Autonomic Remodeling in the Left Atrium and Pulmonary Veins in Heart Failure – Creation of a Dynamic Substrate for Atrial Fibrillation

Running title: Ng et al.; Atrial autonomic remodeling in heart failure

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Abstract:

**Background** - Atrial fibrillation (AF) is commonly associated with congestive heart failure (CHF). The autonomic nervous system is involved in the pathogenesis of both AF and CHF. We examined the role of autonomic remodeling in contributing to AF substrate in CHF.

**Methods and Results** - Electrophysiological mapping was performed in the pulmonary veins (PVs) and left atrium (LA) in 38 rapid-ventricular paced dogs (CHF group) and 39 controls under the following conditions: vagal stimulation, isoproterenol infusion, β-adrenergic blockade, acetylcholinesterase (AChE) inhibition (physostigmine), parasympathetic blockade, and double autonomic blockade. Explanted atria were examined for nerve density/distribution, muscarinic receptor (MR) and beta-adrenergic receptor (βAR) densities, and AChE activity. In CHF dogs, there was an increase in nerve bundle size, parasympathetic fibers/bundle, and density of sympathetic fibrils and cardiac ganglia, all preferentially in the posterior LA/PVs. Sympathetic hyperinnervation was accompanied by increases in β1AR density and in sympathetic effect on ERPs and activation direction. β-adrenergic blockade slowed AF dominant frequency. Parasympathetic remodeling was more complex, resulting in increased AChE activity, unchanged MR density, unchanged parasympathetic effect on activation direction, and decreased effect of vagal stimulation on ERP (restored by AChE inhibition). Parasympathetic blockade markedly decreased AF duration.

**Conclusions** - In this heart failure model autonomic and electrophysiologic remodeling occurs involving the posterior left atrium and pulmonary veins. Despite synaptic compensation, parasympathetic hyperinnervation contributes significantly to AF maintenance. Parasympathetic and/or sympathetic signaling may be possible therapeutic targets for AF in CHF.

**Key Words:** atrial fibrillation, autonomic nervous system, heart failure
Atrial fibrillation (AF) is commonly seen in patients with congestive heart failure (CHF).\textsuperscript{1} Coexistence of AF and CHF results in increased morbidity and mortality as compared to either condition alone.\textsuperscript{2} A variety of structural and electrophysiological changes in the atria contribute to the increased susceptibility to AF in the setting of CHF.\textsuperscript{3,4} Changes in ion-channel expression and gap junction distribution, structural remodeling in the form of fibrosis and oxidative stress are some of the mechanisms that contribute to AF substrate in the setting of CHF.\textsuperscript{3-5} In addition, in both clinical and experimental studies, AF has been shown to be mediated at least in part by the autonomic nervous system.\textsuperscript{6,7} It is likely that no single pathophysiological mechanism in and of itself is sufficient to create adequate AF substrate in the CHF setting, with a combination of mechanisms being required to create conditions for the genesis and maintenance of AF. While the role of ion-channel remodeling and fibrosis has been studied extensively in the creation of AF substrate in CHF, the specific contribution of the autonomic nervous system to AF in the setting of CHF has not been well elucidated.

Parasympathetic and sympathetic stimulation have both been demonstrated to be proarrhythmic in the atrium, through refractory period shortening and increased heterogeneity of repolarization.\textsuperscript{8} Our previous studies have shown the posterior left atrium (PLA) and pulmonary veins (PVs) have a unique autonomic profile which could help sustain AF in the normal heart.\textsuperscript{9} How autonomic effects on atrial electrophysiology are altered by CHF is not completely understood. There is evidence of sympathetic hyperinnervation in patients with AF.\textsuperscript{10} While it has recently been suggested that there are increased vagal and sympathetic discharges in CHF that may provide the triggers of AF,\textsuperscript{11,12} the precise nature of autonomic remodeling in AF and its potential role in the creation of AF substrate in CHF have not been studied. We therefore examined in detail structure-function relationships regarding the nature of sympathetic as well as
parasympathetic remodeling in the left atrium and PVs during CHF. Our results indicate that autonomic remodeling is not only complex, but is strikingly different in the atrium from what has been reported in the ventricle in the setting of CHF, with the parasympathetic nervous system playing an important role in the creation of AF substrate (unlike in the ventricle, where the vagus appears to be protective against arrhythmias). Sympathetic changes in the CHF atrium also differed from the failing ventricle, with both sympathetic nerves and β-receptors being upregulated in the atrium.

**METHODS**

Additional methodological details for each of the sections below are described in the Supplemental Material.

**Canine CHF Model.** Thirty-eight purpose bred hound dogs were used. The details of the pacing model are as previously described. In each dog, sterile surgery for pacemaker implantation was performed. The pacemaker was programmed to pace the right ventricle at a rate of 240 beats per minute. In the first 10 dogs, left ventricular function was assessed during pacing by serial echocardiograms (results for these 10 animals are shown in the Supplemental Material, Figure 1) and clinical symptomology (ascites, tachypnea, reduced physical activity). CHF, indicated by compromised left ventricular function, was confirmed at 3 weeks of pacing. Thirty-nine control dogs (i.e. not paced) were studied for comparison. This protocol conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of
Health (NIH Publication No. 85-23, revised 1996) and was approved by the Animal Care and Use Committee of Northwestern University.

