Supporting Online Materials

Supplemental Methods

SERCA2a activity

SERCA2a activity was measured using the malachite green procedure for phosphate determination, as previously described (1). The reaction was started by the addition of 100 µg of microsomes to 150 µl of reaction mixture [20 mM MOPS/Tris-HCl, pH 6.8. 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 1 mM EGTA, 0.350 mM $CaCl_2$ (free Ca^{2+} concentration of approximately 500 nM as calculated using the CHELATOR program). After 5 min, the reaction was stopped by the transfer of 120 µl of reaction mixture to 80 µl of malachite green reagent mixture in a 96-well microplate. The malachite green reagent mixture was made by mixing 0.122% malachite green hydrochloride in 6.2 N H_2SO_4, 5.76% ammonium paramolybdate tetrahydrate, and 11% Tween 20 in a volume ratio of 100:66:2. Color development was quenched after 10 s by the addition of 45 µl of 15.1% sodium citrate dihydrate. Inorganic phosphate liberated in the ATPase reaction was quantified by comparison of absorbance at 570 nm with standard curves generated with known amounts of Na_2HPO_4 in the reaction buffer.

Cardiomyocyte patch-clamp analyses

Cardiomyocytes were isolated using collagenase digestion. For whole-cell patchclamp recording, the external solution consisted of (in mM): NaCl 130, CsCl 5,HEPES 10, Glucose 5, $MgCl_2$ 1.2, $CaCl_2$ 2 and TTX 0.03, pH 7.4 adjusted with

CsOH. The pipette solution was composed of (in mM): aspartic acid 50, $ATPNa_2$ 5, CsCl 60, EGTA 0.05, HEPES 10, $MgCl_2$ 1 and $CaCl_2$ 0.0215, pH 7.2 adjusted with CsOH. Membrane currents were measured using whole-cell procedures with an Axopatch 200B amplifier (Axon Instruments Inc.). The cells were clamped at -40 mV and depolarized for 500 ms every 4 seconds using 10 mV incremental steps from -40mV to +70mV. Membrane currents were low pass filtered at 2 kHz and sampled at 20 kHz. All measurements were obtained at room temperature (22℃) (2).

