Complex Interactions Between the Sinoatrial Node and Atrium During Reentrant Arrhythmias in the Canine Heart

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Background—Numerous studies implicate the sinoatrial node (SAN) as a participant in atrial arrhythmias, including atrial flutter (AFL) and atrial fibrillation (AF). However, the direct role of the SAN has never been described.

Methods and Results—The SAN was optically mapped in coronary perfused preparations from normal canine hearts (n=17). Optical action potentials were recorded during spontaneous rhythm, overdrive atrial pacing, and AF/AFL induced by acetylcholine (ACh; 0.3 to 3 μmol/L) and/or isoproterenol (Iso; 0.2 to 1 μmol/L). An optical action potential multiple component algorithm and dominant frequency analysis were used to reconstruct SAN activation and to identify specialized sinoatrial conduction pathways. Both ACh and Iso facilitated pacing-induced AF/AFL by shortening atrial repolarization. The entire SAN structure created a substrate for macroreentry with 9.6±1.7 Hz (69 episodes in all preparations). Atrial excitation waves could enter the SAN through the sinoatrial conduction pathways and overdrive suppress the node. The sinoatrial conduction pathways acted as a filter for atrial waves by slowing conduction and creating entrance block. ACh/Iso modulated filtering properties of the sinoatrial conduction pathways by increasing/decreasing the degree of the entrance block, respectively. Thus, the SAN could beat independently from AF/AFL reentrant activity during ACh (49±39%) and ACh/Iso (62±25%) (P=0.38). Without ACh, the AF/AFL waves captured the SAN and overdrive suppressed it. Spontaneous SAN activity could terminate or convert AFL to AF during cholinergic withdrawal.

Conclusions—The specialized structure of the SAN can be a substrate for AF/AFL. Cholinergic stimulation not only can slow sinus rhythm and facilitate AF/AFL but also protects the intrinsic SAN function from the fast AF/AFL rhythm. (Circulation. 2010;122:782-789.)

Key Words: acetylcholine ■ atrial fibrillation ■ atrial flutter ■ sinoatrial node ■ mapping ■ isoproterenol

Both atrial flutter (AFL) and atrial fibrillation (AF) are often associated with sinus node dysfunction.1-3 Sinoatrial node (SAN) structural and functional abnormalities can play an important role in the initiation and maintenance of AF.4-9 On the other hand, the fast AF/AFL rate can lead to SAN dysfunction.10-12 However, because of the lack of mapping data from the human SAN, no one has directly shown how the SAN participates in AF or what the SAN activation is during AF. Do fibrillating waves overdrive suppress the SAN, or does the intrinsic SAN activity remain present and even participate in AF?13 What role does the autonomic nervous system play in the interactions between SAN and AF reentrant waves?14 Although these studies have been widely discussed, no measurements have been published, leaving these questions unanswered until now.14

Clinical Perspective on p 789

The SAN is a specialized, complex anatomic structure.15-17 Anatomic16-18-21 and functional22,23 studies suggest that the canine SAN is a more realistic model for the human SAN4-5 than that of small mammals26,27 (eg, rabbit and mouse). In both humans and canines, conduction barriers are formed by connective tissues surrounding the coronary arteries and SAN tissue and abrupt changes in Connexin 43 expression between connective tissues surrounding the coronary arteries and SAN tissue and abrupt changes in Connexin 43 expression between

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signed to study for the first time the interactions between the SAN and the atria in the canine model of autonomic-induced AF/AFL with high-resolution optical mapping.

Methods

All animals \((n=17)\) used in this study received humane care 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 Washington University Animal Studies Committee. The isolated perfused canine right atrium and the experimental techniques used have been described in detail. Optical mapping of the nodal tissues has been described in our previous studies.

Figure 1A shows an example of the epicardial optical mapping of the canine SAN. The SAN was functionally defined from optical action potential (OAP) morphologies with slow diastolic depolarization and multiple component upstrokes corresponding to asynchronous activation of the SAN tissue layer and atrial layers (Figure 1B through 1D). This study defined the leading pacemaker as the earliest activation observed within the SAN region (SAN OAP component) and atrial breakthrough as the site of earliest activation observed within the atria. On the basis of our previous study, SACPs were defined as areas of preferential conduction, which correspond to narrow muscular bundles containing both Connexin 43-positive and -negative transitional cells. SACPs were located between the superior and inferior borders of the SAN and the atria, respectively, and served as conduction bridges between these 2 structures.

This study used an optimal concentration range of ACh \((1.5\pm0.8\ \mu\text{mol/L})\) to induce sustained AF/AFL episodes without total SAN arrest. If AF/AFL was sustained for \(>10\) minutes, ACh or Iso was transiently washed out, which usually terminated arrhythmias within 1 to 3 minutes.

To analyze atrial and SAN activations during fast atrial pacing and AF/AFL, a fast Fourier transform was applied to determine the dominant frequencies (DFs). To measure the SAN activation during pacing and atrial reentrant arrhythmias, we used low-frequency filters ranging from 10 to 20 Hz, separating the slow-upstroke SAN signals from the fast atria (Figure III and IV in the online-only Data Supplement). For further details, see the online-only Data Supplement.

