SUPPLEMENTAL MATERIAL

Animals, treatment and mortality. Male Sprague-Dawley rats (350-400 g) were used. To induce PH, rats were treated with a single subcutaneous injection of MCT (60 mg/kg, Sigma, St. Louis, MO). This model has been extensively studied by many investigators and has been shown to be reproducible[1;2]. MCT was dissolved in 1N HCl, the pH was adjusted to 7.4, and diluted with PBS before injection. A weight loss of more than 10% per day for 2 consecutive days and arterial oxygen saturation of less than 80% were the criteria required to sacrifice the animals, which were counted as a 'loss' in survival measurements. Both sudden deaths as well as the animals counted as 'loss' were included in the mortality plot. Sudden death was defined as unexpected death of animals without significant hemodynamic compromise, and without reaching the criteria required for sacrifice, and these sudden deaths started as early as day 23. The sudden death animals were only used for mortality plot and the hearts of these animals were not used for molecular, biochemical or electrophysiological experiments. Protocols received institutional review and committee approval. The investigation conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996).

Cardiac and pulmonary hemodynamics. B-mode, M-mode and pulmonary pulsed wave Doppler echocardiography was performed using a VisualSonics Vevo 770 (VisualSonics, Ontario, Canada) equipped with a 30-MHz linear transducer. During the course of the experiments, serial echocardiography was performed to accurately monitor the stage of the disease by measuring cardiac and pulmonary hemodynamic parameters,

as well as RV structure. Peak systolic RV pressure was calculated from pulsed wave Doppler echocardiography of pulmonary artery flow using Mahan's regression equation: MPAP= 79 – 0.45×PAAT, in which MPAP is mean pulmonary artery pressure and PAAT is the pulmonary artery acceleration time[3]. The RV pressure was also measured directly by inserting a catheter (1.4F Millar SPR-671, CO, USA) connected to a pressure transducer (Power Lab, ADInstruments, CO, USA) into the RV right before sacrifice. The values of RV pressure measured by both methods were very similar. The RV ejection fraction, RV free wall thickness and RV cavity dimensions were quantified using M-mode.

Gross histologic evaluation. The RV wall, the left ventricular (LV) wall and the interventricular septum (IVS) were dissected and the weight ratio of the RV to LV plus IVS [RV/(LV+IVS)] was calculated as an index of RV hypertrophy. LV/body weight ratio was calculated as an index of LV hypertrophy.

Isolation of cardiomyocytes

The heart was quickly removed and perfused through the aorta with the following solutions: (i) Ca^{2+}-free Tyrode solution for 5 min, (ii) Ca^{2+} -free Tyrode solution containing 160 U/mL collagenase Type II (Worthington Co., Ltd, Freehold, NJ) and 0.45 U/ml protease Type XIV (Sigma) for ~15 min; and (iii) Krebs solution for 5 min. The solutions were oxygenated with 5% CO_2 and 95% O_2 prior to use and were maintained at 37±1°C. Freshly isolated cardiomycytes were used for electrophysiology or immunocytochemistry.

Immunocytochemistry and imaging. Freshly isolated RV and LV cardiomyocytes were fixed in cold acetone for 10 min at -20˚C. The cells were incubated with 10% normal goat serum (NGS) to block the background and were then stained with primary antibodies in 1% NGS and 0.1% Triton X-100 in PBS at 4˚C overnight. Cells were incubated with goat anti-rabbit IgG-AlexaFluor-488 or goat anti-mouse IgG-AlexaFluor-568 secondary antibodies (Invitrogen). Images were acquired at a spatial resolution of 17391 pixels per μm with a confocal microscope (Olympus Fluoview).

Whole-cell patch-clamp experiments

The recordings were performed with an L/M-PC amplifier (List-Medical, Darmstadt, Germany), set at 3 KHz filtering. pClamp/Clampex8 software (Axon Instruments, Molecular Devices, Sunnyvale, CA) was used for data acquisition and off-line analysis as previously described[4]. The bath solution contained (in mmol/L) NaCl 137, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 10, and glucose 11, pH 7.4, while the pipette solution contained (in mmol/L) Na_2ATP 6, KCl 115, $MgCl_2$ 1, EGTA 5 and HEPES 10, pH 7.4). Pipette resistance was 2.8±0.4 MΩ, seal resistance was 4.1±1.3 GΩ, and series resistance (Rser) was 6.6±3.1 MΩ. Whole-cell recordings after gigasealing were accepted for analysis if the membrane potential stabilized within a few minutes and if the membrane resistance measured in voltage-clamp at –60 mV (Rm(-60)) was >100 MΩ (to avoid leaky membranes). Long duration (2000 ms) depolarizing current pulses promoted triggered activity in cardiomyocytes from control rats as well as from rats with MCT-induced RV failure. Action potential duration (APD) at 90% repolarization (APD90), dispersion of repolarization (DR) as assessed by the difference between the maximal and minimal

APD, frequency and peak amplitudes of triggered activity were measured using pClamp/Clampex8 software.