**Experimental Setup.** Immediately after the 3 weeks of rapid ventricular pacing, a median sternotomy was performed under general anesthesia with isoflurane. High density plaques were applied to the left inferior pulmonary vein (PV; 8 x 5 electrodes; 2.5 mm spacing), the posterior left atrium (PLA; 7 x 3 electrodes, 5 mm spacing) and the left atrial appendage (LAA; 7 x 3 electrodes, 5 mm spacing) for bipolar electrogram recordings and pacing. The PV plaque was placed circumferentially around the vein while the other two plaques were laid flat on the PLA and LAA epicardium. The left cervical vagus nerve was isolated and attached with bipolar electrodes for electrical stimulation.

**Autonomic Maneuvers.** Effective refractory period (ERP) measurement, activation mapping, and arrhythmia induction (to be described) were performed at baseline and in the presence of the parasympathetic and sympathetic maneuvers described below:

**Autonomic blockade**

- Complete parasympathetic blockade with atropine (0.04 mg/kg)
- β-adrenergic blockade with propranolol (0.2 mg/kg)
- Double autonomic blockade with propranolol (0.2 mg/kg) and atropine (0.04 mg/kg)

**Autonomic stimulation**
• Electrical stimulation of the isolated left cervical vagus nerve with a stimulation rate of 20 Hz, pulse width of 5 ms, and amplitude of 10 volts (Grass S44G, Astromed, West Warwick, Rhode Island)

• Acetylcholinesterase (AChE) inhibition through physostigmine infusion IV (3 to 4 mg)

• Combined cervical vagal stimulation in the presence of physostigmine

• Isoproterenol infusion, titrated to increase the sinus rate by 25 percent

In order to exclude M2R desensitization as a cause of the decrease in vagal-induced ERP shortening, we also assessed for ERP shortening by direct application of carbachol (1mmol/L), a non-selective MR agonist, to the PLA. Upon finishing this in vivo portion of the study, the hearts were removed for pathological analysis.

**Effective Refractory Periods.** To test the autonomic effects on refractoriness, ERPs were obtained from 5, 6, and 4 sites on the PV, PLA, and LAA plaque, respectively, during each autonomic intervention.

**Atrial Fibrillation Sustainability and Dominant Frequency.** AF induction was attempted in the LAA during baseline, with atropine, and with double autonomic blockade. This protocol consisted of burst pacing at a cycle length of 180 ms to 100 ms (with 10 ms decrements) for 10 seconds at each cycle length. Current was set at four times the threshold for capture. The maximum durations of the AF episodes induced by the burst pacing were calculated for each intervention.
Electrograms recorded during the maximum duration AF episodes obtained by burst pacing were analyzed with dominant frequency (DF) analysis. This analysis was performed offline using Matlab (Mathworks, Natick, MA). Four four-second segments (16 seconds total) of each channel and AF episode after a stabilization period of four seconds following the cessation of burst pacing were selected for analysis.

**Activation Mapping.** Recordings from the plaques on the PLA and LAA were made during pacing at a cycle length of 400 ms from the left inferior PV at baseline, parasympathetic blockade, and sympathetic blockade. Activation from the PVs was not analyzed due to insufficient signal quality in the majority of these sites. Creation of activation maps from these recordings and the subsequent analysis were performed offline using custom software programmed in Matlab.

We quantified the similarity of activation patterns between two autonomic conditions by calculating the Pearson’s correlation coefficient between two beats, with each beat consisting of activation times obtained by a single multi-electrode plaque. A correlation coefficient of 1 would signify identical activation patterns between a beat at baseline and a beat during autonomic blockade. A correlation coefficient near zero would signify significant change. Correlation coefficients between two repeated beats at baseline and two repeated beats during autonomic blockade were calculated to assess stability of the activation maps. We hypothesized that the similarity in activation patterns between a baseline beat and an autonomic blockade beat would be less than that between two repeated baseline beats. We also hypothesized that the similarity between a baseline beat and an autonomic blockade beat would be different between
normal and heart failure dogs. An example of an activation map construction and reproducibility analysis are presented in the Supplemental Material (Figure 2).

**Immunohistochemistry.** Immunohistochemistry was performed to examine the size and distribution of the parasympathetic (AChE) and sympathetic (dopamine β-hydroxylase, DBH) nerves in the atria. As explained in the expanded Methods section (Supplemental Material), immunostaining was performed on serial sections taken from the PLA, PVs and LAA. Cardiac ganglia were defined as nerve bundles containing one or more neuronal cell bodies. Quantification was performed manually with a light microscope. Mean densities (number per mm²) of nerve bundles and cardiac ganglia cells were quantified using 1x1 mm grids at 10 times magnification. We also counted the number of parasympathetic and sympathetic fibers within individual nerve bundles. Sizes of nerve bundles were quantified using rectangular 1x1 mm grids at 20 times magnification.

