Supporting Information

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SI Materials and Methods

Mouse Strains and Animal Care. All animal procedures were performed in accordance with protocols approved by the University of Colorado Denver-Anschutz Medical Campus Institutional Animal Care and Use Committee. Young (2–3 mo) WT C57BL/6J male mice were obtained from Jackson Laboratories, and old (21–24, 28, and 32+ mo) WT C57BL/6J male mice were obtained from the National Institute on Aging.

Electrocardiography. ECGs were recorded from conscious, nonanesthetized mice using the ECGTunnel device (EMKA Technologies), which passively restrains the animals and positions their feet over footpad electrodes. Signals were amplified with an Animal Bioamp (ADInstruments) connected to a Powerlab amplifier (ADInstruments). ECGs were analyzed offline using Labchart 7 Pro software (ADInstruments). Average heart rates during 30-s recording windows were determined from each animal. Maximum stress-induced heart rate was defined as the maximum value preceding autonomic blockade, and intrinsic heart rate was defined as the minimum rate following i.p. coinjection of atropine and propranolol (2 mg/kg of each; Sigma–Aldrich). Maximum heart rate induced by restraint stress was confirmed by i.p. injection of isoproterenol (ISO; 0.12 mg/kg; Calbiochem) (Fig. S1).

ECG events were automatically detected and averaged over 5-s windows using a LabChart 7 Pro ECG Analysis module (ADInstruments). The PR interval was defined as the time between the peak of the P wave and the peak of the R wave. The QRS interval was defined as the duration between the initiation of the Q wave and the termination of the S wave. The QT interval was defined as the interval between the initiation of the Q wave and the termination of the T wave. The corrected QT interval was calculated using the Fridericia formula (1), which is the QT interval divided by the cube root of the RR interval (defined as the interval between R wave peaks).

Sinoatrial Myocyte Isolation. Sinoatrial myocytes (SAMs) were isolated as previously described (2, 3). Briefly, mice were injected i.p. with heparin, anesthetized with isoflurane, and euthanized via cervical dislocation. Hearts were removed into Tyrode's solution (140 mM NaCl, 5.4 mM KCl, 1.2 mM KH_2PO_4, 5 mM Hepes, 5.55 mM glucose, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$, with the pH adjusted to 7.4 with NaOH) containing 10 U/mL heparin at 35 °C. The atria were separated from the ventricles, and the sinoatrial node region was excised and digested with an enzymatic mixture containing either collagenase type II (Worthington Biochemical), protease type XIV (Sigma–Aldrich), and elastase (Worthington Biochemical) or 3.75 mg of Liberase TM (Roche) and 4.75 U of elastase for 10–30 min at 35 °C in a modified Tyrode's solution (140 mM NaCl, 5.4 mM KCl, 1.2 mM KH_2PO_4, 5 mM Hepes, 18.5 mM glucose, 0.066 mM $CaCl_2$, 50 mM taurine, and 1 mg/mL BSA, with the pH adjusted to 6.9 with NaOH). SAMs were dissociated by mechanical trituration with a fire-polished glass pipette in a modified KB solution (100 mM potassium glutamate, 10 mM potassium aspartate, 25 mM KCl, 10 mM KH_2PO_4, 2 mM $MgSO_4$, 20 mM taurine, 5 mM creatine, 0.5 mM EGTA, 20 mM glucose, 5 mM Hepes, and 0.1% BSA, with the pH adjusted to 7.2 with KOH) at 35 °C. Ca^{2+} was gradually reintroduced to the cell suspension, which was then kept at room temperature for up to 8 h before recording.