Real time PCR

Total RNA from RV and LV was isolated using Trizol (Invitrogen, city, country) and reverse transcribed with gene specific primers using Omniscript RT kit (Qiagen, city, country). Controls were: (1) the reaction without reverse transcriptase, and (2) H_2O instead of cDNA.

Western blot analysis

Standard Western Blot analysis was performed using whole RV and LV lysates. Lysates were prepared by homogenizing the tissue in: 50 mmol/L Tris (pH 7.5), 1 mmol/L EDTA, 5 mmol/L $MgCl_2$, 150 mmol/L NaCl, 1 mmol/L DTT supplemented with phosphatase inhibitor cocktail (Roche, city, country). The samples were then centrifuged at 12,000 g for 10 min and the supernatants were collected. The protein concentrations were measured and 100 μg of protein was treated with SDS/DTT loading buffer prior to gel electrophoresis. The blots were probed with primary and secondary antibodies and visualized with the Odyssey™ Imaging System (Li-Cor). Equal loading of protein onto each lane in the gel was confirmed by analyzing the protein bands of vinculin or GAPDH. Quantification of protein levels was performed using the Metamorph software.

Immunohistochemistry and imaging

Heart was fixed in 4% paraformaldehyde (PFA) in 0.1 mol/L Na_2HPO_4 and 23 mmol/L NaH_2PO_4 (pH 7.4) for 4 h on ice. The tissue was then immersed in ice-cold 20% sucrose overnight to cryoprotect the tissue, mounted using OCT (Sakura Finetek, CA, USA) and transversal 6-7 μm sections were obtained with a cryostat. Tissue sections were stained with immunofluorescence, standard hematoxylin/eosin and Masson trichrome staining. The images were acquired using a light microscope (Axiovert 135, Zeiss Germany) or with a laser scanning confocal microscope (Olympus).

- **Immunofluorescence staining.** Hearts cross-sections (6 μm) were fixed in acetone for 15 minutes at –20°C. The sections were then washed with PBS+0.1% Triton X-100 three times, and incubated with 10% normal goat serum in PBS+0.1% Triton X-100 for 30 min to block the background. The sections were then incubated with primary antibodies in PBS+0.1% Triton X-100+ 1% normal goat serum at 4°C overnight. The sections were then washed with PBS+0.1% Triton X-100 three times, incubated with the appropriate secondary antibodies in PBS+0.1% Triton X-100+ 1% normal goat serum at room temperature for 1 h. For cellular hypertrophy assessment, after washing the secondary antibodies with PBS+0.1% Triton X-100 three times, the sections were incubated with wheat germ agglutinin (WGA, 1:200 dilution) in PBS+0.1% Triton X-100+ 1% normal goat serum for 1 h at room temperature. The sections were then washed with PBS three times and mounted using Prolong gold (Invitrogen) for imaging.

Quantification of cardiac fibrosis

Standard Masson trichrome staining was performed to assess cardiac fibrosis. With the use of a grid that divided the field of view into 100 squares, the number of collagenous tissue (blue stain) at the 100 intersection points in the grid was scored as 1 (present) or 0 (absent). Results are expressed as the percentage occupied by fibrosis to the total area examined.

Reagents

Primary antibodies used were: anti-caspase-3, anti-SERCA-2a, anti-Kv1.5, anti-KCNE2, anti GAPDH and anti-vinculin. Secondary antibodies used were goat anti-rabbit IgG-AlexaFluor-488 (1:1000) and goat anti-mouse IgG-AlexaFluor-568 (1:1000) for immunofluorescence, goat anti-rabbit IgG-AlexaFluor-680 (1: 100,000, all from Invitrogen) and goat anti-mouse IgG-IR Dye 800 CW (1: 100,000, Odyssey, LI-COR) for Western Immunoblotting.

Statistical Analysis

Means were compared between the control and PH groups using t tests as all outcomes were well modelled by Gaussian (normal) distribution. A two-sided *P* value less than 0.05 was considered statistically significant. Means and standard errors of the mean (SEMs) are reported.

