SUPPLEMENTAL DATA

Calmodulin kinase II-mediated sarcoplasmic reticulum Ca^{2+} leak promotes atrial fibrillation in mice

SUPPLEMENTAL MATERIALS AND METHODS

Reverse transcription polymerase chain reaction and Western blot analysis

Total RNA was extracted from atrial tissue using TRIzol (Invitrogen Life Technologies) and 1 μg was used as template for reverse transcription (SuperScript II- reverse transcriptase-H, Invitrogen Life Technologies) to generate first-strand cDNA. RyR2, LTCC, NCX, SERCA2a, and PLN cDNA sequences were detected from the first-strand cDNA mixture using specific primers (1).

Protein extraction and Western blotting was performed as previously described (1). Briefly, mouse atrial whole cell lysates were subjected to electrophoresis on 6% (for RyR2, Cav1.2, NCX1, SERCA2a, and Tubulin) and 20% (for PLN and Tubulin) acrylamide gels, and transferred onto polyvinyl difluoride membranes. Membranes were probed with monoclonal anti-RyR2 (1:5,000; Affinity BioReagents), anti-Cav1.2 (1:200; Sigma), polyclonal anti-NCX1 (1:500; Swant), polyclonal anti-SERCA2a (1: 500; Santa Cruz Biotechnology), polyclonal anti-PLN (1:5,000, Affinity BioReagents), polyclonal anti-Thr17-phosphorylated PLN (1:5,000, Badrilla), polyclonal anti-Thr287-phosphorylated CaMKII (1:5,000; Promega), anti-CaMKIIδ (1:200; Santa Cruz Biotechnology), anti-GAPDH (1:5000; Millipore), polyclonal anti-Tubulin (1:2000; Abcam), anti-calsequestrin (CAQ, 1:2500; Dianova), antibodies, at room temperature (RT) for 5 hours. The pSer2808-RyR2 (1:1000) and pSer2814-RyR2 (1:1000) phosphoepitope-specific antibody were custom generated using the peptide C-RTRRI-(pS)-QTSQV corresponding to the PKA phosphorylation site region at serine 2808 on RyR2 (2) and peptide CSQTSQV-(pS)-VD corresponding to RyR2 CaMKII phosphorylated at Ser2814 (3), respectively. Membranes were then incubated with secondary anti-mouse and anti-rabbit antibodies conjugated to Alexa-Fluor 680 (Invitrogen Molecular Probes) and IR800Dye (Rockland Immunochemicals), respectively, and bands were quantified using infrared visualization and densitometry

(Odyssey System).

Calcium transient measurements

Mouse hearts were perfused in a Langendorff apparatus with Joklik Minimum Essential Medium (JMEM) solution for 5 min, followed by digestion solution containing 0.1 mg/ml Liberase Blendzyme IV (Roche). Single atrial myocytes were isolated from dissected and triturated atria and incubated with 5 μmol/L Fluo-4 AM (Invitrogen) for 15 min at RT in JMEM. Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured using an illumination device (model Lambda DG-4, Sutter Instruments), and an electro-multiplier intensified back-illuminated CCD camera (model Cascade 512B, Photometrics).

Myocytes were preconditioned in 1.8 mM Ca^{2+} normal Tyrode solution for 15 seconds by pacing delivered by platinum electrodes. $[Ca^{2+}]_i$ was measured in paced atrial myocytes for 30 seconds at 1 Hz. Steady state $[Ca^{2+}]_i$ was then monitored after perfusate was switched to a 0 Na^+ 0 Ca^{2+} solution which blocks the Ca^{2+} exchange via the Na^+/Ca^{2+} exchanger and the L-type Ca^{2+} channel. Acute application of tetracaine (5 μmol/L) was used to rapidly and reversibly block RyR2, thus disrupting the SR pump/leak balance, followed by application of caffeine to estimate steady-state SR Ca^{2+}. The tetracaine-dependent shift of Ca^{2+} from cytosol to SR (decrease in cytosolic $[Ca^{2+}]_i$ and increase in SR Ca^{2+} content) is proportional to SR Ca^{2+} leak in the absence of tetracaine (4). The same protocol was repeated for atrial myocytes pre-incubated in normal Tyrode solution supplemented with 1 μmol/L KN-93 or KN-92.

