SUPPLEMENTAL MATERIAL

Supplemental Methods

RT-PCR and Western blot analysis

For this study, $Ca_V1.2$-TS and $Ca_V1.2$-WT channel mRNA was quantified using qPCR as previously described [1]. In addition, protein expression was determined by Western blot, performed as previously described [2]. Briefly, proteins were extracted from WT and TS mice hearts and run on an SDS-page. A WT brain, which has high expression of $Ca_V1.2$, was used as a positive control for the WT band and tsA-201 cells transfected with $Ca_V1.2$-TS plasmids were used as a positive control for the TS band. Electrophoresed proteins were then transferred to PVDF membranes. After blocking with 5% normal donkey serum, membranes were probed with $Ca_V1.2$ primary antibody (Alomone, cat#ACC-003). Alkaline phosphatase-conjugated donkey anti-rabbit secondary antibody (Jackson Immunoresearch) was used for detection using ECF substrate (GE healthcare). $Ca_V1.2$-WT (240 kDa) and TS channels (270 kDa) could be discerned on the blot because $Ca_V1.2$-TS channels are fused to a red fluorescent protein (tagRFP®; Evrogen) (30 kDa).

Electrophysiology

All electrophysiological recordings were performed while cells were superfused with saline solutions at room temperature (≈22 °C). Pipettes were pulled using a Flaming-Brown type puller (Sutter Instruments) with nominal resistance of 1-2 MΩ and filled with solutions as described below.

To record action potentials, the current-clamp mode of the Axopatch 200B amplifier was used. APs were recorded while cells were superfused with Tyrode's solution. The patch-pipette solution used to record APs contained (mM): 110 potassium aspartate, 30 KCl, 10 HEPES, 5 MgATP and 10 NaCl; pH 7.2 with KOH. APs were evoked by a brief (5ms) injection of depolarizing current (7 nA). Membrane voltage records were sampled at 10 kHz and low-pass filtered at 2 kHz.

We used the AP clamp technique to measure $[Ca^{2+}]_i$ during the physiological WT and TS AP by using stored APs as the voltage command to depolarize myocytes. The representative APs used in these experiments were recorded from WT and TS cells at a frequency of 1 Hz as described above. In some experiments, after stimulating the cell to a steady state, 20 mM caffeine was suddenly applied to assay SR Ca^{2+} content.

We also used the AP clamp technique to measure L-type Ca^{2+} currents during the physiological WT and TS AP. Once whole-cell configuration was successfully established in myocytes perfused with Tyrode's solution, the extracellular solution was exchanged for one with the following constituents (mM): 140 NMDG, 5 $CsCl_2$, 2

$CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES; pH 7.4 with HCl, and 10 μM tetrodotoxin was added to block sodium channels. The pipette solution used in these experiments contained (mM): 87 CsAsp, 20 CsCl, 10 EGTA, 1 $MgCl_2$, 5 Mg-ATP and 10 HEPES; pH 7.2 with CsOH. Cells were stimulated with either the WT or TS APs.

To record $I_{Ca,}$ membrane potential was controlled via the patch-clamp technique using an Axopatch 200B amplifier (Molecular Devices). Data were acquired at 10 kHz and low-pass filtered at 2 kHz. Ventricular myocytes were continuously perfused with Tyrode's solution. Once whole-cell configuration was successfully established in myocytes, an extracellular solution with the following constituents (mM) was exchanged: 140 NMDG, 5 $CsCl_2$, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES; pH 7.4 with HCl, and 10 μM tetrodotoxin was added to block sodium channels. The pipette solution used in experiments designed to record I_{Ca} during EC coupling contained (mM): 130 CsCl, 10 TEA-Cl, 5 Mg-ATP and 10 HEPES; pH 7.2 with CsOH. I_{Ca} was evoked by 200 ms long depolarization pulses from -80 mV to potentials ranging from –40 to +60 mV (10 mV increment). In other experiments, 10 mM EGTA was included in the pipette solution to maintain low global $[Ca^{2+}]_i$ and eliminate SR Ca^{2+} release. For these experiments the pipette solution contained (mM): 87 CsAsp, 20 CsCl, 10 EGTA, 1 $MgCl_2$, 5 Mg-ATP and 10 HEPES; pH 7.2 with CsOH.

To calculate the kinetics of I_{Ca}, the 0 mV trace was analyzed in pClamp and fit to the double exponential transient decay equation:

$$y = Ae^{\frac{-x}{\tau_{fast}}} + Be^{\frac{-x}{\tau_{slow}}}$$

where A is the amplitude of the fast decay component (pA), τ_{fast} is the time constant of the fast component (ms), B is the amplitude of the slow decay component (pA), and τ_{slow} is the time constant of the fast component (ms). Current density (pA/pF) was obtained by dividing current (pA) by cell capacitance (pF). Integrals of the current density were calculated in pClamp by integrating the area above the current (and below baseline, since I_{Ca} is inward).

Field Stimulation

Field stimulation was performed via two platinum wires (0.5 cm separation) placed at the bottom of the perfusion chamber. An IonOptix Myopacer (IonOptix Corp, Milton, MA, USA) was used to deliver square voltage pulses (4 ms duration) with an amplitude of 5 V at a frequency of 1 Hz. After reaching steady state, SR Ca^{2+} load was assayed by measuring the caffeine-induced Ca^{2+} transient evoked by rapid application of 20 mM caffeine to the cells via a picospritzer.

