METHODS

Generation of Htr3a targeted knockout mice
Mice were housed in climate-controlled, pathogen-free barrier facilities under a 12-h light/dark cycle. 5-hydroxytryptamine 3a-receptor (Htr3a) targeted knock-out mice (B6.129X1-Htr3atm1jul/J) were purchased from the Jackson Laboratory. Htr3a knock-out (Htr3a −/−) mice were backcrossed with C57BL/6J mice for more than 14 generations, and were used at the age of 8–12 weeks. Mating was confirmed by the presence of a vaginal plug the next morning, and was designated as day 0 of gestation (G0). RNA extraction, RT-PCR, and real time RT-PCR were performed as previously described. The following primer sequences were used: Htr3a primers 5'-AAATCAGGGCGAGTGGGAGCTG-3' and 5'-GACACGATGATGAGGAAGACTG-3', and Tph1 primers 5'-ACCATGATTGAAGACAACAAGGAG-3' and 5'-TCAACTGTTCCTGCGTATC-3'.

Immunohistochemical staining
For histology, five hearts from each group were fixed in 4% (w/v) paraformaldehyde in phosphate buffered saline for 4 hours at 4°C and embedded in paraffin. Tissue slides were stained with Hematoxylin and Eosin. Immunohistochemical staining was performed using polyclonal anti-Htr3a antibody (Sigma-Aldrich, Schnelldorf, Germany) and anti-serotonin antibody (ImmunoStar, Hudson, WI, USA). To visualize the bound antibodies, we used a VECTASTAIN® ABC Kit (Vector labs, Burlingame, CA, USA) and DAB Substrate Kit (Vector laboratories).

Serotonin analysis
Serum samples were collected from wild-type non-pregnant (WT-NP) and wild-type late-pregnant (WT-LP) mice, and serotonin levels were measured using ELISA (Enzo Life Sciences Inc, Ann Arbor, MI, USA), following the manufacturer’s protocol.

Echocardiography
A GE Vivid 5 with a 15L8 15 MHz linear array transducer at a frame rate of 100 frames/s was used to perform echocardiography. Two-dimensional views were acquired in the short-axis and parasternal long-axis views. M-mode recordings were acquired in the short-axis view at the level of the papillary muscles. The left ventricular (LV) chamber dimensions (LV internal dimension diastole, LV internal dimension systole, and wall thickness) were measured from the M-mode recordings. The LV fractional shortening (FS) and ejection fraction (EF) were calculated as follows:

FS (%) = [(LV internal dimension diastole–LV internal dimension systole)/LV internal dimension diastole] × 100
EF (%) = [(LV end-diastolic volume–LV end-systolic volume)/LV end-diastolic volume] × 100

Electrocardiogram
ECG was performed during NP and LP (G16–18) stages. All procedures were performed under inhalation anesthesia using isoflurane/O2. Body temperatures were maintained at 37±0.5°C with a heating pad. ECG leads I, II, and III were recorded using a data acquisition system (MP100 and Acknowledge software, Biopac Systems Inc). The QT interval was measured from the start of the QRS complex to the end of the T wave: the end of T wave was defined as the point of return to the isoelectric line. The corrected QT interval (QTc) was calculated by dividing the QT interval by the square root of the preceding R-R interval. To determine if pharmacologic activation of Htr3 could shorten the QT interval, we measured the QT intervals after an intra-peritoneal injection of serotonin (100 μmol/L) and an Htr3 agonist, m-CPBG (200 nmol/L).

Optical mapping
Optical mapping was performed in six mice from each group. After performing a median sternotomy, the hearts were rapidly excised and immersed in cold Tyrode’s solution containing (mmol/L) 125 NaCl, 4.5 KCl, 0.25 MgCl2, 24 NaHCO3, 1.8 NaH2PO4, 1.8 CaCl2, and 5.5 glucose. The ascending aorta was immediately cannulated and perfused with 37°C Tyrode’s solution equilibrated with 95% O2 and 5% CO2 to maintain a pH of 7.4. For performing optical mapping, the contractility was inhibited by 10–17 μmol/L of blebbistatin. Then, the hearts were stained with RH237 (Invitrogen, Carlsbad, CA, USA), and they were excited with quasi-monochromatic light (520±30 nm) from two green light-emitting diode (LED) lamps. Emitted light was collected by a camera (MiCAM UITIMA, BrainVision, Tokyo, Japan) with a 610-nm long pass filter at 1 ms/frame and 100×100 pixels with a spatial resolution of 0.5×0.5 mm2/pixel. The action potential duration (APD) was measured from the (dF/dt) max to 90% recovery to baseline (APD90). The mean APD was calculated for each heart by averaging the APD90 from a region of the ventricle consisting of 10×10 pixels or 100 APD from each heart. To determine if the activation of Htr3 could shorten the APD, we measured APD90 after infu-
sion of serotonin (100 μmol/L), m-CPBG (200 nmol/L) and the Htr3 antagonist ondansetron (1 μmol/L) in wild mice.