Results

Optical Mapping Aspects of Canine SAN

Figure 1 shows that 2 distinct activation patterns were observed: SAN excitation from the leading pacemaker and atrial excitation from SACPs. The SAN activation map during control (Figure 1E) shows that excitation began in the center of the SAN (OAP 1) and spread inferiorly and superiorly with conduction velocities of 7 to 16 cm/s. SAN excitation did not propagate laterally to the crista terminals or medially toward the septum because of the presence of coronary arteries and connective tissue layers as previously described. Atrial breakthrough (OAP 2) was detected 53 milliseconds after the earliest SAN activation, 6 mm away from the SAN. Thus, the time gap (5 to 40 milliseconds) in activation between the SAN and atria was likely due to a source-sink mismatch when excitation passes through these narrow SACPs.

Effects of ACh and Iso on the SAN Automaticity and Conduction

In 9 preparations, ACh \((0.3\text{ to }3\ \mu\text{mol/L})\) dose dependently increased sinus cycle length (CL) and sinoatrial conduction time from \(519\pm22\) and \(54\pm20\) milliseconds to \(841\pm89\ (P<0.001)\) and \(123\pm40\ (P<0.001)\) milliseconds, respectively, until rhythm disturbance occurred (a pacemaker shift out of the SAN or exit block). Figure 1C shows that 1 \(\mu\text{mol/L} \) ACh slowed sinus rhythm (SR) and nodal conduction but did not change the atrial activation pattern. However, increasing the dose of ACh depressed conduction mainly in the superior SACP rather than in the inferior SACP (Figure 1E). This switch of preferential conduction to an inferior SACP significantly changed the atrial activation pattern (Figure 1E) and was observed in all preparations during perfusion with ACh (1 to 3 \(\mu\text{mol/L})\).
Figure 1D shows that 3\( \mu \text{mol/L} \) ACh could induce exit block within the SAN, preventing activation of the atria. This block allowed the SAN OAPs to be detected without atrial signals. SAN exit block was recorded during the perfusion of ACh or during the recovery from ACh effects in 7 of 9 preparations. At higher ACh concentrations (3 to 10\( \mu \text{mol/L} \)), SAN nodal pacemaker activity was often completely depressed, and latent pacemaker activity could be recorded from the inferior (IVC; \( n=4 \)) and/or superior (SVC; \( n=2 \)) vena cava regions. SAN activity recovered within 1 to 2 minutes after ACh washout.

In 5 preparations, Iso (1\( \mu \text{mol/L} \)) significantly decreased sinus CL and sinoatrial conduction time from 533±110 and 54±20 milliseconds to 450±132 (\( P<0.001 \)) and 37±18 (\( P<0.001 \)) milliseconds. Figure 2 shows an example of the effects of perfusion with Iso on the SAN. During control conditions, the leading pacemaker was located in the inferior SAN, and the atria had a bifocal activation pattern. Iso induced a pacemaker shift inside the SAN superiorly from OAP 1 to 3 (Figure 2) and accelerated SR and intranodal conductions. Thus, Iso switched the preferential SACP from an inferior to superior position and changed the atrial activation pattern from bifocal to unifocal (Figure 2C). In 2 preparations, Iso produced a transient pacemaker shift out of the SAN into the SVC.

ACh also induced a decrease in the action potential duration (APD) of the atrium but not within the SAN region (Figure 1E and Table I in the online-only Data Supplement). Iso decreased APD in both the atria and SAN (Figure 2C and Table I in the online-only Data Supplement).

**SAN Activity During Atrial Pacing**

Figure 3 demonstrates SAN activation during atrial pacing at a CL of 350 milliseconds. SAN activation began from the left superior anatomic border of the SAN. Thus, this area was defined as the left superior SACP. Excitation from this SACPs propagated inferiorly with decrement. No lateral or medial conduction was observed (Figure 3A). During pacing at a CL of 350 milliseconds, there was 1:1 conduction between the atria and the SAN. However, pacing at a CL of 300 milliseconds induced 2:1, Wenckebach-like, entrance block into the SAN (\( n=10 \) preparations).

Figure 3B through 3D shows that increasing the pacing rate results in increased SAN entrance block (as large as 4:1) during control conditions. In all DF maps, the SAN stands out as an oval region of lowest frequency (1.5 to 1.7 Hz), surrounded by the higher-frequency atrial region. In control, the frequency of SAN activity was higher during pacing than without pacing: >1.5 versus 1.45 Hz (sinus CL=688 milliseconds), which means that SAN was overdrive suppressed by atrial pacing. The SAN was always suppressed during control (\( n=10 \)) and during the perfusion with Iso (\( n=5 \)).
A comparison between Figures 3D and 4 shows that perfusion with ACh enhanced entrance block to the SAN (from 4:1 to 7:1). DF and SAN activation maps show that atrial excitation could enter into the SAN only through the inferior SACP, which was a common observation during the perfusion of ACh (n=8). During ACh perfusion, the central SAN region had almost the same rate during pacing and spontaneous SR before pacing, 1.05 versus 1.07 Hz (sinus CL=934 milliseconds), indicating that the SAN was not always overdrive suppressed by atrial pacing. Figure 4C demonstrates that the SAN had intrinsic activity during fast pacing resulting from ACh-induced depression of the conduction through the SACPs.

Effects of ACh and Iso on SAN Recovery Time
Figure 5A shows the effect of overdrive atrial pacing on SAN recovery time (SANRT) under control conditions and after separate perfusions with ACh (0.3 to 3 μmol/L) and Iso (0.5 to 1 μmol/L). Figure 5A represents data obtained only during normal SR before and after pacing. ACh consistently prolonged the corrected SANRT from 131±31 to 199±45 milliseconds (P<0.001), whereas β-adrenergic stimulation with Iso decreased the corrected SANRT to 64±59 milliseconds (P<0.001), consistent with previous observations by Chadda et al.40 (Figure 5B).

