SUPPLEMENTAL MATERIAL

Mouse models and Drugs

All animal procedures were approved by the Animal Care and Use Committee of Vanderbilt University and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

The generation of all transgenic mouse lines expressing human troponin T (TnT-WT (line 3)) or mutant TnT (TnT-I79N (line 8)) has been described previously.[1] A total of 232 age-matched mice (9-14 weeks old) of both genders were used for experiments. All lines had been backcrossed for >10 generations into B6SJLF1/J strain and were bred in-house while continuing to use female B6SJLF1/J breeder mice obtained from Jackson laboratories (stock# 100012). Non-transgenic littermates (NTG) were used for experiments with EMD57033 (3 mmol/L stock solution in DMSO; a generous gift of MERCK KGaA, Darmstadt, Germany). Phosphodiesterase inhibition, often a problem with myofilament Ca^{2+} sensitizing compounds, is not observed at the concentration of 3 µmol/L used here.[2] (-)-Blebbistatin (BLEB) was purchased from Sigma, kept as 30 mmol/L stock solution in DMSO and protected from light. BLEB is an inhibitor of the actin-myosin interaction without any effects on action potential duration.[3] Effects of the TnT-I79N mutation, EMD and BLEB on myofilament Ca^{2+} sensitivity were published previously.[4]

Isolated Heart Perfusion / Volume conducted ECG

Hearts were excised after mice were deeply anesthetized with 1.2% Avertin (30 ml/kg) or 3-5% Isoflurane in 100% O_2 and the aorta was cannulated for retrograde perfusion at a constant pressure of 70 mm Hg as described.[4] The tyrode solution used for perfusion contained in mmol/L: NaCl 139, KCl 4, $NaHCO_3$ 14, NaH_2P0_4 1.2, $MgCl_2$ 1, $CaCl_2$ 1.5, Glucose 10, S-propanolol 0.0002. Tyrode solution was filtered before use and oxygenated with carbogen (95% O_2/5% CO_2) to achieve pH7.4. Hearts were lowered into a warm bath and volume conducted

ECGs recorded using two silver/silver chloride wire electrodes and a ground lead.[5] ECG was recorded using a custom built pre-amplifier and amplifiers connected to a PowerLab station (ADI instruments) and LabChart5 software. Unless described otherwise, a two-prong platinum pacing wire was inserted near the apex into the left ventricle and hearts paced according to the protocol shown in Fig. I. Pacing was applied via an isolated stimulator connected to a PowerLab (AD instruments) and controlled by LabChart5 software. Stimulus duration was 2.5 ms and current was set to 30% above threshold. When applicable, EMD or 0.1% DMSO was added fresh to the perfusion buffer via a syringe pump either to the bubble trap reservoir or heating coil directly above the heart.

Surface ECG

Mice were anesthetized with 1.2% Avertin (30 ml/kg, 2,2,2-tribromoethanol/ tertiary amyl alcohol) and placed in prone position on a warm surface essentially as previously published.[6] 24-gauge electrodes were inserted subcutaneously into all four forelimbs and connected to ECG leads. ECG (lead I and II) was recorded using amplifiers connected to a PowerLab station (AD instruments) and LabChart5 software. After a stable baseline was obtained, isoproterenol (Isuprel, 1.5 mg/kg) was injected i.p. and the ECG recordings continued for 12 min. Afterwards the animal was briefly subjected to 3% Isoflurane in 100% oxygen via nose-cone until loss of foot reflex, the heart harvested and rapidly processed.

ECG Analysis

The entire protocol was evaluated for the presence of ventricular arrhythmias from digitally stored files by the same investigator blinded to the treatment and/or genotype. Heart rate and QRS interval duration were determined by averaging 3 consecutive beats during sinus rhythm (in isolated paced hearts during short pacing breaks when pacing frequency was switched or pacing briefly stopped). Since mice do not show a flat ST-segment like humans to determine the

QRS duration, the S-peak was used. Traces were then examined for the presence of premature ventricular contractions (PVC) and ventricular tachycardia (VT; 3+ consecutive beats). Manual analysis and semi-automatic analysis using a built-in algorithm of LabChart7 software were compared and results closely matched.

