SUPPLEMENTAL MATERIAL

METHODS

All animal procedures were conducted in compliance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the Ohio State University Institutional Animal Care and Use Committee. We studied a canine model of post (6-15 weeks) left ventricular infarction (MI, left anterior descending artery ligation). For optical mapping experiments, five MI (age 2.5±1.4 years, weight 22±2 kg, four females and one male) and four control dogs of similar age and weight were used. For Holter monitoring, an additional five MI and four normal control dogs were used.

Surgical preparation: The animals were anesthetized and instrumented to measure the ventricular electrogram. The left anterior descending coronary artery was also isolated during the instrumentation surgery and a two-stage occlusion of this artery was then performed approximately one-third the distance from its origin in order to produce an anterior wall myocardial infarction ~16% of left ventricular mass.[1] This vessel was partially occluded for 20 min and then tied off. The dogs were given analgesic, antibiotic, and anti-arrhythmic therapy to alleviate post-operative pain, to prevent post-operative infection, and to reduce acute arrhythmias associated with the myocardial infarction as has been previously described.[1, 2]

Holter monitoring: A Holter monitor was placed on the dogs (5 MI and 4 control) with a 2 lead system with electrodes in a standard precordial placement. Before electrode placement, an area was prepared by shaving and cleaning the skin with alcohol. An elastic bandage and a specially designed vest were used to secure the Holter recorder and leads to the dog. All dogs wore the Holter monitor for at least 24 hours.

Dog Heart Isolation: A detailed description of this canine model has been recently published.[2] Dogs were sedated with 5 to 8mg (~25mg/mg) butorphanol IM about 10 minutes prior to induction

of anesthesia. An IV catheter was placed in a peripheral vein and 10mg (0.5mg/kg) of valium was given immediately followed by 30mg (~1.5mg/kg) of etomidate IV. Dogs were intubated and placed on a ventilator with 2% to 5% isoflurane for maintenance of a deep plane of anesthesia. A right thoracotomy was performed either between the 3rd and 4th or 4th and 5th rib. 3000 units of heparin were given IV. Loops were placed around the cranial and caudal vena cava and the azygos vessel was tied off. The pericardium was opened and the heart was exposed. A Satinsky clamp was placed around the aorta but not clamped. The caudal vena cava was tied off and a small opening was cut just above the tie for discharge of the cardioplegia/blood from the heart. The caudal vena cava was tied off and the aorta clamped. A 16-gague needle which was attached to a bag of 1000mL cardioplegia was inserted into the cranial vena cava just below the tie and the cardioplegia was allowed to run into the heart. Ice was also immediately placed in the thoracic cavity. Once 1000mL cardioplegia had been perfused through the coronary arteries, the heart was removed from the thoracic cavity for further processing.

Optical mapping data analysis and interpretations: Optical mapping of the canine SAN has been previously described.[17, 18] The excitation-contraction uncoupler blebbistatin[19] (10-20 μM, Tocris Biosciences, Ellisville, MO) and the near-infrared voltage-sensitive dye, di-4-ANBDQBS[20] (10-40 μM) were added to the perfusate. All preparations tested had uniform staining, consistent with an absence of ischemic (non-perfused) areas throughout the mapped region. Optical mapping was performed at a rate of 1000 frames/sec with the MiCam Ultima-L CMOS camera (SciMedia, Costa Mesa, CA) with an optical field of view of 25x25 mm (250 μm/pixel).

SAN reentry was provoked by different atrial pacing rates ranging from 3.3 Hz up to 10 Hz (3.3, 4, 5, 6, 6.5. 7, 7.5, 8 and 10) for 30 seconds at baseline and after application of acetylcholine (ACh, 0.1, 0.3 and 1 μM) and/or isoproterenol (Iso, 0.01 and 0.1 μM). Concentrations of ACh and Iso (alone or in combination) that were used in the present study to provoke SAN arrhythmias were based upon doses obtained in previous canine *in situ* [21] or *in vitro*[15, 18] mapping studies.

We previously demonstrated that optical mapping, with the use of near infrared dyes, can record fluorescent signals from myocardium layers as deep as 1-3 mm.[3-5] A custom-designed Matlab program was used to analyze multicomponent intramural optical action potentials (OAPs) as previously described.[6] Activation times and corresponding conduction velocities (CV) were defined in the SAN tissue using 50% of the OAP amplitude (AP50%), while activation times of the atrial layer were defined by the dV/dt_{max} for the atrial upstroke component of the OAP. To more precisely analyze the SAN activation pattern during atrial tachypacing, the SAN OAP extraction algorithm was applied to remove atrial components from SAN OAPs (see **Figure 2 D**).

To analyze atrial and SAN activation frequency during fast atrial pacing and atrial flutter and fibrillation (AF/AFL), a Fast Fourier Transform was applied to determine the dominant frequencies (DF).[6, 7] To measure the SAN activation frequency during pacing and atrial reentrant arrhythmias, we used low frequency filters ranging from 10-20 Hz, separating the slow upstroke SAN signals from the fast atrial signals.