**Densities of muscarinic and beta-adrenergic receptors.** Radioligand binding assays were used to determine densities of muscarinic and beta-adrenergic receptors (MRs and βARs, respectively) in PV, PLA, and LAA tissue samples. The frozen samples were first powdered and membranes prepared as previously described.¹⁶ The βAR binding assays were performed as described in the Supplemental Material.

**AChE activity.** The activity of AChE was determined in PV, PLA, and LAA tissue samples. Frozen tissue samples (0.05g) were solubilized and homogenized as described above. Sample supernatants were aliquoted into 96-well microplates and assayed for acetylcholinesterase...
activity via QuantiChrom™ Acetylcholinesterase Assay kit (Ellman’s method) according to the manufacturer’s instructions for tissue assay.

Data Analysis. All values are expressed as mean ± standard error. For most analyses, comparisons between CHF and normal dogs were performed with two-way mixed ANOVA with one within-dogs factor (PV vs PLA vs LAA) and one between-dogs factor (CHF vs normal). Student’s t tests were used for post-hoc testing. Full factorial mixed effects design with measurement site (PV vs PLA vs LAA) as within-dogs variable and presence of CHF as a between-dogs variable was used to analyze the ERP data separately for each of the study stages (baseline, atropine, propranolol, etc). For the physostigmine analysis, physostigmine was also used as a repeated within-dogs variable. To test the effect of autonomic blockade on the correlation coefficients of activation times, repeated measures ANOVA was used. AF duration between interventions was compared using Student’s t test. Statistical significance was taken at p-values less than 0.05.

RESULTS

The breakdown of sample sizes for each intervention/analysis is listed in Table 1. The tissue and molecular assay data are presented first, followed by the in vivo electrophysiological data.

Immunohistochemistry

Qualitative observations. Nerve bundles were found to be predominately located in the fibrofatty tissue overlying the epicardium (Figures 1A.i and 1A.ii) in both CHF and normal dogs.
Nerve bundles contained either neuronal cells (cardiac ganglia) and/or nerve fibers that arose from the ganglion cells (nerve trunks). Figure 1A.iii shows an example of cardiac ganglia in the PLA; parasympathetic nerve fibers are seen to arise from one of the ganglia (left sided ganglion). Figure 1A.iv shows an example of a ganglion and a nerve trunk side by side. Sympathetic and parasympathetic were found to be co-localized inside the bundles of both the CHF and normal dogs with the amount of parasympathetic fibers dominating over the sympathetic fibers (Figures 1A.i-1A.v).

**Quantitative analysis.** Figure 1A.v shows an example of a grid over a nerve bundle at 20X magnification. Nerve bundle and nerve fiber density for the PV, PLA, and LAA for the CHF and normal dogs is shown in Figure 1B.i. Nerve bundle density in the PLA was significantly greater than in the PV and LAA. Nerve bundle density was significantly higher in CHF dogs than in normals in the LAA but not in the PV and PLA. Nerve bundle size was significantly larger in the PLA of CHF than of normal dogs (Figure 1B.ii). In addition, the number of parasympathetic fibers/nerve bundle was significantly increased with CHF in the PLA (Figure 1B.iii). The number of sympathetic fibers/nerve bundle did not significantly change with CHF (Figure 1B.iv). Nerve fiber density within nerve bundles was not analyzed in the LAA, due to the very low bundle count in that region. CHF significantly increased the density of cardiac ganglia in the PLA (Figure 1B.v). The number of cell bodies within each ganglion showed a small but non-significant increase (Figure 1B.vi). Sympathetic nerve fiber density was significantly increased in the PV (Figure 1B.vii). There was no significant change in parasympathetic nerve fiber density in either the PV or PLA (Figure 1B.viii).
**β-Adrenergic and Muscarinic Acetylcholine Receptor Densities**

β₁AR density was increased in the PLA with CHF (Figure 2A), but not in the PV and LAA. There was no change in β₂AR density with CHF in any region (Figure 2B). There was no detected change in MR density with CHF in any region (Figure 2C).

**Acetylcholinesterase Activity**

Since immunostaining for parasympathetic nerves showed a significant increase in AChE due to an increase in parasympathetic nerve bundle size and ganglion density, we also assessed for AChE activity in the left atrium. As shown in Figure 2D, CHF increased AChE activity in the PV, PLA, and with a trend in the LAA.

**Effective Refractory Periods**

**Baseline.** At baseline, the ERPs in CHF dogs were significantly longer than in normal dogs in all regions (Figure 3A).

**Autonomic blockade.** ERP increase due to propranolol was significantly greater in CHF dogs than in normals in the PV (Figure 3B). ERP increase with atropine was significantly less in CHF compared to normals in the PLA and LAA (Figure 3C). ERP increase with double blockade (atropine and propranolol) was not different between CHF and normals in any region (Figure 3D).