Figure 5B presents SANRT data separately for all cases (15 cases from 9 different preparations) of pacing-induced SAN exit block observed during perfusion with ACh (0.3 to 3 μmol/L). Figure 5C shows an example of SAN exit block induced during pacing. SAN exit block resulted from conduction failure within SACP(s) promoted by both pacing and ACh. In all cases of exit block, there was no significant difference in sinus CL before or after pacing: 883±82 versus 841±89 milliseconds (P>0.05). These findings support the observation shown in Figure 4C that ACh-induced bidirectional block within the SACP can prevent SAN from overdrive suppression by pacing. Iso (n=3) or atropine (3 μmol/L; n=3) recovered ACh-induced and pacing-induced SAN exit blocks (see Figure V in the online-only Data Supplement).

SAN Activity During Sustained AF/AFL
Under control conditions, only 1 episode of sustained AFL was induced by atrial pacing (Figure 6A). However, in preparations with ACh (4.8±3.9 μmol/L; n=10), Iso (1 μmol/L; n=2), and a mixture of ACh (1 μmol/L) and Iso (0.2 to 1 μmol/L; n=3), atrial pacing induced multiple episodes of sustained AFL and AF (>3 minutes; Figures 6 and 7 and Figures I and II in the online-only Data Supplement).

The mechanism of sustained AFL (26 episodes from 10 preparations) was consistently observed to be macroreentrant excitation around the SAN, which functioned as a conduction barrier (Figure 6 and Figures I and II in the online-only Data Supplement). Figure 6A demonstrates that under control conditions, AFL could overdrive suppress the SAN through the SACPs, causing 3:1 to 4:1 entrance block into the SAN (Movie I in the online-only Data Supplement). Iso alone could induce sustained AFL (n=2) but not AF. Iso-induced reentry activated
the SAN from both the superior and inferior pathways with entrance block (4:1; Figure 6B). The perfusion of ACh during atrial pacing induced AFL in 8 of 10 preparations with a CL of 97–1100 ms (10.2 ± 3.5 Hz). ACh prevented overdrive suppression of the SAN by inducing entrance block into the SAN (up to 11:1), permitting intrinsic SAN pacemaker activity. The SAN activation map in Figure 6C demonstrates the presence of intrinsic SAN pacemaker activity during ACh-induced AFL (Movie II in the online-only Data Supplement).

ACh usually sustained AFL, but during ACh washout, AFL converted to AF (Figure 6D) and preserved the SAN intrinsic pacemaker activity. Before ACh washout terminated AF/AFL, SAN activity significantly accelerated from 1.18 ± 0.36 to 1.63 ± 0.33 Hz (P < 0.01; n = 10). Several recorded cases from 3 different preparations demonstrated the conversion from AFL to AF during washout from ACh. SCL indicates sinus CL. Other abbreviations as in Figure 1.

Figure 6. SAN activation during AFL in control and ACh and Iso perfusions. A through C, Atrial activation maps, DF maps, and examples of SAN activation during different AFL episodes. All of the atrial activation maps clearly show a single macroreentrant circuit of AFL (dotted white arrow) around the SAN structure. A and B are from the same preparation. A, Interaction between the SAN and atria during control AFL B. Interaction between the SAN and atria during Iso-induced AFL. The SAN activation maps in A and B show no intrinsic pacemaker activity. C, Interaction between the SAN and atria during ACh (1 μmol/L) washout. Unlike control (A) or Iso (B) conditions, the SAN had its own intrinsic pacemaker activity as depicted in the SAN activation and DF maps. D, Conversion of AFL (C) to AF during washout from ACh. SCL indicates sinus CL. Other abbreviations as in Figure 1.

Figure 7. The complex interaction between reentrant atrial waves and SAN intrinsic activity during sustained ACh-induced AF and ACh/Iso-induced AF. A and B, The complicated interaction between the atria and SAN during sustained ACh (3 μmol/L)-induced AF (A) and sustained ACh (1 μmol/L) and Iso (0.5 μmol/L)-induced AF (B) (same preparation as for Figure 1). Left, DF maps; right, SAN and atrial OAP recordings and the corresponding frequency power spectrum. C, OAP recording during ACh/Iso-induced AF (B) from the SAN region broken up into 3 components: central SAN (blue, 1), superior SACP (green, 2), and inferior SACP (red, 3). D, Enlarged views of the SAN activation map (pink dotted rectangle in A) during the times shown by the black dotted rectangle in C. Abbreviations as in Figure 1.

ACh-induced AF (47 episodes from 10 preparations) was usually associated with reentrant excitations around the pectinate muscles and/or SVC and IVC regions with a maximum DF of 22.32 ± 5.86 Hz and atrial DF around the SAN of 11.26 ± 5.23 Hz (Figure 7 and Table II in the online-only Data Supplement). Figure 7A and 7B illustrates that AF episodes induced during the perfusion of a mixture of ACh and Iso had a higher frequency of SAN activity than during the perfusion of ACh only (1.64 ± 0.22 versus 1.17 ± 0.21 Hz; P < 0.01; n = 6; Figure 7). Figure 7C and 7D shows an example of 3 possible interactions observed between the atria and the SAN: (1) intrinsic SAN pacemaker activity, which escapes into the atria; (2) entrance block in the SACP, leaving the intrinsic SAN pacemaker activity intact; and (3) both exit and entrance block in the SACPs. These various scenarios occurred in random order. The percentages of total recorded SAN beats that were not paced by the atria during ACh-induced and ACh/Iso-induced AF/AFL were 49 ± 39% and 62 ± 25%, re-
spectively. The frequency of the SAN activities during all analyzed episodes of AF/AFL was close to the control SR values before or after the arrhythmia (1.38±0.51 versus 1.30±0.31 Hz; P=0.65).