Optical mapping

Hearts were stained with di-4-ANEPPS (Invitrogen, 0.5 mg/ml in DMSO) slowly injected through a port into the bubble trap above the perfusion cannula and fluorescence was excited by a diode-pumped, solid-state laser (Verdi, Coherent, CA) at a wavelength of 532 nm delivered to the heart via optical fibers.[7] The left ventricle was stimulated in the center of the field of view with a platinum pacing electrode (0.1-1 mA, 2 ms duration, 10 Hz). To prevent tissue damage at the contact site, stimulation via this electrode was limited to 3 min. Images were recorded with a high-speed CCD camera (14-bit, 80x80 pixels, 1000 fps, RedShirt Imaging) equipped with a 52 mm standard lens and the emitted light passed through a high pass filter. The imaging area was adjusted and typically about 12×12 mm. Custom developed "C"-based software was used to control data acquisition, stimulus timing and laser illumination as described.[8] Fast (longitudinal) and slow (lateral) CVs were identified by plotting local CVs against orientation. Local CVs were calculated after applying a Gaussian 3x3 spatial filter and 3 ms moving average temporal filter. Isochronal maps were computed by a custom Matlab-based program and activation times plotted versus distance from the center for each angle, plotted and the two fast and slow CVs averaged. No assumptions about the fiber orientation were necessary. Motion artifacts did not play a significant role, because the activation is fast and precedes contraction.

Sample Fractionation/Homogenization

Heart samples for protein analysis had been flash frozen and stored at -80°C. Samples were fractionated following a modified protocol according to Smyth et al. [9] Each heart was pulverized

in a metal mortar cooled in liquid N_2 (Bessman tissue pulverizer) and transferred into a 2 ml Dounce homogenizer flask. Each sample was homogenized with the same number of strokes in 15 vol of homogenization buffer containing in mmol/L: Tris 50, Triton-X 100 1%, sodium fluoride 10, sodium-ortho-vanadate 5, sodium pyrophosphate 1, β-glycerophosphate 10, pH 7.5 with protease inhibitors (Sigma, P8340) added fresh before use. Afterwards homogenates were agitated for 2 hrs at 4°C. SDS was added to a small aliquot to a final concentration of 1% and samples flash frozen (= **total**). The remaining sample was centrifuged at 500×g for 10 min at 4°C and an aliquot of the supernatant supplemented with SDS and flash frozen (= **low spin**). The remaining supernatant was transferred into a new tube and centrifuged at 7000×g for 20 min at 4°C. One last aliquot was supplemented with SDS and flash frozen (= **soluble**). Protein concentration was determined according to Lowry.

Western Blotting

SDS polyacrylamid gels according to Sambrook et al. [10] were made fresh before electrophoresis essentially as described before.[11-13] Samples were diluted in homogenization buffer to achieve the same protein concentration, mixed with one volume of Laemmli sample buffer (BioRad 161-0737) containing 5% beta-mercaptoethanol and 40 mmol/L ditheiothreitol and heated to 55°C for 5 minutes. Proteins were transferred overnight to nitrocellulose (0.45 µm pore size, BioRad) and membranes incubated in 6% fat free dry milk in Tris-buffered saline containing 0.2% Tween20. The following antibodies were applied in Odyssey Blocking Buffer (LICOR Biosciences, NE): anti-Connexin43 (SIGMA, C6219 polyclonal rabbit), anti-alpha-tubulin (Abcam ab7291 mouse monoclonal), anti-AMP-kinase alpha (Cell Signaling 2603 (23A3) rabbit monoclonal) and anti-phospho Thr 172 AMP-kinase (Cell Signaling 2535 (40H9) rabbit monoclonal). Secondary fluorescent antibodies recognizing rabbit (IRDye 800 conjugated anti-rabbit IgG) or mouse IgG (IRDye 700DX conjugated anti-mouse IgG, both Rockland) were

detected with a LICOR Odyssey near-infrared scanner and signals were analyzed using Odyssey 3.0 software.