Confocal microscopy
Confocal microscopy was performed on isolated mouse ventricular myocytes, as previously described. Briefly, ventricular myocytes were plated on laminin (10 μg/mL)-coated coverslips for 3 h at 4°C; fixed with 4% formaldehyde in PBS on ice; permeabilized in 2% donkey serum in PBS with 0.1% Triton X-100 (30 min); and incubated with primary antibodies against Kv4.3 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Htr3a (SR-3A, 1:200, Santa Cruz Biotechnology), Hsc70 (1:200, Santa Cruz Biotechnology), or HSP90 (1:200, Santa Cruz Biotechnology) overnight at 4°C, followed by incubation with Alexa Fluor 633-conjugated goat anti-mouse, Alexa Fluor 633-conjugated goat anti-rabbit, or Alexa Fluor 488-conjugated donkey anti-goat secondary antibodies, at a 1:200 dilution for 1 h at room temperature. Confocal laser scanning microscopy was performed using a LSM 700 apparatus (Carl Zeiss, Thornwood, NY, USA). Cells were randomly selected and used for imaging and analysis, and experiments were repeated at least five times.

Cell fractionation and immunoprecipitation assays
Membrane isolation was conducted as previously described. All procedures were performed at 4°C. Ventricular myocytes were homogenized in homogenization buffer [20 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, protease inhibitor cocktail (2.5 μL/mg, Sigma)]. Nuclei and debris were pelleted by centrifugation at 500×g for 10 min. The supernatant was centrifuged at 25000×g for 30 min. The pellet was resuspended in homogenization buffer and centrifuged again at 25000×g for 30 min. The final pellet was stored at -80°C. The protein concentration was determined using the Pierce BCA Protein Assay (Sigma-Aldrich).

Co-immunoprecipitation was conducted as previously described. Cells were lysed in lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L PMSF, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate]. Following homogenization and rotation for 1 h, cell lysates were centrifuged at 3000×g for 5 min. The supernatant was pre-cleared with 25 µL of protein-G beads (PROTGA-RO Roche, Sigma-Aldrich) for 30 min. The beads were pelleted at 1000×g for 1 min, and the supernatant was used for immunoprecipitation, using 1 µg of the respective antibody. Thirty microliters of protein-G beads were added, and the samples were incubated for 4 h, followed by another centrifugation at 500×g for 1 min. After additional washes, the beads were loaded on 8% and 10% acrylamide gels.

Antibodies and immunoblot analysis
The primary antibodies used were anti-Kv1.5 (1:1000, Santa Cruz Biotechnology), anti-Kv1.4 (1:1000, Santa Cruz Biotechnology), anti-Kv4.2/Kv4.3 (1:200, Santa Cruz Biotechnology), anti-HERG (1:1000, Santa Cruz Biotechnology), anti-Htr3a (SR-3A, 1:500, Santa Cruz Biotechnology), anti-Hsc70 (1:500, Santa Cruz Biotechnology), anti-HSP90 (1:200, Santa Cruz Biotechnology), and anti-GAPDH (1:1000, Cell Signaling, Beverly, MA, USA).

Cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Richmond, CA, USA). After blocking with 5% non-fat dry milk in 1× TBS containing 1% Tween 20 (for 1 h), immunoblotting was performed with each of the indicated primary antibodies, followed by HRP-conjugated anti-rabbit or anti-goat IgG secondary antibodies. Detection was performed using an enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical analysis
Data are expressed as means±SEMs. An analysis of variance with post hoc test, a Turkey’s test, was used to compare the means of continuous variables that were approximately normally distributed among the groups. Categorical variables are reported as counts (percentage), and were compared using Fisher’s exact test. The SPSS statistical package (ver. 9.0; SSPS Inc., Chicago, IL, USA) was used to perform all statistical evaluations. A p value of ≤0.05 was considered statistically significant. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agreed to the manuscript as written.

REFERENCES