Discussion

Canine Model of the Interactions Between the SAN and Atria

Figure 8 shows a 3-dimensional structural model of the canine SAN and region of atria mapped (modified from Fedorov et al24) summarizing the principal findings of this study. By using ACh, Iso, and atrial pacing, the present study found functional evidence for at least 4 SACP S. The main new findings from our present study are as follows. First, atrial excitation waves can enter into the SAN through the SACP S and overdrive suppress the node. The SACP S act like a low-pass filter for atrial waves by slowing conduction and creating entrance block. Second, ACh and Iso modulate these filtering properties of the SACP S by increasing or decreasing the degree of the entrance block, respectively. Third, conduction properties of the superior SACP S have a higher sensitivity to autonomic stimulation than the inferior SACP S. Fourth, the entire SAN structure creates a substrate for macroreentry (typical AFL). Fifth, during cholinergic stimulation, the SAN can beat independently from AF/AFL reentrant activity. During control and with Iso, the AF/AFL waves capture the SAN and overdrive suppress it. Finally, spontaneous SAN activity can terminate or convert AFL to AF during cholinergic withdrawal.

Effects of Sympathetic and Parasympathetic Stimulation on SAN Function

In the present study, perfusion with Iso and ACh generally resulted in a preferential use of superior and inferior SACP S, respectively, as a result of a shift in the location of the leading pacemaker and/or inhomogeneous changes in conduction within SAN (Figures 1, 2, and 8D). We propose that the inferior and superior SACP S have different conduction properties and different sensitivities to ACh and Iso resulting from spatial differences in muscarinic and β-adrenergic receptors.41 The distance of the pacemaker shift inside the SAN varies from 1 to 15 mm (Figures 1E and 2C). However, even with a negligible pacemaker shift, there are changes in the preferential SACP caused by inhomogeneous conduction changes within the SAN (Figure 1E). Thus, the atrial breakthrough site can be moved by drugs even more, up to 25 mm, without significant shift of the leading pacemaker inside the SAN. These results explain previous canine studies34,42,43 that demonstrated that sympathetic stimulation accelerated SR and superiorly shifted the area of earliest atrial activation, but cholinergic stimulation slowed SR and inferiorly shifted the breakthrough site 10 to 20 mm away.

In addition, ACh can depress conduction in all of the SACP S, resulting in SAN exit block (Figures 1, 3, and 8E), or induce SAN inexcitability (Figure 8F).44 Under these conditions, the present study observed pacemaker shifts into the IVC or SVC regions (Figure 1D). Iso can also accelerate these or other latent pacemakers and cause transient pacemaker shift outside the SAN.
The study shows that slow atrial pacing (350 to 400 milliseconds) preferentially conducts through the left superior SACP (Figures 3A and 8C) and that faster pacing rates (CL <300 milliseconds) and ACh preferentially cause conduction through the inferior SACPs (Figures 4, 8C, and 8D).

**SAN Role in AF/AFL Mechanisms**

In their classic microelectrode study of the rabbit SAN, Kirchhof and Allessie\(^{13}\) discovered a high degree of SAN entrance block (5:1) during low-potassium–induced AF. However, it is uncertain whether the conclusions made from small-animal models (e.g., rabbit) about the function of the SAN during AF/AFL are the same as in large, more complex 3-dimensional structures such as canine\(^{26}\) and human SANs.\(^{3,22}\) Neither the study of Kirchhof and Allessie\(^{13}\) nor any other studies\(^{42}\) directly demonstrated how the SAN functionally and structurally participates in atrial reentrant arrhythmias.

The present study demonstrated for the first time that the SAN structure is a substrate for macroreentry. Sustained AFL reentry circuit under all conditions (control, ACh, and Iso) tends to anchor around the SAN structure (see Figure 6), as was observed in a previous rabbit study.\(^{27}\) Our recent optical mapping studies confirm that the human SAN structure could also be a substrate for the typical AFL mechanism in humans.\(^{25}\) In the present study, AF is maintained by a single or multiple reentry circuits anchored mostly to the pectinate muscles as previously demonstrated (Figures 7 and 8 and Figure VI in the online-only Data Supplement).\(^{46-48}\)

The present study demonstrated that SACPs play a major role in creating SAN entrance blocks during atrial pacing (see Figure 4), AFL (see Figure 6), or AF (see Figure 7). ACh can promote conduction block in the SACP and thus prevent AF/AFL impulses from suppressing the intrinsic SAN activity. Some SAN impulses can escape through these SACPs during AF/AFL and partially activate the surrounding atria during washout from ACh (Figure 6D) or during the addition of Iso (Figure 7D). Thus, SAN impulses might perpetuate the arrhythmogenic process (Figure 6D and Movie II in the online-only Data Supplement). In the present canine model, AF can be converted to AFL and back to AF or to SR during washout from ACh. The SAN can also participate in these conversions because of faster recovery of pacemaker activity and conduction in the SACPs (Figures 6D, 8J, and 8K). Moreover, the observation in the present study of accelerated SAN activity during AF induced by a mixture of ACh and Iso (Figure 7) can explain our previous observation as to how Iso potentiates the initiation and maintenance of ACh-mediated AF.\(^{33}\)

**Potential Implications**

Disturbances in the autonomic nervous system can be a cause of both SAN dysfunctions\(^{5-22}\) and paroxysmal AF/AFL.\(^{28}\) Thus, the cholinergic-induced SAN dysfunctions (exit blocks and depressed intranodal conduction and automaticity) make the canine model of AF clinically relevant. Moreover, parasympathetic stimulation can preserve the SAN function from the fast AF rate by enhancing the filtering properties of the SACP, resulting in entrance block. Thus, it can prevent overdrive pacing–induced remodeling of the SAN during atrial tachycardias.\(^{12}\) We propose that the present comprehensive study of the interaction between the SAN and atria will help to explain the role of an increased/reduced sympathetic and vagal tones in patients with early AF recurrence\(^{49}\) and suggest how autonomic control may contribute to the initiation and maintenance of AF/AFL.