Immunohistochemistry

Heart tissue was embedded horizontally in OCT and flash frozen in liquid N_2 cooled 2-methylbutane. 10-16 µm thick frozen cross-sections (frontal, four chamber view) were fixed in 4% formaldehyde for 30 min and then washed in phosphate buffered solution (PBS) 3 times for 10 min. After pre-incubation in PBS containing 5% goat serum and 0.4 % Triton X-100, primary antibodies were incubated overnight at 4°C. In addition to the antibodies listed under "Western Blotting", a mouse monoclonal anti-connexin 43 antibody specific for the P0 isoform was used (Invitrogen 13-8300). This Cx43-P0 antibody was directly labeled with AlexaFluor 488 using the APEX kit according to the manufacturer's instructions (Invitrogen A10468) or purchased pre-labeled (Invitrogen, 13-8388). The secondary anti-rabbit antibody (Alexa Fluor 568 Goat Anti-Rabbit IgG, Invitrogen) was applied in PBS containing 0.4 % Triton X-100 for 1 hr at RT. Sections were mounted in ProLong Gold antifade reagent (Invitrogen) and cured for 24 hrs before coverslip edges were sealed with nail polish.

Imaging and Image Analysis

Two channel composite images of immunostained sections were acquired using a Zeiss LSM 510 inverted confocal scanning microscope (part of the VUMC Cell Imaging Shared Resource (supported by NIH grants CA68485, DK20593, DK58404, HD15052, DK59637 and EY08126)) by an investigator blinded to the treatment and genotype of the sample. Settings to acquire images were identical within sets of samples and only minor adjustments were made between sets. At least five images (0.45x0.45 mm) per section were acquired from all regions of the left ventricle (LV free wall, apex and septum) while avoiding areas containing large vessels and regions where the pacing wire was attached. Image sampling was stratified that if areas with

Cx43-P0 accumulation were found after visual inspection, these areas were included during image acquisition. This reduced the number of images necessary to document these regions ("patches") and prevented false negative results.

Quantitative analysis was performed using ImageJ 1.43 (NIH) using only raw images as illustrated in Fig. II. Background signal was negligible in buffer perfused hearts, but was subtracted from non-perfused hearts. Signal area and signal integrated density both normalized to the area covered by tissue were determined for Cx43 and Cx43-P0 for each image by "thresholding" and conversion into binary images.

Lucifer Yellow Dye transfer (modified scrape loading)

Gap junctional function was assessed essentially as described previously.[14, 15] Isolated perfused hearts were subjected to a standardized pacing protocol (Fig. IA) and then washed for 3 min with Ca^{2+}-free Dulbecco's PBS. The left ventricle was punctured at multiple sites with a 27-gauge needle and a solution injected containing 0.5% Lucifer yellow DH (LY) and 0.3% Tetramethylrhodamine dextran (TMR, MW 10.000, both Invitrogen) in 120 mM LiCl. LY will diffuse through gap junctions, while TMR is not permeable. After 15 min, the left ventricle was fixed in 4% formaldehyde for 30 min, embedded in OCT and flash frozen in liquid N_2 cooled 2-methylbutane. 18 µm sections were immunostained using Cx43-P0 specific antibody and analyzed using a Zeiss LSM 510 inverted confocal scanning microscope. For each needle puncture, three images were obtained detecting LY, TMR and Cx43-P0. Dye spread distance was analyzed using ImageJ, measuring the distance between the border of the TMR signal to the border of the LY signal. Four measurements per puncture were obtained, two for the maximal transverse spread and two for the maximal longitudinal spread on each side, which were then averaged.

Statistics

GraphPad Prism 5.0 (GraphPad Software Inc.) or tools from the website http://vassarstats.net/ were used for statistical analysis. Analysis was performed using unpaired two-tailed t-test with Welch's correction, one-way ANOVA followed by Tukey's multiple comparisons test, repeated measures ANOVA (mixed model) or Fisher exact probability test as appropriate. All values are mean ± SEM. P values of less than 0.05 were considered significant.