References

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Legends for Supplemental Figures

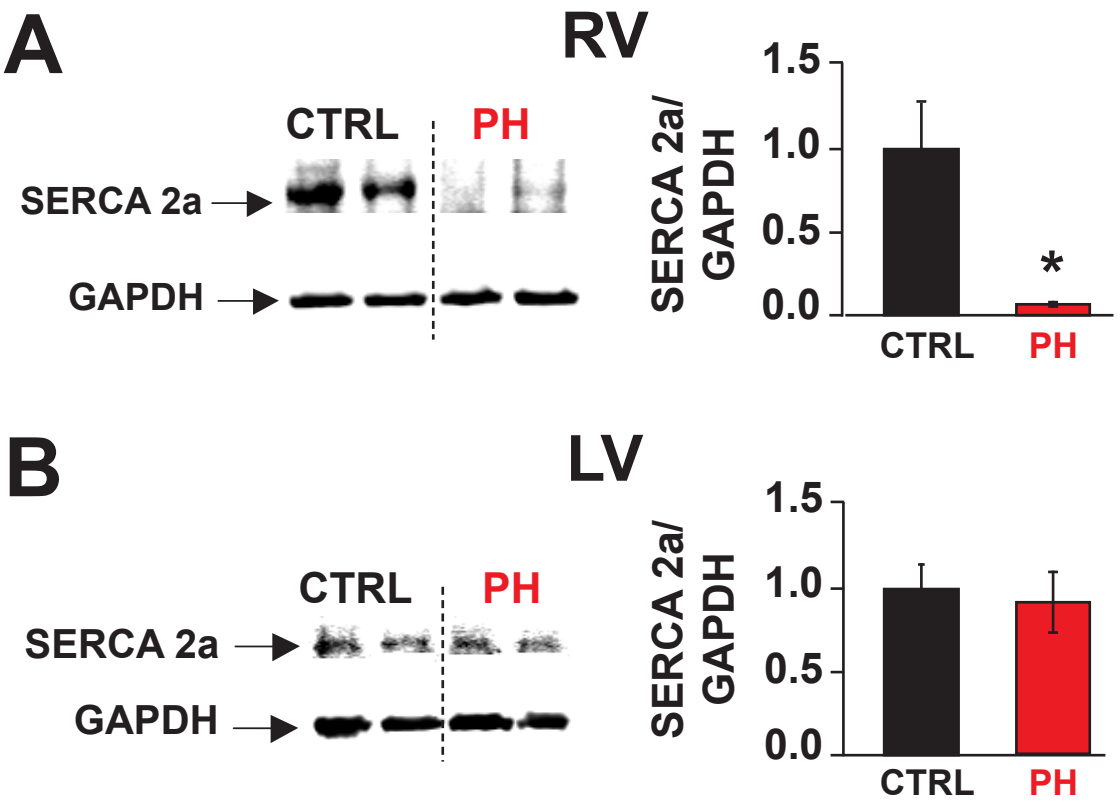
Figure 1. Selective downregulation of SERCA-2a protein in the RV of PH rats. Representative immunoblots of RV (**A**) and LV (**B**) from CTRL and PH-groups labelled with anti-SERCA2a and anti-GAPDH antibodies. Mean levels of SERCA2a protein normalized to GAPDH in CTRL (black bar) and PH (red bar) are shown at the right ($^{*}p<0.05$ *vs.* CTRL, n=3-4 rats/group).

Figure 2. Snap shots during VF initiated in a heart isolated 29 days after MCT injection. Panel A shows snap shots of activation during 212 ms of VF during a period shown by the double-headed arrow in panel C, with arbitrarily chosen 0 ms in the first snap shot shown in panel A. Asterisks indicate focal activation sites and arrows show the direction of the propagating wave with incomplete rotating patterns. Panel B is a schematic OAP with red denoting depolarization and blue repolarization. Panel C shows seven consecutive OAPs recorded from the RV epicardial surface at sites indicated 1 to 7 with white arrow in snap shot 0 ms in panel A. Notice the irregular OAP reflecting mixed focal along with incomplete reentrant wavefronts and irregularly propagating wavefronts.