**Conclusions**

This study demonstrates that the SACPs play an important role in the protection of the SAN against overdrive pacing from AF/AFL. However, the specialized functional structure of the SAN can be a substrate for both the initiation and maintenance of AF/AFL.

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**Disclosures**

None.

**References**

CLINICAL PERSPECTIVE

Although significant progress has been made in elucidating basic mechanisms of atrial fibrillation (AF) and flutter (AFL) over the years, the role of the sinoatrial node (SAN) in inducing and maintaining these clinically prevalent arrhythmias has yet to be explored. We have previously identified discrete sinoatrial conduction pathways located within the canine and human SAN that mediate conduction between the SAN and the surrounding atrial myocardium. For the first time, we identify unique interactions and dependency between the canine SAN, sinoatrial conduction pathways, and surrounding atrial myocardium during normal rhythm and AF/AFL with autonomic nervous system influence using high-resolution optical mapping. We found the sinoatrial conduction pathways to play an important role in the protection of the SAN against overdrive pacing from AF. Interestingly, the anatomic structure of the SAN itself was also noted to be a substrate for AF/AFL by functioning as an obstacle around which atrial reentrant activity can be pinned. Parasympathetic stimulation preserves SAN function from the fast rate of the surrounding atria during AF by enhancing entrance block in the sinoatrial conduction pathways, preventing the depression of the SAN during atrial tachyarrhythmias. From this study, the complex relationship between the SAN, sinoatrial conduction pathways, autonomic nervous system, and surrounding atria is displayed, providing new insight into the mechanisms involved in AF/AFL formation and possible underlying mechanisms involved in the tachycardia-bradycardia syndrome. Continued studies of the role of the sinoatrial conduction pathways in the mechanisms of AF and SAN dysfunctions will contribute to the development of novel genetic, cellular, or device therapies.
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SUPPLEMENTAL MATERIAL

Title: Complex interactions between the sinoatrial node and atrium during reentrant arrhythmias in the canine heart.

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SUPPLEMENTAL METHODS

This protocol was approved by the Washington University Animal Care and Use Committee. The isolated, perfused canine right atrium\(^1\) as well as the experimental techniques\(^2\) have been previously described in detail.

**In vitro optical mapping studies**

Optical mapping studies were conducted on isolated, coronary-perfused preparations of the canine right atrium.\(^{1;3;4}\) Normal, healthy, young (10-12 month old) mongrel dogs (n=17) weighing between 18 and 25 kg, without any history of disease were anesthetized (IV 7.5 mg/kg propofol), intubated, and placed on a positive pressure respirator with 2% to 3% isoflurane for anesthesia throughout the procedure. A median sternotomy was performed. The heart was cradled in the pericardium and the azygous vein was ligated and divided. The intra-atrial groove was dissected, separating the left (LA) and right atria (RA). The RA was dissected from the rest of the heart and divided through the superior vena cava (SVC) down to the inferior vena cava (IVC). The right coronary artery was cannulated with a 16-gauge catheter. The RA preparations were positioned in a temperature-controlled glass chamber with the epicardium (Figure 1) or endocardium facing the optical apparatus (Online Figure 1). Two bipolar pacing and recording electrodes were placed on the RA free wall and intra-atrial septum (IAS) regions. The RA preparations were superfused (50 mL/min) and coronarily perfused under a constant pressure of 55±5 mmHg with oxygenated (95% O\(_2\)-5% CO\(_2\)) modified Tyrode’s solution containing (in mM): 128.2 NaCl, 1.3 CaCl\(_2\), 4.7 KCl, 1.05 MgCl\(_2\), 1.19 NaH\(_2\)PO\(_4\), 25 NaHCO\(_3\), and 11 glucose. Temperature and pH were continuously maintained at 36±0.5°C and 7.35±0.05, respectively.
Preparations were equilibrated during normal sinus rhythm in the tissue chamber for 60-90 min before the measurements. During this time, the preparations were stained with voltage-sensitive dye, di-4-ANEPPS or RH237, and Blebbistatin (10-20 µM) was used to suppress motion artifacts in the optical signals caused by muscle contraction.\(^5\) The SAN preparations were restained with the dye during the experiment as needed. No measurements were performed until 5 min after the restaining procedure. Stability of the preparation was periodically verified by measuring sinus cycle length.

1) In the preliminary experiments, a 16×16 photodiode array (Hamamatsu, Japan) with a spatial resolution of 2.25 mm at a rate of 1,500 frames/s recorded optical fluorescent signals from an optical field of view 36×36 mm\(^2\) (OFV) at the epicardium, endocardium (Online Figure 1), or both (n=5). The OFVs contained part of the SVC, crista terminalis (CT) and IAS regions.