Histological examination and anatomic correlation: Histology was performed as previously described.[3] The SAN preparations were paraffin-embedded and sectioned parallel to the epicardium. To determine the role of the cardiac structure in SAN excitation, we used Masson's trichrome staining (International Medical Equipment, San Marcos, CA, USA) and immunostained with Rb-Cx43 (1:400; Sigma, St. Louis, MO) as previously described[6] to examine 5 μm thick by 35±5 mm x 25±5 mm sections, that were centered around the leading pacemaker site. Following optical mapping, pins (0.25 mm in diameter) were inserted around the SAN to precisely register optical mapping with histology data. Anatomic structures of the canine SAN pacemaker complex were identified as described previously.[3]

Statistics: Quantitative data are shown as mean ± SD. Hypothesis testing was performed using an unpaired Student's t-test or non-parametric Mann-Whitney test (Minitab 16) dependent on normality assumptions which were tested using Anderson-Darling test. For reentry cycle length vs post-reentry sinus cycle length in the same dog, a paired t-test was used. Analysis of fibrosis data

was done using PROC MIXED procedure in SAS 9.2. Model included SAS as a response and treatment and dog as predictors; treatment was used as fixed effect, dog as random effect nested within the treatment. The significance of between-group SAN reentry incidence was tested between MI and control animals using Fisher's exact test in SAS 9.2. A $P<0.05$ was considered to be statistically significant.

The following statistical significance of results has been observed:

Sinus cycle length

Comparison: Control vs. MI

Anderson-Darling test for normality: Control p-value = 0.519 ; MI p-value = 0.157

No significant departures from normality; t-test can be used

T-test p-value = **0.031**

SAN conduction time

Comparison: Control vs. MI

Anderson-Darling test for normality: Control p-value = **0.032**; MI p-value = 0.061

Departures from normality; Mann-Whitney test was used

Mann-Whitney test p-value = **0.0233**

Pauses

Comparison: Control vs. MI

Anderson-Darling test for normality: Control p-value = 0.787; MI p-value = **0.006**

Departures from normality; Mann-Whitney test was used

Mann-Whitney test p-value = **0.0200**

Reentry cycle length vs. post-reentry sinus cycle length

Comparison: reentry vs. sinus rhythm

Paired data; Anderson-Darling test for the differences: p-value = 0.372

No significant departures from normality for differences

Paired t-test p-value: **0.001**

Fibrosis

Comparison: Control vs. MI

Treatment effect p-value **<.0001**

Estimated difference between control and MI is 14.97 units (this is a difference between least squares means, means adjusted for dog effects)

