Supplemental materials

Stress induced ventricular arrhythmias in three CPVT mouse models

Mice harboring CPVT mutations (RyR2-R2474S+/−, RyR2-N2386I+/−, and RyR2-L433P+/−) were studied using two conventional stress protocols to demonstrate CPVT: 1, treadmill exercise plus epinephrine (EPI) injection1 and 2, isoproterenol (Iso) plus caffeine injection2. Following treadmill exercise plus EPI (2 mg/kg i.p.) injection, all (6/6) RyR2-R2474S+/− mice displayed typical bidirectional VT, while the RyR2-N2386I+/− and RyR2-L433P+/− mice failed to exhibit any VT (Supplemental Figure IA). We further examined arrhythmogenesis by treating the mice with Iso (0.5 mg/kg i.p.) plus caffeine (120 mg/kg i.p). Under these conditions, mice with CPVT mutations exhibited ventricular bigeminy and again, all RyR2-R2474S+/− mice developed bidirectional VT (Supplemental Figure IB). Thus, the 3 CPVT mouse models recapitulated the CPVT phenotype.

Generation of RyR2-N2386I knock-in mouse

The targeting vector for homologous recombination consisted of an 8.9-kb genomic DNA fragment including exons 44-51 of the RyR2 genomic sequence. The 5’ and 3’ flanking regions were amplified from 129S mouse genomic DNA using PCR with the following 2 sets of primers: 5’-ATGCGGCCGCAGGAATCCTAACATCCCCTTGC-3’ and 5’-TCATCGATGCACCTTATGAGATTTCTTGCCAACC-3’ (3’ flanking region) and 5’-TCAACAGCACTGAAAGGTGCCACC-3’ with 5’-TCCTGCCACAGCATAGGCACAGAGACC-3’ (5’ flanking region). The resulting PCR fragments were subcloned into the pBlueScript SK plasmid (Stratagene). Mutagenesis was performed in the 5’ flanking region using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions to introduce the codon change leading to the N2366I variant in exon 47.
(Supplemental Figure IIA). To facilitate screening for the mutation a BspH1 site was deleted. The two arms were subsequently cloned into SalI sites of pACN \(^3\). The resulting plasmid constitutes the TVN2386l targeting vector consisting of 2 arms separated by the pACN cassette. TVN2386l was linearized with SacII and transfected by electroporation into ES cells. Transfected ES cells underwent positive selection with G418 (200 μM) for 10 days. Genomic DNA from resistant clones was analyzed for occurrence of homologous recombination by Southern blotting. Recombinant ES cells were injected into C57BL/6 blastocyst-stage mouse embryos. Chimeric male mice were bred to C57BL/6 female mice to establish a hybrid line. Germline transmission generated RyR2+RyR2-N2368I (RyR2-N2386I\(^{+/−}\)) mice. The genotypes from the F1 generations were determined by Southern blotting and PCR on DNA using tail biopsy specimens. Genomic DNA extracted from the tail was digested with EcoRV and analyzed by hybridization with an external 5’probe. The 9.1-kb band corresponds to the WT allele, whereas the 6.0-kb band corresponds to the mutant allele (Supplemental Figure IIB). Mouse genotyping was performed using the following primers: 5’ - GGAATCAATTATTGGTGGAACA-3’ and 5’-ATCCTAACATGCCCTTGCAG-3’. A PCR product of 688 bp was digested with BspH1 which results in 2 specific bands at 409 bp and 279 bp from the WT but not the mutant allele (Supplemental Figure IIC).

**Generation of RyR2-L433P knock-in mouse**

A targeting construct for homologous recombination was constructed using the BAC modification system \(^4,5\). A 200 kb fragment of C57BL/6 BAC clone containing RyR2 genomic sequence was purchased from Cori.org. It was designed to introduce a leucine to proline mutation in E15 by galk selection \(^6\), along with a loxp-neo-loxp cassette and DTA cassette insertion in upstream of E15 by Red system (Supplemental Figure IIIA). This targeting construct containing BAC was linearized and electroporated into C57BL/6 129 sv hybrid embryonic stem cells. Targeted clones were identified by PCR using primer: 5’-TGGTTTCCTTTGCTGTGTTT
located in the region where the RyR2 sequences were replaced by DTA cassette, and 3’ primer: 5’-CCTACCGGTGGATGTGGAAT located in the neo cassette (Supplemental Figure IIIB). Two of these targeted clones were injected into blastocytes. Heterozygous mice carrying the targeted allele were obtained. They were mated with Ell2a Cre transgenic mice to remove the neo cassette flanked by a pair of loxp sites. Mice carrying the L433P knock-in allele were identified by a PCR genotyping using the following primer: 5’-AGCAAATGTCAGTGCTTGAAGA and 3’-CCTGAATCACAGCAAACACTTC (Supplemental Figure IIIC).