SAM Electrophysiology. An aliquot of the SAM cell suspension was transferred to a heated, glass-bottomed recording chamber on the stage of an inverted microscope. Cells were constantly perfused (1–2 mL/min) with extracellular solution at 35 ± 1 °C during all experiments. A 1-mM stock of (−) ISO HCl (in 1 mM ascorbic acid) was made fresh daily and was added to the Tyrode's extracellular solution to a final concentration of 1 nM [for control current-clamp experiments (4)] or 1 μM, as indicated. SAMs were identified by their characteristic morphology, small size (20–45 pF), action potential (AP) shape, presence of hyperpolarization-activated funny current (I_f), and/or spontaneous contractions in accordance with previous studies (2, 5, 6).

Amphotericin-perforated patch current-clamp recordings for AP waveform analysis. Spontaneous APs were recorded from isolated SAMs in the perforated-patch configuration using an intracellular pipette solution composed of 135 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM NaCl, 10 mM EGTA, 4 mM Mg-ATP, and 10 mM Hepes, with the pH adjusted to 7.2 with KOH. Amphotericin B (Sigma–Aldrich) was added to the pipette solution to a final concentration of 200 μg/mL and vortexed for ∼1 min. The final solution was protected from light and was refreshed hourly. AP events were detected using the "template search" function of ClampFit software (Molecular Devices), and average firing rates were computed from 30-s recording windows. Basal AP firing rates were determined in the presence of 1 nM ISO to stabilize the firing rate as previously reported (4). Maximum AP firing rates were determined in the same cells >2 min after wash-on of 1 μM ISO.

AP parameters were calculated for each cell from average waveforms from 5-s recording windows using a method modified from that of Bucchi et al. (7). Access resistance (R_a) was monitored using ClampEx acquisition software (Molecular Devices), and APs for waveform analysis were collected within 30 s of achieving a stable R_a below 10 MΩ to control for a time-dependent "drift" in the AP waveform [in which the maximum diastolic potential (MDP) became progressively more negative and the AP upstroke faster, without a change in firing rate]. MDP and V_{max} were defined as the most negative and positive membrane potentials, respectively, and the take-off potential (TOP) was defined as the membrane potential when the first derivative of voltage with respect to time (dV/dt) reached 10% of its maximum value. Cycle length was defined as the interval between MDPs in successive APs. The maximum rates of the AP upstroke and repolarization were taken as the maximum and minimum values of the first derivative (dV/dt_{max} and dV/dt_{min}, respectively). Action potential duration (APD) was defined as the interval between the TOP and the subsequent MDP. APD_{50} and APD_{90} were defined as the interval between the TOP and 50% and 90% repolarization, respectively. The diastolic duration was defined as the interval between MDP and TOP. The early diastolic depolarization rate was estimated as the slope of a linear fit between 10% and 50% of the diastolic duration, and the early diastolic duration was the corresponding time interval. The nonlinear late diastolic depolarization phase was estimated as the duration between 1% and 10% dV/dt.

Whole-cell voltage-clamp recordings. Whole-cell Ca^{2+} currents were recorded from acutely dissociated SAMs using borosilicate glass pipettes with resistances of ∼1.5–3.0 MΩ when filled with an intracellular solution consisting of 130 mM CsCl, 1 mM $MgCl_2$, 10 mM Hepes, 10 mM EGTA, 4 mM Mg-ATP, and 0.1 mM Na-GTP, with the pH adjusted to 7.2 with CsOH. Cells were constantly perfused at 35 ± 1 °C with an extracellular solution

containing 130 mM tetraethylammonium chloride, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM 4-aminopyridine, and 10 mM HEPES, with the pH adjusted to 7.4 with CsOH. Total Ca^{2+} current was elicited by depolarizing voltage steps between −70 and +60 mV in 10-mV increments from a holding potential of −90 mV. L-type Ca^{2+} current ($I_{Ca,L}$) was elicited by depolarizing steps between −50 and +60 mV from a holding potential of −60 mV, where T-type Ca^{2+} current ($I_{Ca,T}$) is largely inactivated (8, 9). $I_{Ca,T}$ was calculated by subtracting $I_{Ca,L}$ from I_{Ca} in each cell. Linear components of leak and capacitive current were corrected for using −P/4 subtraction. All voltages were corrected for a calculated −9-mV liquid junction potential. Individual and average current-voltage plots were fit with a modified Boltzmann equation:

$$I = \frac{G_{max} * (V - V_{rev})}{\left\{1 + \exp\left[-\frac{\left(V - V_{1/2}\right)}{k_G}\right]\right\}}, \quad \textbf{[S1]}$$

where I is the peak current at a given voltage (V), V_{rev} is the reversal potential, G_{max} is the maximum conductance, $V_{1/2}$ is the half-maximal activation potential, and k_G is the slope factor. $I_{Ca,L}$ and $I_{Ca,T}$ inactivation rates were estimated from double exponential fits of current sweeps at −10 and −30 mV, respectively.

Whole-cell voltage-clamp recordings of I_f from SAMs were performed as previously described (2, 3). Cells were perfused with Tyrode's solution containing 1 mM $BaCl_2$, and recording pipettes had resistances of ∼1.5–3.0 MΩ when filled with an intracellular solution consisting of 135 mM potassium aspartate, 6.6 mM sodium phosphocreatine, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes, 10 mM EGTA, and 4 mM Mg-ATP, with the pH adjusted to 7.2 with KOH. Cells were held at −50 mV, and I_f was elicited by 3-s test pulses from −60 to −160 mV in 10-mV increments (reported voltages were corrected for a calculated −14-mV junction potential error). Conductance was calculated from inward currents using the equation

$$G = I/(V - V_r), \quad \textbf{[S2]}$$

where G is conductance, I is the time-dependent inward current at a given voltage V, and V_r is the reversal potential for I_f [−30 mV (2, 6)]. Conductances were subsequently plotted as a function of voltage and fit with a Boltzmann equation to determine the midpoint activation voltage ($V_{1/2}$):

$$f(V) = V_{\min} + \frac{V_{\max} - V_{\min}}{1 + e^{\frac{Z_d F}{RT}\left(V - V_{1/2}\right)}}. \quad \textbf{[S3]}$$

Statistics. All data are presented as mean ± SEM. Individual data points greater than 3 SDs from the mean were considered outliers and were excluded from analysis. Comparisons were made using unpaired Student t tests or one-way ANOVAs with Holm–Sidak post hoc tests as noted. Statistical significance was indicated by $P < 0.05$.

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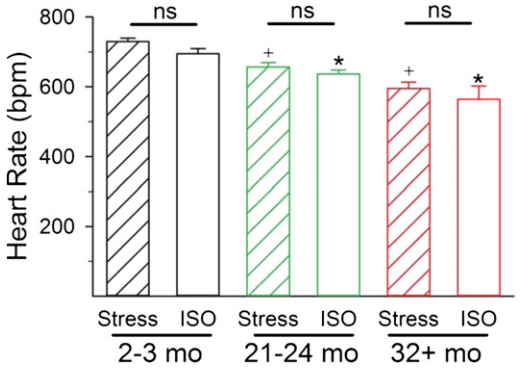

Fig. S1. Equivalent maximum heart rate in response to restraint stress and ISO. Average (±SEM) heart rates recorded from mice aged 2–3 mo (black, $n = 5$), 21–24 mo (green, $n = 12$), and 32+ mo (red, $n = 3$–5) in response to restraint stress (hatched bars) or restraint plus i.p. injection of 0.12 mg/kg of ISO (open bars). Not significant (ns) indicates $P > 0.05$, t tests; $+P < 0.05$ vs. 2–3 mo of stress, $*P < 0.05$ vs. 2–3 mo of ISO; ANOVA with a Holm–Sidak posttest.