SAN Reentry incidence

Comparison: Control vs. MI

Fisher's exact test p-value: **0.025**

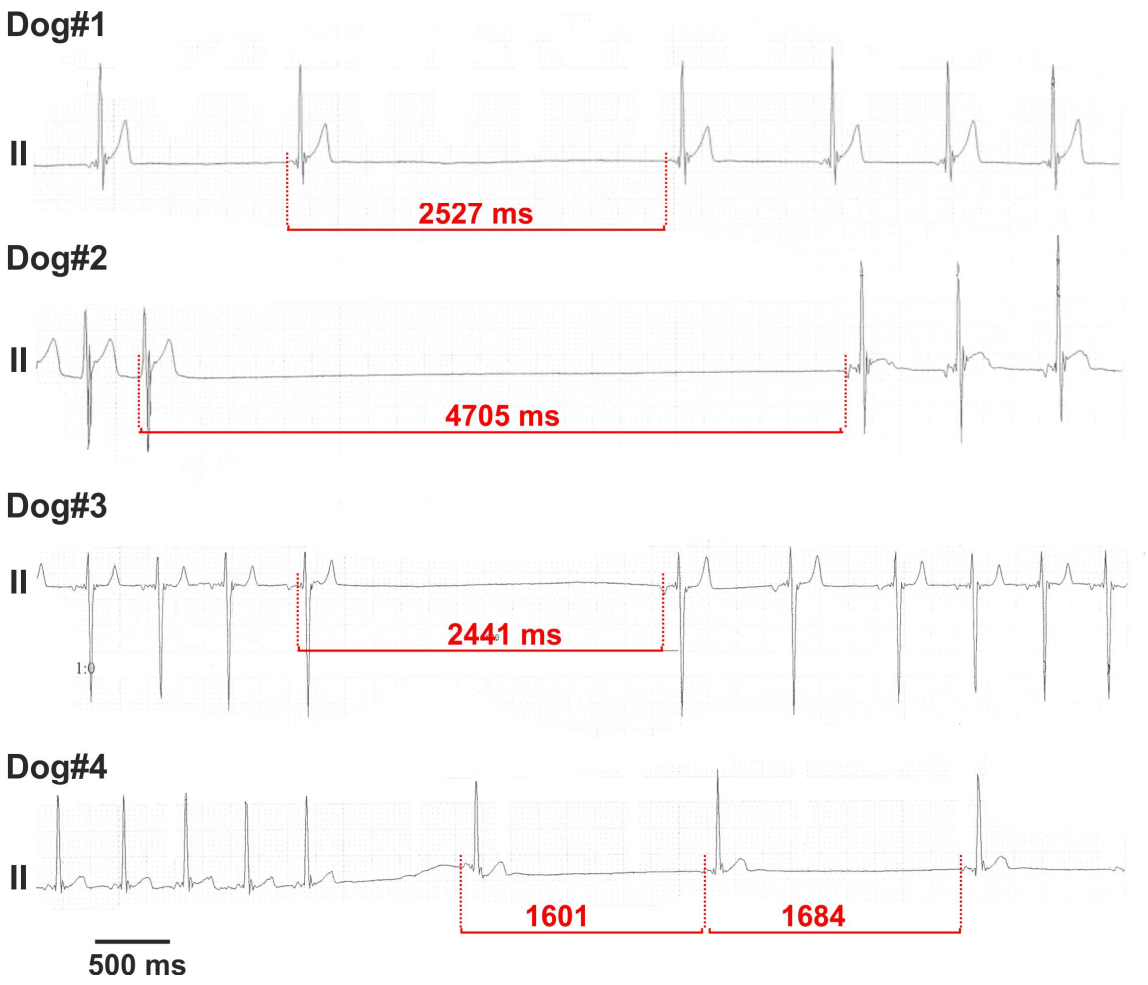
Reference List

1. Billman GE. A comprehensive review and analysis of 25 years of data from an in vivo canine model of sudden cardiac death: Implications for future anti-arrhythmic drug development. *Pharmacology & therapeutics*. 2006;111:808-835.
2. Billman GE, Nishijima Y, Belevych AE, Terentyev D, Xu Y, Haizlip KM, Monasky MM, Hiranandani N, Harris WS, Gyorke S, Carnes CA, Janssen PM. Effects of dietary omega-3 fatty acids on ventricular function in dogs with healed myocardial infarctions: In vivo and in vitro studies. *Am J Physiol Heart Circ Physiol*. 2010;298:H1219-1228.
3. Fedorov VV, Schuessler RB, Hemphill M, Ambrosi CM, Chang R, Voloshina AS, Brown K, Hucker WJ, Efimov IR. Structural and functional evidence for discrete exit pathways that connect the canine sinoatrial node and atria. *Circ Res*. 2009;104:915-923.
4. Fedorov VV, Glukhov AV, Chang R, Kostecki G, Aferol H, Hucker WJ, Wuskell JP, Loew LM, Schuessler RB, Moazami N, Efimov IR. Optical mapping of the isolated coronary-perfused human sinus node. *J Am Coll Cardiol*. 2010;56:1386-1394.
5. Fedorov VV, Ambrosi CM, Kostecki G, Hucker WJ, Glukhov AV, Wuskell JP, Loew LM, Moazami N, Efimov IR. Anatomic localization and autonomic modulation of atrioventricular junctional rhythm in failing human hearts. *Circulation. Arrhythmia and electrophysiology*. 2011;4:515-525.

6. Fedorov VV, Chang R, Glukhov AV, Kostecki G, Janks D, Schuessler RB, Efimov IR. Complex interactions between the sinoatrial node and atrium during reentrant arrhythmias in the canine heart. *Circulation*. 2010;122:782-789.

7. Berenfeld O, Zaitsev AV, Mironov SF, Pertsov AM, Jalife J. Frequency-dependent breakdown of wave propagation into fibrillatory conduction across the pectinate muscle network in the isolated sheep right atrium. *Circ Res*. 2002;90:1173-1180.

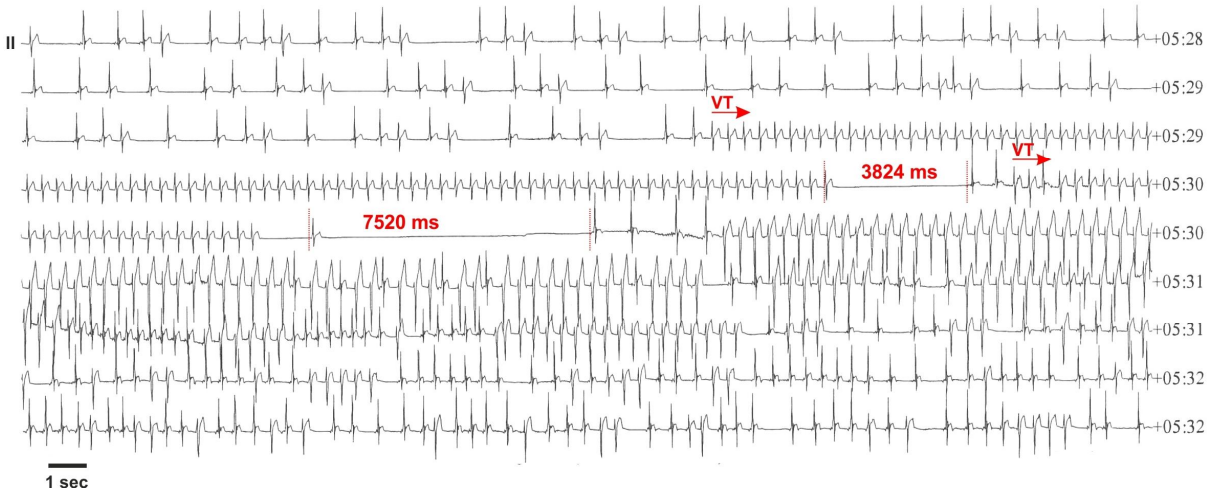
Supplement Figure 1.



