Supporting Information

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SI Materials and Methods

ECG Recording and Analysis. Mice were monitored by ECG performed with a signal transmitter-receiver (RPC-1; DSI) connected to a data acquisition system (IOX2; EMKA Technologies). The data were collected continuously over 24 h at a sampling rate of 1,000 Hz. Continuous digital recordings were analyzed offline using the ECG-auto, version 1.5.12.22 (EMKA Technologies). Telemetric data were scanned using software by blinded observers to measure heart rate variability (HRV) parameters, as described previously (1). In brief, ECG signals were digitally filtered between 0.1 and 1,000 Hz and analyzed manually to detect arrhythmias identified by RR intervals two times greater or smaller than the mean, and then quantified. RR intervals were calculated from nocturnal ECG recordings.

HRV was analyzed similarly on the same recordings. For HRV analyses, the RR values not included between RR ± 2 SD (95.5% confidence intervals) were removed from the analyses. They were not replaced by any averaged or interpolated beat (2). The indexes used were the mean RR interval and SDNN, which reflects total autonomic variability.

RyR2 and NOS Biochemistry. Hearts from WT and mdx age-matched littersmates were lysed isotonically in 1.0 mL of a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 20 mM NaF, 1.0 mM Na3VO4, and protease inhibitors. The levels of nNOS, eNOS, and iNOS were compared between mdx and WT muscle homogenates by immunoblot analysis. Heart homogenates (50 μg) were separated by 10% PAGE, and the immunoblots were prepared using antibodies against nNOS, eNOS, and iNOS (all 1:2,000 dilution; BD Biosciences). All immunoblots were developed and quantified using the Odyssey infrared imaging system (LICOR Biosystems) and infrared-labeled secondary antibodies.

An anti-RyR antibody (4 mg; 5029 Ab) or the NOS isoform antibodies (4 mg; BD Biosciences) were used to immunoprecipitate RyR2, nNOS, eNOS, or iNOS from 500 μg of heart homogenate. The samples were incubated with the appropriate antibody in 0.5% of a modified RIPA buffer [50 mM Tris-HCl (pH 7.4), 0.9% NaCl, 5.0 mM NaF, 1.0 mM Na3VO4, 1% Triton- X100, and protease inhibitors] for 1 h at 4°C. The immune complexes were incubated with protein G Sepharose beads (Sigma-Aldrich) at 4°C for 1 h, after which the beads were washed three times with buffer. Proteins were separated on SDS/PAGE gels (6% for RyR2; 10% for NOS enzymes) and transferred onto nitrocellulose membranes for 1 h at 200 mA (SemiDry transfer blot; Bio-Rad). Immunoblots were prepared as described above. RyR2 S-nitrosylation was measured by developing immunoblots with both a RyR antibody (Affinity Bioreagents) and an anti–Cys-NO antibody (Sigma-Aldrich).

Enzymatic Cell Dissociation. Cardiac ventricular myocytes were enzymatically dissociated using standard procedures (3). In brief, the mouse was euthanized by cervical dislocation, after which the heart rapidly was excised and retrogradely perfused at 37°C for 6–8 min with a modified tyrode solution [113mM NaCl, 4.7 mM KCl, 0.6 mM KH2PO4, 0.6 mM Na2HPO4, 1.2 mM MgSO4, 12 mM NaHCO3, 10 mM KHCO3, 10 mM Hepes, 30 mM Taurine (pH 7.4)] containing 0.1 mg/mL of liberase (Roche). Isolated myocytes were then transferred to the same enzyme-free solution containing 1 mM CaCl2.