Generation of RyR2-R2474S knock-in mouse was as previously described ¹.

**Murine atrial myocytes isolation**

Atrial myocytes were isolated from WT and CPVT mice according to a modified version of AfCS Procedure Protocol PP00000125. Briefly, the heart was perfused with AfCS perfusion buffer, includes (mM): NaCl 113, KCl 4.7, KH₂PO₄ 0.6, Na₂HPO₄ 0.6, MgSO₄ 1.2, NaHCO₃ 12, KHCO₃ 10, Hepes 10, taurine 30, glucose 1.5 and BDM 10, for 5 min at a speed of 3ml/min after rapidly excised and canulated. The isolated heart was then perfused with digestion buffer (0.65 mg/mL Collagenase type 2 and 50 µM CaCl₂ in AfCS) for 10~15 min. After enzyme digestion of the heart was complete (heart appeared swollen, pale and flaccid), the atria were excised and teased into small pieces in stop 1 buffer (0.65 mg/mL Collagenase type 2, 0.065 mg/mL Protease XIV, 15 mg/mL BSA and 50 µM CaCl₂ in AfCS) and bath at 37°C for 10 min. Pipets were used to dissociate the heart tissue gently until all large pieces were dispersed. After separation from the enzyme by centrifugation for 4 min at 200 rpm, cells were resuspended in stop 2 buffer (15 mg/mL BSA and 50 µM CaCl₂ in AfCS) and recovered [Ca²⁺] to 1.2 mM. The cells were maintained in stop 2 buffer until use.

**Intracellular calcium measurements**
Atrial myocytes were loaded with 5 µM fluo-4 AM for 15 min at room temperature, then washed and maintained in K-H solution (mM): NaCl 125, KCl 4.75, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.2, HEPES 30, glucose 10, taurine 50, CaCl\(_2\) 1.2, titrated to pH 7.4. A Leica TCS SP2 confocal microscopy with 63x, 1.4 NA oil immersion objectives was used for confocal linescan imaging. The scan zoom was adjusted to fit the cells, and the scan line was along the long axis of cells. The excitation for Fluo-4 is 488 nm, and emission was collected at 505-530 nm. For Ca\(^{2+}\) sparks measurement, cells were scanned at 400 Hz for 20 s immediately after pacing at 0.5 Hz > 20 beats. For SR Ca\(^{2+}\) content measurement, cells were exposed to 10 mM caffeine immediately after pacing at 0.5 Hz for 1 min, and sampling started 10 s before caffeine treatment. For S107 treatment, cells were pre-treated with 10 µM S107 for 2 hrs before loading and imaging. Ca\(^{2+}\) sparks detection and analyses used algorithms described previously 7.

**Total SR Ca\(^{2+}\) leak**

The background noise of a confocal laser scan images follows a Poisson distribution. For a normalized linescan image, the background noise can be fit into a modified Gaussian distribution:

\[
N = N_0 \times e^{\frac{(x-1)^2}{b_i}}
\]

where, \(x\) is normalized fluorescence. \(b_i\), \(i=1\) for \(x<1\) and \(i=2\) for \(x>1\) (\(b_2\) is usually a bit greater than \(b_1\)). As shown in Supplemental Fig. IX, for a linescan fluo-4 fluorescence image (A) without Ca\(^{2+}\) sparks (which means rare Ca\(^{2+}\) release events), the histogram can be fit according to equation S1 (B), while for images with Ca\(^{2+}\) sparks, only the background can be fitted into Gaussian curves (C). The differences between the real curves and fitted background noises curves represent all the Ca\(^{2+}\) release signals pixels. Thus, we define a parameter for total Ca\(^{2+}\) release flux (total SR Ca\(^{2+}\) leak):
where, \( x \) is normalized fluorescence and \( \Delta N \) is the difference between real and fitted curves.