**Autonomic stimulation.** ERP shortening due to β-adrenergic stimulation with isoproterenol was not significantly different between CHF and normals (Supplemental Material, Figure 3). ERP shortening due to vagal stimulation was significantly less in CHF than in normals in all three regions (Figure 3E). Because immunostaining showed a significant increase in AChE in...
CHF atria (see Immunohistochemistry section above), and previous data have shown that changes in AChE activity in the synaptic cleft can contribute to vagal desensitization in the heart, we re-assessed vagal effect on ERPs after infusion of physostigmine, an AChE inhibitor. The shortening of CHF ERPs due to vagal stimulation was significantly increased after infusion of physostigmine (Figure 3F), with ERP shortening being equivalent to that noted in normal animals (figure 3E). In contrast, physostigmine infusion did not significantly affect vagal-induced ERP shortening in normal animals (see Supplemental Material, Figure 4). Taken together, the AChE expression/activity data presented in the previous section and the electrophysiology data presented in this section indicate that vagal induced ERP shortening is significantly attenuated in the CHF left atrium due to an increase in AChE activity (in CHF). AChE inhibition restores vagal-induced ERP shortening to normal levels.

In order to exclude M2R desensitization as a cause of the decrease in vagal-induced ERP shortening, we also assessed for ERP shortening by direct application of carbachol, a non-selective MR agonist, to the PLA. Carbachol application on the CHF PLA also resulted in ERP shortening that was equivalent to that noted in normal dogs (see Supplemental Material, Figure 5).

**Atrial Fibrillation Duration**

The AF duration results are shown in Figure 4A. The maximum duration of AF episodes induced in these dogs was significantly shorter during parasympathetic blockade with atropine than during baseline. Maximum AF duration with double blockade was also shorter than during baseline. There was no significant effect on AF duration after adding propranolol to atropine than with atropine alone.
Atrial Fibrillation Dominant Frequency

The DF results of the maximum duration AF episodes are shown in Figure 4B. There was no significant difference in mean DF between baseline and with atropine. However, the addition of propranolol to atropine significantly reduced the DF. Figure 4C shows examples of electrograms from the PLA and the corresponding power spectrum and DF for baseline, atropine, and double blockade. The electrograms with double blockade were slower and more organized in this figure.

Activation mapping

Two paced beats obtained at baseline and two beats obtained in the presence of atropine/propranolol were used to analyze the parasympathetic/sympathetic effect on activation maps in the PLA and LAA of CHF and normal dogs. Reproducibility results are presented in the Supplemental Material. Correlation coefficients were significantly less when baseline maps were correlated with atropine maps in both normal (Figure 5A.i) and CHF dogs (Figure 5A.ii) compared to the correlation coefficients from repeated beats. This indicates a significant change in activation with atropine. Propranolol caused a similar change in activation (Figures 5B.i and 5B.ii). The correlation coefficients between baseline and atropine maps were not different in the CHF PLA and LAA than in the normal PLA and LAA (Figure 5A.iii) suggesting that parasympathetic effect on activation is maintained in CHF. However, the correlation coefficients between baseline and propranolol maps were significantly less in both the CHF PLA and LAA than in the normal PLA and LAA (Figure 5B.iii) suggesting an enhanced sympathetic effect on conduction with CHF in
both these regions. Figures 5A.iv and 5B.iv show examples of a PLA activation map of a CHF
dog during baseline and the altered activation map after atropine and propranolol, respectively.

DISCUSSION

Summary of results

The main findings of this study are that in CHF both parasympathetic and sympathetic
remodeling occur in the left atrium, with an increase in both parasympathetic and sympathetic
innervation. Notably, neural remodeling was more pronounced in the PLA and PVs than in the
rest of the left atrium. Evidence of sympathetic remodeling was an increase in sympathetic nerve
fiber density and a concomitant increase in $\beta_1$AR. This was accompanied by an increase in
sympathetic responsiveness in the PLA and PV, as evidenced by an increase in ERP
responsiveness and an enhanced effect on conduction characteristics of these regions.

Parasympathetic remodeling was more complex. There was a pronounced increase in
parasympathetic innervation, with an increase in nerve bundle size, number of parasympathetic
fibers/bundle, cardiac ganglia density, and the number of cell bodies in the ganglia. Vagal
hyperinnervation was accompanied by an increase in AChE activity, and a decrease in vagal
ERP responsiveness in the left atrium. The increased AChE activity likely represents a
compensatory response to vagal hyperinnervation. However, this apparent synaptic
compensation was only partial, as parasympathetic contribution to conduction/activation was
maintained in the left atrium. Most importantly, parasympathetic blockade led to a significant
decrease in AF duration, indicating that parasympathetic activity is an important contributor to
AF substrate in CHF. While, double autonomic blockade did not result in a further decrease in AF duration, it did decrease AF dominant frequency, thus indicating the additional influences of sympathetic activity on AF characteristics.

Figure 6 summarizes the results of this study and proposes a working model of how autonomic remodeling in CHF may contribute to the creation of AF substrate.

