frequency (Fig. 1a, purple and blue traces and bars). Notably, consistent with previous reports,<sup>25,26</sup> none of the aforementioned interventions (i.e., TTX, riluzole or flecainide) was associated with alterations in the SR  $\text{Ca}^{2+}$  content (Fig. 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  $\text{Na}^+$  current ( $I_{\text{Na}}$ ) in CPVT and WT cardiomyocytes. Exposure to ISO (100nM) elicited persistent  $I_{\text{Na}}$  both in CPVT and WT myocytes (Fig. 1b, Supplemental Fig. 2, respectively, red traces and bars). Notably, this current was sensitive to 100nM TTX (Fig. 1b, Supplemental Fig. 2, green traces and bars), riluzole as well as flecainide (Fig. 1b, purple

and blue traces and bars, respectively), despite the two former agents exhibiting only a fraction of flecainide's total peak  $I_{Na}$  blocking potential (Supplemental Fig. 3).

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

### **TTX-sensitive $nNa_v$ -mediated persistent $I_{Na}$ augmentation and increased SR $Ca^{2+}$ load are necessary and sufficient for arrhythmias in CPVT**

To determine whether augmentation of  $nNa_v$ -mediated  $Na^+$  influx alone (independent of  $\beta$ -AR stimulation) is sufficient for inducing arrhythmogenic DCR, we induced persistent  $I_{Na}$  via  $nNa_v$  augmentation with  $\beta$ -Pompilidotoxin ( $\beta$ -PMTX)<sup>40</sup> in CPVT cardiomyocytes (Fig. 2a). Persistent  $I_{Na}$  induced by  $40\mu M$   $\beta$ -PMTX (Alomone) was completely reversed by TTX (100 nM), riluzole (10  $\mu M$ ) as well as flecainide (2.5  $\mu M$ ; Fig. 2a). Stimulation of  $nNa_v$  channels by  $\beta$ -PMTX (30 mg/kg I.P.) in the absence of catecholamine challenge, however, failed to induce VT *in vivo* (Fig. 2d). Of note,  $\beta$ -PMTX further promoted DCR in the presence of ISO on the cellular level (Fig. 2b). This resulted in over 90% VT incidence in the CPVT mice undergoing concomitant  $\beta$ -PMTX treatment and catecholamine challenge (Fig. 2c, orange ECG and bar). Confirming the involvement of  $nNa_v$  in this pro-arrhythmic process,  $Na^+$  channel blockade – both selective and non-selective – significantly reduced DCR and VT incidence in  $\beta$ -PMTX exposed, catecholamine challenged myocytes and animals, respectively which was independent of changes in SR  $Ca^{2+}$  load (Fig. 2b, 2c and Supplemental Fig. 4a, green, purple and blue bars and ECGs). Thus, stimulation of  $nNa_v$  alone, although necessary, is not sufficient to reproduce the proarrhythmic action of catecholamines in CPVT.

To test whether increased SR  $Ca^{2+}$  content is another necessary condition for arrhythmogenesis in CPVT we performed experiments in CPVT mice that conditionally overexpress SERCA2a (CPVT-SERCA).<sup>35</sup> Even without  $\beta$ -AR stimulation, CPVT-SERCA myocytes evidenced comparable SR  $Ca^{2+}$  load to ISO-exposed CPVT myocytes (Supplemental Fig. 4a) and significantly more arrhythmic DCR events relative to ISO-naive CPVT myocytes (Fig. 1a and 2b). However, this was insufficient to promote VT *in vivo* (Fig. 2d, red ECG and bar). Importantly, augmentation of  $Na^+$  flux through  $nNa_v$  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. (Fig. 2b) This, in turn, precipitated VT in all the CPVT-SERCA mice exposed to  $\beta$ -PMTX (Fig. 2d, orange ECG and bar). Of note, in two instances when SERCA2a overexpression was reversed in CPVT-SERCA mice by stopping doxycycline-rich diet for 14 days, exposure to  $\beta$ -PMTX failed to induce VT. Taken together, these results suggest that  $nNa_v$ -mediated



persistent  $I_{Na}$ , combined with genetically impaired RyR2 function and enhanced SR  $Ca^{2+}$  refill, are necessary and sufficient for the arrhythmogenic phenotype responsible for CPVT.

### **Proarrhythmic effect of $\beta$ -AR stimulation on TTX-sensitive persistent $I_{Na}$ augmentation involves CaMKII phosphorylation of $nNa_v$ and is independent of RyR2 phosphorylation**

The aforementioned finding that  $\beta$ -AR stimulation promotes  $Na^+$  influx through  $nNa_v$  suggests that catecholamines may modulate  $nNa_v$  function through phosphorylation. Recently,  $Na^+$  channels have been shown to be subject to phosphorylation by CaMKII.<sup>41,42</sup> To investigate the role of CaMKII-mediated modulation of  $Na^+/Ca^{2+}$  signaling in CPVT, we pharmacologically or genetically perturbed CaMKII signaling in CPVT cardiomyocytes. First, we observed that pharmacological blockade of CaMKII with KN93 (1 $\mu$ M; Sigma) prevented ISO-induced persistent  $I_{Na}$  (Fig. 3a). Secondly, KN93 significantly reduced ISO-promoted DCR in CPVT myocytes (Fig. 3b, black and red bars, respectively). These results suggested that CaMKII promotes aberrant  $Na^+/Ca^{2+}$  signaling by augmenting  $Na^+$  influx through  $nNa_v$ . To examine the potential direct effects of CaMKII phosphorylation on RyR2 function in CPVT, we used CPVT-S2814A mice in which RyR2 is rendered non-phosphorylatable by CaMKII at S2814.<sup>36</sup> Cardiomyocytes isolated from CPVT-S2814A mice evidenced similar frequency of ISO-promoted DCR relative to those isolated from CPVT mice (Fig. 3b red and gray bars, respectively). Furthermore, the frequency of these aberrant DCRs was significantly reduced by  $Na^+$  blockade with riluzole (Fig. 3b, purple bar). Notably, none of the aforementioned interventions affected SR  $Ca^{2+}$  load (Supplemental Fig. 4b). Thus CaMKII-mediated  $Na^+$  influx through  $nNa_v$  can modulate DCR independently of RyR2 phosphorylation.