Sinoatrial nodal cells

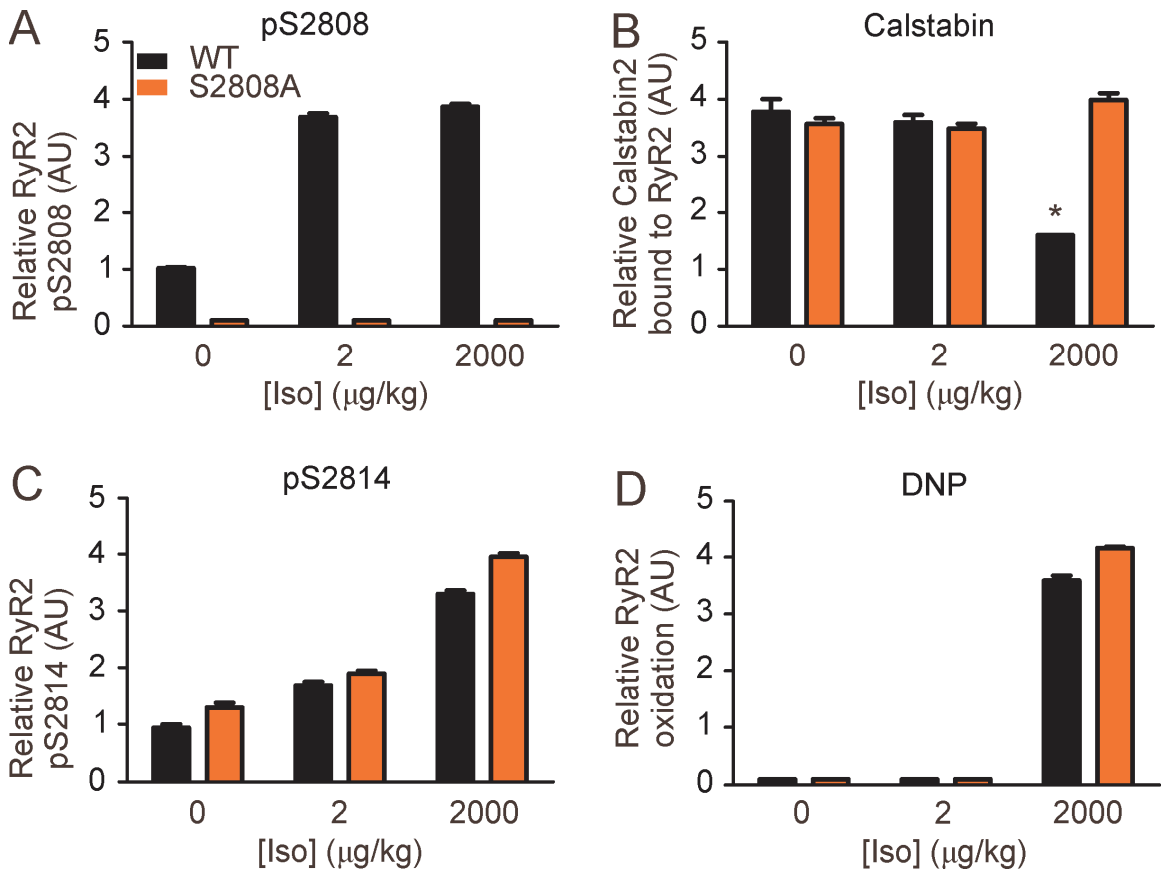
SANC were isolated from mice as previously described (3-4). Briefly, following treatment with heparin, mice were anesthetized and sacrificed. The heart was placed in chilled Tyrode's solution (4ºC): (in mmol/L) 140 NaCl, 5.4 KCl, 1.2 KH_2PO_4, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 5.55 glucose, and 5 HEPES, with pH adjusted to 7.4 (NaOH). The SAN region was identified, cut into small strips, and transferred to a second solution: (in mmol/L) 140 NaCl, 5.4 KCl, 1.2 KH_2PO_4, 0.2 $CaCl_2$, 50 taurine, 18.5 glucose, 5 HEPES and 1 mg/ml bovine serum albumin (BSA), with pH adjusted to 6.9 (NaOH). Small tissue strips were transferred into a warmed bath (35º C) of the same solution containing protease (type XIV), collagenase (type II), and elastase for exactly 30 min. The resulting digested tissue was moved to KB solution: (in mmol/L) 100 potassium glutamate, 10 potassium aspartate, 25 KCl, 10 KH_2PO_4, 2 $MgSO_4$, 20 taurine, 5 creatine, 0.5 EGTA, 20 glucose, 5 HEPES, and 0.1% BSA, with pH adjusted to 7.2 (KOH) and agitated with a glass pipette. After moving the cells into normal Ca^{2+}, individual SANCs

were isolated based on morphology and spontaneous contractions. Spontaneous action potentials (APs) were recorded using the perforated patch clamp technique on single SANC. APs were recorded at 35±1ºC. The effects of Isoproterenol (50-100 nM) on spontaneous AP frequency were also investigated. HCN4 antibody staining on isolated SANC was performed as previously reported (5).

Evaluation of apoptosis

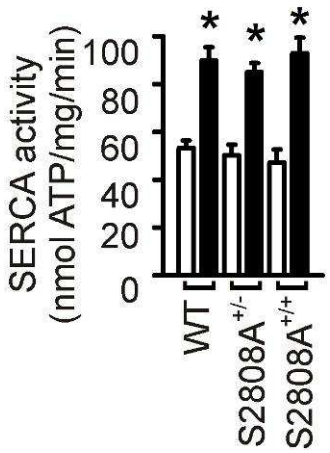
Apoptotic cell death was evaluated using the terminal deoxynucleotidyl transferase enzyme for nick end labeling (TUNEL) method using in Situ Cell Death kit (Roche Diagnostics, IN) (6). In brief, three sections were made from each sample to make a clear judgment for each experiment. Before tissues were analyzed for apoptosis, tissue sections were deparaffinized with xylene and washed in succession with different concentrations of ethanol (absolute, 95%, and 70%). Tissues were then treated with proteinase K for 15 min at room temperature, excess liquids were carefully blotted around the sections, 1×equilibrium buffer was applied directly on the specimens, and specimens were placed in a humidified chamber for 5 min at room temperature. The specimens were then washed twice with PBS, stained with the TUNEL reaction mixture for 60 min at 37℃, washed twice with PBS, and labeled with peroxidase-conjugated goat Ab for 30 min at 37℃. DNA fragmentation was d etected by staining with diaminobenzidine and observed under a microscope (Olympus IX51, NY).