Measurement of Intracellular Ca2+ and Ca2+ Sparks. Cells were loaded for 30 min at RT with fluo-4 AM (5 μmol/L; Molecular Probes), then field-stimulated at 1 Hz with 1-ms current pulses delivered via two platinum electrodes, one on each side of the perfusion chamber. Changes in fluo-4 AM fluorescence were recorded using an LSM510 Meta confocal microscope equipped with a 63× water-immersion objective (numerical aperture: 1.2; Zeiss). All measurements were performed in line-scan mode (1.5 ms/line), and scanning was carried out along the long axis of the cell. An excitation wavelength of 488 nm was used, and emitted light was collected through a 505-nm long-pass filter. The laser intensity was used (3%–6% of the maximum) had no noticeable deleterious effect on the fluorescence signal or cell function over the course of the experiment. To enable comparisons between cells, the change in fluorescence (ΔF) was divided by the fluorescence detected immediately before the 0.5-Hz stimulation pulse (F0). Spontaneous Ca2+ sparks were recorded in quiescent cells following 5-min stimulations to reach steady-state SR-Ca2+ content.

Calcium Imaging. To quantitatively monitor intracellular Ca2+ concentration, cardiomyocytes were loaded for 30 min at RT with 10 μM indo-1 AM (Invitrogen), then field-stimulated at 1 Hz (20 V, 1 ms), and simultaneously illuminated at 305 nm using a xenon arc bulb light. Indo-1 AM fluorescence emitted at 405 nm and 480 nm was recorded simultaneously using IonOptix acquisition software (Hilton). To record spontaneous Ca2+ waves in the resting condition, stimulation was stopped for 30 s once a Ca2+ transient steady-state was reached. Indo-1 AM calibration was performed as described previously (4), using the following equation:

\[
\left[Ca^{2+}\right] = K_f \times \beta \times (R - R_{min}) / (R_{max} - R),
\]

where \(R\) is the fluorescence ratio (F405/F480), \(R_{min}\) is the fluorescence ratio in the absence of [Ca2+], \(R_{max}\) is the fluorescence ratio in the presence of [Ca2+], \(K_f\) is the dissociation constant, and \(\beta\) is the ratio of the F480 values at 0 and saturating level of Ca2+.

Measurement of Membrane Potential. Action potentials (APs) were recorded in rod-shaped Ca2+-tolerant myocytes under current-clamp conditions using a whole-cell patch-clamp technique at 22 ± 2°C with an Axopatch 200B (Axon Instruments), as described previously (5). Pipettes (2–3 MΩ) were filled with a recording solution [130 mM KCl, 25 mM HEPES, 3 mM ATP(Mg), 0.4 mM GTP(Na), 0.5 mM EGTA, pH adjusted to 7.2 with KOH]. The myocytes were superfused with Tyrode's solution (135 mM NaCl, 1 mM MgCl2, 4 mM KCl, 11 mM glucose, 2 mM HEPES, 1.8 mM CaCl2, pH adjusted to 7.4 with KOH) (6). APs were elicited by 0.2-ms current injections of suprathreshold intensity. Signals were acquired at 10 KHz and filtered at 5 KHz using low-pass Bessel filter. Cells were stimulated routinely at 0.1 Hz until APs stabilized (3–5 min). Once steady state was established, myocytes were paced at 5 Hz for 1 s every 5 s, to record DADs. Data acquisition and analyses were performed using Pelamp version 10.1 (Axon Instruments). Resting membrane potential (RMP) and AP duration (APD) at 20% (APD20%), 30% (APD30%), 50% (APD50), 90% (APD90), and 95% (APD95) of repolarization were measured.

Statistical Analysis. Statistical comparison were performed with Sigmasat version 3.5 (Systat Software). For spatiotemporal properties of Ca2+ sparks, normality tests failed, and thus data were statistically compared using Kruskal-Wallis one-way ANOVA on ranks. Other statistical comparisons were done with ANOVA. Data are expressed as mean ± SEM, and differences were considered significant at \(P < 0.05\).