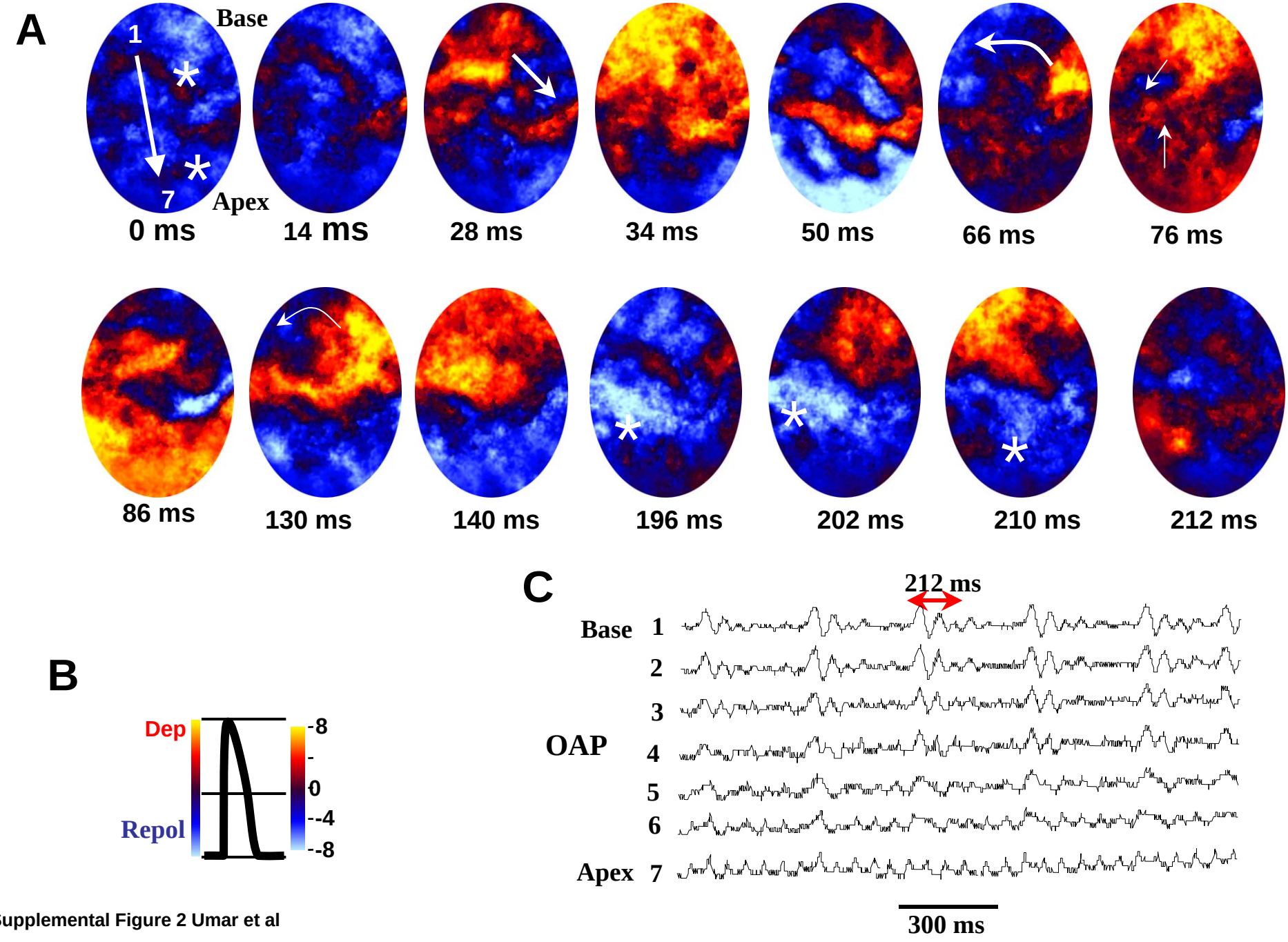
Figure 3. Direct effects of MCT on RV APD90. Bar graph showing mean action potential duration at 90% repolarization (APD90) in RV cardiomyocytes from normal healthy rats without (black bar) or with MCT (60mg/L) in the bath solution (red bar).

Legend for the Supplemental Movie

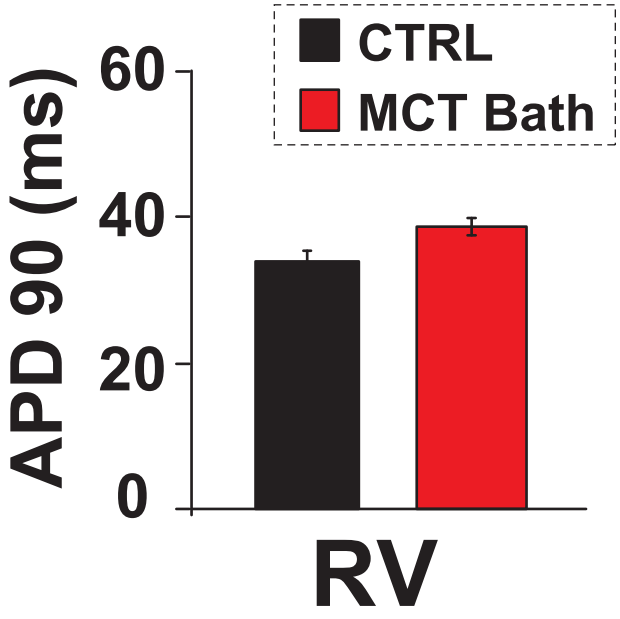
Movie showing activation wavefront dynamics during VF that becomes maintained in the failed RV by mixed focal and incomplete reentrant mechanisms.



Supplementary Figure 1 Umar et al



Supplemental Figure 2 Umar et al



Supplementary Figure 3 Umar et al