Confocal Imaging of Ca^{2+} signals

For ratiometric data, Asante Calcium Red (ACaR) was prepared and calibrated as described by Ljubojevic *et al.* [3]. Briefly, ACaR was suspended in a solution of 0.02% pluronic F-127 in DMSO and added to cells to a concentration of 10 μM. Cells

were incubated for 1 hour to pellet, then resuspended in Tyrode's solution for use. Excitation occurred at 488 nm and emission was collected at >650 nm (F_1) and 525 nm (F_2). Absolute [Ca^{2+}] was calculated according to the equation:

$$\left[Ca^{2+}\right] = K_d \beta \frac{\left(R - R_{\min}\right)}{\left(R_{\max} - R\right)}$$

where R = F_1/F_2, R_{min} = F_1/F_2 in the absence of Ca^{2+}, R_{max} = F_1/F_2 in the presence of 20 mM [Ca^{2+}] and 10 μM ionomycin, K_d = 1336 ± 38 nM in the cytoplasm, and β = the proportionality factor determined by the ratio of the F_2 intensities of free and Ca^{2+}-bound dye, respectively [3].

Ventricular myocytes were loaded with the membrane-permeable acetoxymethyl-ester form of Fluo-4 (Fluo-4 AM) for measurement of $[Ca^{2+}]_i$ as previously described (Santana et. al. 2002). Experiments to measure Ca^{2+} waves and sparks were performed on the stage of an Olympus Fluo View 1000 confocal microscope. All other experiments were performed using a BioRad Radiance 2000 confocal system coupled to a Nikon TE300 inverted microscope equipped with a Nikon 60x oil immersion lens. All images were analyzed using ImageJ. Ca^{2+} sparks were identified using the computer algorithm SparkMaster on ImageJ [4]. Fluorescence signals were calibrated using the F_{max} equation [5]:

$$\left[Ca^{2+}\right] = K_d \frac{\left(\frac{F}{F_{\max}} - \frac{1}{R_f}\right)}{\left(1 - \frac{F}{F_{\max}}\right)}$$

where F is fluorescence, F_{max} is the fluorescence intensity of Fluo-4 AM in the presence of a saturating free Ca^{2+} concentration, K_d is the dissociation constant of Fluo-4 AM (1100 nM), and R_f (255) is this indicator's F_{max}/F_{min}. F_{min} is the fluorescence intensity of Fluo-4 in a solution where the Ca^{2+} concentration is 0. K_d and R_f values for Fluo-4 were determined in vitro using standard methods [6].

To determine diastolic Ca^{2+} for TS cells, F_{max} was determined by (1) obtaining stable field transients in Tyrode's; (2) exposing cells to Tyrode's solution with 20 mM 2,3-butanedione monoxime (BDM) and 0 mM Ca^{2+} until no transients could be induced; (3) exposing cells to 20 mM Ca^{2+} and 10 μM ionomycin until the fluorescence signal was saturated.

To quantify sarcolemmal leak for WT and TS myocytes, cells incubated with 1μM thapsigargin were exposed to solution containing (in mM): 140 NMDG, 5 KCl, 10 HEPES, 10 glucose, 10 EGTA, and 1 $MgCl_2$ until stable calcium levels were reached. Cells were then exposed to the same solution except without EGTA and with 2 mM $CaCl_2$ added until the cell reached a new steady state level of calcium.

Supplemental Figure Legend

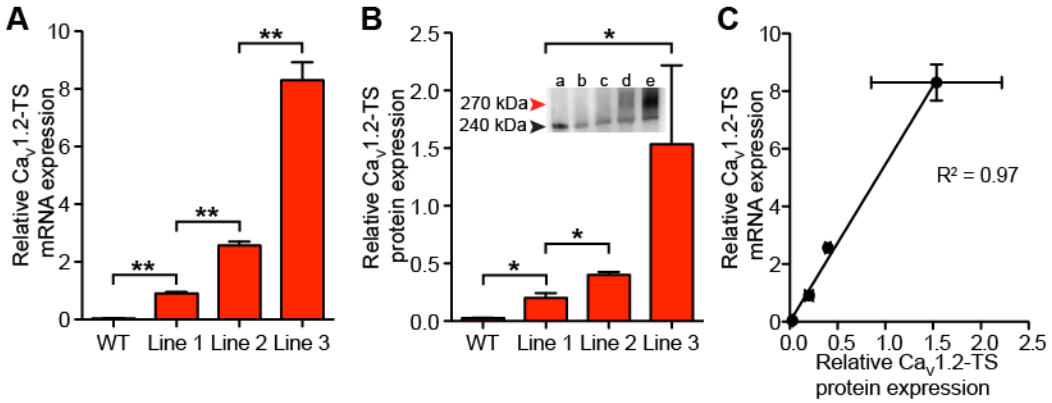

Figure S1. Linear relationship of relative mRNA and protein expression of Ca_V-1.2-TS. A, plot of relative Ca_V1.2-TS mRNA expression normalized to expression of β-actin. **B**, plot of relative Ca_V1.2-TS protein expression normalized to expression of Ca_V1.2-WT protein. *Inset*, example Western blot: *a*, WT mouse brain (high Ca_V1.2-WT expression); *b*, WT mouse heart; *c*, TS line 1 mouse heart; *d*, TS line 3 mouse heart; *e*, tsA-201 cells transfected with Ca_V1.2-TS plasmids. Black arrow at 240 kDa indicates expected Ca_V1.2 channel, red arrow at 270 kDa indicates expected Ca_V1.2-TS channel fused with tagRFP. **C**, linear fit of relative Ca_V1.2-TS protein expression, x-axis, vs. relative Ca_V1.2-TS mRNA expression, y-axis, $R^2 = 0.97$.

Supplemental References

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