Atrial mapping studies

Mice (25~30 g) were anesthetized with 250 mg/kg i.p. tribromoethanol and anticoagulated with 1,000U/kg i.p. heparin. Hearts were rapidly removed and retrogradely perfused with oxygenated Tyrode's solution (containing (mM): 136 NaCl, 5.4 KCl, 1 MgCl2, 0.33 NaH2PO4 1.2, 10 HEPES, 10 glucose, pH adjusted to 7.4 with HCl) containing 1.8 mM CaCl2 at 37°C in a Langendorff apparatus. The perfusion pressure was maintained constant at ~60-65 mmHg and monitored using an in-line physiological pressure transducer connected to a Bridge Amplifier (AD Instruments Inc).

Staining for intracellular Ca^{2+} was achieved with 0.2 mg Rhod-2 AM dissolved in 0.2 mL dimethyl sulfoxide containing Pluronic F-127 (20% wt/vol). This solution, diluted in 150 ml of Tyrode's solution to achieve a final Rhod-2 concentration of 1.18 μmol/L, was infused into the heart over a 30-minute period. To prevent motion artifacts, blebbistatin at a final concentration of 5 μmol/L was applied as an excitation-contraction uncoupler (5). The heart was then stained with the voltage-sensitive dye RH237 (0.33 μM) for 10 minutes to monitor changes in membrane potential. Both dyes were from Invitrogen. After staining atria were cut and imaged in a chamber perfused with Tyrode containing 1.8 mM CaCl2. Green light (520±20nm) from a 532 nm laser light source (B&W TEK Inc) was shone directly onto the atrial tissue. Emitted fluorescence was split by a 630 nm dichroic mirror and collected using 2 electron-multiplying CCD cameras (Cascade 128+, Photometrics) covering the same mapped field. A grid was used to calibrate the field of view of the 2 CCD cameras. For intracellular Ca^{2+}, reflected fluorescence was collected through a 585±20 nm filter. For membrane voltage, passed fluorescence was collected through a 710 nm long pass filter.

SUPPLEMENTAL REFERENCES

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1. R176Q/+ knock-in mice do not develop spontaneous atrial fibrillation. **A.** Example of Lead I ECG recording in conscious R176Q/+ mice by telemetry. **B.** Example of Lead II surface ECG in R176Q/+ and WT anesthetized mice. P- P wave; QRS- QRS complex. **C.** Example of intracardiac atrial electrogram in R176Q/+ and WT anesthetized mice during EP studies. A- atrial wave; V- ventricular wave. **D.** Example of intracardiac ventricular electrogram in R176Q/+ and WT anesthetized mice during EP studies. A- atrial wave; V- ventricular wave.

Figure S2. Baseline CaMKII-Thr287 phosphorylation in R176Q/+ atria is similar to WT atria. Examples of CaMKII and phosphorylated CaMKII-Thr287 in atria of R176Q/+ and WT mice. Bottom: Mean ± SEM protein-band intensities phosphorylated CaMKII-Thr17 relative to total CaMKII (n>4).

Figure S3. Pacing does not induce an increase in RyR2 phosphorylation at the PKA site Ser2808. Representative Western blots of total RyR2 and Ser2808 phosphorylated RyR2 at baseline and after pacing in WT and R176Q/+ atria. Bar graph showing quantification of Western blots band densities. The ratios (of RyR2-pSer2808 density divided by RyR2 density) presented in the bar graph represent average data of 4 mice in each group. Cardiac pacing was performed as described in the Methods section.

Figure S4. R176Q/+ knock-in mice exhibit normal atrial dimensions and morphology. A. Representative four-chamber section of a WT heart stained with hematoxylin-eosin. Scale represents 1 mm. **B.** Representative four-chamber section of a R176Q/+ heart stained with hematoxylin-eosin. **C.** Representative section of WT right atria stained with Mason Trichrome. Scale represents 200 μm. **D.** Representative section of R176Q/+ right atria stained with Mason Trichrome.

Figure S5. Atrial expression levels of major Ca^{2+} handling proteins are unaltered by the R176Q

mutation in RyR2. **A.** Expression levels of RyR2 (cardiac ryanodine receptor), Cav1.2 (L-type voltage sensitive Ca2+ channel), SERCA2a (sarcoplasmic/endoplasmic reticulum Ca^{2+} ATP-ase), PLN (phospholamban), and NCX1 (Na^{+}/Ca^{2+} exchanger) were determined by RT-PCR and densitometry and normalized to L7. **B.** Representative examples of RT-PCR products for RyR2, Cav1.2, SERCA2a, PLN, and NCX1 from atria of R176Q/+ and WT mice. **B.** Representative examples of Western blots for RyR2, Cav1.2, SERCA2a, PLN, NCX1, Tubulin from atria of R176Q/+ and WT mice. **D.** Quantification of protein **e**xpression levels of RyR2 Cav1.2, SERCA2a, PLN, and NCX1 by densitometry and normalization to the Tubulin.

