Upregulation of Adenosine A1 Receptors Facilitates Sinoatrial Node Dysfunction in Chronic Canine Heart Failure by Exacerbating Nodal Conduction Abnormalities Revealed by Novel Dual-Sided Intramural Optical Mapping

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Background—Although sinoatrial node (SAN) dysfunction is a hallmark of human heart failure (HF), the underlying mechanisms remain poorly understood. We aimed to examine the role of adenosine in SAN dysfunction and tachy-brady arrhythmias in chronic HF.

Methods and Results—We applied multiple approaches to characterize SAN structure, SAN function, and adenosine A1 receptor expression in control (n=17) and 4-month tachypacing-induced chronic HF (n=18) dogs. Novel intramural optical mapping of coronary-perfused right atrial preparations revealed that adenosine (10 μmol/L) markedly prolonged postpacing SAN conduction time in HF by 206±99 milliseconds (versus 66±21 milliseconds in controls; P=0.02). Adenosine-induced SAN intranodal conduction block or microreentry in 6 of 8 dogs with HF versus 0 of 7 controls (P=0.007). Adenosine-induced SAN conduction abnormalities and automaticity depression caused postpacing atrial pauses in HF versus control dogs (17.1±28.9 versus 1.5±1.3 seconds; P<0.001). Furthermore, 10 μmol/L adenosine shortened atrial repolarization and led to pacing-induced atrial fibrillation in 6 of 7 HF versus 0 of 7 control dogs (P=0.002). Adenosine-induced SAN dysfunction and atrial fibrillation were abolished or prevented by adenosine A1 receptor antagonists (50 μmol/L theophylline/1 μmol/L 8-cyclopentyl-1,3-dipropylxanthine). Adenosine A1 receptor protein expression was significantly upregulated during HF in the SAN (by 47±19%) and surrounding atrial myocardium (by 90±40%). Interstitial fibrosis was significantly increased within the SAN in HF versus control dogs (38±4% versus 23±4%; P<0.001).

Conclusions—In chronic HF, adenosine A1 receptor upregulation in SAN pacemaker and atrial cardiomyocytes may increase cardiac sensitivity to adenosine. This effect may exacerbate conduction abnormalities in the structurally impaired SAN, leading to SAN dysfunction, and potentiate atrial repolarization shortening, thereby facilitating atrial fibrillation. Atrial fibrillation may further depress SAN function and lead to tachy-brady arrhythmias in HF. (Circulation. 2014;130:315-324.)

Key Words: adenosine ■ adenosine A1 receptor ■ atrial fibrillation ■ heart failure ■ optical imaging ■ sick sinus syndrome ■ sinoatrial node

Sinoatrial node (SAN) dysfunction is common in heart failure (HF), leading to bradycardia-related death, which may account for up to 43% of sudden cardiac deaths in chronic HF. Improving SAN function could potentially prevent the progression of HF. Despite the importance of SAN function, the complex 3-dimensional (3D) heterogeneous structure of the SAN presents an as-yet unresolved obstacle in determining the molecular and structural remodeling underlying SAN dysfunction.

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Adenosine, an endogenous metabolite of the heart, regulates SAN function primarily via its action on the adenosine A1 receptor (A1R). Under metabolic stress, adenosine release is increased,
which may slow the heart rate and reduce energy consumption.\textsuperscript{6,9,10} Although plasma levels of adenosine increase progressively with the severity of chronic HF (up to 7-fold),\textsuperscript{11,12} it is unknown whether this elevated level of adenosine directly affects SAN function. Moreover, alterations of adenosine signaling, including A1R expression in the SAN pacemaker complex, are poorly elucidated in HF.

On the basis of the prevalence of bradycardia-related deaths\textsuperscript{3,4} and significant increases in the endogenous levels of adenosine in HF,\textsuperscript{11,12} we hypothesized that pathophysiological concentrations of adenosine may lead directly to SAN dysfunction in HF. To test this hypothesis, we applied an integrated approach combining high-resolution optical mapping in parallel with immunohistological analyses and immunoblotting techniques in a clinically relevant canine chronic HF model.\textsuperscript{13}

To resolve impulse generation and conduction within the 3D SAN pacemaker complex, we developed a novel dual-sided, high-resolution optical mapping system to map the intramural electric activity of the entire SAN. Here, we define for the first time the heterogeneous expression of A1R within specific SAN compartments (head, center, tail) and surrounding atrial myocardium as the molecular mechanism for adenosine-induced SAN dysfunction in HF.

### Methods

An expanded Methods section can be found in the online-only Data Supplement.

### Canine Chronic HF Model

An established canine model of nonischemic chronic irreversible HF\textsuperscript{13} (n=18; weight, 20.6±3.3 kg) and age-matched control dogs (n=17; weight, 19.1±2.3 kg) were used. All animal procedures were approved by The Ohio State University Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication No. 85-23, revised 1996). Table I in the online-only Data Supplement shows how each canine heart was used for different experimental protocols.

### Coronary-Perfused SAN-Atrial Preparations

Coronary-perfused canine SAN-atrial preparations\textsuperscript{14–17} (n=10 HF; Figure 1A) were immobilized by 10 to 20 μmol/L blebbistatin\textsuperscript{18} and stained by di-4-ANBDQBS (10–40 μmol/L).\textsuperscript{15,17} Adenosine (1, 10, and 100 μmol/L), the nonselective adenosine receptor blocker theophylline (50 μmol/L; n=4), and the selective A1R blocker 8-cyclopentyl-1,3-dipropylxanthine (DPCPX;19 1 μmol/L; n=4) were applied to the perfusate. The detailed sequential drug treatments for each canine SAN preparation are given in Table II in the online-only Data Supplement.