**Dynamic vs. Fixed Substrate for AF**

Adequate electrophysiological substrate for AF may be characterized by areas of short refractory periods and slow conduction, and in particular, heterogeneity in repolarization or conduction. In addition to alterations in the ion-channel and gap junction expression that occur in AF, structural abnormalities also contribute to the electrophysiological changes that are necessary for the genesis and maintenance of AF. An important structural abnormality that has been studied extensively for its role in creating electrophysiological abnormalities in the atrium is fibrosis. Fibrosis promotes heterogeneity of conduction and pathways facilitating micro and macroreentry. Our findings suggest that autonomic remodeling may also play a significant role in the creation of AF substrate. It appears that while fibrosis may lead to conduction heterogeneity in the atrium and create a fixed substrate for reentry and consequent AF, parasympathetic and sympathetic remodeling in the PLA and PVs contribute to a more dynamic AF substrate that is dependent on the autonomic state of the left atrium. In fact, even after 3 weeks of rapid ventricular pacing - which typically leads to marked fibrosis in the left atrium - autonomic blockade led to a significant reduction in duration of AF, thereby indicating an important role for the autonomic remodeling in the maintenance of AF. The likely synergism
between fibrosis and parasympathetic effect on the creation of AF substrate is as shown in the schematic model proposed in Figure 6.

**Relative role of sympathetic vs. parasympathetic remodeling in creating AF substrate in heart failure**

Clinical studies suggest that at least in some patients, the sympathetic and/or the parasympathetic nervous system play a role in the genesis of AF. Autonomic fluctuations before the onset of AF have been recognized in several studies. Earlier studies suggested that exercise-induced AF may be sympathetically driven. In contrast, the parasympathetic nervous system may contribute AF in young patients without structural heart disease—i.e., vagal AF. The physiologic studies conducted by Patterson et al suggest that adrenergic influences may be playing an important modulatory role in creating adequate substrate for AF, helping provide a necessary ‘catalyst’ for the emergence of focal drivers in the presence of an increased vagal tone.

The results from our study support the notion that the parasympathetic nervous system is the dominant autonomic limb contributing to AF in CHF, with the sympathetic system playing a more modulatory role. Neural remodeling was characterized by increase nerve bundle size and cardiac ganglia density, both of which primarily consist of parasympathetic nerves. Parasympathetic blockade also significantly shortened the duration of AF. A more modulatory role for the sympathetic system is supported by the observation that double autonomic blockade had no additional effect on AF duration, but instead significantly decreased the dominant frequency of AF compared to parasympathetic blockade alone.

Our findings clearly highlight the importance of the parasympathetic nervous system in establishing AF substrate in the setting of CHF. However, previous studies have suggested that
there is a decrease in parasympathetic tone (which accompanies an increase in sympathetic tone) in CHF, as indicated by a decrease in heart rate variability.\textsuperscript{22} It must be remembered however, that heart rate variability is a measure of autonomic modulation on the sinus node, and does not reliably quantify sympathetic and parasympathetic activity.\textsuperscript{23} Our study therefore sheds important new light on the autonomic changes induced in this CHF model and their role in genesis of AF.

\textbf{Differences in parasympathetic and sympathetic remodeling in the atrium versus the ventricle in the setting of heart failure}

The sympathetic nervous system appears to promote both atrial and ventricular fibrillation. On the other hand, the parasympathetic nervous system is protective against ventricular fibrillation\textsuperscript{24}, but is pro-fibrillatory in the atrium, both in normal hearts\textsuperscript{25} as well as in CHF (as shown in the current study).\textsuperscript{1} This difference is in part due to a differential effect of parasympathetic stimulation on atrial and ventricular repolarization. Parasympathetic stimulation significantly shortens action potential duration in the atria but either lengthens it\textsuperscript{26} or shortens it by a significantly smaller amount in the ventricle\textsuperscript{27,28}. One possible explanation for this difference is the heterogeneity of $\text{IK}_{\text{Ach}}$ (Kir3.1/3.4) expression in ventricular myocytes, with some ventricular myocytes having little or no $\text{IK}_{\text{Ach}}$.\textsuperscript{29} In contrast, as we have recently shown, most atrial myocytes – at least in normal hearts - appear to have $\text{IK}_{\text{Ach}}$ (as demonstrated their sensitivity to acetylcholine and tertiapin Q\textsuperscript{16}.

Our study also shows that unlike in the failing ventricle\textsuperscript{13,14,30}, where there is a downregulation of $\beta$-receptors in the ventricle, there is an upregulation of both sympathetic
nerves and β-receptor binding in the CHF atrium (with an accompanying increase in sympathetic responsiveness). The increase in sympathetic innervation we demonstrate in our model is consistent with that previously noted in human AF. Differences in sympathetic remodeling between the atrium and ventricle may be attributed at least in part to CHF being typically more severe in the studies evaluating ventricular autonomic remodeling.