Supplement Figure 1. Heart rhythm alternations in MI dogs.

ECG recordings demonstrate maximum atrial pauses obtained from non-anesthetized post-MI dogs *in vivo*. The dogs repeatedly exhibited evidence of apparent "SAN block" as well as apparent episodes of accelerated heart rhythm with the same ECG P-wave morphology as seen in normal sinus rhythm (these changes did not coincide with respiratory activity).

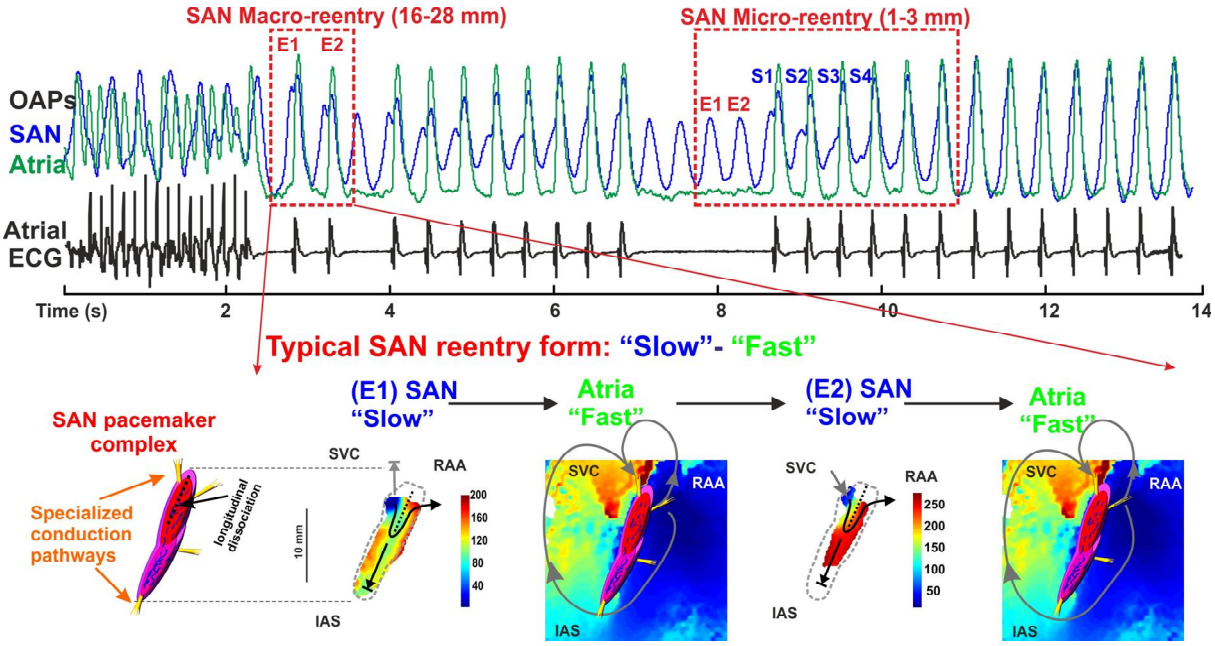
Supplement Figure 2.



Supplement Figure 2. Atrial pauses caused by either ventricular or supraventricular tachycardia in MI dogs.

Continuous ECG recording obtained from MI dog is shown. In this dog, the significantly prolonged atrial pauses appeared after spontaneous termination of tachyarrhythmias. Notice a different morphology of ECG during both tachyarrhythmias (5.29-5.30 versus 5.30-5.31) and during heart rhythm alternations (5.28-5.29 and 5.32).