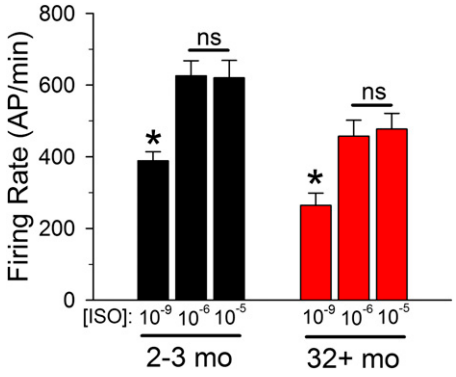

Fig. S2. Saturating firing rate response to 1 μM ISO in SAMs isolated from young and old mice. Average (±SEM) AP firing rates from perforated-patch recordings in young (2–3 mo, black, $n = 14$) and old (32+ mo, red, $n = 10$) SAMs in response to 1 nM, 1 μM, and 10 μM ISO. *$P < 0.05$, ns indicates $P > 0.05$; one-way ANOVA with a Holm–Sidak posttest.

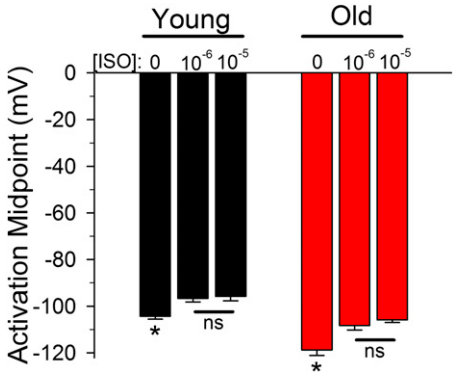

Fig. S3. Saturating I_f response to 1 μM ISO in SAMs isolated from young and old mice. Average (±SEM) $V_{1/2}$ values for I_f in the absence of ISO (control) compared with 1 μM or 10 μM ISO in whole-cell recordings from young (2–3 mo, black) and old (29+ mo, red) SAMs. Young cells: control ($n = 20$), 1 μM ($n = 13$), and 10 μM ($n = 11$). Old cells ($n = 6$ for all concentrations). *$P < 0.05$; ns indicates $P > 0.05$; one-way ANOVA with a Holm–Sidak posttest.

Table S1. ECG parameters from awake restrained mice of different ages

	Intrinsic			Maximum		
	2–3 mo	21–24 mo	32+ mo	2–3 mo	21–24 mo	32+ mo
PR interval, ms	32.7 ± 1.1 (5)	39.5 ± 1.2 (12)*	43.8 ± 0.9 (5)*	28.2 ± 3.7 (5)	36.3 ± 1.3 (12)	39.5 ± 1.1 (5)*
QRS interval, ms	12.2 ± 1.4 (5)	14.8 ± 0.4 (12)	16.4 ± 2.1 (5)	9.8 ± 0.6 (5)	14.5 ± 0.5 (12)*	15.9 ± 2.0 (5)*
Corrected QT, ms	62.1 ± 6.7 (5)	70.2 ± 3.6 (12)	74.6 ± 5.9 (5)	63.6 ± 1.9 (5)	80.1 ± 4.1 (12)	93.7 ± 10.6 (5)*

Average PR, QRS, and corrected QT intervals (±SEM) were determined at intrinsic heart rate or maximum heart rate from awake restrained mice.
*$P < 0.05$ vs. 2–3 mo (ANOVA with a Holm–Sidak posttest).

Table S2. Properties of spontaneous APs in SAMs isolated from mice of different ages