### Dual-Sided Optical Mapping

To unmask the intramural activation of the entire SAN complex, we developed a dual-sided optical mapping system (Figure 1A) that offers a powerful approach to record optical action potentials simultaneously from both the epicardium and endocardium with 2 CMOS cameras (MiCAM Ultima-L, SciMedia). Because the thickness of the canine SAN preparation (including atria and the SAN) is 1 to 4 mm\textsuperscript{14} and the near-infrared dye (di-4-ANBDQBS) allows collection of signals from a depth of at least 2 mm,\textsuperscript{20} our dual-sided mapping approach can collect signals from the entire 3D SAN structure. This approach also allowed us to reveal the intrinsic transmural differences in SAN activation. Figure 1D illustrates a validation of the novel imaging technique in which the epicardial amplitude of the SAN optical action potential is bigger at the SAN head compared with the SAN tail, suggesting that the head is closer.
to the epicardium than the tail, which is consistent with canine and human 3D SAN anatomies.14,15

Electrophysiology Data Analysis
SAN signals were extracted from the background atrial signals as previously described.17 SAN conduction time (SACT) was measured during sinus rhythm (SACTsr; Figure 2A) and the first postpacing SAN beat (SACTppb; Figure 3A). Indirect (SNRTi) and direct (SNRTd) sinus node recovery times15 were measured (Figure 3A). Corrected SNRT (cSNRT) was calculated by subtracting the preceding sinus cycle length (SCL) from SNRT. Functional results from HF preparations were compared with the results from 7 control SAN experiments conducted in our previous study with the same protocols.17 More details are provided in the online-only Data Supplement.

Histology, Immunoblotting, and Immunostaining
SAN preparations (n=8 HF and n=8 control) were sectioned for immunostaining and Masson trichrome staining. Freshly frozen tissues from various locations of the SAN and atria were collected (n=5 HF and n=5 control). Immunoblot images were acquired and quantified as previously described.22 Protein expression was normalized to GAPDH.

Statistical Analysis
Data are presented as mean±SD. Differences between the HF and control and differences between adenosine treatments and baseline were evaluated with the PROC MIXED procedure in SAS 9.2 (SAS Institute, Cary, NC) for maximum atrial pause, SACT, SCL, cSNRTi, action potential duration (APD), SACTppb, the number of leading pacemaker sites, fibrosis, and A1R protein expression. The following comparisons were made: control versus HF and adenosine treatment versus baseline for control and for HF. P values for multiple comparisons were adjusted with Tukey-Kramer method. The significance of between-group differences in the incidence of SAN exit block and AF was tested with the Fisher exact test in SAS 9.2. A value of P<0.05 was considered statistically significant. The online-only Data Supplement provides more details.

Results
Functional Remodeling of the SAN in HF at Baseline
Compared with control dogs, SCL in HF animals was markedly prolonged (from 477±62 milliseconds17 to 637±78 milliseconds in control and HF, respectively; P=0.039), indicating that intrinsic automaticity was depressed in HF. cSNRTi significantly increased in HF after the pacing of 3.3 Hz (215±65 versus 65±28 milliseconds; P<0.001). SAN conduction was compromised in HF with SACTsr significantly lengthened (68±31 versus 41±11 milliseconds; P=0.03).

Adenosine Depresses SAN Function in Failing Hearts
Adenosine dose-dependently prolonged SCL and SACTsr in HF (Table). Adenosine at the pathophysiological concentration (10 μmol/L) slowed the intrinsic automaticity in all HF preparations (Figure I in the online-only Data Supplement). Figure 2A and 2C shows that 10 μmol/L adenosine increased the SCL, shortened atrial APD, prolonged SACTsr, and therefore slowed conduction within the SAN.

Adenosine also significantly prolonged cSNRTi (Table), a clinical marker for SAN dysfunction.23 Although a high...
in atrial pauses (>1 second) in 63% of HF preparations (versus 0% control; P=0.03), suggesting an increased risk of adenosine-induced SAN dysfunction in HF.

Prolonged cSNRTi may result from slow recovery of automaticity (overdrive suppression) and failed sinoatrial conduction (exit block). Our intramural optical mapping system allowed us to clearly distinguish SAN conduction impairments from automaticity depression. Figure 2B shows that at baseline 3.3 Hz pacing captured the SAN (1:1) and the termination of pacing led to moderate depression of automaticity (cSNRTi=123 milliseconds; cSNRTId=72 milliseconds). In contrast, after the application of 10 μmol/L adenosine, cessation of 3.3 Hz pacing was followed by a long atrial pause of 6252 milliseconds (Figure 2B). The SAN recording in Figure 2B indicated that the SAN had recovered long before the first recovered atrial beat and was active during the atrial pause. Indeed, cSNRTd (an indicator of overdrive suppression) was only 84 milliseconds, suggesting that overdrive suppression of SAN automaticity was not the main reason for the prolonged cSNRTi in this HF preparation. Instead, failed conduction from the SAN to atria, that is, SAN exit block, was responsible for the long atrial pause (Figure 2B and 2C). Importantly, SAN entrance blocks resulting from adenosine also occurred during 3.3 Hz pacing in HF hearts (Figure 2B), further reducing overdrive suppression.

The effects of adenosine on postpacing-induced suppression of SAN automaticity and conduction (cSNRTi and cSNRTd versus SACTppb) in all control and HF preparations are summarized in Figure 3. The definitions of SNRTi, SACTppb, and SACTbd are illustrated graphically in Figure 3A. Figure 3B shows that adenosine led to a 2-fold increase in SACTppb in HF, which contributed to a prolonged cSNRTi. In contrast, adenosine had no significant effects on cSNRTd (Figure 3C), especially in HF preparations, indicating limited effects on pacing-induced depression of automaticity. Examination of individual preparations revealed that 3 of 8 HF preparations showed significant overdrive suppression of automaticity (cSNRTd>250 milliseconds; see also the blue lines in Figure IA in the online-only Data Supplement), which did not occur in the other 5 HF preparations. Importantly, SAN exit block occurred in only those 5 preparations.

Impaired SAN conduction may protect the leading pacemaker clusters from overdrive suppression. Indeed, SACTppb

### Table. Effects of Adenosine on Canine HF SAN

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Baseline (n=8)</th>
<th>Adenosine 1 μmol/L (n=7)</th>
<th>Adenosine 10 μmol/L (n=8)</th>
<th>Adenosine 100 μmol/L (n=4)</th>
<th>Theophylline 50 μmol/L (n=4)</th>
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<tr>
<td>SCL, ms</td>
<td>637±78</td>
<td>684±74*</td>
<td>829±117*</td>
<td>1064±258*</td>
<td>631±94</td>
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<td>SACT during</td>
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<td>106±60*</td>
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<td>215±65</td>
<td>287±45*</td>
<td>841±1079*</td>
<td>549±260</td>
<td>264±97</td>
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<tr>
<td>Maximum atrial pause, s</td>
<td>1.8±2.2</td>
<td>0.9±0.1</td>
<td>17.1±28.9*</td>
<td>3.9±4.5*</td>
<td>0.96±0.2</td>
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<tr>
<td>APD80%, ms</td>
<td>168±13</td>
<td>162±10</td>
<td>156±12*</td>
<td>139±10*</td>
<td>168±16</td>
</tr>
</tbody>
</table>

AF indicates atrial fibrillation; APD80%: action potential duration at 80% recovery; cSNRTi, indirect corrected sinoatrial node recovery time; cSNRTd, 3.3 Hz pacing; HF, heart failure; SACT, sinoatrial node conduction time; SAN, sinoatrial node; and SCL, sinus cycle length.