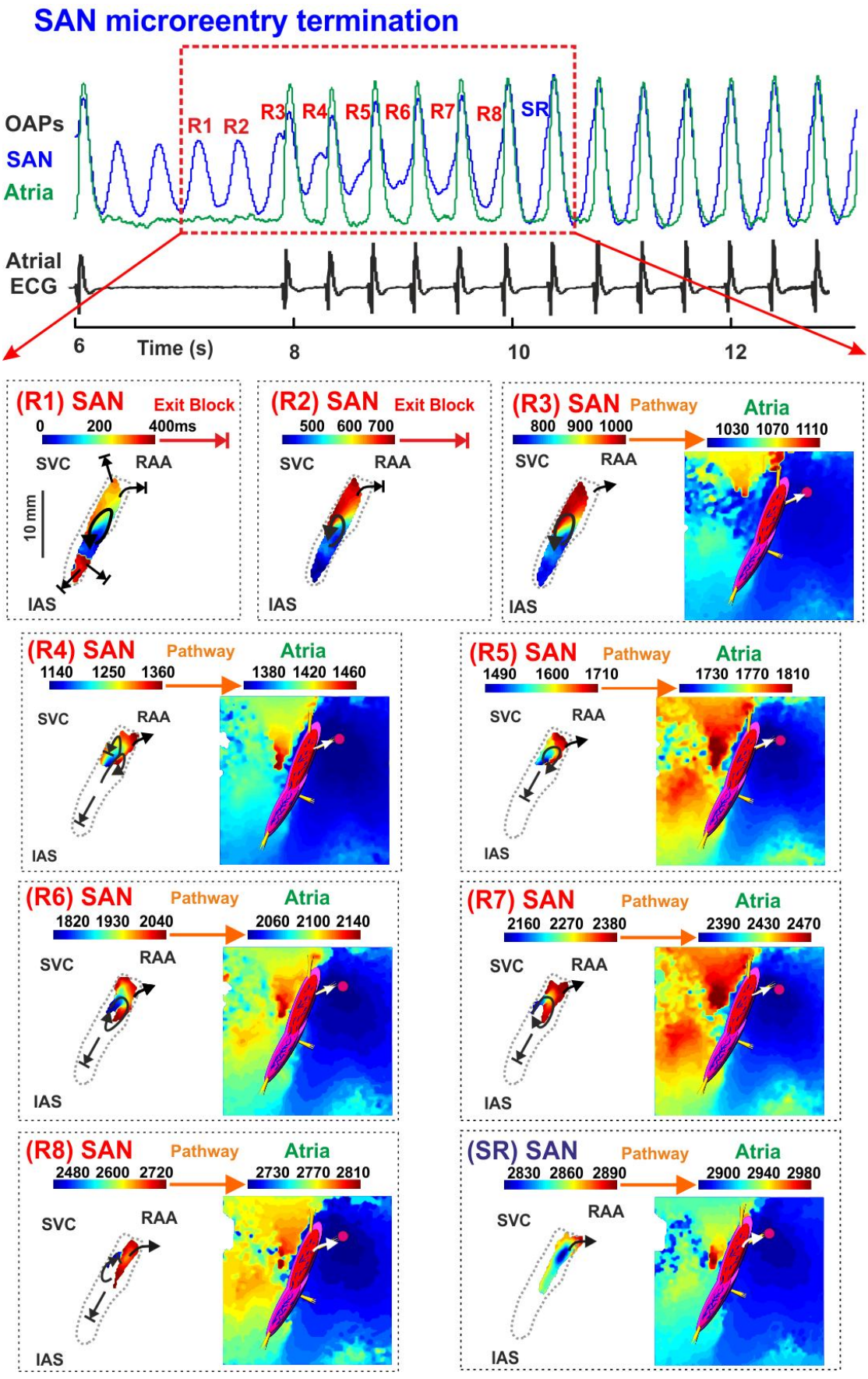
Supplement Figure 3.



Supplement Figure 3. Typical SAN Macro-reentry.

Termination of atrial tachypacing (7.5 Hz) during perfusion of 0.01 μM Iso induced multiple heart rate abnormalities characterized by irregular rhythm and profound pauses on the ECG. Optical mapping of the SAN revealed several forms of reentries associated with the SAN, such as SAN macro-reentry. The first form of reentry observed was macro-reentry through the SAN. During circus movement, the SAN was activated by the atria through the left superior sino-atrial conduction pathway (SACP) and then successively re-activated the atria through the right superior SACP. After two consecutive marco-reentrant beats, the propagation through the SACP was blocked and macro-reentry transformed into a sustained counterclockwise micro-reentry within the SAN. This figure corresponds to **Movie 3 and 4**. SVC and IVC – superior and inferior vena cava; RAA – right atrial appendage; PV – pulmonary veins; IAS – interatrial septum.