### **Arrhythmogenesis in CPVT depends on $Na_v1.6$ -mediated persistent $I_{Na}$**

As we have previously demonstrated,<sup>26</sup> cardiac myocytes contain several types of  $Na^+$  channels, including TTX-sensitive  $nNa_v$  ( $Na_v1.1$ ,  $Na_v1.3$  and  $Na_v1.6$ ) as well as the TTX-resistant  $Na_v1.5$ . The former are located in the vicinity of RyR2 in the junctional microdomain, and the latter, in the lateral membrane and the intercalated discs (Fig.4a). To more precisely examine the localization of these channels with respect to RyR2 in CPVT we performed a proximity ligation assay (PLA).<sup>37</sup> We found that all  $Na^+$  channel isoforms were closely co-localized (within 40 nm<sup>37</sup>) with RyR2 (Fig.4b); however,  $Na_v1.5$  appeared to be primarily co-localizing with RyR2 in the cell periphery, while the  $nNa_v$ s exhibited a more diffuse pattern of co-localization. Specifically,  $Na_v1.6$  evidenced the highest degree of co-localization with RyR2 relative to the other  $nNa_v$  isoforms (Fig.4c). The pattern and degree of co-localization of  $Na_v1.6$  with RyR2 were similar between myocytes isolated from WT and CPVT hearts whereas this was not the case for  $Na_v1.1$  and 1.3 (Supplemental Fig.5). These data, in the context of recent work suggesting a role for  $Na_v1.6$  in progression of demyelinating disease,<sup>43</sup> led us to hypothesize a mechanistic role for  $Na_v1.6$  in CPVT. Further, the  $nNa_v$  inhibitor riluzole may exert a therapeutic effect in Amyotrophic Lateral Sclerosis (ALS), a demyelinating disorder, through the blockade of  $Na_v1.6$ .<sup>44</sup> We therefore examined the functional role of  $Na_v1.6$  in CPVT. To test this, first we conducted a dose response experiment with  $\mu$ -conotoxin SmIIIa, which can discriminate between TTX-sensitive  $Na^+$  channel isoforms.<sup>45</sup> Specifically, at very low nM concentrations  $\mu$ -conotoxin SmIIIa inhibits  $Na_v1.1$  and  $Na_v1.3$ .<sup>45</sup> Despite the putative inhibition of  $Na_v1.1$  and  $Na_v1.3$ ,

50nM  $\mu$ -conotoxin SmIIIa (Cusabio Biotech) did not significantly alter ISO-induced persistent  $I_{Na}$  in CPVT myocytes (Fig.5). On the other hand, a concentration of  $\mu$ -conotoxin SmIIIa near the  $IC_{50}$  for  $Na_v1.6$  (100nM),<sup>45</sup> partially reduced ISO-induced persistent  $I_{Na}$ , while 300nM  $\mu$ -conotoxin SmIIIa virtually abolished this ISO-induced phenomenon (Fig.5). These data suggest that  $Na_v1.6$  can potentially contribute to the ISO-induced persistent  $I_{Na}$  and arrhythmias in CPVT. To examine this possibility further in both cardiac myocytes and *in vivo* we used a selective  $Na_v1.6$  inhibitor, 4,9-anhydro-TTX (Focus Biomolecules).<sup>46,47</sup> Notably, ISO-induced persistent  $I_{Na}$  in CPVT cardiomyocytes was sensitive to 300 nM 4,9-anhydro-TTX (Fig.6a), suggesting that this ISO-promoted persistent  $I_{Na}$  is for the most part carried by  $Na_v1.6$ . Furthermore, addition of 300 nM 4,9-anhydro-TTX to CPVT myocytes reduced the frequency of ISO-promoted DCRs (Fig.6b). Similarly, pretreatment of CPVT mice with 4,9-anhydro-TTX (750  $\mu$ g/kg) reduced markedly VT vulnerability during catecholamine challenge (Fig.6c, blue ECG and bar). Notably, this intervention had no significant effect on SR  $Ca^{2+}$  load (Supplemental Fig.6). We further addressed the role  $Na_v1.6$  in CPVT by siRNA approach to selectively target  $Na_v1.6$  (Supplemental Fig.7). CPVT mice injected with siRNA against  $Na_v1.6$  showed a marked decrease in arrhythmia episodes during catecholamine challenge (Fig.6c, purple ECG and bar). Taken together, these results suggest that  $Na_v1.6$  may be in part involved in CPVT-related arrhythmogenesis, which likely involves  $Na^+$ - $Ca^{2+}$  exchange (NCX).