Figure S6. Sequential voltage isochronal maps showing reentry in R176Q/+ atria. Simultaneous recordings of voltage (Fv) and Ca^{2+} fluorescence (FCa) traces in R176Q/+ mouse atria at pacing cycle length of 140 ms, demonstrating an ectopic beat (beat 3) occurring during the decline of the Ca^{2+} transient corresponding to beat 2. The ectopic beat initiates reentry that sustains for 5 rotations as shown in sequential voltage isochronal maps corresponding to beats 3-7 (bottom). Note the subtle variations in activation pattern and reentrant cycle length.

Figure S7. Ser2808 phosphorylation of RyR2 is increased in patients with chronic AF and CREM-IbΔC-X transgenic mice. A. Western blot analysis of total RyR2 and Ser2808 phosphorylated RyR2 in atrial biopsies of patients in sinus rhythm (SR) or chronic atrial fibrillation (AF), showing increased Ser2808 phosphorylation in chronic AF. **B.** Western blot analysis showing no increase of Ser2808 phosphorylation in goals with lone AF or goats in sinus rhythm (SR). **C.** CREM-IbΔC-X transgenic mice with spontaneous AF exhibit increased Ser2808 phosphorylation on RyR2. Bar graphs show mean ± SEM protein-band intensities of phosphorylated RyR2-Ser2808 relative to total RyR2. 'n' inside bar indicates number of mice tested. * $P < 0.05$.

Supplemental Movie. 1. Ectopic activity and reentry in paced R176Q/+ atria. Movie corresponds to the

second paced beat and ensuing ectopic activity and reentry from Fig 5C.

SUPPLEMENTAL TABLES

Table S1. Measurement of surface ECG and electrophysiology parameters in WT and R176Q/+ mice. PR- interval from beginning of P waves to the peak of R wave; RR- interval between two consecutive R wave peaks; QRS - duration of interval between beginning of Q wave to peak of S wave; QTc - duration of QT interval corrected for heart rate; HR- heart rate; SNRT- sinus node recovery time after 30 s rapid atrial pacing; AERP – atrial effective refractory period; AVNERP - atrioventricular nodal effective refractory period. NS, non-significant.

	WT (n=15)	**R176Q/+ (n=14)**	**T-test**
PR (ms)	41.7±1.5	43.4±1.2	NS
RR (ms)	115±3.2	120.2±3.1	NS
QRS (ms)	10.0±0.3	10.3±0.2	NS
QTc (ms)	23.9±0.7	25.9±1.3	NS
HR (bpm)	530±12	509±12	NS
SNRT (ms)	153.6±4	161.1±7	NS
AERP (ms)	42.0±0.8	43.1±1.3	NS
AVERP (ms)	49.2±0.7	51.1±0.9	NS

Table S2. Measurement of surface ECG and electrophysiology parameters in R176Q/+ mice before and after administration of 10-30 µmole/kg KN-93 i.p. PR- interval from beginning of P waves to the peak of R wave; RR- interval between two consecutive R wave peaks; QRS- duration of interval between beginning of Q wave to peak of S wave; QTc- duration of QT interval corrected for heart rate; HR- heart rate; SNRT- sinus node recovery time after 30 s rapid atrial pacing; AERP – atrial effective refractory period; AVNERP- atrioventricular nodal effective refractory period. NS, non-significant.