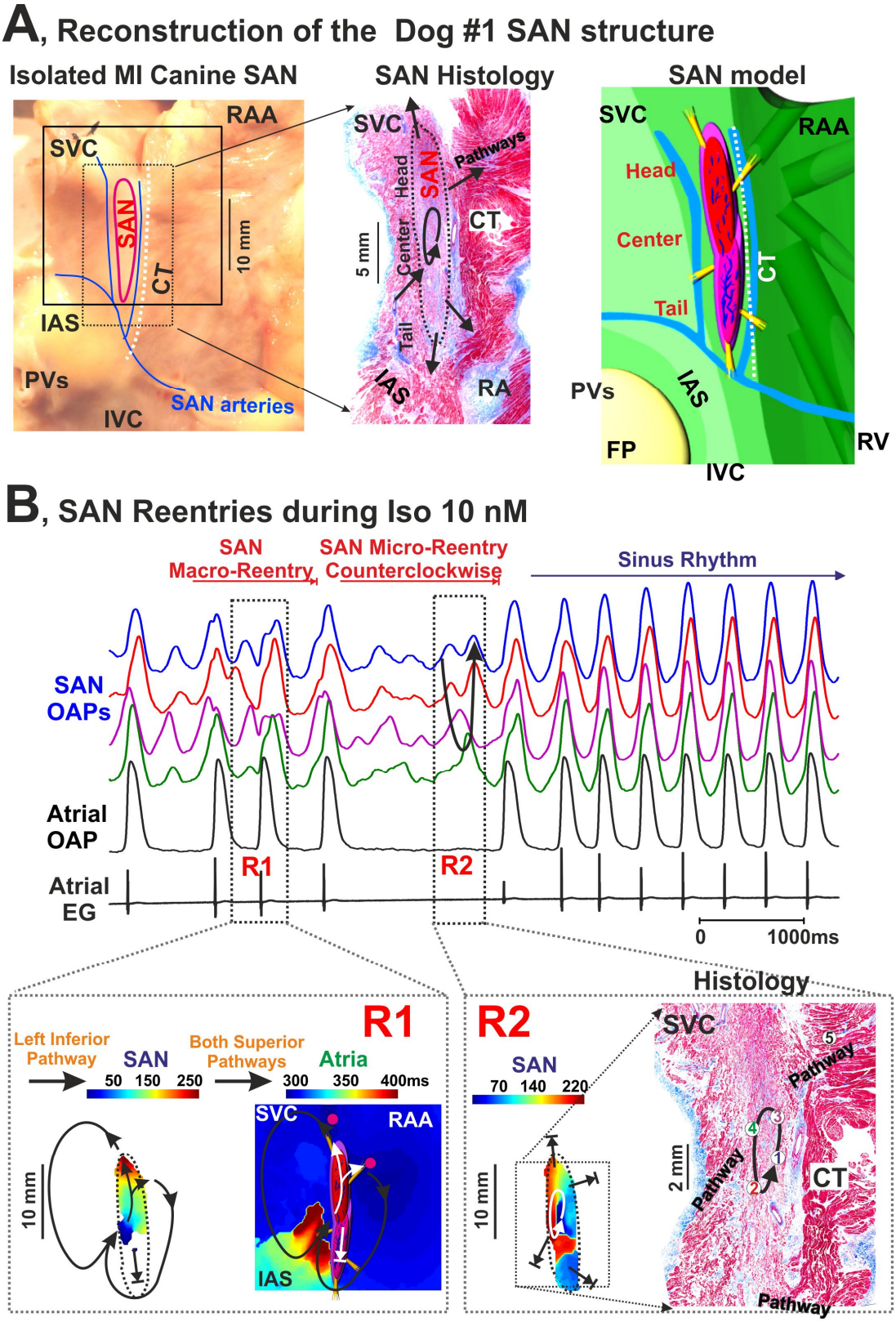
Supplement Figure 4.



Supplement Figure 4. Counterclockwise to clockwise reversal of the reentry direction inside the post-MI canine SAN.

The same arrhythmia episode as shown in **Figure 5.** Below the OAPs and ECG, the switching process is shown in detail. The black circuits indicate the direction of reentry waves inside the SAN while white arrows show the exit of conduction to the atria through superior SACPs. This figure corresponds to **Movie 4**.

Supplement Figure 5.