Lastly, to assess the potential role of NCX in the  $Na^+$ / $Ca^{2+}$  signaling, we examined structural correlation between NCX and  $nNa_v$ s as well as functional effect of NCX inhibition on aberrant DCR. We found with the aid of PLA that NCX co-localizes with the TTX-sensitive  $nNa_v$  isoforms (Supplemental Fig.8). Furthermore, NCX inhibition with SEA0400<sup>48</sup> (1  $\mu$ M; Medchemexpress LLC) had a similar effect on DCR relative to that observed with 4,9-anhydro-TTX (Fig. 6b). Therefore, these data suggest that NCX may be a component of the pro-arrhythmic interaction between  $nNa_v$ 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.<sup>2-5</sup> Specifically, in the normal heart after each systolic  $Ca^{2+}$  release RyR2s become refractory via a process that involves a decrease in the SR luminal  $Ca^{2+}$ .<sup>10</sup> An intra-SR  $Ca^{2+}$  buffering protein, CASQ2, has been implicated in this process, acting as a  $Ca^{2+}$  buffer and a luminal  $Ca^{2+}$  sensor that regulates RyR2 gating.<sup>7-9,11,12,15</sup> 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.<sup>10,13-15</sup> This defective RyR2 gating and the resulting DCR, which are evidenced in CPVT, are enhanced by  $\beta$ -AR stimulation. Despite the critical role of  $\beta$ -AR stimulation as an arrhythmia trigger, the precise mechanisms that link  $\beta$ -AR signaling to arrhythmogenesis remain elusive. Here we demonstrate that augmented  $Na^+$  entry via  $nNa_v$  in the settings of the genetically compromised RyR2s and enhanced SR  $Ca^{2+}$  refill are

essential and necessary for the arrhythmogenesis during  $\beta$ -AR stimulation in CPVT. Furthermore, we show that augmentation of  $\text{Na}^+$  entry involves  $\beta$ -AR-mediated activation of CAMKII subsequently leading to  $\text{nNa}_v$  augmentation. Importantly, selective inhibition of  $\text{Na}_v1.6$  effectively prevents arrhythmia *in vivo*, thus potentially presenting a clinically useful antiarrhythmic approach.

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

Stimulation of  $\beta$ -AR has been previously shown to affect intracellular  $\text{Na}^+$  influx both early and late after a depolarizing stimulus.<sup>53,54</sup> In the case of peak  $I_{\text{Na}}$ , Yarbrough et al.<sup>53</sup> suggested that this phenomenon is coordinated by caveolin-3. Recently, caveolin-3 has been demonstrated to coordinate local nanodomain  $\beta_2$ -AR-mediated regulation of L-type  $\text{Ca}^{2+}$  channels in the T-tubules.<sup>55,56</sup> However, future studies will need to address the role of caveolin-3 compartmentation on regulation of  $\beta$ -AR-mediated signaling of subpopulations of  $\text{Na}^+$  channels in various cellular compartments.

Our structural and functional studies make a very compelling case for the involvement of  $\text{nNa}_v$  in the arrhythmogenic process. However, this does not preclude the cardiac isoform of the  $\text{Na}^+$  channels ( $\text{Na}_v1.5$ ) from contributing to arrhythmogenesis. In fact, early reports have described late  $I_{\text{Na}}$  as a component of the cardiac  $I_{\text{Na}}$  that can be inhibited by ranolazine.<sup>54</sup> This late  $I_{\text{Na}}$  was presumably carried by  $\text{Na}_v1.5$  and is a reflection of cell-wide sarcolemmal  $\text{Na}_v1.5$  activity. Here we show that  $\text{Na}_v1.5$  is present in the core-compartment of cardiomyocytes presumably in the T-tubules, albeit its presence in that compartment is very limited (Fig. 4). On the other hand,  $\text{nNa}_v$ , which include  $\text{Na}_v1.6$ , are the predominant isoforms present within these distinct nanodomains (Fig. 4) and are responsible for the persistent  $I_{\text{Na}}$  phenotype during  $\beta$ -AR stimulation (Fig. 1, 6 and Supplemental Fig. 2). In this vein, non-selective ( $\text{Na}_v1.5$  and  $\text{nNa}_v$ ) inhibition with flecainide,<sup>57</sup> despite having similar effect on persistent  $I_{\text{Na}}$ , more profoundly affected peak  $I_{\text{Na}}$  relative to 100 nM TTX (Supplemental Fig. 3), a concentration that completely blocks  $\text{nNa}_v$  while mostly sparing  $\text{Na}_v1.5$ .<sup>24,27–29</sup> These data would further suggest that since 10 $\mu\text{M}$  riluzole inhibits both peak and persistent  $I_{\text{Na}}$  to similar extent as 100nM TTX, that it perhaps may 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 three isoforms of  $n\text{Na}_v$  have been identified in the heart,  $\text{Na}_v1.1$ ,  $1.3$  and  $1.6$  (Fig. 4).<sup>26,30–32,58</sup> In order 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 employed structural and functional assays. PLA as well as pharmacological and silencing approaches (Fig. 4b, Supplemental Fig. 5 and Fig. 5 & 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 (Fig. 4 and Supplemental Fig. 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,<sup>26,30–32,58</sup> its substantial persistent current<sup>59,60</sup> and localization in the T-tubules in the vicinity of the RyR2 (Fig. 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 (Fig. 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 which correspond to DI-II linker conforming to Arg/Lys-X-X-Ser/Thr.<sup>61</sup> 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  $I_{\text{Na}}$ .