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Fig. I

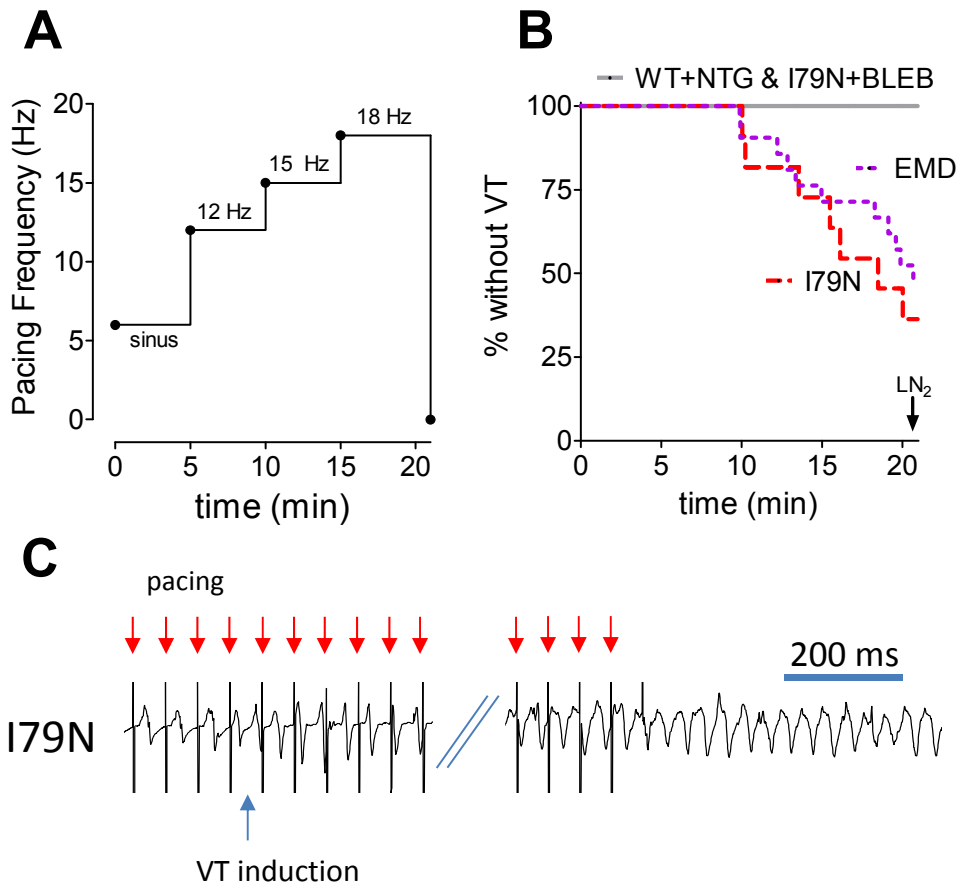

Fig. I: Rapid pacing induces VT in Ca^{2+} sensitized isolated perfused hearts. (A) Experimental pacing protocol to generate samples for all biochemical assays. All isolated perfused hearts were processed (e.g. frozen) after 21 min of perfusion time. ECG was monitored during all experiments and traces analyzed for occurrence of VT. (B) The incidence of VT during pacing ("arrhythmia survival") of isolated hearts. VT was not observed in TnT-WT or NTG and there were also no arrhythmias in TnT-I79N hearts treated with BLEB, but in 63% of TnT-I79N hearts and 52% of EMD treated hearts. (C) Representative ECG trace of a TnT-I79N heart with VT. VT induction in this heart occurred during pacing at 18 Hz.