2) During the main experimental series (n=12), a 100x100 Ultima-L CMOS camera (SciMedia, Japan) with a spatial resolution of 300-400 µm/pixel at a rate of 1,000 frames recorded fluorescent signals from the epicardial OFV ranging in size from 30x30 to 40x40 mm\(^2\) (Figure 1).

**Experimental protocol**

Programmed atrial stimulation was used to measure atrial conduction properties and SAN recovery time at different cycle lengths (CL) from 350 ms to 175 ms. S1S2 protocol (Online Figure 2) or progressive overdrive atrial pacing up to CL=90 was used to induce atrial fibrillation (AF)/atrial flutter (AFL) (Figure 3) before and after perfusion with acetylcholine (ACh) or isoproterenol (Iso).

In the preliminary series of experiments (n=5), we wanted to induce sustained AF and AFI during perfusion with Acetylcholine (ACh, 0.1 - 10 µM) without completely...
depressing the SAN as previously described for this model. Depending on the individual preparation, 0.3-3 µM ACh could promote sustained AF/AFL without completely depressing SAN intrinsic pacemaker activity. Thus, for the remaining experiments (n=12), we used ACh concentrations in this range. To block ACh effects, 3 µM Atropine was used in three preparations (Online Figure 5D). In 5 out of 12 preparations, we also perfused with Isoproterenol (Iso, 0.2-1 µM) to investigate the effects of beta-adrenergic stimulation on the SAN activity during spontaneous rhythm or AF/AFL.

Optical mapping data analysis and interpretation

The SAN tissue was electrically isolated from the atrial myocardium except through the sinoatrial exit pathways (SACPs). Based on our previous study, the SAN conduction pathways were defined as areas of preferential conduction between SAN and atrial myocardium which correspond to narrow muscular bundles containing both Connexin 43 positive and negative transitional cells. These bundles spread from the SAN into larger muscular bundles found in the atria, such as the CT. SACPs were located between the superior and inferior borders of the SAN and the atria and served as conduction bridges between these two structures.

There were two separate activation patterns: SAN excitation from the leading pacemaker, and atrial excitation from the exit pathways. While both activations were electrically separated, the SAN and atrial excitations were related to each other. This study defined the leading pacemaker as the earliest activation in the SAN region (by the SAN OAP component) and “atrial breakthrough” as the earliest activation in the atria (by the atrial OAP component). Sinoatrial conduction time (SACT) was defined as the time between the earliest excitation in the SAN and the atrial breakthrough.
A custom Matlab computer program was used to analyze the optical signals in the SAN and the atria as previously described.\(^7\) Optical recordings contained signatures of cardiac excitation to a depth of 1-3 mm.\(^8\text{-}11\) Therefore, our optical recordings contained signals from both the SAN and atrial tissue, which we separated as described in our recent study of the canine SAN.\(^7\)

Using this method, we located the conduction block area around the SAN. This area cannot be detected using surface electrogram recordings.\(^12\) The separation of these components allowed for the measurement of the sequential SAN and atrial activation patterns, and conduction velocity in individual tissue layers (Figure 1).

Activation times and corresponding conduction velocities were defined in the SAN layer at 50% of the SAN OAP component (AP50\%).\(^2\text{-}7,13\) Atrial activation times and patterns were defined by traditional $dF/dt_{\text{max}}$.

To estimate the changes in action potential duration at 80% of repolarization APD (APD80) over the atria and SAN, we selected several regions corresponding to notable anatomic features (Figure 1) and computed summary statistics for the APDs within these regions. The three regions used were: 1) the right atrial free wall, the right atrial tissue anterior to the sulcus terminalis, which contained the trabeculated part of the right atrial wall; 2) the IAS, the right atrial tissue between septal border and SAN block zone; 3) SAN, the central part of the SAN.

Optical signals were filtered using low-pass Butterworth filters (10-200 Hz). During normal sinus rhythm, we distinguished the preceding SAN component of the optical signals from the consequent atrial upstroke (Figures 1 and 2). Figure 3 shows that slow atrial pacing paced the SAN 1:1 (S1S1 = 350 msec); but faster atrial pacing (120 ms – 200 ms) may cause up to even 4:1 Wenckebach block-like interaction between the atria and the SAN. Since SAN excitation came after the atrial component
(Figure 3), reconstruction of the SAN activation during atrial pacing required a different algorithm.\(^\text{14}\)

Moreover, during fast atrial pacing and atrial reentrant arrhythmias, the atrial activation frequency was much higher (6 Hz – 32 Hz) than the SAN's (0.5 Hz – 3.5 Hz). This significant difference in frequencies and amplitude masked the SAN component. To measure the SAN activation 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 atria. **Online Figure 3** shows the success of the filtering process in separating low-frequency SAN signals (dark blue) from the high frequency atrial signals (green) during AF/AFI.

We also used Fourier transform analyses to produce the Dominant Frequency (DF) maps. Using the custom Matlab program, we analyzed the power spectrum for each recording site and labeled the frequency with the largest amplitude as the dominant frequency, similar to past studies.\(^\text{15}\) These individual DF values from each recording site were then compiled to create DF maps of the entire OFV. **Figure 3** shows the DF maps with the 16 Hz low-pass filtering during atrial pacing CL. Using this DF analysis, we were able to associate the region of low DF as the SAN anatomical region. **Online Figure 4** shows that decreasing the low–pass frequency from 200Hz to 16 Hz improved the quality of the SAN optical recordings and DF maps.