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<sup>40</sup> 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 (Fig. 7): Mainly, the cross-talk between  $n\text{Na}_v$ , 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.<sup>34,62</sup> Further, aberrant  $\text{Ca}^{2+}$  release events are also observed in atria of patients with various forms of atrial fibrillation.<sup>63</sup> 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  $\text{Na}^+/\text{Ca}^{2+}$  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,<sup>21,23</sup> 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.<sup>23</sup> 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.<sup>64</sup> Here we propose an additional and novel antiarrhythmic mechanism for flecainide in CPVT: 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,<sup>65</sup> inhibition of nNa<sub>v</sub> can potentially be applied to treat these diverse conditions. Interestingly, while 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,<sup>20</sup> it has proven to be pro-arrhythmic and enhance the risk of arrhythmic death in patients with structural heart disease evidently due to reduced electrical excitability of the myocardium.<sup>66,67</sup> In this context, nNa<sub>v</sub> appear 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> channel as well as β-AR blockers under one 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.

### COMPETENCY IN MEDICAL KNOWLEDGE

In a mouse heart, catecholamines promote proarrhythmic 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>). Thus, these form the functional basis for catecholamine-induced polymorphic ventricular tachycardia (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, while 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> appear 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations and Acronyms

<b>ALS</b>	amyotrophic lateral sclerosis
<b>AmsF<sup>-1</sup></b>	Amp.ms/F
<b>β-PMTX</b>	β-Pompilidotoxin
<b>β-AR</b>	β-adrenergic receptor
<b>CaMKII</b>	Ca <sup>2+</sup> /calmodulin-dependent protein kinase II
<b>CASQ2</b>	calsequestrin
<b>CPVT</b>	Catecholaminergic Polymorphic Ventricular Tachycardia
<b>DAD</b>	delayed afterdepolarizations
<b>DCR</b>	diastolic Ca <sup>2+</sup> release
<b>I<sub>Na</sub></b>	Na <sup>+</sup> current
<b>ISO</b>	isoproterenol
<b>nNa<sub>v</sub></b>	neuronal Na <sup>+</sup> Channels
<b>NCX</b>	Na <sup>+</sup> /Ca <sup>2+</sup> exchange
<b>PLB</b>	phospholamban
<b>PKA</b>	protein kinase A
<b>RyR2</b>	ryanodine-receptor Ca <sup>2+</sup> release channels
<b>SR</b>	sarcoplasmic reticulum
<b>SERCA2a</b>	sarcoplasmic reticulum Ca <sup>2+</sup> -ATPase 2a
<b>TTX</b>	tetrodotoxin
<b>VT</b>	ventricular tachycardia
<b>WT</b>	wild type

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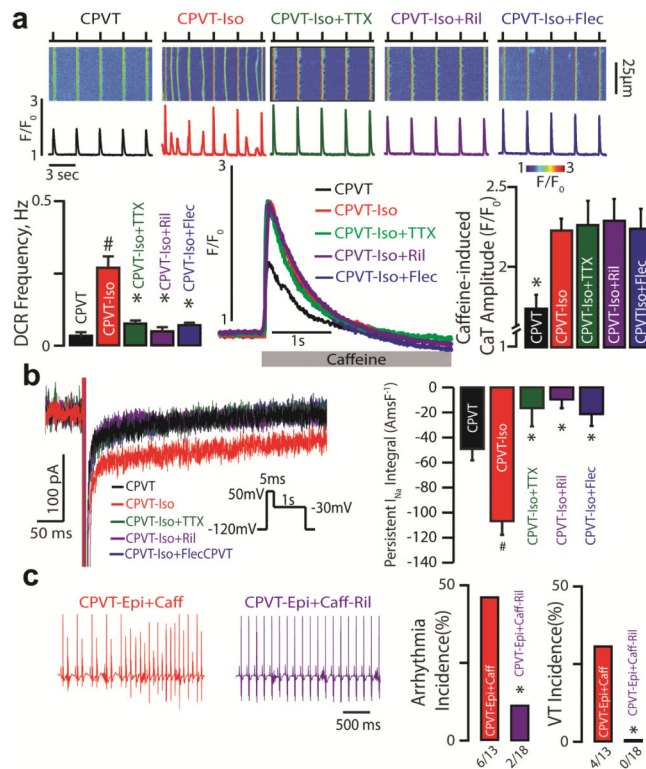
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**Fig. 1.  $\beta$ -AR stimulation increases propensity for CPVT by augmenting TTX-sensitive  $n\text{Na}_v$ -mediated late  $I_{\text{Na}}$**

(a) Effect of  $\beta$ -AR stimulation on  $n\text{Na}_v$  blockade and  $\text{Ca}^{2+}$  handling. (Top) Representative examples of the line-scan images and corresponding  $\text{Ca}^{2+}$  transients (CaT) recorded in 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 nM) and tetrodotoxin (TTX, 100 nM), riluzole (Ril, 10  $\mu\text{M}$ ) or flecainide (Flec, 2.5  $\mu\text{M}$ ).  $\beta$ -AR stimulation with Iso promotes DCR events in the form of  $\text{Ca}^{2+}$  waves relative to untreated CPVT cardiomyocytes ( $n=166$  and 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,  $p<0.001$  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-induced (20 mM) CaT. ISO significantly increased caffeine-induced CaT relative to untreated CPVT cardiomyocytes ( $n=13$  and 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  $n\text{Na}_v$  blockade on persistent  $I_{\text{Na}}$ . Representative traces of persistent  $I_{\text{Na}}$  elicited using the protocol shown in the inset. Iso enhanced persistent  $I_{\text{Na}}$  in CPVT cardiomyocytes ( $n=18$  and 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 groups vs. ISO). Summary data presented as persistent  $I_{\text{Na}}$  integral amp-msec/ $F$  ( $\text{AmsF}^{-1}$ ). (c) Effect of  $\beta$ -AR stimulation on  $n\text{Na}_v$ -mediated ventricular arrhythmias *in vivo*. Representative ECG recordings of CPVT