**Preferential autonomic remodeling in the PLA/PVs: Therapeutic Implications of Current Findings**

Our previous work in normal hearts has shown that the PLA and the PVs have a unique autonomic profile compared to other areas of the atrium. Interestingly, in the current study, the greatest changes in autonomic innervation and related autonomic responsiveness in the setting of CHF were also localized to the PVs and PLA, thus underscoring the importance of this region in the genesis and maintenance of AF, especially in the setting of CHF.

These findings have important implications on the treatment of AF in the setting of CHF. Catheter ablation in and around the PV and PLA has recently emerged as a viable therapy for focal AF. However, in patients with structural heart disease e.g. CHF, significantly more extensive ablation has to be performed, in order to increase ‘cure’ rates for AF. Yet, success rates in the setting of structural heart disease do not appear to exceed 50-60%. Our study indicates that the autonomic nervous system contributes to the creation of AF substrate, with the parasympathetic nervous system being involved in the maintenance of AF, and the sympathetic nervous system affecting the frequency characteristics of AF. Targeted approaches to selectively interrupt autonomic signaling in the PLA may therefore help increase the success rates of current...
ablative or surgical therapies for AF. We have recently shown that inhibition of G\textsubscript{i} – the G-protein responsible for downstream parasympathetic signaling in the atrium – by novel G\textsubscript{αi} C-terminal inhibitory peptides can allow selective parasympathetic inhibition in the left atrium, with a resulting decrease in vagal induced AF in normal canine hearts\textsuperscript{16}. A similar, G-protein targeted approach can potentially be used to selectively inhibit parasympathetic and/or sympathetic signaling in the atrium in the setting of CHF.

Limitations

The time course over which autonomic remodeling occurs was not studied. Thus, it is unknown whether the autonomic remodeling begins shortly after the initiation of pacing or only after structural remodeling has taken place. Furthermore, we did not systematically study the correlation between the echocardiographic/clinical parameters and the extent of autonomic remodeling. The mechanism of burst pacing induced AF may be different from spontaneous clinical AF (spontaneous AF was not observed in this study). As parasympathetic tone is higher in dogs than in humans and the duration of induced CHF is relatively brief, the degree to which these conclusions can be extrapolated to human CHF is as yet undefined. Interpretation of the atrial response to propranolol and atropine may be complicated by the sympathovagal interactions that occur with autonomic blockade. Autonomic remodeling in the right atrium and in the ventricles was not assessed in this study.

Conclusions
A multifaceted approach was used to demonstrate autonomic remodeling of the atria due to CHF. Further study is necessary to determine whether targeted autonomic blockade in the PLA is capable of inhibiting AF in the setting of CHF.

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**Conflict of Interest Disclosures:** None

**References:**


Table 1. Sample sizes for analyses performed in this study.

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Figure Legends:

**Figure 1.** Panel A: Examples of sympathetic and parasympathetic nerve staining. A.i. – example of a nerve bundle located in the fibrofatty tissue overlying the epicardium (EPI) (10X). ENDO – endocardium. A.ii. – example of nerve bundles located in fibrofatty tissue on the epicardial aspect of PV (4X). Sympathetic fibers are in blue (arrows). A.iii. – examples of cardiac ganglia, with parasympathetic fibers arising from cardiac ganglion on the left side (20X). A.iv. - example of cardiac ganglia on the left and nerve bundle on the right; nerve fibers showing co-localized sympathetic (blue) and parasympathetic fibers (brown) (20X). A.v. - illustration of the use of 1 mm x 1 mm grids to quantify the cross-sectional area of a nerve bundle at 20x magnification. Panel B: Quantitative analysis of nerve staining. B.i. – nerve bundle density, B.ii. – nerve bundle size, B.iii. – number of parasympathetic nerve fibers/bundle, B.iv. – number of sympathetic nerve fibers/bundle, B.v. – density of cardiac ganglia, B.vi. – number of cell bodies/cardiac ganglion, B.vii. - density of sympathetic fibers, B.viii. – density of parasympathetic fibers.

**Figure 2.** Comparison of beta-adrenergic receptors (βAR), muscarinic receptors (MR), and acetylcholinesterase (AChE) in the PV, PLA and LAA for CHF and normal dogs.

**Figure 3.** Result of ERP testing obtained from the PVs, PLA, and LAA for CHF and normal dogs. A - ERPs at baseline; B – increase in ERP with propranolol; C – increase in ERP with atropine; D – increase in ERP with double blockade (combined propranolol and atropine); E – decrease in ERP with vagal stimulation effect; F – decrease in ERP with vagal stimulation effect for CHF dogs with and without physostigmine.
Figure 4. Results of AF analysis in CHF dogs. A – Maximum AF durations obtained by burst pacing at baseline, with atropine, and with double blockade, B – Average dominant frequencies (DF) in the PVs, PLA and LAA at baseline, with atropine, and with double blockade. C – examples of PLA electrograms and corresponding power spectrum during baseline, with atropine, and with double blockade.