Fig. II

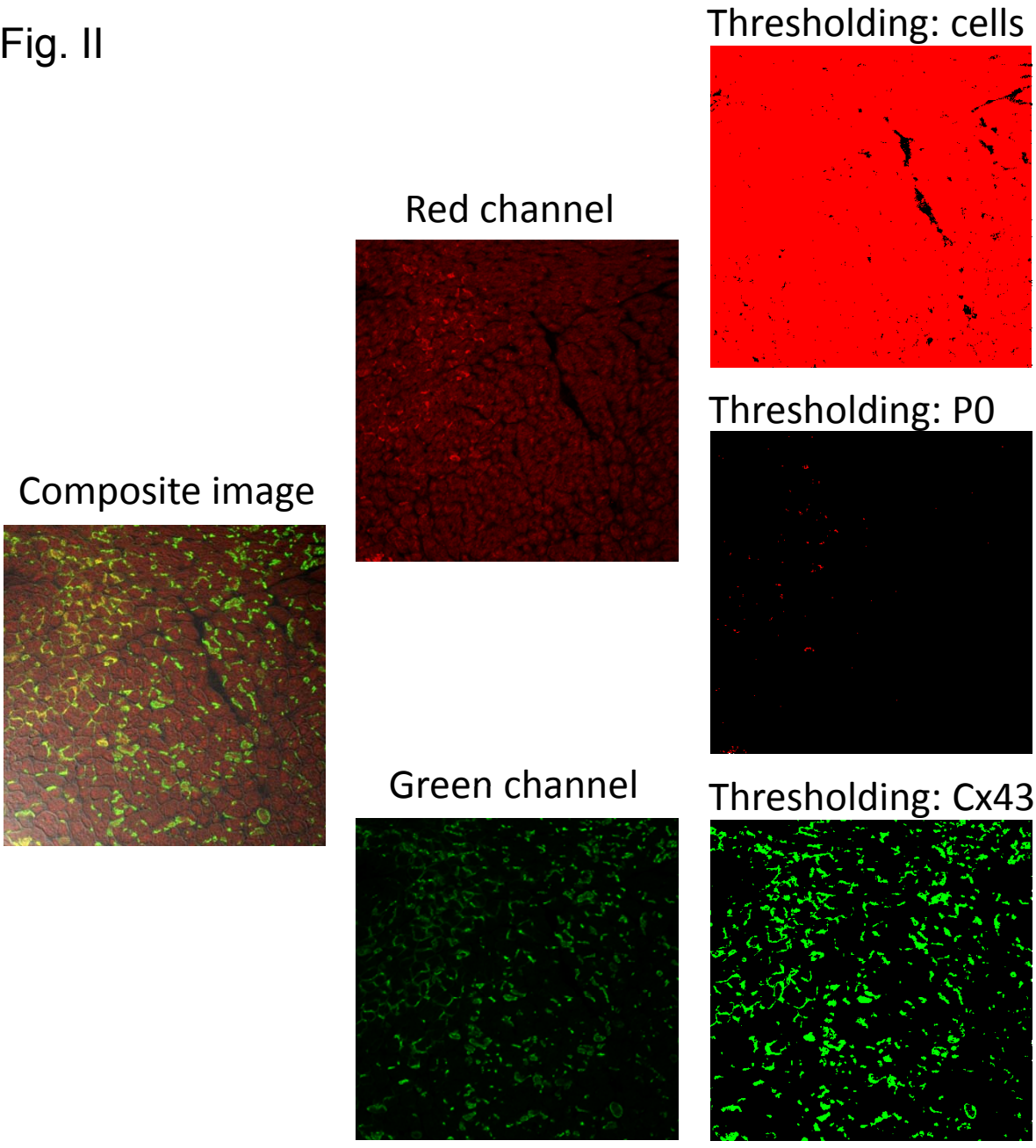

Fig. II: Quantitative analysis of confocal images. All images were recorded with a Zeiss 510 confocal scanning microscope and analyzed using ImageJ 1.43 (NIH). Each channel was converted into a binary image using the “threshold” function (fixed threshold for each experimental set). The integrated signal density per channel is then calculated from the number of pixel sand mean intensity per pixel determined using the histogram function and normalized to the area occupied by cells.