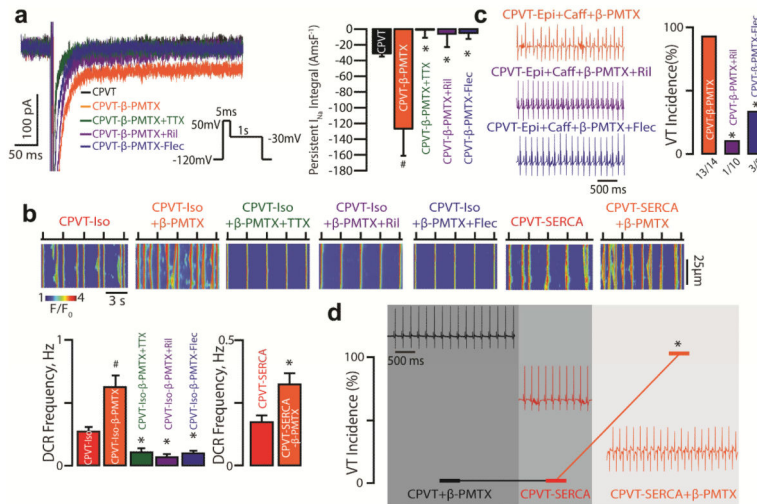
mice after catecholamine challenge with intraperitoneal injection (i.p.) of epinephrine (1.5 mg/kg) and caffeine (120 mg/kg; red ECGs). A subset of mice was pretreated with Ril (15 mg/kg; purple ECGs). Arrhythmia and ventricular tachycardia (VT) incidence (%) in CPVT mice exposed to catecholamine challenge during Na<sup>+</sup> channel blockade with riluzole (n = 13 vs 18 CPVT-Epi+Caff vs CPVT-Epi+Caff-Ril treated mice. \*,  $p=0.043$  and \*,  $p=0.023$  Fisher's exact test for Arrhythmia and VT incidence, respectively).

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**Fig. 2. TTX-sensitive  $nNa_v$ -mediated persistent  $I_{Na}$  augmentation in conjunction with increased SR  $Ca^{2+}$  load contribute to CPVT**

(a) Slowed inactivation of  $nNa_v$  with  $\beta$ -PMTX results in TTX-sensitive persistent  $I_{Na}$  in CPVT mice. Representative traces of persistent  $I_{Na}$  recorded in CPVT cardiomyocytes. Direct augmentation of  $nNa_v$ -mediated persistent  $I_{Na}$  with  $\beta$ -PMTX (40  $\mu$ M) in CPVT myocytes increased persistent  $I_{Na}$  relative to control (n = 12 and 31 cells, respectively, #  $p=0.004$  Wilcoxon rank-sum test).  $\beta$ -PMTX-induced persistent  $I_{Na}$  was reduced by 100 nM TTX, 10  $\mu$ M Ril and 2.5  $\mu$ M Flec (n = 8, 6 and 5 cells,  $p < 0.001$  Kruskal-Wallis test; \*,  $p=0.002$ ,  $p=0.039$ ,  $p=0.003$  Wilcoxon rank-sum test vs  $\beta$ -PMTX alone, respectively). (b) Pharmacological augmentation of  $nNa_v$ -mediated persistent  $I_{Na}$  promotes DCR in CPVT. Representative line-scan images obtained from CPVT cardiomyocytes and those conditionally overexpressing SERCA2a (CPVT-SERCA) that were loaded with  $Ca^{2+}$  indicator, Fluo-3 AM and paced at 0.3 Hz. Concomitant application of Iso (100 nM) and  $\beta$ -PMTX (40  $\mu$ M) further promoted DCR frequency in CPVT cardiomyocytes relative to CPVT myocytes exposed to Iso alone (n = 79 and 166 cells, respectively, #  $p=0.001$  Wilcoxon rank-sum test). Addition of TTX (n = 38), Ril (n = 61) or Flec (n = 70) significantly reduced Iso/ $\beta$ -PMTX-promoted DCRs ( $p < 0.001$  Kruskal-Wallis test; \*,  $p < 0.001$  Wilcoxon rank-sum test for each experimental group vs ISO+ $\beta$ -PMTX). In the absence of catecholamines, CPVT-SERCA cardiomyocytes exposed to  $\beta$ -PMTX evidenced greater DCR frequency relative to the untreated ones (n = 80 and 83 cells, respectively;\*,  $p=0.01$  Wilcoxon rank-sum test). (c) Representative ECG recordings of CPVT mice after catecholamine challenge with i.p. injection of epinephrine (1.5 mg/kg) and caffeine (120 mg/kg) and pretreatment with,  $\beta$ -PMTX (30mg/kg),  $\beta$ -PMTX+Ril (15 mg/kg) or  $\beta$ -PMTX +Flec (20 mg/kg). VT incidence in CPVT mice exposed to catecholamine challenge during various interventions (n = 14, 10 and 9 mice for CPVT- $\beta$ -PMTX, CPVT- $\beta$ -PMTX+Ril and CPVT- $\beta$ -PMTX+Flec, respectively. \*  $p < 0.001$  and  $p=0.005$  Fisher's exact test for CPVT- $\beta$ -PMTX vs. CPVT- $\beta$ -PMTX+Ril and CPVT- $\beta$ -PMTX vs. CPVT- $\beta$ -PMTX+Flec, respectively). (d) Representative ECG recordings and summary VT incidence of CPVT +SERCA mice before and after doxycycline-induced SERCA2a overexpression and in the presence or absence of  $\beta$ -PMTX (30mg/kg; n = 6, \* $p = 0.031$  McNemar's test for CPVT-SERCA vs. CPVT-SERCA+ $\beta$ -PMTX). All experiments in CPVT-SERCA mice were