**SANRT**

Sinus node recovery time (SANRT) was used as a measure of the SAN function before and after perfusion with ACh and Iso. SANRT was defined as the delay between the last paced beat and the first spontaneous beat (**Online Figure 5**). Direct SANRT, SANRTd, was measured from the last paced atrial beat to a 50% rise in the SAN optical upstroke. The rate corrected SANRT was then computed as the difference between the
measured SANRTd and the recorded sinus rhythm taken before pacing began. Indirect SANRT, SANRTi, was computed as the difference between the last paced atrial beat and a 50% rise in the first atrial optical upstroke (Online Figure 5B).

**Histological examination and anatomic correlation**

Histology was performed as previously described. After optical mapping experiments, canine SAN preparations (n=8) were perfused with 3.7% formaldehyde for 5 minutes and left in solution overnight. The SAN preparations were then transferred to 20% sucrose for two days before the tissue was frozen. SAN preparations were embedded in Tissue-Tek OCT compound (Histo Prep; Fisher Scientific, Fairlawn, NJ, USA), frozen in isopentane, cryosectioned parallel (n=4) or perpendicular (n=4) to the epicardium, and stored at -80°C until staining and imaging was performed.

Online Figure 2 shows that slow conduction areas, conduction block, and the core of the re-entrant wave front were correlated with the macroscopic anatomic and histological findings of the SAN structure.

**Statistics**

Statistical analysis was done using SAS 9.2 (Cary, NC). Mixed linear models included treatment as fixed effect and sample as random effect. Least squares estimates of the ACh and Iso treatments were compared to control with Dunnett’s adjustment. For SCL and SACT variables, each treatment had its own control, thus differences between treatments and controls were used as responses. Mixed linear models were modified to compare these differences to zero and included differences as fixed effect and sample as random effect. For comparison of AF and AF/AFL groups, a paired t-test was used.
Study Limitations

We used denervated preparations from young mongrel dogs without any structural diseases. SAN dysfunctions and AF/AFL were very often caused by structural diseases. It is possible that the suppression of mechanical contractions by Blebbistatin prevented the activation of stretch-activated channels, which could play a role in the pacemaker activity of the SAN\(^{16}\) as well as in the cholinergic AF/AFL mechanism.\(^{17}\) The optical recordings conducted from the epicardium or endocardium surface were likely to carry contributions from both the atrial and SAN intramural layers and represented a weighted average of the transmembrane recordings throughout the canine atrial wall.\(^{7}\) However, the ratio of the atria input amplitude to the SAN signals could be unpredictable due to the variability in nodal anatomy and locations of the leading pacemakers. Thus, it was still not possible to clearly distinguish conduction in the SACPs due to their size and intramural nature.\(^ {7}\)
SUPPLEMENTAL TABLES

Online Table 1. Effects of Acetylcholine and Isoproterenol on the main electrophysiological parameters of canine right atria (n=12)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>ACh</th>
<th>Iso</th>
<th>p-value (ACh vs. Control)</th>
<th>p-value (Iso vs. Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCL (ms)</td>
<td>519 ± 21.9</td>
<td>841.4 ± 88.7</td>
<td>373.17 ± 70.5</td>
<td>0.035</td>
<td>0.027</td>
</tr>
<tr>
<td>SACT (ms)</td>
<td>54.0 ± 20.3</td>
<td>123.2 ± 40.2</td>
<td>36.7 ± 17.8</td>
<td>0.0036</td>
<td>0.023</td>
</tr>
<tr>
<td>SANRT (ms)</td>
<td>628.0 ± 56.6</td>
<td>981.5 ± 181.6</td>
<td>439.0 ± 96.0</td>
<td>0.031</td>
<td>0.031</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>ACh</th>
<th>Iso</th>
<th>p-value (ACh vs. Control)</th>
<th>p-value (Iso vs. Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APD80% IAS (ms)</td>
<td>166.9 ± 11.3</td>
<td>95.31 ± 25.3</td>
<td>108.9 ± 32.8</td>
<td>&lt;0.001</td>
<td>0.0015</td>
</tr>
<tr>
<td>APD80% RAFW (ms)</td>
<td>163.4 ± 14.4</td>
<td>90.6 ± 21.1</td>
<td>110.3 ± 32.8</td>
<td>&lt;0.001</td>
<td>0.0006</td>
</tr>
<tr>
<td>APD80% SAN (ms)</td>
<td>204.1 ± 21</td>
<td>207.7 ± 20.2</td>
<td>170.3 ± 17.4</td>
<td>0.953</td>
<td>0.019</td>
</tr>
</tbody>
</table>

Sinus cycle length (SCL), sinoatrial node conduction time (SACT), sinoatrial node recovery time (SANRT), right atrial free wall (RAFW), intraatrial septum (IAS), and sinoatrial node (SAN). APD80% - action potential duration at 80% repolarization.

Online Table 2. SAN and atrial frequency during AFL and AF.