*P<0.05 versus baseline.
in those 5 HF preparations was significantly longer compared with the other 3 HF preparations (see red lines in Figure IB in the online-only Data Supplement). SAN exit block occurred only when SACTppb was >212 milliseconds, which we refer to as the exit block threshold. Activation maps of the SAN also showed much slower SAN conduction during rapid pacing in the preparation with SAN exit block (Figure IIA in the online-only Data Supplement) compared with the preparation with overdrive suppression (Figure IIB in the online-only Data Supplement). As expected, larger depression of SAN conduction was associated with more intranodal conduction blocks during rapid pacing, as indicated in the dominant-frequency maps and SAN recordings (explanation of dominant-frequency maps is given in Figure II and the Methods section in the online-only Data Supplement).

To differentiate between the roles of pacing and adenosine in conduction depression, we quantified the difference between SACTppb (baseline and adenosine) and SACTsr at baseline (Figure 3D). At baseline, in control preparations, SACTppb did not differ from the SACTsr (Figure 3D), reflecting a large resistance of SAN conduction to pacing-induced depression. However, in HF preparations at baseline, SACTppb was significantly longer than SACTsr in HF, suggesting a reduced SAN conduction reserve (or increased sink-source mismatch) that brought the SACTppb closer to the exit block threshold. After adenosine in HF, SACTppb was 206±99 milliseconds longer than SACTsr at baseline (versus 66±21 milliseconds in control; P=0.02; Figure 3D). The combined effect of adenosine and pacing (206±99 milliseconds) was approximately twice the sum of the individual increases in SACT caused by adenosine alone (43±29 milliseconds) and by pacing alone (62±79 milliseconds). These data demonstrate the amplification of the depressant effects of adenosine on SAN conduction by pacing in HF.

Conduction Suppression Reduces the Number of Functional Conduction Pathways but Increases the Number of Functional Pacemaker Clusters

We found an increase in the number of leading pacemaker sites in the HF SAN compared with control, particularly after adenosine and pacing (2.1±0.8 versus 1.3±0.5; P=0.001; Figure 4). This increase reflects an enhanced competition among different SAN pacemaker clusters and thus reduced mutual entrainment and synchrony. Despite an increase in the number of leading pacemaker sites, there were fewer atrial breakthrough sites in HF during all conditions (Figure 4), which is consistent with the clinical observations in HF patients during sinus rhythm.24 This reduction was due to a decrease in the number of functional exit conduction pathways from 4 to 3 in HF (Figure 4). Specifically, the midlateral conduction pathway in the crista terminalis (Figure 4) was not functional in all 8 HF SANs.

Adenosine Aggravates Tachy-Brady Arrhythmias in Failing Hearts

Adenosine (10 μmol/L) facilitated induction of AF by rapid atrial pacing (6–9 Hz) in 6 of 7 HF preparations but not in control hearts. Termination of AF resulted in SAN exit blocks and atrial pauses in 4 HF preparations, therefore exhibiting tachy-brady arrhythmias. Figure 5A illustrates such an example from an HF preparation in which at baseline rapid atrial pacing (7.5 Hz) failed to induce neither AF nor SAN exit block. In contrast, with 10 μmol/L adenosine, atrial pacing at the same frequency induced AF that lasted for ≈1.5 minutes. Furthermore, cessation of AF was followed by an atrial pause of 14 seconds. Importantly, the SAN remained active during the atrial pause, suggesting the presence of SAN exit block. Overall, 10 μmol/L adenosine significantly increased the maximum duration of atrial pauses after atrial pacing or AF (17.1±28.9 versus 1.8±2.2 seconds, adenosine versus baseline; P<0.001). All severe atrial pauses (>1.5 second) were due to SAN exit block rather than automaticity depression. In 1 HF preparation, 10 μmol/L adenosine in the absence of atrial pacing caused persistent SAN exit block of up to 143 seconds (Figure III in the online-only Data Supplement).

Stable macroreentry around the SAN was consistently observed during AF in right atrial HF preparations (Figure 5B and Figure IV in the online-only Data Supplement). At

<table>
<thead>
<tr>
<th>Control</th>
<th>Heart Failure</th>
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<tbody>
<tr>
<td>Dorg: #1 #2 #3 #4 #5 #6 #7</td>
<td>Dorg: #1 #2 #3 #4 #5 #6 #7</td>
</tr>
<tr>
<td>Baseline SR</td>
<td>Baseline SR</td>
</tr>
<tr>
<td>SVC</td>
<td>SVC</td>
</tr>
<tr>
<td>RAA Head Center</td>
<td>RAA Head Center</td>
</tr>
<tr>
<td>Tail</td>
<td>Tail</td>
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<tr>
<td>IAS</td>
<td>IAS</td>
</tr>
<tr>
<td>RAFW</td>
<td>RAFW</td>
</tr>
<tr>
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<td>Post-pacing</td>
</tr>
<tr>
<td>Ado 10μM SR</td>
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<tr>
<td>SVC</td>
<td>SVC</td>
</tr>
<tr>
<td>RAA Head Center</td>
<td>RAA Head Center</td>
</tr>
<tr>
<td>Tail</td>
<td>Tail</td>
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<tr>
<td>IAS</td>
<td>IAS</td>
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<tr>
<td>RAFW</td>
<td>RAFW</td>
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</table>

![Figure 4](https://example.com/figure4.png) Leading pacemaker sites and atrial breakthrough sites before and after adenosine and pacing. Leading pacemaker sites (○) and atrial breakthrough sites (∗) from control preparations (n=7) and heart failure (HF) preparations (n=8) are shown for baseline (sinus rhythm [SR]), baseline postpacing, 10 μmol/L adenosine, and 10 μmol/L adenosine postpacing. Within each group (HF or control), results for different preparations are shown in different colors. Blocked arrows indicate nonfunctional midlateral conduction pathway in HF. The fifth pathway, extending toward the superior vena cava (SVC) in all hearts, was observed only as an entrance pathway, never as an exit pathway, during baseline and adenosine. CT indicates crista terminals; IAS, interatrial septum; RAA, right atrial appendage; and RAFW, right atrial free wall.)
10 μmol/L adenosine, AF inducibility was much higher in HF compared with controls (Figure 5C). This higher AF inducibility might be associated with greater shortening of repolarization by 10 μmol/L adenosine in HF compared with control hearts. At baseline, average APD at 80% recovery in the right atrial free wall was not different between HF and control (Figure 5D). However, 10 μmol/L adenosine significantly shortened the atrial APD at 80% recovery in HF but not in control (Figure 5D).