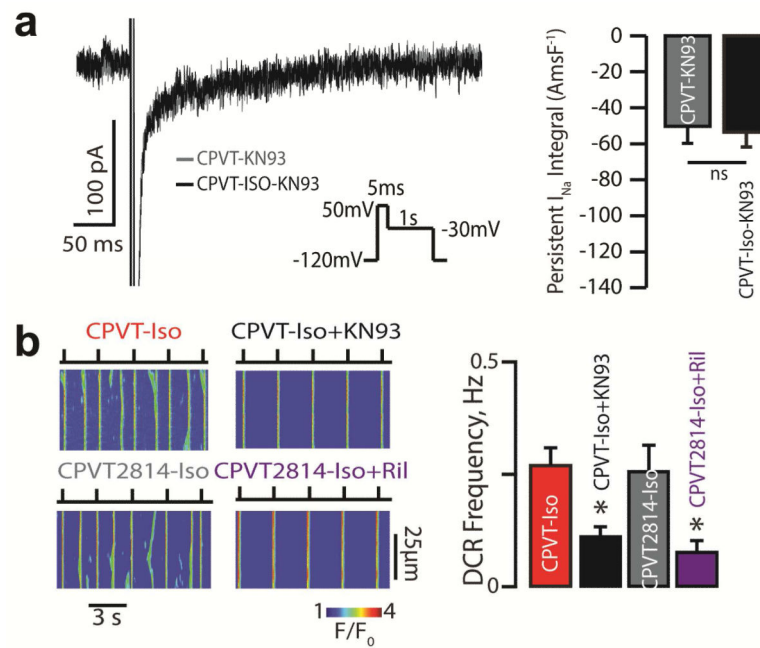
conducted in the absence of epinephrine and caffeine. Before induction of SERCA2a overexpression all 6 mice were exposed to  $\beta$ -PMTX. After 2-3 weeks of doxycycline-supplemented diet, all 6 mice (horizontal line connecting the hash marks that represent VT incidence) were assessed for arrhythmias after which they were again exposed to  $\beta$ -PMTX.

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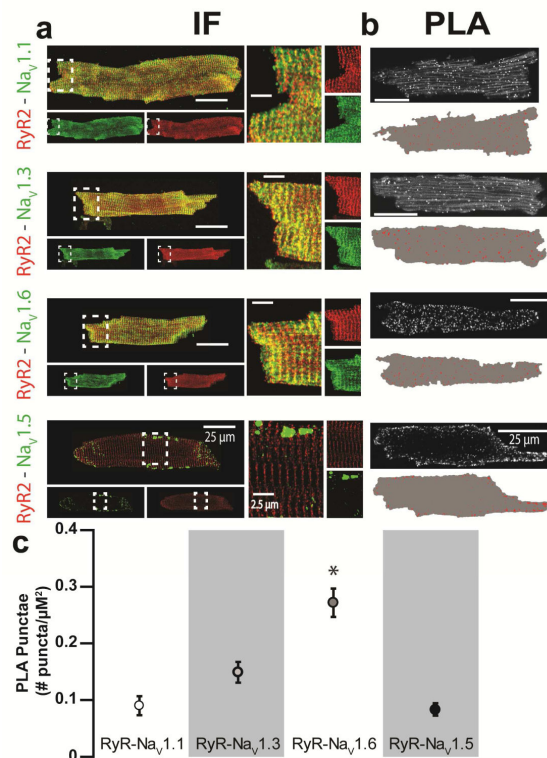
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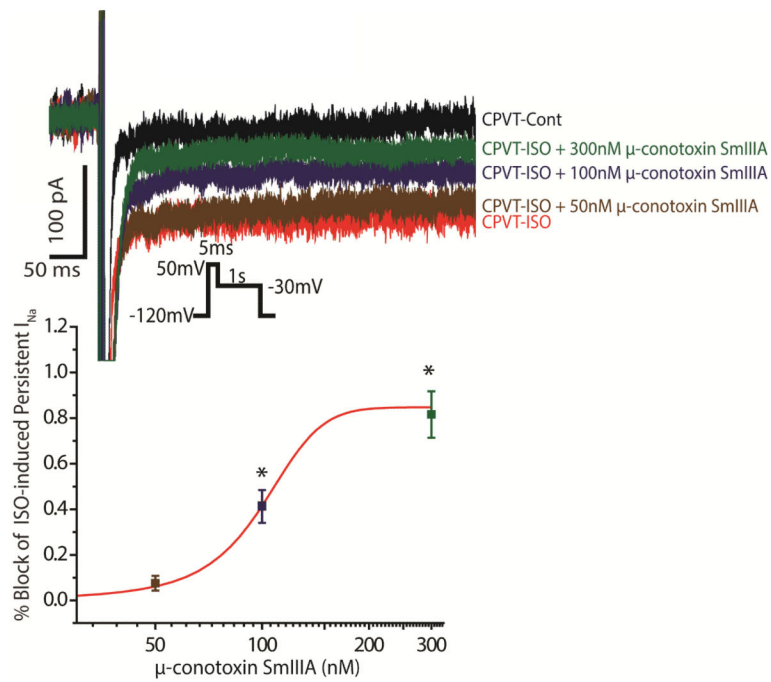
**Fig. 3. Proarrhythmic effect of  $\beta$ -AR stimulation on TTX-sensitive persistent  $I_{Na}$  involves CaMKII phosphorylation of  $nNa_v$  and is independent of RyR2 phosphorylation**