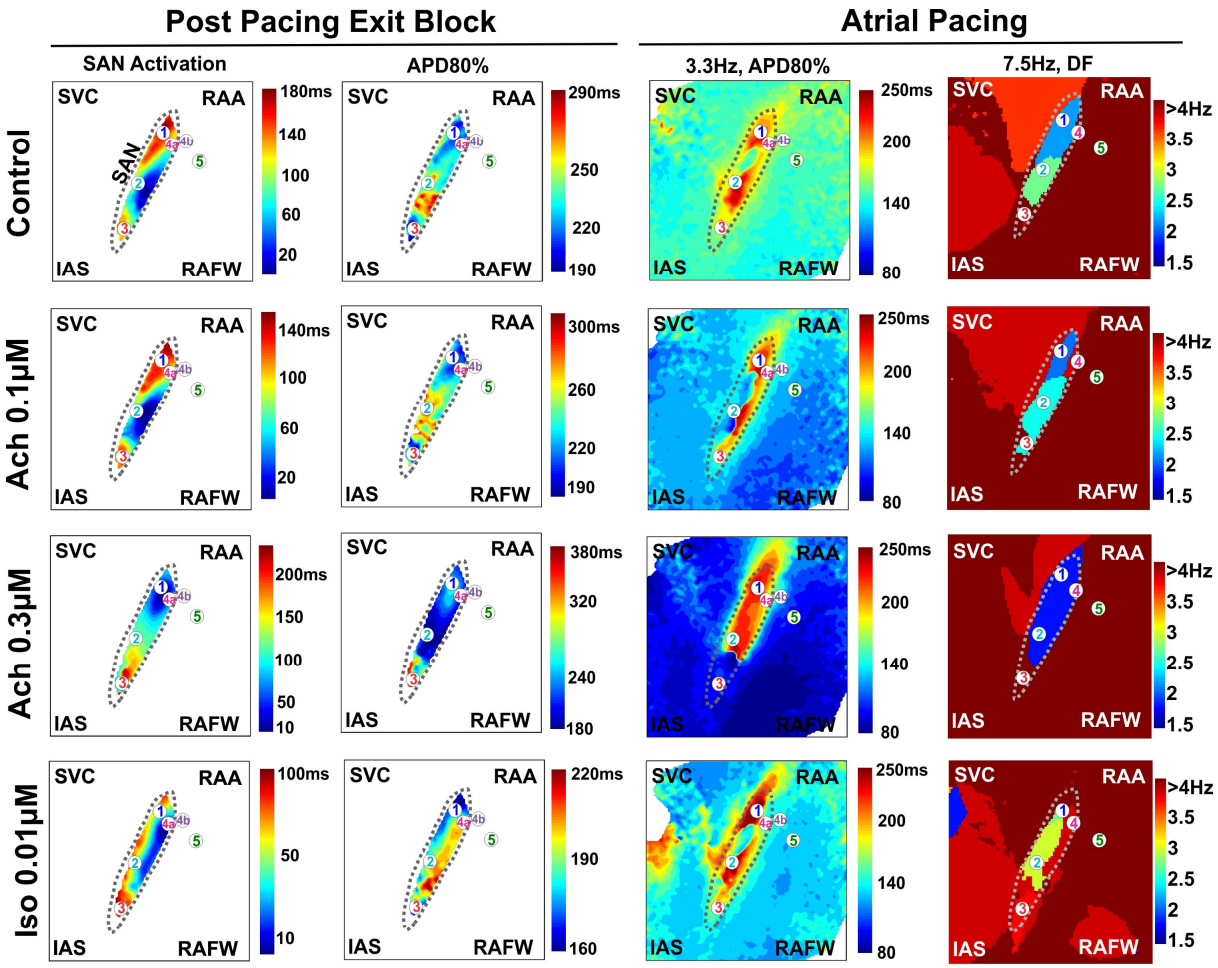


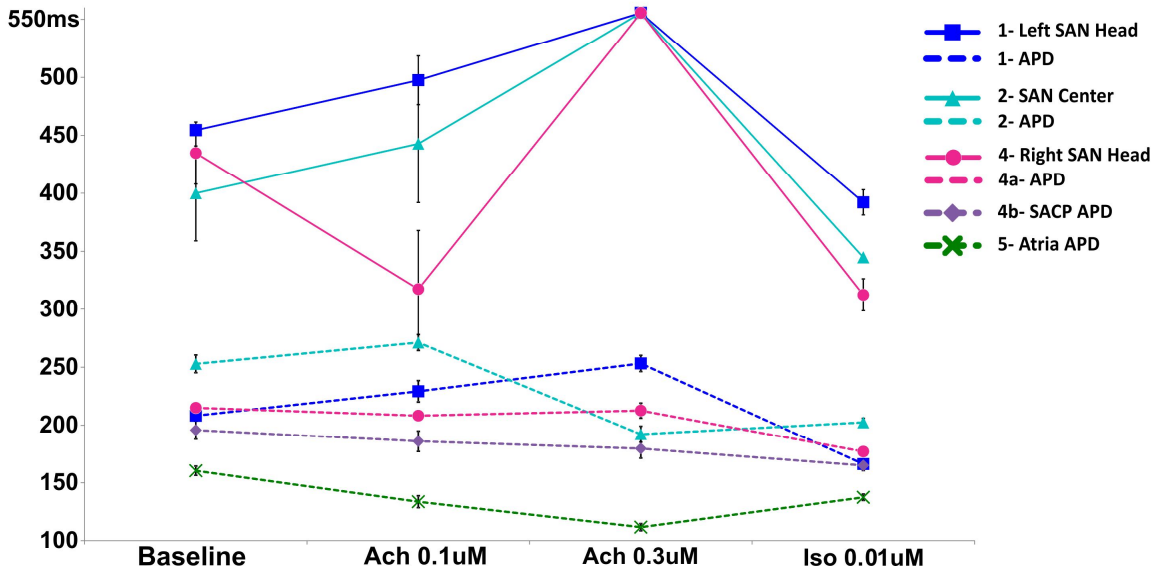
Supplement Figure 5. Evidence of multiple reentrant circuits inside the post MI canine SAN.

(**A)** Anatomical reconstruction of the post infarction (MI) canine SAN (6 wks post MI) from dog #1. **Left panel:** Epicardial photograph of a perfused canine atria preparation with a 25 x 25 mm optical field of view. The white dotted line shows the location of the crista terminalis (CT). The SAN was characterized by histological staining (**middle panel**). Dotted lines in the middle and right panels demarcate the border of the SAN. **Right panel:** Model of SAN pacemaker complex for dog #1 similar to that shown in **Figure 2A**. Abbreviations are the same as in **Supplement Figure 3**.