To confirm that the observed changes were due to adenosine, we applied theophylline (a nonselective adenosine 1 receptor [A1R] blocker). Representative reentrant activation around the SAN during pacing-induced atrial fibrillation (AF). Summary data of AF inducibility at baseline, 10 μmol/L adenosine, and additional 50 μmol/L theophylline (a nonselective adenosine 1 receptor [A1R] blocker). Figure 5B shows connexin43 (Cx43) staining of a neighboring atrial myocardium (Table, Figure 5A, and Figure V in the online-only Data Supplement). To demonstrate further that the action of adenosine was mainly through A1R,7,9 we applied DPCPX (a selective A1R blocker19; 1 μmol/L; n=4), which also prevented adenosine-induced changes in SCL and SACT (baseline versus DPCPX+adenosine: SCL, 557±79 versus 562±76 milliseconds, P=0.84; SACTsr, 66±25 versus 73±15 milliseconds; P=0.70).

**Figure 5.** Adenosine induced tachy-brady arrhythmias in heart failure (HF). A, Sinoatrial node (SAN) optical action potentials (OAPs) and atrial ECG recordings during and after 7.5 Hz atrial pacing at baseline, 10 μmol/L adenosine, and additional 50 μmol/L theophylline (a nonselective adenosine 1 receptor [A1R] blocker). B, Representative reentrant activation around the SAN during pacing-induced atrial fibrillation (AF). C, Summary of AF inducibility at baseline, 10 μmol/L adenosine, 100 μmol/L adenosine, and A1R blocker (50 μmol/L theophylline or 1 μmol/L 8-cyclopentyl-1,3-dipropylxanthine [DPCPX]) in HF vs control. D, Changes in atrial action potential duration at 80% recovery (APD80%) by adenosine and A1R blocker in HF vs control.

**HF-Associated Structural Remodeling of the SAN**
Structural remodeling might also contribute to conduction impairment. We found that fibrosis was significantly increased throughout the entire SAN pacemaker complex. Figure 7A presents histological sections from a control SAN and an HF SAN. The magnified views of the same histology sections in Figure 7B demonstrate increased fibrosis within the SAN head and a SAN conduction pathway in HF compared with control. Figure 7C shows connexin43 (Cx43) staining of a neighboring HF SAN section, confirming the locations of the SAN (lack of Cx43 or Cx43 negative) and the pathway (transition between the Cx43-negative and Cx43-positive regions). Summary data (Figure 7D) indicate that fibrosis was significantly increased throughout the SAN complex in HF compared with control. We also observed a substantial increase in fibrosis at the intranodal microreentry, which is evident from the activation map from the epicardium in Figure 6A (Movie I in the online-only Data Supplement). As shown in the DiffEPI END map in Figure 6A. Similar transmural heterogeneity was observed in another HF preparation (Figure VI in the online-only Data Supplement).}

**A1R Protein Expression Is Increased in the SAN and Atria From Failing Hearts**
Immunoblot data in Figure 8 indicate that A1R protein expression was significantly increased in all compartments of the SAN pacemaker complex (head, center, and tail) and throughout the surrounding atria (crista terminalis, intratral septum, and right atrial free wall) in HF compared with control. Interestingly, A1R expression in controls was significantly higher in SAN
compartments than in the right atrium. We also immunostained for A1R in SAN and atrial cardiomyocytes (Figure 8B). To confirm further that the A1R upregulation is due to an increased expression in the cardiomyocytes (eg, fibroblasts), we excluded other cell types during the quantification of A1R signal intensity (Figure VII in the online-only Data Supplement). Figure VIII in the online-only Data Supplement shows that, similar to immunoblot results, A1R signal intensity was approximately twice as high in HF SAN and atrial cardiomyocytes compared with control.

Because the outward potassium current ($I_{K,Ado}$) is the main downstream effector of the A1R,7,9 we also quantified protein expression of the 2 main subunits of the $I_{K,Ado}$ channel: GIRK1 and GIRK4.26 We found that although GIRK1 was not significantly altered, GIRK4 was upregulated in the SAN pacemaker tissue (by 39±20%; $P=0.056$; Figure IX in the online-only Data Supplement) and surrounding atrial myocardium (by 70±18%; $P=0.016$; Figure IX in the online-only Data Supplement).

**Discussion**

In this study, we investigated the effects of adenosine on SAN function in correlation with the heterogeneous molecular and structural remodeling in a clinically relevant large animal model of chronic HF. Our major finding is that chronic HF is associated with an upregulation of A1R protein expression in the SAN and atria, which leads to enhanced suppression of SAN function and facilitation of AF. Importantly, using our novel dual-sided intramural optical mapping of the entire 3D SAN pacemaker complex, we were able to reveal that severe SAN dysfunction in HF results predominantly from nodal conduction abnormalities.
A1 Receptor Upregulation Increases Risk of Adenosine-Induced Brady and Tachy-Brady Arrhythmias in HF

Plasma levels of adenosine increase with the progression of HF in patients. Whether this increased level of adenosine is sufficient to produce common SAN dysfunction in HF was yet to be defined. Here, we show that the sensitivity to adenosine was increased in failing SAN, leading to SAN dysfunction and AF under the pathophysiological concentrations (10 μmol/L) of adenosine. It is known that adenosine regulates heart rate primarily via the A1R. We demonstrate for the first time that A1R protein expression is significantly upregulated in both the SAN and atria during chronic HF (Figure 8). Overexpression of A1Rs in mice produced a reduction in heart rate, consistent with a causal relationship between A1R protein expression and the suppression of SAN function.