**(a)** Effect of  $\beta$ -AR stimulation on persistent  $I_{Na}$  is mediated through CaMKII. Representative traces of persistent  $I_{Na}$  recorded in CPVT cardiomyocytes exposed to CaMKII inhibitor KN93 (1  $\mu$ M) before and after treatment with 100 nM Iso ( $n = 9$  cells for both groups,  $p=0.14$  Wilcoxon signed-rank test). **(b)** CaMKII modulates DCR independent of RyR2 phosphorylation at S2814. Representative line-scan images recorded in CPVT ventricular cardiomyocytes as well as those expressing RyR2 that cannot be phosphorylated by CaMKII at site 2814 (S2814A). Myocytes were loaded with  $Ca^{2+}$  indicator, Fluo-3 AM and paced at 0.3 Hz. CaMKII inhibition with 1  $\mu$ M KN93 reduced DCR frequency in Iso treated CPVT cardiomyocytes ( $n=166$  and 105, respectively; \*,  $p = 0.001$  Wilcoxon rank-sum test). CPVT2814 cardiomyocytes did not evidence altered DCR frequency relative to Iso-treated CPVT myocytes; however, exposure of Iso-treated CPVT2814 cardiomyocytes to Ril 10  $\mu$ M significantly reduced DCR frequency relative to Iso alone ( $n = 57$  and 61 cells, respectively; \*,  $p=0.003$  Wilcoxon rank-sum test).



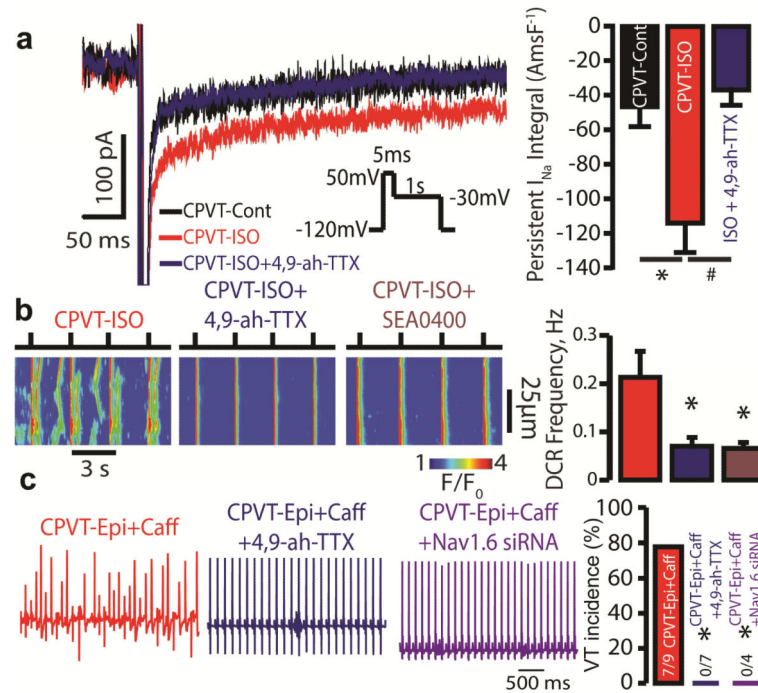


**Fig. 4. Neuronal Na<sup>+</sup> channels and RyR2 colocalize to the same discrete subcellular regions**  
**(a)** Representative confocal micrographs of isolated CPVT ventricular myocytes labeled for RyR2 (red) with various Na<sub>v</sub> isoforms (Na<sub>v</sub>1.x; green) often resulted in an overlap between the immunofluorescent (IF) signals (yellow) when overlaid. Panels on the right show 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 nNa<sub>v</sub> isoforms (Na<sub>v</sub>1.x). Below each image, are shown the results of digital segmentation with the cell mask in grey and PLA signal in red. **(c)** Plot of average number of PLA punctae per μm<sup>2</sup> ( $p < 0.001$  Kruskal-Wallis test; \*,  $p=0.002$ ,  $p=0.019$ ,  $p<0.001$  Wilcoxon rank-sum test for Na<sub>v</sub>1.6 vs. Na<sub>v</sub>1.1, 1.3 and 1.5, respectively;  $n = 1231$ ,  $1223$ ,  $1291$  and  $2848$  punctae from  $7$ ,  $6$ ,  $12$  and  $7$  cells for Na<sub>v</sub>1.1, 1.3, 1.5 and 1.6, respectively).