	Age		
	2–3 mo	21–24 mo	32+ mo
Cycle length, ms	147.8 ± 7.3 (10)	192.4 ± 8.0* (12)	218.3 ± 14.5* (9)
MDP, mV	−59.4 ± 1.7 (14)	−64.6 ± 1.2* (13)	−67.5 ± 1.2* (8)
TOP, mV	−45.9 ± 1.3 (13)	−48.7 ± 1.6 (13)	−52.2 ± 1.0* (8)
Diastolic duration, ms	51.1 ± 5.6 (14)	72.5 ± 4.3* (13)	73.4 ± 1.9* (8)
Early diastolic duration, ms	22.7 ± 2.9 (14)	31.3 ± 1.2* (12)	35.4 ± 2.8* (8)
Late diastolic duration, ms	17.7 ± 2.6 (14)	20.3 ± 1.5 (13)	20.6 ± 3.2 (8)
Early DDR, mV/s	133.7 ± 15.4 (13)	86.7 ± 6.2* (13)	62.1 ± 16.3* (8)
dV/dt_{min}, V/s	−2.3 ± 0.2 (14)	−2.1 ± 0.2 (13)	−2.3 ± 0.2 (8)
dV/dt_{max}, V/s	24.3 ± 4.4 (13)	22.8 ± 3.8 (13)	33.5 ± 6.7 (8)
V_{max}, mV	11.1 ± 1.6 (13)	10.9 ± 1.1 (13)	16.0 ± 1.8 (7)
Amplitude, mV	73.5 ± 3.2 (14)	75.4 ± 1.7 (13)	85.3 ± 2.5* (8)
APD, ms	81.9 ± 7.3 (14)	98.2 ± 7.7 (13)	93.8 ± 10.6 (8)
APD_{50}, ms	35.6 ± 3.3 (14)	42.1 ± 3.3 (13)	40.2 ± 2.5 (8)
APD_{90}, ms	50.8 ± 2.8 (14)	62.4 ± 4.3 (13)	63.6 ± 5.8 (8)

Average parameters determined from APs recorded in amphotericin perforated-patch recordings from acutely isolated SAMs from mice of the indicated ages. The number of cells is shown in parentheses. DDR, diastolic depolarization rate; DV/dt_{max}, maximum value of the first derivative; dV/dt_{min}, minimum value of the first derivative; V_{max}, positive membrane potential.

*$P < 0.05$ vs. 2–3 mo (one-way ANOVA with a Holm–Sidak posttest).

Table S3. Properties of $I_{Ca,L}$, $I_{Ca,T}$, and I_f in SAMs isolated from mice of different ages