**Intra-esophageal burst pacing in mouse**

Intra-esophageal pacing was performed using either a 1.1-Fr octapolar catheter (EPR-800, Millar Instruments, Houston, Texas) or 1-Fr bipolar pacing catheter (model EP118-2, NuMED; Hopkinton, NY) placed in the esophagus close to the left atrium using an external stimulator (STG-3008, MultiChannel Systems, Reutlingen, Germany). A computerized data acquisition system (EMKA Technologies, Falls Church, VA) was used to record a 3-lead body surface ECG, and up to 4 intra-esophageal bipolar electrograms. Inducibility of atrial arrhythmias was tested by applying a series of 2-second bursts. The first 2-second burst had a cycle length (CL) of 40 ms, and CL was decreased in each successive 2-ms decrements until reaching a CL of 10 ms. These series of bursts were repeated once. AF was defined as a period of rapid irregular atrial rhythm lasting at least 1 sec. If 1 or more bursts in the 2 series of bursts evoked an AF episode, AF was considered to be inducible in that animal; otherwise, AF was considered to be non-inducible. All data were analyzed by two independent board certified electrophysiologists blinded to the genotype and treatment groups of the animals.

**Intra-cardiac burst pacing and ECG recording in mouse**

Mice were anesthetized with isoflurane (1.5-2%) in 100% oxygen. Animals were placed in supine position on a temperature-controlled surgical table to maintain rectal temperature at 37°C and were allowed to breathe spontaneously. Under a dissecting microscope (model SZ61, Olympus; Tokyo, Japan), the right jugular vein was isolated, and a 1.1-Fr octapolar catheter was
inserted (EPR-800, Millar Instruments, Houston, Texas). Intra-cardiac ECG was recorded showing typical ventricular, atrioventricular node, and atrial ECG. Atrial and ventricular pacing was established using a stimulator (STG-3008, MultiChannel Systems, Reutlingen, Germany).

**Immunoprecipitation and immunoblot analyses**

RyR2 was immunoprecipitated from cardiac homogenates (100 µg) using anti-RyR antibody (2 µl 5029 Ab) in 0.5 ml of a modified RIPA buffer (50 mM Tris-HCl pH 7.4, 0.9% NaCl, 5.0 mM NaF, 1.0 mM Na$_3$VO$_4$, 0.5% Triton-X100, and protease inhibitors) for 2 hrs at 4°C. The samples were incubated with protein A sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ) at 4°C for 1 hr and washed five times with 1.0 ml RIPA. Samples were heated to 95°C and size fractionated by PAGE (6% for RyR2, 15% for calstabin2). Proteins were transferred to nitrocellulose membranes and immunoblots were developed using the following antibodies: anti-calstabin (1:1,000), anti-RyR (5029; 1:3,000), anti-phospho-RyR2-pSer$^{2808}$ (1:5,000). Levels of RyR2 bound proteins were normalized to the total RyR2 immunoprecipitated (arbitrary units). All immunoblots were developed using the Odyssey system (LI-COR, Inc., Lincoln, NE) with IR labeled anti-mouse and anti-Rabbit IgG (1:10,000 dilution) secondary antibodies.