Fig. III

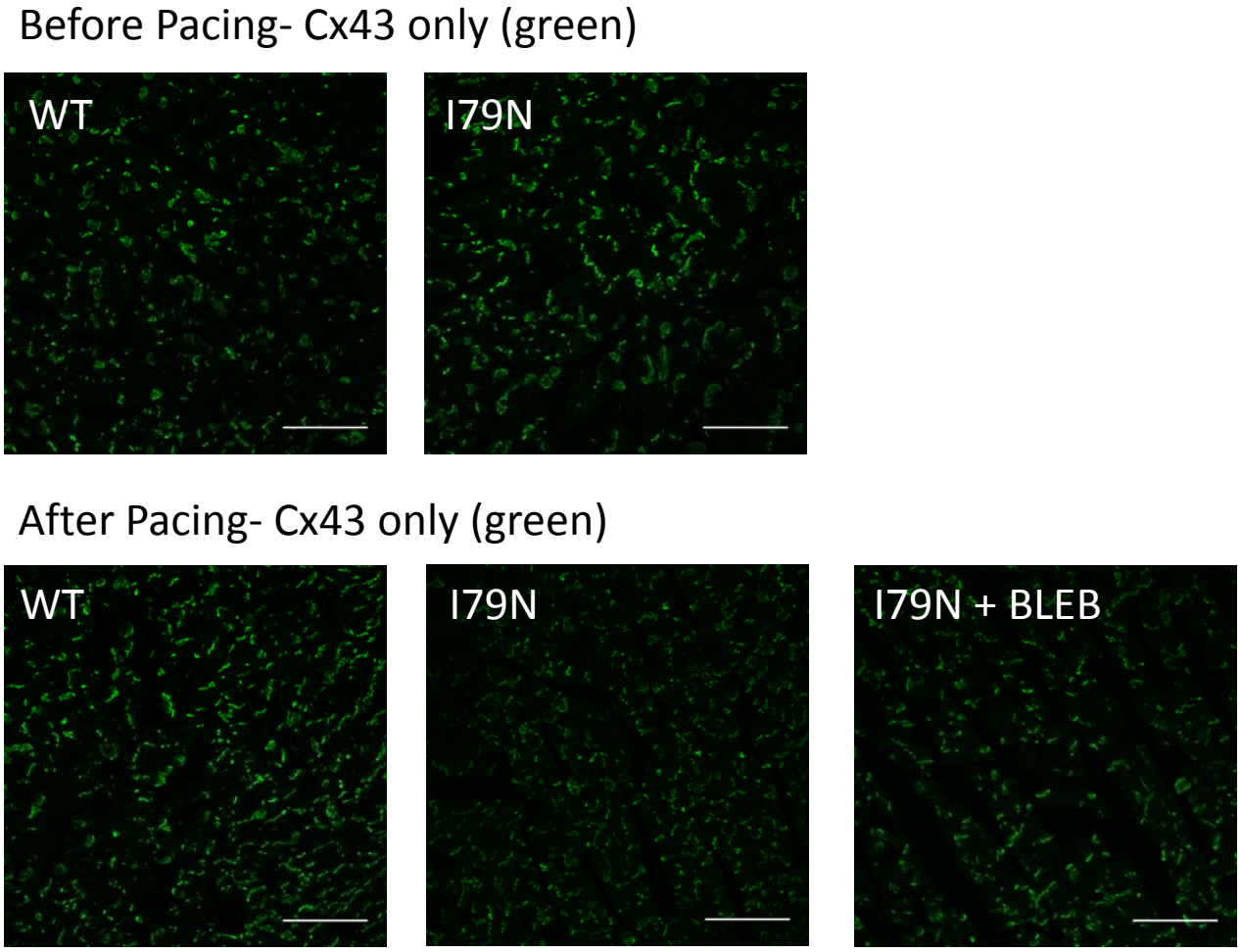

Fig. III: Effect of pacing on Cx43 ("green"). Shown is Cx43 staining only. Representative images for WT and I79N after 3 min of perfusion without pacing (top) and after pacing (bottom) including I79N treated with BLEB. Pacing protocol as shown in Fig. S1A. Summary data are shown in Fig. 3.