Fig. S1. Treatment with NAC prevents RyR2 S-nitrosylation and depletion of calstabin2 in mdx hearts. RyR2 was immunoprecipitated from heart homogenate of 35-day-old mdx mice and WT littermates. In addition, a group of 3-week-old mdx mice were treated for 2 weeks with NAC. Immunoblots were prepared for RyR2, S-nitrosylation of cysteine residues on RyR2 (Cys-NO), and calstabin2 bound to RyR2. The blots are representative of three independent experiments.

Fig. S2. Effect of NAC on RyR function in mdx mice. Ventricular cardiomyocytes were isolated from 35 day-old mdx mice (n = 3) and separated into two batches. One batch was maintained in a normal external buffer, and the other was maintained for 1 h before use in experiments in the same medium containing the ROS/RNS scavenger NAC (20 mM). Cells were then loaded for 30 min with fluo-4 AM, with fluorescence monitored by laser scanning confocal microscopy. RyR2-induced diastolic SR Ca2+ leak was estimated based on the average frequency of sparks. The high sparks frequency observed in mdx cells was reversed to a value comparable to that in WT cells (Fig. 2D). *P < .05 mdx vs. NAC-treated mdx. n = 651 sparks in 30 cells in mdx; n = 175 sparks in 30 cells in NAC-treated mdx.
**Fig. S4.** Isoproterenol-induced ectopic Ca$^{2+}$ transients in mdx mice. Isolated cardiomyocytes were paced at 1 Hz. Ca$^{2+}$ fluorescence was recorded in line-scan mode (x vs. time; 1.5 ms/line) using confocal microscopy. (A) Typical sequences of Ca$^{2+}$ transients obtained in cardiomyocytes after acute application of 100 nM isoproterenol. (B) This treatment significantly increased the number of ectopic Ca$^{2+}$ transients, which were rare in WT and S107-treated mdx mice. Data are expressed as mean ± SEM. *P < .05 WT vs. mdx; #P < .05 mdx vs. S107-treated mdx.

**Fig. S3.** Consequences of SR Ca$^{2+}$ leak on AP duration and RMP. APs were recorded on isolated cardiomyocytes from 35-day-old mice using the patch-clamp technique with an internal medium containing a low EGTA concentration to avoid excessive Ca$^{2+}$ buffering, which could interfere with Ca-dependent regulatory mechanisms on RMP. APs were recorded at 5 Hz. The stimulation was interrupted to challenge resting membrane potential and occurrence of DADs. (A–C) Typical train of APs followed by a rest period recorded in mdx cardiomyocytes (A), S107-treated mdx cardiomyocytes (B), and cardiomyocytes incubated with 20 mM NAC (C, Left). The arrow in A indicates a DAD in the mdx cardiomyocytes. This behavior was not observed after S107 treatment or NAC incubation. The first APs recorded in a train are shown in the right panel in an expanded time scale. AP durations were measured at different phases of AP repolarization. (D) Data summary showing a shortening effect of both S107 treatment and NAC incubation that appear to be significant at 90% and 95% repolarization, as denoted by the arrow in A (Right). RMP depolarization occurring in mdx cardiomyocytes was prevented by S107 treatment or NAC incubation. Data are expressed as mean ± SEM. n = 15 in mdx (black), n = 11 in S107-mdx (red), and n = 10 in NAC-mdx (blue). *P < .05 mdx vs. S107-treated mdx; #P < .05 mdx vs. NAC-treated mdx.

**Fig. S5.** Isoproterenol-induced ectopic Ca$^{2+}$ transients in mdx mice. Isolated cardiomyocytes were paced at 1 Hz. Ca$^{2+}$ fluorescence was recorded in line-scan mode (x vs. time; 1.5 ms/line) using confocal microscopy. (A) Typical sequences of Ca$^{2+}$ transients obtained in cardiomyocytes after acute application of 100 nM isoproterenol. (B) This treatment significantly increased the number of ectopic Ca$^{2+}$ transients, which were rare in WT and S107-treated mdx mice. Data are expressed as mean ± SEM. *P < .05 WT vs. mdx; #P < .05 mdx vs. S107-treated mdx.