**RyR2 Oxidation**

To detect RyR2 protein oxidation, SR membrane samples (50 µg) were immunoprecipitated as described above. Immunoprecipitate was treated with 2, 4-dinitrophenyl hydrazine (DNPH) and the derivatized carbonyls were detected using an OxyBlot™ Protein Oxidation Detection Kit (Cat # S7150, Chemicon International, Inc., Temecula, CA). Proteins were size fractionated on 6% SDS-PAGE gels and transferred onto nitrocellulose membranes and immunoblots were developed with an anti-RyR antibody (Affinity Bioreagents, Bolder, CO)
1:2,000). The DNP signal associated with RyR was determined using an anti-DNP antibody (1:2000).
Supplemental figure I. Ventricular arrhythmias induced by exercise plus epinephrine and epinephrine plus caffeine in 3 CPVT mice. A) Upper panel: representative ECG traces recorded by telemetry transducers from WT (n=10), RyR2-R2474S+/− (n=16), RyR2-N2386I+/− (n=11), and RyR2-L433P+/− (n=5) after maximal treadmill exercise and epinephrine (2 mg/kg) injection. Lower panel: incidence of PVCs, ventricular bigeminy and bidirectional ventricular tachycardia (BVT) in WT and mice with RyR2 mutations. B) Upper panel: representative ECG traces recorded by surface ECG from WT (n=11), RyR2-R2474S+/− (n=11), RyR2-N2386I+/− (n=8), RyR2-L433P+/− (n=11) mice after ISO (0.5 mg/kg) plus caffeine 120 (mg/kg) injection. Lower panel: incidences of PVC, ventricular bigeminy and bidirectional ventricular tachycardia (BVT) in WT and mice with RyR2 mutations. *, P<0.05 vs. WT.
**Supplemental figure II. Generation of RyR2-N2386I knock-in mouse model.** A) Top line: the wild-type locus of the murine RyR2 gene containing Exon 44 -50. Second line: The targeting construct containing of 5’ homologous arm (4.1 kb) and 4.8 kb of 3’ homologous arm. The N2386I mutation was engineered in Exon 47. Third line: the homologous recombinant mutant allele containing the RyR2-N2386I mutation and ACN selection marker cassette. Bottom line: final RyR2-N2386I allele after excision of ACN selection marker. B) Southern blot result in screening RyR2-N2386I knock-in mice: genomic DNA extracted from tail of F1 mice digested with EcoRV and analyzed by hybridization with an external 5-probe. The 9.1 kb band corresponds to the WT allele whereas the 6kb band corresponds to the Mutant allele. C) PCR result in screening RyR2-N2386I knock-in mice: mouse genotyping was performed using the following primers: 5’-TCCTAACATGCCCTTGAG-3’ and 5’-GGAATCAATTATTGGTGGAAACA-3’. PCR product of 688bp was digested with BspHI and resulted in 2 specific band at 409bp and 279bp from WT but not the mutant.
Supplemental figure IV. Echocardiography and histology studies showed no difference between WT and 3 CPVT mouse models. A) Echocardiography results in 6-8 months old WT (n=12), RyR2-R2474S^{+/−} (n=8), RyR2-N2386I^{+/−} (n=9), RyR2-L433P^{+/−} (n=8). B) Representative histology sections using Masson’s trichrome stain for atrium, left and right ventricles from WT, RyR2-R2474S^{+/−}, RyR2-N2386I^{+/−}, and RyR2-L433P^{+/−} mice. Bar = 10 μm.
Supplemental figure V. Preventing CaMKII phosphorylation of RyR2 does not impact the prevalence of burst pacing-induced AF in heart failure. Prevalence of burst pacing-induced AF in WT (n=10) and RyR2-S2814A mice (n=11) before (Pre-MI) and 4 weeks post myocardial infarction (HF). The heart function data was published previously. 

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Supplemental figure VI. Metoprolol treatment for 4 weeks does not decrease the prevalence of burst pacing-induced AF in all 3 CPVT mouse models. Prevalence of burst pacing-induced AF in RyR2-R2474S\textsuperscript{+/-} (n=10), RyR2-N2386I mice (n=14), and RyR2-L433P (n=9) treated by vehicle or metoprolol (30 mg/kg/d) for 4 weeks.
Supplemental figure VII. DTT treatment decreased Ca\(^{2+}\) leak in atrial myocytes from RyR2-R2474S\(^{+/-}\) mice. Atrial myocytes were bathed in imaging buffer with 5 mM DTT for 3 min before sampling. (DTT is membrane-permeable, and the experiment are done with intact cells). \(n = 20\text{-}25\) cells in each group. **, \(p<0.01\) vs WT group; # and ##, \(p<0.05\) and 0.01 vs R2474S group.
Supplemental figure VIII. Line scan of atrial myocytes isolated from RyR2-R2474S+/− mice showed increased DAD/EADs comparing to WT and S107 treated animals. Atrial myocytes were bathed in 2mM [Ca^{2+}] imaging buffers. n = 20–24 cells from 3 hearts in each group. **, p<0.01 vs WT group; ##, p<0.01 vs R2474S group.
Supplemental figure IX. Line scan of atrial myocytes isolated from RyR2-R2474S+/− mice showed spontaneous Ca^{2+} waves comparing to WT and S107 treated animals. Atrial myocytes were bathed in 2mM [Ca^{2+}] imaging buffers and pacing at 3 Hz for at least 1 minute. After stop pacing, 8 out of 21 atrial myocytes in R2474S group occurred Ca^{2+} wave, which is obviously higher than WT (0/9) and R2474S+S107 (2/14) groups.
Supplemental figure X. Total SR Ca^{2+} leak measurement. A) A confocal line scan image for cardiac myocyte loading with fluo-4, which shown rare Ca^{2+} release. B) Histogram of normalized images in A (blue) can be well fitted into Gaussian distribution (red). C) Histogram curves (blue) and fitted curves (blue) for Ca^{2+} images of atrial myocytes from WT (upper) and RyR-R2474S^{+/−} (lower) mice. The differences between real and fitted curves show the total leak.
Supplemental figure XI. Amplitudes and Spatio-temporal characters of Ca^{2+} sparks in atrial myocytes isolated from different mice models.
Supplemental figure XII. CaMKII phosphorylation of RyR2 in atrial tissues freshly isolated from both WT and RyR2-R2474S+/− mice immediately after atrial burst pacing.
References