Role of Conduction Impairment in SAN Dysfunction in HF

Sanders et al have previously demonstrated prolonged indirect SACT and SNRT in HF patients. This study emphasized the importance of conduction impairment in SAN dysfunction, although SAN conduction was evaluated only indirectly and the molecular and structural mechanisms for these abnormalities were not elucidated. Our novel dual-sided optical mapping allowed us to define for the first time the intramural conduction within the 3D SAN structure. Our findings suggest that all severe postpacing atrial pauses (>1.5 seconds) were due to SAN conduction abnormalities in HF (Figures 2–6), which could result from both structural (increased fibrosis; Figure 7) and molecular (A1R and GIRK4 upregulation; Figure 8 and Figure IX in the online-only Data Supplement) remodeling within the SAN.

Thery et al have previously demonstrated a direct correlation between fibrosis in human SAN and the occurrence of tachy-brady syndrome in older patients. Our results also show structural remodeling (increased fibrosis) within the SAN pacemaker complex during HF (Figure 7), which could contribute to the intrinsic SAN conduction abnormalities, including SAN intranodal and exit blocks, as well as SAN reentry. Moreover, the increased interstitial fibrosis could provide additional insulation around the SAN pacemaker clusters, thereby reducing mutual entrainment, and facilitate competition between different SAN and atrial pacemakers (Figure 4), causing heart rhythm disorders.

Our results also show that as a result of A1R upregulation, adenosine could significantly amplify intrinsic conduction abnormalities in failing SAN (Figure 3). Increased GIRK4 expression suggested that adenosine could suppress SAN conduction through activating A1R and the downstream outward potassium current (I_{K.Ado}), which hyperpolarizes both the SAN pacemaker and atrial myocytes. The hyperpolarization, by increasing the sink-source mismatch between the SAN and surrounding atrial myocardium, could suppress conduction and excitability in SAN conduction pathways. Our preliminary findings in failing human hearts also showed a similar increase in A1R and GIRK4 expression in the right atrial myocardium, emphasizing the relevance of A1R and GIRK4 upregulation in the context of human HF.

Additionally, because adenosine is known to antagonize adrenergic stimulation by inhibiting cAMP production, it could have a further effect on conduction by suppressing the I_{Ca,L} current and L-type calcium current, especially if they were enhanced by adrenergic stimulation. Future studies including computer simulations are warranted to test these hypotheses.

Adenosine Increases AF Incidence in HF

We observed a high occurrence of adenosine-induced AF in right atrial HF preparations (Figure 5C), a finding that is in agreement with the close association between AF and HF. Importantly,
our results suggested increased sensitivity to adenosine as a new mechanism for increased risk to AF in HF. AF and SAN dysfunction often co-occur in the same patient.66–68 Adenosine could be responsible for both SAN dysfunction and AF in HF. AF could further suppress SAN function, as demonstrated by the long post-AF atrial pauses (Figure 5A). SAN dysfunction, in turn, could also contribute to the occurrence of AF. First, increased fibrosis within the SAN may facilitate induction and maintenance of reentry around the SAN.30,39 Second, modeling39 and experimental41 studies have shown that increased fibrosis within the SAN may facilitate reentry circling through the SAN via 2 separate conduction pathways. Third, atrial pauses resulting from SAN depression may unmask ectopic activity from atrial latent pacemakers, which could compete with SAN pacemakers and trigger AF.10,42,43

Limitations
In the present study, we investigated the role of the adenosine-associated pathway in SAN dysfunction in failing hearts. However, HF is a complex disease wherein multiple molecular mechanisms could be altered, ultimately leading to pump failure.44 Several other mechanisms have been implicated in the origin of SAN dysfunction, including an imbalance in the autonomic control of the heart,45 alterations in the “pacemaker” current (Ih),46,47 and calcium-handling abnormalities.50 Reduced response to β-adrenergic stimulation in HF could also contribute to SAN dysfunction.48

Potential Implications and Future Directions
By improving SAN function, the A1R blocker theophylline has been demonstrated to prevent the development of HF in patients with SAN dysfunctions.5 Our results further indicate that A1R blockade may prevent and restore adenosine-induced atrial pauses in HF subjects. However, because A1Rs are expressed in a variety of tissues in addition to cardiac muscle, long-term use of theophylline can produce off-target side effects such as seizures49 and therefore should be evaluated with caution.

Although adenosine leads to SAN dysfunction, it also ameliorates metabolic stress.50 The double-edged role of adenosine suggests that neither complete suppression nor activation of the adenosine signaling pathway will be more beneficial than harmful. Therefore, targeting local expression/activity of A1Rs within the SAN conduction pathways could offer an alternative approach to prevent severe bradycardia without losing the beneficial effects of adenosine in reducing metabolic stress in failing human hearts.

Conclusions
Here, we demonstrated for the first time that chronic HF increases the risk of adenosine-induced SAN dysfunction and AF as a result of molecular (A1R upregulation) and structural (fibrosis) remodeling. Specifically, upregulation of A1R expression in both the SAN and atria leads to increased sensitivity of SAN conduction and atrial repolarization to adenosine, which aggravates SAN conduction abnormalities in the structurally impaired SAN and facilitates the development of AF and tachy-brady syndrome.

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Disclosures
None.

References
the adenosine A1 receptor. Our integrative optical mapping study provides the first direct demonstration that adenosine A1 receptor antagonists such as theophylline or 8-cyclopentyl-1,3-dipropylxanthine can successfully prevent tachy-brady arrhythmias without losing the beneficial effects of adenosine in failing human hearts.

Future investigations should also focus on modulating adenosine A1 receptor signaling to specifically restore SAN conduction and to prevent tachy-brady arrhythmias without losing the beneficial effects of adenosine in failing human hearts.