		2–3 mo		21–24 mo		28+ mo	
		Basal	ISO	Basal	ISO	Basal	ISO
Capacitance, pF		36.1 ± 1.0 (64)	—	45.9 ± 1.2 (51)*	—	48.2 ± 1.0 (37)*	—
$I_{Ca,L}$	G_{max} density, pS/pF	134 ± 11 (17)	270 ± 26 (17)†	103 ± 8 (19)*	182 ± 16 (19)*,†	92 ± 8 (13)*	186 ± 13 (13)*,†
	G_{max}, nS	4.6 ± 0.5 (17)	9.3 ± 1.0 (17)	4.3 ± 0.3 (18)	8.2 ± 0.8 (19)	4.6 ± 0.5 (13)	9.2 ± 0.7 (13)
	βAR response, % increase G_{max}	—	102.1 ± 16.7 (17)	—	84.5 ± 7.5 (17)	—	92.9 ± 9.1 (12)
	$V_{1/2}$, mV	−27.9 ± 1.2 (17)	−28.6 ± 1.5 (17)	−28.8 ± 1.6 (19)	−29.9 ± 0.9 (19)	−25.9 ± 1.3 (13)	−27.5 ± 1.2 (13)
	Slope factor (K), mV	6.5 ± 0.2 (17)	6.4 ± 0.2 (17)	6.3 ± 0.3 (19)	6.2 ± 0.2 (19)	6.3 ± 0.4 (13)	6.7 ± 0.3 (13)
	τ_{fast}, ms	5.7 ± 0.30 (16)	6.9 ± 0.42 (17)	5.2 ± 0.19 (19)	6.3 ± 0.25 (19)	5.5 ± 0.41 (13)	7.0 ± 0.48 (13)
	τ_{slow}, ms	27.5 ± 1.6 (17)	26.8 ± 1.4 (17)	25.5 ± 1.1 (19)	26.0 ± 0.88 (19)	29.8 ± 0.9 (13)	27.0 ± 1.5 (13)
$I_{Ca,T}$	G_{max} density, pS/pF	105 ± 14 (16)	177 ± 20 (16)†	53 ± 9 (19)*	103 ± 15 (19)*,†	48 ± 6 (13)*	81 ± 13 (13)*,†
	G_{max}, nS	3.7 ± 0.5 (16)	6.2 ± 0.8 (16)	2.3 ± 0.4 (19)*	4.1 ± 0.6 (18)	2.3 ± 0.3 (13)*	3.9 ± 0.6 (13)
	βAR response, % increase in G_{max}	—	74.4 ± 19.2 (17)	—	67.5 ± 20.9 (19)	—	75.7 ± 17.5 (12)
	$V_{1/2}$, mV	−41.9 ± 1.0 (16)	−40.0 ± 1.5 (17)	−42.5 ± 1.0 (18)	−43.1 ± 1.4 (19)	−41.6 ± 1.4 (13)	−39.6 ± 1.9 (13)
	Slope factor (K), mV	5.1 ± 0.5 (17)	4.9 ± 0.4 (17)	4.8 ± 0.2 (19)	4.6 ± 0.2	5.0 ± 0.3 (13)	5.1 ± 0.4 (13)
	τ_{fast}, ms	4.9 ± 0.33 (17)	4.9 ± 0.26 (16)	4.4 ± 0.27 (18)	4.5 ± 0.24 (19)	4.5 ± 0.24 (12)	5.0 ± 0.43 (13)
	τ_{slow}, ms	31.9 ± 2.1 (17)	30.7 ± 2.1 (17)	30.6 ± 1.0 (19)	31.0 ± 2.2 (19)	35.3 ± 2.5 (13)	29.7 ± 2.6 (13)
I_f	Peak current density, pA/pF	−27.7 ± 2.1 (20)	−30.9 ± 3.3 (13)	−18.2 ± 2.8 (9)*	−19.8 ± 3.2 (10)*	−13.2 ± 2.4 (8)*	−13.4 ± 2.2 (6)*
	Peak current amplitude, pA	−1,108.6 ± 121.2 (20)	−1,189.6 ± 170.1 (13)	−960.4 ± 214.2 (10)	−937.1 ± 161.2 (10)	−617.6 ± 115.5 (8)	−641.1 ± 122.4 (6)
	$V_{1/2}$, mV	−104.3 ± 1.3 (20)	−96.7 ± 1.5 (13)†	−110.3 ± 1. 7 (9)*	−101.5 ± 1.0 (10)*,†	−117.3 ± 2.0 (8)*	−108.3 ± 1.6 (6)*,†
	βAR response (shift in $V_{1/2}$), mV	—	7.6	—	9.0	—	9.0

Data from whole-cell voltage-clamp recordings in isolated SAMs from mice of the indicated ages. Maximal conductance (G_{max}) and midpoint activation voltages ($V_{1/2}$) for $I_{Ca,L}$, $I_{Ca,T}$, and I_f were determined from Boltzmann fits to current-voltage plots (*SI Materials and Methods*). Peak I_f amplitudes and density were determined at −150 mV. β-adrenergic receptor (βAR) responsiveness for $I_{Ca,L}$ and $I_{Ca,T}$ is presented as the average percentage increase in G_{max} in response to ISO, and βAR responsiveness for I_f is presented as the shift in $V_{1/2}$ in response to ISO. Fast and slow inactivation time constants (τ_{fast} and τ_{slow}) for $I_{Ca,L}$ and $I_{Ca,T}$ were determined from double exponential fits of currents at −10 mV and −30 mV, respectively.

*$P < 0.05$ vs. 2–3 mo cells (one-way ANOVA with a Holm–Sidak posttest).

†$P < 0.05$, paired or unpaired *t* tests.