Figure 5. Effect of autonomic blockade on activation patterns in the PLA and LAA for CHF and normal dogs. The corresponding results for atropine and propranolol are shown in panels A and B, respectively. A.i and B.i show the effect of autonomic blockade on conduction in the normal dog by comparing correlation coefficients of activation times from the repeated beats compared with the correlation coefficients obtained between baseline vs. autonomic blockade beats, A.ii and B.ii show the effect of autonomic blockade on conduction in the CHF dogs. A.iii and B.iii compare the correlation coefficients of activation times of the normal dogs vs. the CHF dogs. A.iv and B.iv show examples of activation maps from the PLA before and after autonomic blockade in a CHF dog.

Figure 6. Proposed model of creation of autonomic substrate for AF in CHF. The model suggests the likely presence of synergistic interactions between structural changes (fibrosis) and autonomic remodeling in the creation of AF substrate in heart failure.

ACh = acetylcholine, AChE = acetylcholinesterase, β1AR – beta-1 adrenergic receptor, ERP = effective refractory period
Baseline ERP

ERP Increase with Propranolol

ERP Increase with Atropine

ERP Increase with Double Blockade

ERP Decrease with Vagal Stimulation

Physostigmine Effect on CHF ERP Decrease with Vagal Stimulation

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Autonomic Remodeling in the Left Atrium and Pulmonary Veins in Heart Failure - Creation of a Dynamic Substrate for Atrial Fibrillation

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SUPPLEMENTAL MATERIAL

DETAILED METHODS

CHF Model Validation. The CHF model was validated in 10 consecutive dogs with respect to its effect on left ventricular and left atrial size. These data, as seen in Supplemental Figure 1, show that there is a progressive increase in left atrial volume (LA Vol), left ventricular end diastolic diameter (LVEDD), and left ventricular end systolic diameter (LVESD) with rapid right ventricular pacing.

Supplemental Figure 1.

Effective Refractory Periods. For each ERP, the pacing protocol consisted of a drive train (S1) of eight beats with a cycle length of 400 ms followed by an extrastimulus (S2). The S2 was decremented by 10 ms until loss of capture. The longest S2 which did not capture was considered the ERP for that particular site. Pacing was performed at an output current twice the threshold required for consistent capture of the tissue. The mean ERP was used as the representative ERP for each of the three sites as well as the entire left atrium.

Atrial Fibrillation Sustainability and Dominant Frequency. Current was set at four times the threshold for capture. The maximum durations of the AF episodes induced by the burst pacing were calculated for each intervention. Electrograms were pre-processed with 40 to 250 Hz band pass filtering, rectification, and 20 Hz low pass filtering. Power spectra of the processed electrograms were obtained using the Fast Fourier Transform. The DF, defined as the frequency with the highest power in the power spectrum, was used as the estimation of the local AF activation rate. A composite DF from each region was calculated by averaging the values spatially across all channels of the plaque and temporally across the four four-second segments.

Activation Mapping. Activation times for each electrode recording for a specific paced beat were selected at the peak voltage of the bipolar electrograms after band pass filtering (40 to 250 Hz), rectification, and low pass filtering (20 Hz). The filtering and rectification steps were used to account for the different morphologies of bipolar electrogram recordings. Two-dimensional activation maps for the PLA and LAA were then constructed from the activation times.

The left panel of the Supplemental Figure 2 shows an example of raw electrograms of one sinus beat obtained from the PLA. The middle panel shows the electrograms after band pass filtering, rectification, and low pass filtering. The dots indicate the detected peaks of the processed electrograms. The right panel shows the activation map constructed with the activations times determined from the second panel. The isochrones, indicated by the white lines, show the progression of the wavefront every 3 milliseconds.
Supplemental Figure 2.

Good reproducibility of the baseline and atropine maps was evident with high correlation coefficients of activation times for both the normal (PLA: 0.981±0.005, LAA: 0.985±0.004) and CHF (PLA: 0.977±0.003, LAA: 0.970±0.007) dogs. There was also good reproducibility of the baseline and propranolol maps evident by the high correlation coefficients for both normal (PLA: 0.968±0.007, LAA: 0.992±0.001) and CHF (PLA: 0.963±0.009, LAA: 0.966±0.009) dogs.

Tissue Sample Preparation. For both normal and CHF dogs, immediately following the termination of the in vivo electrophysiology assays, the heart was promptly excised out of the chest and immersed in ice-cold cardioplegia solution containing (mmol/L) NaCl 128, KCl 15, HEPES 10, MgSO4 1.2, NaH2PO4 0.6, CaCl2 1.0, glucose 10, and heparin (0.0001 U/mL); pH 7.4. The heart was quickly cannulated via the aorta and perfused with ice cold cardioplegia solution containing protease inhibitors (Sigma cocktail cat#P8340) until vessels were clear of blood and tissue was cold. The atria were separated from the associated ventricles, and tissue samples were taken from the PLA, PV, and LAA regions of the left atrium and snap frozen in liquid nitrogen, and subsequently stored at -80°C.