(**B**) Multiple reentrant circuits inside the SAN during 0.01 μM Iso perfusion. Atrial pacing induced multiple heart rate abnormalities characterized by irregular rhythm and profound pauses on the right atrial electrogram (EG). Optical mapping of the SAN revealed non-sustained macro-reentry through the SAN (panel R1) and non-sustained micro-reentry within the SAN. Below the OAPs, these arrhythmias are shown in greater detail. The pink circles indicate superior atrial breakthroughs. Abbreviations are the same as in **Supplement Figure 3.**

Supplement Figure 6.





Supplement Figure 6. Action potential duration at 80% of repolarization (APD80%) measurements in the SAN subjected to atrial tachypacing (3.3 Hz and 7.5 Hz) at control and

during autonomic stimulation.

(A) Representative color contour maps of SAN intranodal activation (left) and corresponding repolarization pattern (right) reconstructed from the most susceptible to SAN reentry MI preparation #3 at control and during autonomic stimulation. Five different areas were selected for representative average values of APD80% shown in panel C below.

(B) Atrial APD80% (left) and dominant frequency (DF, right) distribution maps reconstructed for the same canine preparations as shown in panel A. APD80% was measured during 3.3 Hz atrial tachypacing. DF was measured during 7.5 Hz atrial tachypacing. Five different areas were selected for representative traces shown in panel C below.

(C) Representative comparison between functional refractoriness and APD80% measured in five areas selected from the SAN and atrium. Note that the functional refractory period of the SAN compartments exceeds APD80% and this difference is exaggerated during autonomic stimulation. Abbreviations are the same as in **Supplement Figure 3.**

DATA SUPPLEMENT MOVIE LEGEND

Movie 1. Macro-reentry through the SAN.

The color map movie shows the spatial and temporal changes in amplitude of normalized optical action potentials (OAPs). The middle panel shows two OAP recordings (blue – SAN center, green – Crista terminalis) from the corresponding color point in color map movies. The bottom panel shows corresponding atrial ECG. This movie corresponds to **Figure 4B**, panel R1 (see also **Supplement Figure 3**). Termination of fast atrial pacing (7.5 Hz) during perfusion of 0.01 μM Iso induced multiple heart rate abnormalities characterized by irregular rhythm and profound pauses on the ECG. Optical mapping of the SAN revealed several types of reentries associated with the SAN, such as the SAN macro-reentry seen in the movie. During circus movement, the SAN was activated by the atria through the left superior sino-atrial conduction pathway (SACP) and then repeatedly re-activated the atria through the right superior SACP. After two consecutive marco-reentrant beats, the propagation in the SACP was blocked and macro-reentry transformed into a sustained counterclockwise micro-reentry within the SAN. SVC – superior vena cava; RAA – right atrial appendage; IAS – inter-atrial septum.

Movie 2. Counterclockwise and clockwise SAN micro-reentries.

The color map movie shows the spatial and temporal changes in amplitude of normalized optical action potentials (OAPs). The middle panel shows two OAP recordings (blue – SAN center, green – Crista Terminalis) from the corresponding color point in both color map movies. The bottom panel shows corresponding atrial ECG. This movie corresponds to **Figure 4B**, panels R2 and R3. This movie is a continuation of **Movie 1.** After two consecutive macro-reentrant beats, macro-reentry transformed into a sustained counterclockwise micro-reentry within the SAN. After five seconds of counter-clockwise micro-reentry within the SAN, dissociation in the intra-nodal conduction between the inferior compartment and the rest of the SAN appeared. Subsequent activation of the pacemaker located in the inferior SAN compartment led to competition between the pacemaker and the micro-reentry circuit that, in turn, resulted in a change of direction of the

micro-reentry circuit from counterclockwise to clockwise. SVC – superior vena cava; RAA – right atrial appendage; IAS – inter-atrial septum.

Movie 3. Clockwise SAN micro-reentry.

The color map movie shows the spatial and temporal changes in amplitude of normalized optical action potentials (OAPs). The middle panel shows two OAP recordings (blue – SAN center, green – Crista terminalis) from the corresponding color point in all color map movies. The bottom panel shows corresponding atrial ECG. The movie corresponds to **Figure 6B** and demonstrates two successive clockwise micro-reentrant circuits registered within the superior compartment of the SAN during perfusion of 0.1 μM ACh. SVC – superior vena cava; RAA – right atrial appendage; IAS – inter-atrial septum.

Movie 4. Counterclockwise SAN micro-reentry.

The color map movie shows the spatial and temporal changes in amplitude of normalized optical action potentials (OAPs). The middle panel shows two OAP recordings (blue – SAN center, green – Crista terminalis) from the corresponding color point in both color map movies. The bottom panel shows corresponding atrial ECG. The movie corresponds to **Figure 7C** and demonstrates several successive counterclockwise micro-reentrant circuits registered within the superior compartment of the SAN during perfusion of 0.01 μM Iso. Note that SAN micro-reentry is associated with paradoxical heart rhythm slowing and pauses. SVC – superior vena cava; RAA – right atrial appendage; IAS – inter-atrial septum.