CLINICAL PERSPECTIVE

Sinoatrial node (SAN) dysfunction and atrial fibrillation (AF) are common heart rhythm disorders in failing human hearts. The successful diagnosis and treatment of the specific mechanisms responsible for SAN dysfunction are critical to prevent both heart failure progression and AF development. Adenosine, a cardiac metabolite, is known to regulate SAN function via its action on the adenosine A1 receptor. Our integrative optical mapping study provides the first direct demonstration that adenosine A1 receptor antagonists such as theophylline or 8-cyclopentyl-1,3-dipropylxanthine can successfully prevent atrial pauses and AF in failing hearts. The findings from our study suggest that targeting the adenosine signaling pathway in a clinical setting should be considered a powerful strategy to simultaneously treat both SAN dysfunction and AF associated with heart failure. However, because adenosine A1 receptor upregulation could also be a favorable compensatory mechanism to meet energy deficits in heart failure, targeted approaches to locally modify specific gene/cell populations should be explored. Future investigations should also focus on modulating adenosine A1 receptor signaling to specifically restore SAN conduction and to prevent tachy-brady arrhythmias without losing the beneficial effects of adenosine in failing human hearts.
Upregulation of Adenosine A1 Receptors Facilitates Sinoatrial Node Dysfunction in Chronic Canine Heart Failure by Exacerbating Nodal Conduction Abnormalities Revealed by Novel Dual-Sided Intramural Optical Mapping

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SUPPLEMENTAL MATERIAL

Upregulation of adenosine A1 receptors facilitates sinoatrial node dysfunction in chronic canine heart failure by exacerbating nodal conduction abnormalities revealed by novel dual-sided intramural optical mapping.

Qing Lou, PhD,* Brian J. Hansen, BSc,* Olga Fedorenko, PhD, Thomas A. Csepe, Anuradha Kalyanasundaram, PhD, Ning Li, MD, PhD, Lori Hage, BSc, Alexey V. Glukhov, PhD, George E. Billman, PhD, Raul Weiss, MD, Peter J Mohler, Ph.D, Sándor Györke, PhD, Brandon J. Biesiadecki, PhD, Cynthia A. Carnes, PharmD, PhD, Vadim V. Fedorov, PhD

*Q.L. and B.J.H. contributed equally to this study

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SUPPLEMENTAL METHODS

Canine Chronic Heart Failure (HF) Model
An established canine model of non-ischemic chronic HF produced by 4 months of rapid pacing (n=17) and age-matched control dogs (n=18) were used for our studies. The chronic HF dog model was produced by 4-month right ventricular tachypacing as previously described.1,2 This model recapitulated key clinical features (systolic and diastolic left ventricular dilation, low contractility and ejection fraction < 35%, elevated catecholamine level and atrial chambers dilations) of end-stage dilated cardiomyopathy in humans.1,2 Table I shows how each canine heart was utilized for different experimental protocols. All the animal procedures were approved by The Ohio State University Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of
Health (NIH publication N. 85-23, revised 1996). Pacemakers and leads were provided for the development of the canine HF model by St. Jude Medical, Inc., USA.

**Canine Heart Isolation**

Dogs were sedated with 5-8 mg butorphanol IM before the induction of anesthesia. 10 mg (0.5mg/kg) of diazepam was administered via an IV catheter in a peripheral vein, and was followed immediately by 30mg (~1.5mg/kg) of etomidate. Dogs were intubated and ventilated with 2-5% isoflurane to maintain deep anesthesia. A right thoracotomy was performed either between the 3rd and 4th or the 4th and 5th ribs. Heparin (3000 units) was administered by IV. Loops (cotton umbilical tape) were placed around the superior and inferior vena cava and the azygous vein was tied off. The pericardium was opened widely and the heart was exposed. A Satinsky clamp was placed around the aorta but not clamped. The inferior 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 superior vena cava was tied off and the aorta was then clamped. A 16-gauge needle attached to a bag of 1000ml cardioplegia was inserted into the superior vena cava just below the tie to allow cardioplegia to run into the heart. Ice was also immediately placed in the thoracic cavity. Once 1000ml of cardioplegia had been perfused through the coronary arteries, the heart was removed from the thoracic cavity for further processing.

**Coronary-Perfused SAN-Atrial Preparations**

HF Canine SAN preparations were isolated (n=10), coronary-perfused and superfused with oxygenated Tyrode's solution at 37°C under controlled pH and pressure.3-6 The preparations were immobilized by 10-20 μM blebbistatin,7 and stained by di-4-ANBDQBS (10-40 μM).4, 6 A typical canine SAN preparation from a HF dog is shown in Figure 1B. SAN preparations were sequentially perfused by regular Tyrodes' solution, 1, 10 and 100 μM adenosine followed by the non-selective adenosine receptor blocker theophylline (50 μM). A selective A1R blocker DPCPX8 (1μM) was used in four HF preparations. Specifically, DPCPX was added before adenosine in two experiments and added after adenosine in another two experiments.

**Dual-Sided Optical Mapping**

To unmask intramural activation of the entire SAN complex, we developed a novel dual-sided optical mapping system (Figure 1A) which offers a powerful approach to
simultaneously record optical action potentials (OAPs) from both the epicardium and endocardium using two CMOS cameras (MiCAM Ultima-L, SciMedia, CA). Specifically, since the thickness of the canine SAN preparation (including atria and the SAN) is 1 - 4 mm$^3$ and the near-infrared dye (di-4-ANBDQBS) allows collection of signals from a depth of at least 2mm,$^9$ our dual-sided mapping approach can collect signals from the entire 3D SAN structure. In fact, the size of the functionally mapped SAN complex (Figure 1D) exactly matched the anatomical size of the SAN defined by subsequent histological examination of the same preparation (Figure 1C). By using bilateral optical imaging of the same area using the two-camera system, we were able to reveal the intrinsic transmural differences in SAN activation.$^{10}$ In addition, after normalization by the total signal (SAN+atria), the amplitude of the SAN signal indicated the relative depth of the SAN beneath the superficial atrial tissue. Figure 1D illustrates a validation of the novel imaging technique where the epicardial amplitude of SAN OAP is bigger at the SAN head compared to that of the SAN tail, suggesting that the head is closer to the epicardium than the tail, which is consistent with the canine and human 3-D SAN anatomy.$^{3,4}$