Supplemental Figure 1: Quantification of immunoblots from Figure 1C



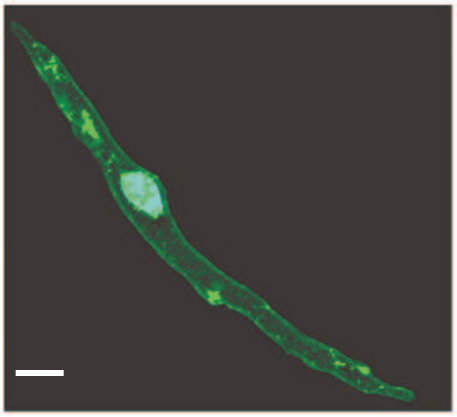
Bar graphs summarizing degree of (A) PKA phosphorylation of RyR2-Ser2808, (B) calstabin2 associated with the channel, (C) CaMKII phosphorylation of RyR2-Ser2814, (D) Oxidation status of the channel. RyR2 were immunoprecipitated from 100 μg cardiac lysates *, $P < 0.05$.

Supplemental Figure 2: SERCA2a activity in response to Iso



SERCA2a activity was increased to the same degree by Iso treatment in WT, RyR2-S2808A$^{+/-}$ and RyR2-S2808A$^{+/+}$ mice.

Supplemental Figure 3: Immunocytochemistry of SANC



Identity of sino-atrial nodal cells were confirmed by staining with anti-HCN4 antibody (white bar = 10μm)

Supplemental Table 1: ß-AR densities in WT and RyR2-S2808A$^{+/+}$ mice

Mouse	B_{max} (fmol/mg)	K_d (nmol/L)
WT	17.5 ± 1.4	5.3 ± 0.4
RyR2-S2808A$^{+/+}$	18.2 ± 1.8	5.1 ± 0.4

β-adrenergic receptor density in hearts from WT and RyR2-S2808A$^{+/+}$ mice.

Supplemental Table 2: Cardiac response to Iso in WT and RyR2-S2808A$^{+/+}$ mice

Genotype	WT	*S2808A*$^{+/+}$
Baseline		
n	16	17
BW (g)	29.88 ± 2.0	30.12 ± 2.1
LVEDD (mm)	3.02 ± 0.10	2.91 ± 0.05
LVESD (mm)	1.64 ± 0.09	1.68 ± 0.02
EF (%)	80.11 ± 0.94	81.03 ± 0.45
Heart rate (min^{-1})	486 ± 11.92	498 ± 11.49
dP/dt$_{max}$ (mmHg/s)	7410 ± 352.9	7616 ± 397.2
dP/dt$_{min}$ (mmHg/s)	-5489 ± 418.5	-6347 ± 347.3
(dP/dt$_{max}$)/P$_{id}$ (s^{-1})	82.30 ± 2.65	79.07 ± 2.70
(dP/dt$_{min}$)/P$_{id}$ (s^{-1})	-60.23 ± 3.11	-65.44 ± 1.98
LVESP (mmHg)	88.76 ± 4.89	94.88 ± 3.04
ISO 100 ng kg^{-1} min^{-1}		
Heart rate (min^{-1})	574.67 ± 11.30†	520 ± 8.62#
dP/dt$_{max}$ (mmHg/s)	14365 ± 594.4†	9814 ± 402.1†#
dP/dt$_{min}$ (mmHg/s)	-9355 ± 771.3†	-8015 ± 364.6‡
(dP/dt$_{max}$)/P$_{id}$ (s^{-1})	133.41 ± 4.31†	91.77 ± 4.28‡#
(dP/dt$_{min}$)/P$_{id}$ (s^{-1})	-85.16 ± 4.48†	-73.83 ± 1.94‡*
LVESP (mmHg)	105.97 ± 9.12	97.13 ± 2.34

†P<0.001 vs. baseline; ‡P<0.05 vs. baseline; #P<0.001 vs. WT; *P<0.05 vs. WT

Summary of *in vivo* cardiac function studies in RyR2-S2808A$^{+/+}$ and age-matched wild-type (WT) littermate control mice in the absence and presence of isoproterenol (Iso). Average data of left ventricular contractility measurements, BW, body weight; heart rate in beats per minute (bpm); LVEDD, left ventricular end diastolic dimension; LVESD, left ventricular end systolic dimension; EF, ejection fraction; LVESP, left ventricular end systolic pressure (measure of systolic blood pressure); dP/dt$_{max\ or\ min}$, maximum or minimum rate change in LV

pressure, $dP/dt_{max\ or\ min}$/Pid, $dP/dt_{max/min}$ normalized to instantaneous developed pressure.