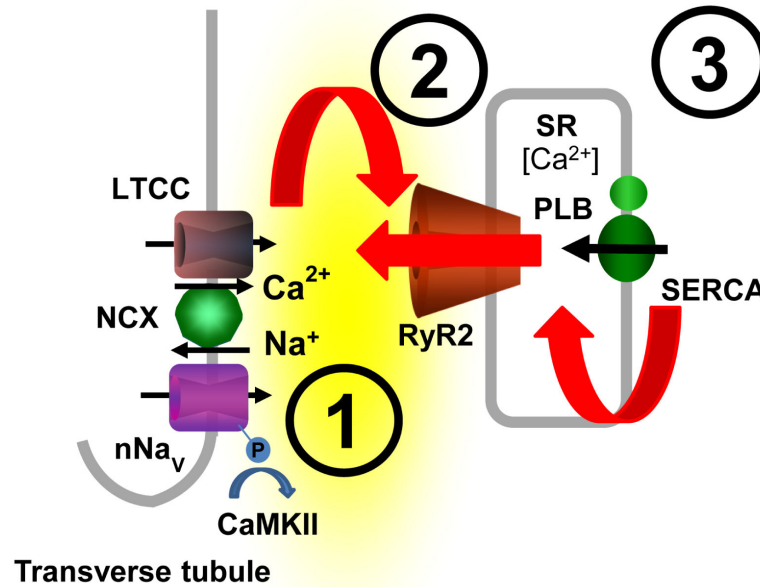


**Fig. 5. Dose response of ISO-induced persistent  $I_{Na}$  in CPVT cardiomyocytes to  $nNa_v$  blockade with  $\mu$ -conotoxin SmIIIA**

(Top) Representative traces of persistent  $I_{Na}$  recorded in CPVT cardiomyocytes exposed to ISO (100 nM and subsequent increasing concentrations of  $\mu$ -conotoxin SmIIIA (50, 100 and 300 nM). (Bottom) Summary of ISO-induced persistent  $I_{Na}$  dose response to  $\mu$ -conotoxin SmIIIA. ISO-induced persistent  $I_{Na}$  was not significantly affected by 50 nM  $\mu$ -conotoxin SmIIIA ( $n = 10$  for both CPVT-ISO and CPVT-ISO+50nM  $\mu$ -conotoxin SmIIIA,  $p = 1$  Wilcoxon rank-sum test), partially inhibited by 100 nM ( $n = 9$ ; \*,  $p = 0.048$  Wilcoxon rank-sum test vs. CPVT-ISO) and almost completely blocked by 300 nM ( $n = 6$ ; \*,  $p = 0.003$  Wilcoxon rank-sum test vs. CPVT-ISO).



**Fig. 6. Arrhythmogenesis in CPVT depends on  $Na_v1.6$ -mediated persistent  $I_{Na}$**   
**(a)** Effect of  $Na_v1.6$  blockade on ISO-induced persistent  $I_{Na}$ . Representative traces of persistent  $I_{Na}$  pre- and post-exposure to 100nM ISO in CPVT cardiomyocytes ( $p = 0.007$  Kruskal-Wallis test,  $n = 6$  for both; \*,  $p = 0.035$  Wilcoxon rank-sum test vs. CPVT-control). The persistent  $I_{Na}$  response to ISO was abolished by 300nM 4,9-anhydro-TTX (4,9-ah-TTX;  $n = 5$ ; #,  $p = 0.016$  Wilcoxon rank-sum test vs. CPVT-ISO). **(b)**  $Na_v1.6$  and NCX blockade reduce DCR. Representative line-scan images recorded in CPVT ventricular cardiomyocytes that were loaded with  $Ca^{2+}$  indicator, Fluo-3 AM and were paced at 0.3 Hz.  $Na_v1.6$  inhibition with 300nM 4,9-ah-TTX as well as NCX inhibition with SEA0400 (1  $\mu$ M) reduced DCR frequency in ISO treated CPVT cardiomyocytes ( $n = 99$ , 123 and 74 cells for ISO-4,9-ah-TTX, ISO-SEA0400 and ISO treated cells, respectively).  $p = 0.026$  Kruskal-Wallis test; \*,  $p = 0.031$  and \*,  $p = 0.032$  Wilcoxon rank-sum test for ISO-4,9-ah-TTX and ISO-SEA0400 vs. ISO, respectively). **(c)** Representative ECG recordings and summary VT incidence of CPVT mice exposed to catecholamine challenge (epinephrine and caffeine) as well as those with pharmacological or genetic inactivation of  $Na_v1.6$ . Pretreatment with 4,9-ah-TTX (750  $\mu$ g/kg I.P.) or administration of siRNA selectively targeting  $Na_v1.6$  prevented VT during catecholamine challenge ( $n = 9$ , 7 and 4 mice for CPVT-Epi+Caff, CPVT-Epi+Caff+4,9ah-TTX and CPVT-Epi+Caff+ $Na_v1.6$  siRNA, respectively). \*,  $p = 0.003$  and \*,  $p = 0.021$  Fisher's exact test for CPVT-Epi+Caff+4,9ah-TTX and CPVT-Epi+Caff+ $Na_v1.6$  siRNA vs. CPVT-Epi+Caff, respectively).



**Fig.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.<sup>68</sup> Based on 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 genetic defects in the RyR2 complex (i.e., RyR2, CaM, CASQ2, TRD and/or calstabin),<sup>2-5</sup> These along with **3**) increased SR  $\text{Ca}^{2+}$  load play a critical role in triggering aberrant DCR during  $\beta$ -AR stimulation in CPVT.