**Electrophysiology Data Analysis**

Details of the data analysis have been described elsewhere.$^{3-6}$ SAN signals free from atrial signal contamination were extracted from the background atrial signals as previously described.$^6$ Sinus cycle length (SCL) and SAN conduction time (SACT) were measured before and after each treatment. Corrected SAN recovery time (cSNRT) was measured after 30 seconds of atrial pacing (3.3-9 Hz). As an indicator of conduction abnormality, SACT was defined as the time from the first activation within the SAN (leading pacemaker) to the first atrial activation (exit point in the atria or atrial breakthrough). SACT was measured during sinus rhythm (SACTsr, Figure 2A) as well as during the first post-pacing atrial beat. In the presence of post-pacing SAN exit block where SAN activity recovered before atrial activity, SACT of the first recovered atrial beat did not reflect the SAN conduction immediately following overdrive suppression. We therefore defined a new parameter to quantify the SACT of the first post-pacing beat of SAN (SACTppb, Figure 3A). If there is no post-pacing exit block, SACTppb was equivalent to the SACT defined above; otherwise, SACTppb was considered to be the complete SAN activation time of the first post-pacing SAN beat. Indirect SNRT (SNRTi, Figure 3A), the traditional SNRT, was measured from the last paced atrial beat to the
first recovered atrial beat. To quantify the recovery of SAN pacemaker function, direct SNRT (or SNRTd)\(^{11}\) was measured as the time from the last paced atrial beat to the first recovered SAN beat (Figure 2B). Corrected SNRT\(_i\) (cSNRT\(_i\)) and corrected SNRTd (cSNRTd) were calculated by subtracting the preceding SCL from SNRT\(_i\) or SNRTd. To determine whether the sensitivity to adenosine was altered due to HF, we compared the results from HF preparations with the results from seven control SAN experiments, which were conducted in our previous study with the same protocols.\(^6\)

**Analysis of conduction pattern in the SAN pacemaker complex**

As we have previously shown,\(^3,5\) the SAN is insulated from the atria except at 3-5 exit or entrance regions, namely the conduction pathways. We could functionally identify these pathways during a regular sinus beat (Figure 2C) or a paced SAN beat (Figure II) using activation maps. If the SAN activation reached the conduction pathways but did not exit to the atria, then **SAN exit block** occurred at those conduction pathways. If the SAN activation was blocked before it reached any conduction pathway, then it was an **intra-nodal block**. Similarly, during atrial pacing, if the atrial excitation reached the conduction pathway but did not enter the SAN, then **entrance block** occurred at those conduction pathways. It is likely that the atrial excitation entered the SAN through pathway(s) but was blocked by intra-nodal block before it reached the center of the SAN.

Other than activation maps, we could also define the regions of conduction block during fast atrial pacing and AF based on dominant frequencies (DF) maps.\(^5,6\) The DF at each individual location indicates the dominant rate of activation at this region during a recording and depends on both conduction and refractoriness of that region. When conduction is repetitively blocked at one line, this line of conduction block will be manifested as the border line between areas with different DF. **Online Figure II** shows DF maps during atrial pacing of 7.5 Hz from two preparations. It can be seen that DF within the SAN pacemaker complex is lower compared to the surrounding atrial tissues, suggesting that entrance block occurred. The DF map in **Online Figure IIA** also shows multiple DFs within the SAN, indicating that conduction blocks also occurred within the SAN between pacemaker clusters.

**Histology, Immunostaining and Immunoblot**
SAN preparations (n=5 HF and n=5 control after experiments, and n=3 HF and n=3 control freshly-fixed) were formalin-fixed, paraffin-embedded, and then sectioned parallel to the epicardium. The resultant rectangular sections (~35±5 mm × 25±5 mm), centered around the SAN pacemaker complex, were subjected to standard immunostaining technique. Lack of Connexin 43 (Cx43) expression, distinct cell morphology and fiber organization, and an increased ratio of fibrotic/connective tissue to cardiomyocytes3, 6 were used to identify the SAN pacemaker complex. This was followed by a quantification of fibrosis (with Masson’s trichrome staining) reported as a percentage of fibrotic area out of the total tissue area in different compartments of the SAN pacemaker complex, SAN conduction pathway and the right atria (see Figure 7).

To quantify spatial expression of A1R, GIRK1 and GIRK4, tissue from various locations of the SAN and atria was frozen in liquid nitrogen immediately after cardiologic arrest of the heart and collected for Western blot (additional n=5 HF, n=5 control). Specifically, we collected tissues from the SAN head, center, and tail (~1x1 mm²), as well as crista terminalis (CT), inter-atrial septum (IAS), and right atrial free wall (RAFW) from the atria (~2×2 mm²). All membranes were imaged on a Typhoon 9410 imager (GE Healthcare) with appropriate excitation and emission filters as described previously.12 Resultant Western blot images were quantified by analysis with ImageQuant TL 7.0 (GE Healthcare).12 Protein expression was first normalized to GAPDH, and then normalized to the expression at the IAS for each heart. To analyze the fine details of A1R expression and distribution of fibroblasts in the SAN, immunostaining of A1R, vimentin (marker of fibroblast), Cx43, and alpha-actinin were also performed on the tissue sections (n=6 HF, n=6 control). Primary antibodies included rabbit anti-A1 polyclonal antibody (Abcam, 1:2000 for Western, 1:50 for immunostaining), rabbit anti-Kir3.1 (GIRK1) polyclonal antibody (Alomone labs, 1:500), rabbit anti-Kir3.4 (GIRK4) polyclonal antibody (Alomone labs, 1:500), mouse anti-GAPDH polyclonal antibody (Sigma, 1:50,000), mouse anti-vimentin monoclonal antibody (Sigma, 1:400), rabbit anti-Cx43 polyclonal antibody (Sigma, 1:400), and mouse anti-α-Actinin monoclonal antibody (Sigma, 1:200). Secondary antibodies included Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, 1:250) and Alexa Fluor 555 goat anti-mouse IgG1 (Molecular Probes, 1:200).

**Statistical Analysis**
Data are presented as mean ± SD. The differences between the HF and controls and differences between adenosine treatments and baseline were evaluated using PROC MIXED procedure in SAS 9.2 (SAS Institute, Cary, NC) for maximum atrial pause, SACT, SCL, cSNRTi, action potential duration (APD), SACTppb, the number of leading pacemaker sites, fibrosis, and A1R protein expression. Analysis was done for each adenosine concentration separately. The following model was used: Measure = Group + Level + Group * Level + Subject, where factor Group is either HF or control, factor Level - either adenosine treatment or Baseline, Group * Level is interaction term, and Subject is the subject ID. Subject was treated as random factor nested within the group. The following comparisons were made: Control vs. HF for adenosine treatment; adenosine treatment vs. baseline for control and for HF. P-values for multiple comparisons were adjusted using Tukey-Kramer method. The significance of between-group differences in the incidence of SAN exit block/atrial pauses and AF were tested using Fisher’s exact test in SAS 9.2. The significance between control (n=5) and HF (n=5) for GIRK1 and GIRK4 protein expression was tested 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. A p<0.05 was considered to be statistically significant.