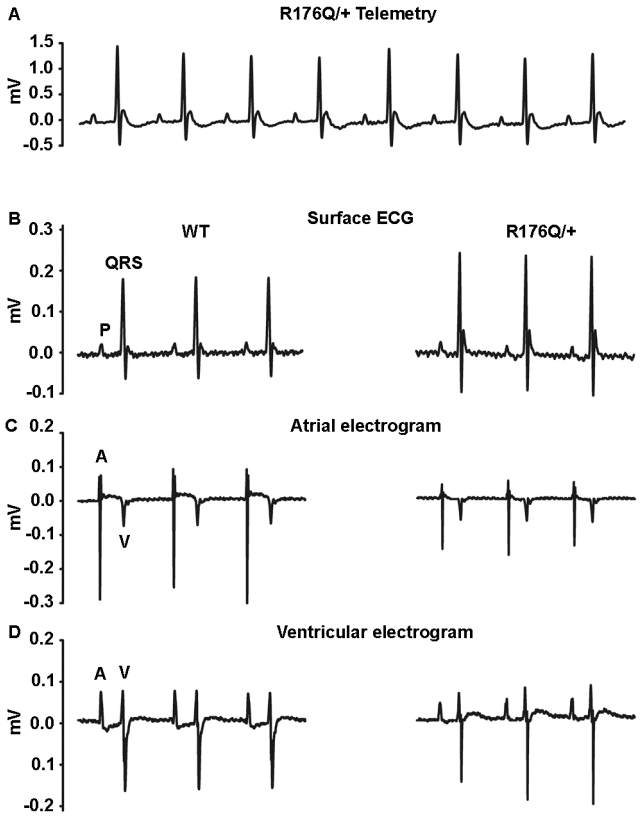
	Before KN-93 (n=8)	**After KN-93 (n=8)**	**T-test**
PR (ms)	44.1±2.0	42.6±1.2	NS
RR (ms)	113.4±2.4	115.1±2.1	NS
QRS (ms)	10.0±0.3	10.4±0.3	NS
QTc (ms)	25.8±1.9	26.6±2.3	NS
HR (bpm)	530±12	523±10	NS
SNRT (ms)	153.6±4	167.6±9.2	NS
AERP (ms)	44.5±2.3	46.4±1.8	NS
AVERP (ms)	50.5±1.5	52.4±2.2	NS

Table S3. Patient characteristics.

	SR	cAF
Patients, n	7	7
Gender, M/ F	4 / 3	4 / 3
Age, years	61.4±3.3	69.0 ± 4.7
Body mass index, kg/m^2	30.8 ± 2.5	26.7 ± 0.9
CAD, n	2	1
MVD/AVD, n	2	4
CAD + MVD/AVD, n	3	2
Hypertension, n	4	6
Diabetes, n	4	0
Hyperlipidemia, n	5	5
LVEF, %	49.6 ± 4.2	50.1 ± 5.9
LVEDD, mm	51.9 ± 3.1	52.6 ± 5.1
LA, mm	43.7 ± 1.5	47.0 ± 4.6
Digitalis, n	0	0
ACE inhibitors, n	5	2
AT1 blockers, n	1	1
β-Blockers, n	3	6
Ca^{2+}-antagonists, n	0	3
Diuretics, n	5	3
Nitrates, n	1	3
Lipid-lowering drugs, n	4	2

SR, sinus rhythm; cAF, chronic atrial fibrillation; CAD, coronary artery disease; MVD, mitral valve disease requiring valve replacement; AVD, aortic valve disease requiring valve replacement; LVEF, left ventricular ejection fraction; LVEDP, left ventricular end-diastolic pressure; LA, left atria diameter; LVEDD, left ventricular end-diastolic diameter; ACE, angiotensin-converting enzyme; AT, angiotensin receptor. $P<0.05$ calculated from nonpaired Student's t test for continuous variables and from χ^2 test for categorical variables.

Figure S1. R176Q/+ knock-in mice do not develop spontaneous atrial fibrillation.



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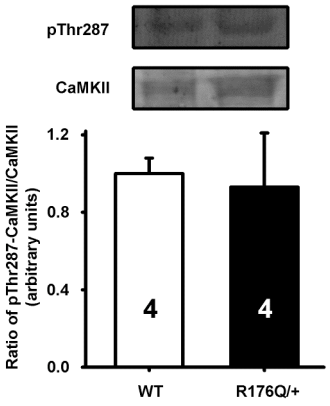

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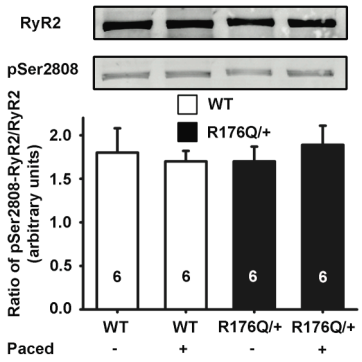