Supplemental Table 3: Cardiac response to DBcAMP in RyR2-S2808A$^{+/+}$ mice

Genotype	WT	RyR2-S2808A$^{+/+}$
Baseline		
n	7	8
Heart rate (min^{-1})	471 ± 15.26	486 ± 18.09
E_{es} (mmHg/µl)	2.04 ± 0.14	2.06 ± 0.08
dP/dt_{max} (mmHg/s)	7990 ± 112.7	8303 ± 262.2
dP/dt_{min} (mmHg/s)	-7215 ± 221.9	-7480 ± 228.6
$(dP/dt_{max})/P_{id}$ (s^{-1})	81.62 ± 3.65	82.47 ± 3.54
$(dP/dt_{min})/P_{id}$ (s^{-1})	-73.15 ± 2.38	-74.09 ± 2.56
DBcAMP 10 mg kg^{-1}		
Heart rate (min^{-1})	577 ± 11.74*	541 ± 4.50#
E_{es} (mmHg/µl)	12.75 ± 1.69	3.26 ± 0.38‡
dP/dt_{max} (mmHg/s)	14022 ± 137.7*	9710 ± 607.8‡
dP/dt_{min} (mmHg/s)	-10144 ± 328.5*	-7861 ± 449.9#
$(dP/dt_{max})/P_{id}$ (s^{-1})	117.16 ± 8.31†	92.04 ± 5.09#
$(dP/dt_{min})/P_{id}$ (s^{-1})	-85.38 ± 8.05	-74.40 ± 3.39

*P<0.001 vs. baseline; †P<0.05 vs. baseline; ‡P<0.001 vs. WT; #P<0.05 vs. WT

Summary of *in vivo* cardiac function studies in RyR2-S2808A$^{+/+}$ and age-matched wild-type (WT) littermate control mice in the absence and presence of db-cAMP. Average data of left ventricular contractility measurements; Heart rate in beats per minute (bpm); E_{es}, systolic elastance as determined from the endsystolic pressure-volume relationship; $dP/dt_{max\ or\ min}$, maximum or minimum rate change in LV pressure; $dP/dt_{max\ or\ min}$/Pid, $dP/dt_{max/min}$ normalized to instantaneous developed pressure.

Supplemental Table 4: Heart rates in *Ex vivo* study

	Baseline	100nM Iso
WT	394±16	573±14
RyR2-S2808A	385±15	566±15

Heart rates of WT and RyR2-S2808A$^{+/+}$ isolated hearts in absence and presence of 100nM Iso. (*P*=NS)

Supplemental Table 5: RyR2-S2808A$^{+/+}$ mice exhibit cardioprotection from chronic β-adrenergic receptor activation

Genotype	WT	S2808A$^{+/+}$
n	6	6
Baseline		
Heart rate (min^{-1})	503 ± 11.52	509 ± 8.05
Echo LV-HW/BW (mg/g)$^{\$}$	1.53 ± 0.06	1.49 ± 0.13
Chronic Iso treatment for 56 days		
Heart rate (min^{-1})	502 ± 39.77 n.s.	538 ± 10.30 n.s.
Echo LV-HW/BW (mg/g)$^{\$}$	2.61 ± 0.12*	2.10 ± 0.10 *#
HW/BW (mg/g)	6.96 ± 0.20	5.99 ± 0.24 #

After 56 days of continuous Iso treatment, both WT and RyR2-S2808A$^{+/+}$ exhibited no significant heart rate differences between baseline and 56-day treatment either within the group or between groups. Although both groups showed significant increased LV mass normalized for body weight (BW) by echocardiography, the LV mass and heart weight (HW) were significantly decreased in RyR2-S2808A$^{+/+}$ mice compared to WT littermates (* P<0.001 versus baseline; # P<0.05 versus WT; n.s., no significant difference versus baseline). LV-HW/BW, left-ventricular heart weight/bodyweight, determined by echocardiographic measurements of the LV chamber dimensions, and LV mass (LVM) calculated using the following two-dimensional, area-length method: LVM = 1.05 [(5/6) A1(L + T) (5/6) A 2L], where 1.05 is the specific gravity of muscle, A1 and A2 are the epicardial and endocardial parasternal short-axis areas, respectively, L is the parasternal long-axis length, and T is the wall thickness. HW/BW, total heart weight normalized to body weight.

References

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