Reference List


### SUPPLEMENTAL TABLES

#### Online Table I. Experimental utilization of individual canine tissues

<table>
<thead>
<tr>
<th>HF Dog</th>
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<th>Histology /Immuno</th>
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* Control mapping experiments have been previously reported.⁶
Online Table II. Sequential drug treatments in individual canine experiments

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* Control mapping experiments have been previously reported.
SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

Online Figure I

**A** SCL or SNRTd (ms)  

- **Exit block** (n=5)  
- **no Exit block** (n=3)  
- **Average** (n=8)

**B** SACTsr or SACTppb (ms)  

- **Exit block** (n=5)  
- **no Exit block** (n=3)  
- **Average** (n=8)

**Online Figure I.** Sinus cycle length [SCL, (A)] and post-pacing-beat sinoatrial node conduction time [SACTppb, (B)] of each HF preparation at baseline and 10µM adenosine (Ado) before and after pacing. Red lines indicate that the preparation experienced SAN exit block (exit-block group); blue lines indicate that the preparation did not experience SAN exit block (no-exit-block group). The green line indicates the averaged values from all HF preparations. At 10µM adenosine, the exit-block group was associated with less overdrive suppression of automaticity and significantly slower SAN conduction time. The no-exit-block group, on the contrary, was associated with considerable overdrive suppression and less depression in SAN conduction. The black dotted line indicates a threshold for occurrence of SAN exit block. When the SAN conduction impairment was above this threshold, SAN exit block occurred; otherwise overdrive suppression of automaticity occurred.
Online Figure II

A. HF preparation with exit block

B. HF preparation with overdrive suppression

Online Figure II. Difference in SAN conduction during 7.5Hz atrial pacing for one HF example with exit block (A) and another HF example with overdrive suppression of automaticity (B). Shown for each example are 1) an activation map showing how the SAN was paced by waves entering from both superior and inferior conduction pathways at one particular beat during 7.5Hz pacing, 2) dominant frequency (DF) map showing the dominant rate of activation during atrial pacing (boundaries of each color are indicative of the lines of conduction block), 3) frequency spectrums illustrating the dominant frequencies from five local regions labeled in each DF map, and 4) optical action potentials (OAPs) during the same recording of 7.5Hz atrial pacing from the same five regions. SAN: sinoatrial node; SVC: superior vena cava; IVC: inferior vena cava; IAS: inter-atrial septum; RAA: right atrial appendage; RV: right ventricle; CT: crista terminalis.
Online Figure III. Adenosine-induced atrial pauses due to SAN exit block. (A) Atrial ECG recordings before and after the addition of adenosine. Adenosine led to bradycardia, which became more and more severe until complete atrial arrest occurred. (B) SAN activity during the bradycardia indicated in the green square in Panel A. SAN remained active between atrial diastole, suggesting that the bradycardia was due to SAN exit block, although the sinus cycle length (SCL) was slower compared to the SCL before the application of adenosine.
Online Figure IV. Clock-wise macro-reentry around SAN during an episode of atrial fibrillation in a HF preparation during 10µM adenosine.
Online Figure V. The reversal of adenosine-induced SAN dysfunction by theophylline in a HF preparation. Atrial ECG and SAN OAP during and after 3.3Hz pacing at baseline, 10µM adenosine, and additional 50µM Theophylline.
Online Figure VI. An example of SAN micro-reentry. (A) SAN OAPs and atrial ECG during and after 3.3Hz pacing under 10µM adenosine in a HF preparation. SAN micro-reentry (labeled as R1, R2, and R3) occurred during SAN exit block.
(B) Activation maps during the first two reentrant beats (R1 and R2) within the SAN. Three maps are shown for each beat, including activation maps at the epicardium (Epi) and endocardium (Endo), and the transmural activation time difference pattern (Epi minus Endo). In the time difference maps, red color indicates that Epi activated first, blue color indicates that Endo activated first. This sustained SAN micro-reentry is also shown in Online Movie II.

(C) Epi and Endo OAPs during the SAN micro-reentry. The time window for this micro-reentry is indicated in the dotted rectangle in panel A. The spatial origins of these recordings are marked in the first map in panel B and panel D.

(D) Histology of the same SAN preparation shows that increased interstitial fibrosis strands were the structural substrate of the micro-reentry.

Online Figure VII

A1R expression in cardiomyocytes vs. fibroblasts

Online Figure VII. A1R expression quantification in cardiomyocytes. High-magnification (60x) images from dual-staining of A1R and Vimentin of SAN conduction pathway show A1R expression in cardiomyocytes and fibroblasts. Cardiomyocytes are outlined in purple. Fibroblasts are outlined in white and are excluded from the quantification for A1R expression in cardiomyocytes.
**Online Figure VIII.** A1R signal density in cardiomyocytes. (A) Summarized A1R expression in cardiomyocytes in Control vs. HF hearts based on the analysis of immunolabeling of A1R in the SAN, Pathway, and crista terminalis (CT). HF increased A1R signal density in cardiomyocytes. *: p < 0.05; **: p < 0.001. (B) Immunostaining of A1R (green) and vimentin (red) of the canine SAN Head, Pathway, and CT (20x images) in control and HF sections.
Online Figure IX. Protein expression of GIRK1 and GIRK4. (A) Representative Western blot of A1R, GIRK1 and GIRK1-Gly (glycosylated), GIRK4, and GAPDH of SAN head and inter-atrial septum (IAS) tissues from control and HF hearts. (B) Protein expression summary.
SUPPLEMENTAL MOVIE LEGENDS

**Online Movie I.** Sustained SAN micro-reentry during adenosine in a canine HF preparation. Up-left panel shows the electrical activations (red) of SAN at the epicardium. Up-right panel shows the signal derivative, with the red color indicating the activation wave front. Bottom panel shows the optical action potentials (OAPs) from selected regions marked by color-coded asterisks in upper panels. RAA: right atrial appendage; SVC: superior vena cava; IAS: inter-atrial septum. This recording is also presented in detail in Figure 6.

**Online Movie II.** SAN micro-reentry during adenosine in another canine HF preparation. Panel description is the same as Online Movie I. Detailed analysis of this recording can be found in Online Figure VI.