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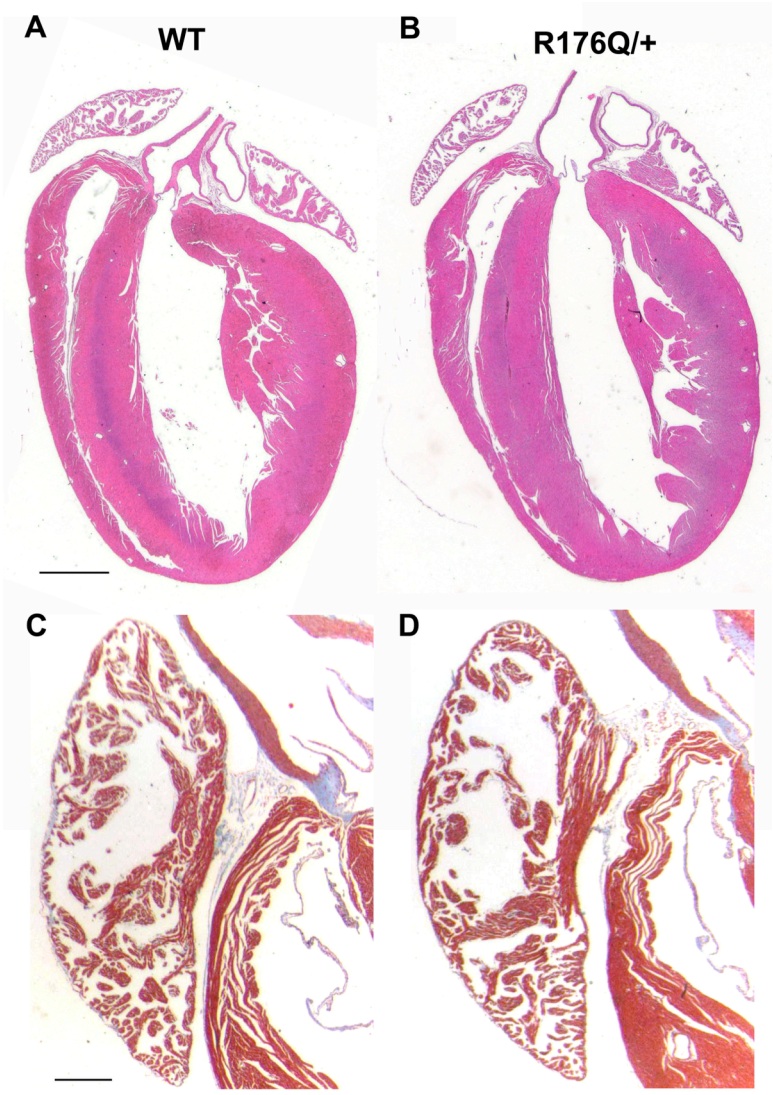

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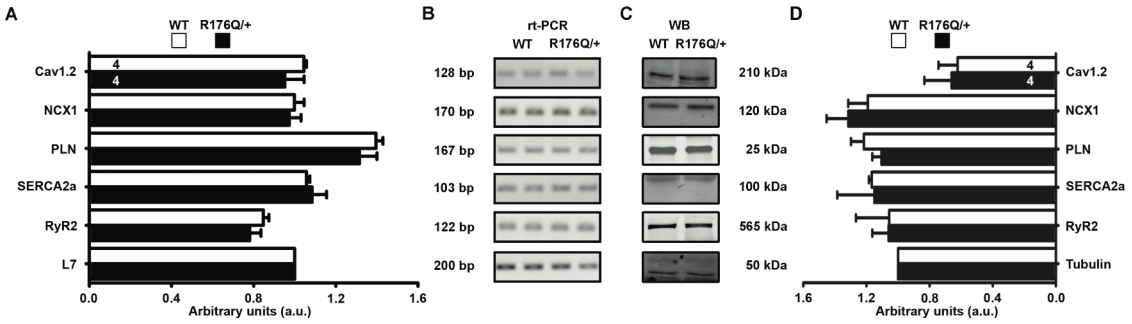

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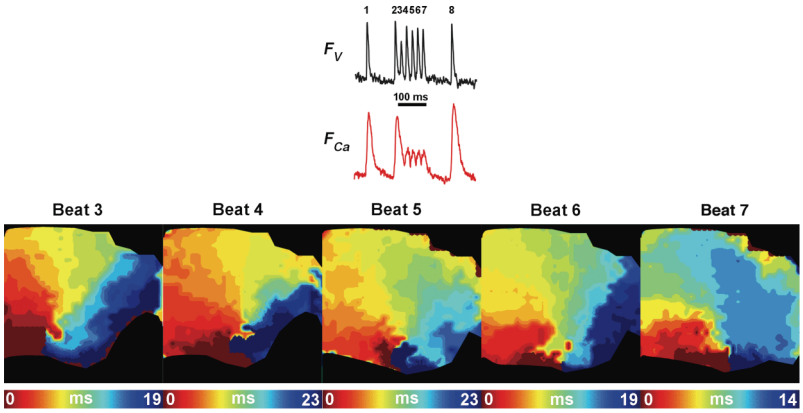

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