Fig. IV

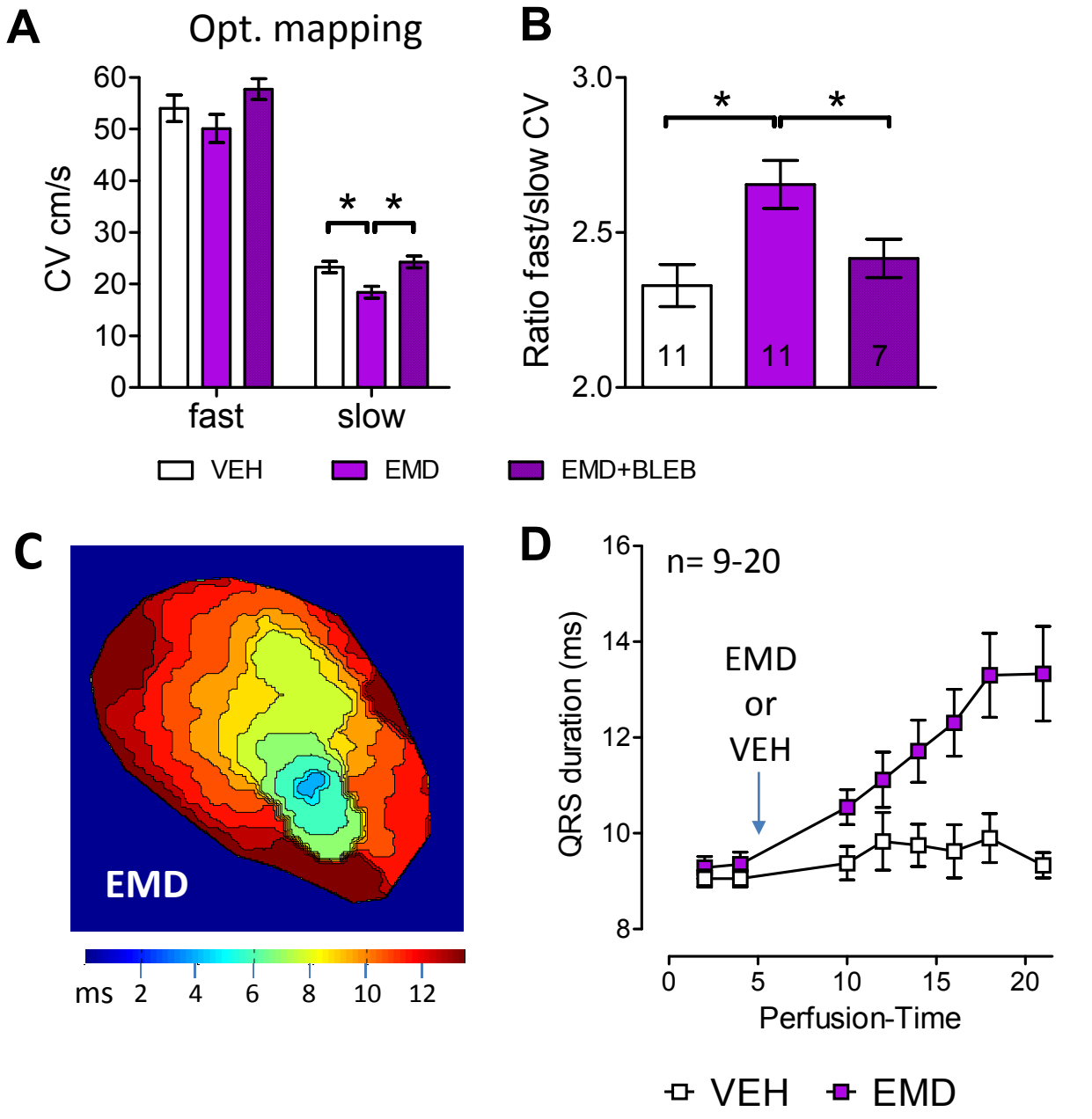

Fig. IV: Slowed lateral CV, QRS prolongation and arrhythmias in EMD treated control hearts. Longitudinal (fast) and transverse (slow) conduction velocity (A) and anisotropy ratio (B) in control (vehicle, VEH, 0.1% DMSO) and hearts treated with EMD. N numbers are indicated in columns. (C) Abnormal isochronal map observed after a few minutes of perfusion with EMD. (D) QRS prolongation observed in NTG hearts after perfusion with EMD. QRS duration was measured from sinus beats during short pacing breaks. EMD (3 μM) or vehicle (VEH, DMSO 0.1% final) were added after 5 min of equilibration (as indicated by arrow).