Immunohistochemistry. Immunohistochemistry was performed to examine the anatomy of the parasympathetic and sympathetic nerves in the atria. For the PVs, cross sections were cut serially from proximal to distal. The sections from the PLA and LAA were cut parallel to the plane of the mitral annulus as to include both the epicardial and endocardial aspect of the myocardium in each slice. Sections were air-dried and fixed in acetone for ten minutes before being washed in Tris-buffered saline (TBS). Hydrogen peroxidase block (Dako, Carpenteria, CA) was placed on the sections for ten minutes, and the slides were washed in TBS. Protein block was placed on the sections for thirty minutes. Primary antibodies were then incubated overnight at 4°C. Antibodies for dopamine β-hydroxylase (DBH; Chemicon, Temecula, CA) were used to stain sympathetic nerves, whereas antibodies for acetylcholinesterase (AChE; Millipore;) were used to stain parasympathetic nerves. The specificity of
DBH for sympathetic nerve fibers was confirmed by the use of a sympathetic nerve marker, i.e., tyrosine hydroxylase (TH; Chemicon), which stained the same nerve elements that were stained by DBH. Similarly, the specificity of parasympathetic staining was confirmed using a second parasympathetic nerve marker, i.e., cholineacetyl transferase (ChAT; Chemicon), which stained the same nerve elements that were stained by AChE. Some sections were double-stained for AChE and DBH. After incubation, the slides were washed in TBS, and the appropriate secondary antibody (Chemicon) was placed on the sections for 30 min. The sections were again washed in TBS, and the appropriate chromagen was added to each specimen. Sympathetic nerves were stained blue with 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and parasympathetic nerves were stained brown with 3,3’-diaminobenzidine (DAB). Cell nuclei were marked by placement of the specimens in methyl green (Dako) for 10 min. The specimens were then dehydrated in alcohol, mounted, and examined under light microscopy. Cardiac ganglia were defined as nerve bundles containing one or more neuronal cell bodies. The presence of neuronal cell bodies within nerve bundles was confirmed by staining these bundles for hematoxylin; all neuronal cell bodies stained positive for hematoxylin, thereby excluding the presence of fat cells within these bundles. Specimens taken from the cervical vagus nerve served as a positive control for parasympathetic nerve fibers but a negative control for sympathetic nerves. Specimens from the stellate ganglia served as a positive control for sympathetic fibers but a negative control for parasympathetic nerve fibers. Quantification was performed manually with a light microscope. Mean densities (number per mm²) of nerve bundles and cardiac ganglia cells were quantified using 1x1 mm grids at 10 times magnification. We also counted the number of parasympathetic and sympathetic fibers within individual nerve bundles. Sizes of nerve bundles were quantified using rectangular 1x1 mm grids at 20 times magnification.

**Densities of muscarinic and beta-adrenergic receptors.** The βAR binding assays were performed in duplicate at 37°C for one hour in 250 µL of binding buffer containing 50 to 100 µg of membrane proteins and increasing concentrations of 125I-iodocyanopindolol (0.01 to 1 nmol/L) in the absence or presence of either Betaxolol (β₁-antagonist; 1 µmol/L) or ICI-118,551 (β₂-antagonist; 1 µmol/L). Following incubation, 4 ml ice-cold buffer was added to the reaction mixture and rapidly filtered under vacuum on GF/C filters. The filters were washed with 4 x 5 ml of ice-cold buffer, air dried and radioactivity was counted in gamma counter. The specific counts were obtained by subtracting non specific counts from total counts. The receptor density (Bmax) was determined from Scatchard plots. For MRs, the experiments were same as described above except 3H-quinuclidynl benzilate (1 to 20 nmol/L) was used as radioligand and atropine sulfate (1 µmol/L) as the competitive cold ligand. The air dried GF/C filters were counted in scintillation counter with 5 ml scintillation fluid.

**AChE activity.** The Ellman’s method acetylcholinesterase hydrolysis products are detected at 412 nm absorbance, which was read every 2 min for 30 min via UV-VIS spectrophotometer (BioRad microplate reader). Absorbance was plotted against time and enzyme activity was calculated from the slope of the line so obtained and expressed as a percentage compared to an assay using a buffer without an inhibitor.

**Isoproterenol effect on ERPs.** The ERP shortening due to sympathetic stimulation with isoproterenol compared to propranolol was not significantly different between CHF and normals (Supplemental Figure 3).
Vagal Stimulation effect on ERPs with and without physostigmine in normal dogs. The decrease in ERP from vagal stimulation with physostigmine was not different from the decrease in ERP from vagal stimulation at baseline for normal dogs (Supplemental Figure 4). This is in contrast with CHF dogs where the ERP decrease from vagal stimulation was significantly more with physostigmine than at baseline (see main manuscript). This finding is consistent with the increase in AChE with CHF.

Carbachol effect on ERPs. The shortening of ERPs due to application of 1 mmol/L Carbachol (CCh) to the PLA was not significantly different between CHF and normal (Supplemental Figure 5). Thus, it can be concluded that M2R desensitization was not responsible for the decrease in vagal-induced ERP shortening.
REFERENCES