<table>
<thead>
<tr>
<th>Maximal frequency (Hz)</th>
<th>AF/AFl (ACh) (n=47)</th>
<th>AF (ACh and Iso) (n=6)</th>
<th>AF/ AFL washout from ACh (n=10)</th>
<th>AFL (Iso) (n = 5)</th>
<th>AFL (Control) (n = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAN (Hz)</td>
<td>1.17 ± 0.21</td>
<td>1.64±0.22*</td>
<td>1.63 ± 0.33*</td>
<td>2.42± 0.36</td>
<td>2.0</td>
</tr>
<tr>
<td>Atrial around SAN (Hz)</td>
<td>11.26 ± 5.19</td>
<td>11.44 ± 1.5</td>
<td>9.28 ± 3.72</td>
<td>9.98 ± 0.87</td>
<td>6.8</td>
</tr>
<tr>
<td>DF (Hz)</td>
<td>22.32 ± 5.86</td>
<td>21.38 ± 2.49</td>
<td>17.8± 7.27</td>
<td>9.98 ± 0.87</td>
<td>6.8</td>
</tr>
<tr>
<td>% Spontaneous activation of SAN</td>
<td>48.6 ± 38.9</td>
<td>62.3 ±24.8</td>
<td>39.5± 34.6</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Atrial fibrillation (AF), atrial flutter (AFL), Acetylcholine (ACh), Isoproterenol (Iso). DF –dominant frequency for AF cases.
* p<0.01 vs AF/AFL (ACh) group (paired t-test)
SUPPLEMENTAL MATERIAL

Figure 1_Online
A. Sinus Rhythm (2uM ACh)

LPF 200Hz

Atrial

SAN

SCL = 792ms

B. AFL

LPF 10Hz

LPF 200Hz

SAN APs

Atrial APs

AFLCL = 72 ms

SCL = 804 ms

AFL/SCL = 11:1

C. AF

LPF 20Hz

LPF 200Hz

SAN APs

Atrial APs

AFCL = 44 ms

SCL = 812 ms

AF/SCL = 18:1
Figure 4 online

S1S1 = 200 ms

DF, Hz

LPF 200Hz

OAPs

1 - SAN
2 - Atria

Power

Time (s)

DF, Hz

LPF 64Hz

OAPs

Power

DF, Hz

LPF 16 Hz

OAPs

Power

Frequency (Hz)
Figure 5-Online

A. Control, SCL=579ms

B. 0.3μM ACh, SCL=806ms

C. 1 μM ACh, SCL=961ms, Full exit block

D. 3μM Atropine, SCL=560ms
ONLINE FIGURE LEGENDS

Online Figure 1 - Fluorescent optical mapping of the right atrial endocardium during ACh-induced AFI and AF in the canine isolated atria.
Panel A - Photo of the preparation with an epicardial optical field of view (OFV).
Panels B and C - Atrial activation maps and optical action potentials (OAP) during AFL and AF, which were induced by atrial burst pacing and ACh 2 µM.

Online Figure 2 - Fluorescent optical mapping of the canine right atrial epicardium during sinus rhythm and pacing-induced reentry.
Panel A - Photo of the preparation with an epicardial optical field of view (OFV).
Panel B - Parallel histology section close to the epicardial surface corresponding to the dotted rectangle on panel A. This panel shows a single 2D histology section through different layers of tissue, which also includes the SAN (pink oval) and coronary arteries (light blue arrows).
Panels C and D - Optical action potentials from the recording sites 1-5 in Panel A and activation maps during sinus rhythm and atrial pacing S1-S2=300ms-160ms induced reentry. Abbreviations are the same as in Figure 1.

Online Figure 3 - The low-pass filtering (LPF) used to unmask SAN optical signals during atrial arrhythmias.
Panel A – Endocardial optical recording from the central part of SAN shows the double component upstroke (SAN and Atrial) of the OAPs during sinus rhythm (ACh 2 µM).
Panel B – The optical recording from the same location in Panel A during atrial flutter (AFL) used the high (200Hz - green) and low frequency (10 Hz - dark blue) filters to unmask the SAN signal.
Panel C - The optical recording from the same location in Panel A and B during atrial fibrillation (AF) used the high (200Hz - green) and low frequency (20 Hz - dark blue) filters to unmask the SAN signal.
Online Figure 4 - The low-pass filtering (LPF) unmask SAN signals and improves Dominant Frequency maps.

The left panels show DF maps of OAPs recorded during atrial pacing CL at 200 ms (same preparation as Figure 1). The optical signals were filtered with three different filtering frequencies 200Hz, 64 Hz and 16 Hz. The right panels show OAPs recordings and their Frequency Power spectrums from the SAN center (1-Blue) and right atria free wall (2-Red).

Online Figure 5 - SAN recovery time in control and after ACh and Atropine.

Panels A and B - Examples of the SANRTd and SANRTi measurements in control and after ACh, respectively. Both panels show two optical recordings from the center of the SAN (blue) and the crista terminalis CT (green). Panel B represents an example of the pacing-induced transient SAN exit block.

Panel C - An example of the full SAN exit block during 1 µM ACh perfusion.

Panel D – SAN recovery after 3 µM Atropine.
ONLINE MOVIE LEGEND

Online Movie 1. AFL Suppresses SAN
The color map movies show the spatial and temporal changes in amplitude of normalized OAP (upper left), and their derivative (upper right) during AFL, respectively. The bottom panel shows two OAP recordings (blue – SAN center, green – Crista terminalis) from the corresponding color point in both color map movies. This movie corresponds to Figure 6A. It shows sustained AFL and its termination by atrial pacing (CL = 145 ms), and the corresponding recovery of the first normal sinus beat.

Online Movie 2. SAN activation during ACh-induced AFL/AF
The color map movies show the spatial and temporal changes in amplitude of normalized OAP (upper left), and their derivative (upper right) during ACh-induced AFL/AF, respectively. The bottom panel shows two OAP recordings (blue- SAN center, green – Intratrial septum) from the corresponding color point in both color map movies. The movie shows distinguishable coronary arteries surrounding the SAN. It shows conversions from AFL to AF due to SAN intrinsic activity at approximately Time=2.5 sec. This movie corresponds to Figures 6C and D.
Reference List


