Vagal stimulation targets select populations of intrinsic cardiac neurons to control neurally-induced atrial fibrillation

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Abbreviations: AF, atrial fibrillation; AOR, descending aortic occlusion; ECG, electrocardiogram; IVC, inferior vena cava; IC, intrinsic cardiac; ICNS, intrinsic cardiac nervous system; LCN, local circuit neuron; LCV, left cervical vagosympathetic complex; LSS, left stellate ganglion stimulation; LV, left ventricle; LVP, left ventricular pressure; MNS, mediastinal nerve stimulation; RAE, right atrial electrogram; RAGP, right atrial ganglionated plexus; RCV, right cervical vagosympathetic complex; RSS, right stellate ganglion stimulation; RV, right ventricle; VNS, Vagus nerve stimulation.
Abstract:

Background: Mediastinal nerve stimulation (MNS) reproducibly evokes atrial fibrillation (AF) by excessive and heterogeneous activation of intrinsic cardiac (IC) neurons. This study evaluated whether pre-emptive vagus nerve stimulation (VNS) impacts MNS-induced evoked changes in IC neural network activity to thereby alter susceptibility to AF. Methods: IC neuronal activity in the right atrial ganglionated plexus was directly recorded in anesthetized canines (n=8) using a linear microelectrode array concomitant with right atrial electrical activity in response to: 1) epicardial touch or great vessel occlusion vs (2) stellate or vagal stimulation. From these stressors, post-hoc analysis (based on the Skellam distribution) defined IC neurons so recorded as afferent, efferent or convergent (afferent and efferent inputs) local circuit neurons (LCN). The capacity of right-sided MNS to modify IC activity in the induction of AF was determined prior to and after pre-emptive right (RCV) vs left-sided (LCV) VNS (15 Hz, 500µsec; 1.2x bradycardia threshold). Results: Neuronal (n=89) activity at baseline (0.11±0.29Hz) increased during MNS-induced AF (0.51±1.30Hz; p<0.001). Convergent LCN’s were preferentially activated by MNS. Pre-emptive RCV reduced MNS-induced changes in LCN activity (by 70%), while mitigating MNS-induced AF (by 75%). Pre-emptive LCV reduced LCN activity by 60%, while mitigating AF potential by 40%. IC neuronal synchrony increased during neurally-induced AF, a local neural network response mitigated by pre-emptive VNS. These anti-arrhythmic effects persisted post-VNS for, on average, 26 min. Conclusions: VNS preferentially targets convergent LCNs and their interactive coherence to mitigate the potential for neurally-induced AF. The anti-arrhythmic properties imposed by VNS exhibit memory.
New and noteworthy: Focal and excessive neural inputs to the intrinsic cardiac (IC) nervous system increases activity and coherence among IC neurons in association an increased potential for atrial fibrillation; pre-emptive VNS prevents such neurocardiac effects. The anti-arrhythmic effects imparted by VNS have memory.
Introduction

Atrial fibrillation (AF) affects more than three million people a year in the United States, a prevalence that is projected to reach 5.6 - 12.1 million by 2050 (26, 47). Despite such prevalence, the underlying mechanisms of AF are not fully understood. Current treatments consist of pharmacological therapies that have been combined with localized atrial catheter-based or surgical ablation (18, 61). Ablation procedures are associated with complications such as the left atrial stiffness syndrome (24), micro-embolic episodes (58), and a risk of symptomatic or silent cerebral ischemia (21). Such drawbacks have increased the research focus on defining specific neural and cardiac substrate interactions underlying AF and with such information evolving novel non-pharmacologic therapeutic options for its management (79). Bioelectric neuromodulation therapies for AF represent a novel approach to such management. Among these, vagus nerve stimulation (VNS) (43, 60, 62) and spinal cord stimulation (23, 66, 71) target various aspects of the cardiac neuronal hierarchy to reduce the arrhythmia potential.

The cardiac nervous system includes reflex networks located in the insular cortex, brain stem, spinal cord, intrathoracic sympathetic ganglia and the intrinsic cardiac nervous system (ICNS) (3, 7, 81). It has been proposed that its ICNS component acts as the final coordinator of regional cardiac indices, doing so under the influence of intrathoracic, spinal cord and brainstem reflexes (7). Neural activity within the ICNS is influenced by afferent (mechanosensitive, chemosensitive and ischemia sensitive) and efferent neuronal inputs (7, 9, 80). These afferent and efferent inputs are processed by local circuit neurons (LCNs) in peripheral ganglia to modulate
sympathetic and parasympathetic efferent postganglionic projections to all regions of the heart (3, 20, 33, 45). Neuronal imbalances within the ICNS can exert deleterious effects on cardiac function including, arrhythmia induction (8, 11, 57, 59). To date, which populations of neurons within the ICNS are so involved remains unresolved.

The model of atrial fibrillation utilized in this study is intermittent focal mediastinal nerve stimulation (MNS) (11). MNS elicits ICNS network hyper-excitability (23) that in turn deranges efferent neuronal outflows to atrial tissues thereby causing heterogeneities in atrial electrical indices (11, 56). Such heterogeneities in the atrial electrical substrate rapidly degenerate into self-limiting episodes of AF (11, 56). The MSN-induced AF episodes occur with a latency of ~1s from stimulation onset, have a duration of ~30 sec and are reproducible over hours of experimentation (11, 23, 56). This model provides a reproducible experimental platform whereby anti-arrhythmic therapies can be evaluated and optimized (11, 23, 40, 56). Prior work has demonstrated that MNS-induced AF can be eliminated by atropine (11), modified by timolol (11), and blunted by alpha adrenoceptor blockade (56). Hexamethonium likewise reduces the number of AF responses to MNS stimulation from 90% baseline to 10% post treatment (56). These data substantiate fundamental aspects of ICNS neural activity in relationship regulation of the AF potential.

Vagal nerve stimulation modulates cardiac electrical indices (42, 73) and, as such, has the potential to either increase or decrease the propensity to arrhythmias (18). Higher intensity stimulations tend to increase atrial fibrillation inducibility (76, 78); lower intensity vagal stimulation can stabilize atrial electrical function (17, 67). In order to understand the efficacy of VNS therapy with respect to atrial arrhythmia suppression,
we first defined the response characteristics of functionally delineated subpopulations of intrinsic cardiac (IC) neurons to MNS-evoked AF and then the capacity of cervical VNS (right vs left) to modify neural network and cardiac electrical responses to such destabilizing inputs. To this purpose, we directly recorded the activity of multiple neurons in the canine right atrial ganglionated plexuses (RAGP), an aggregate of IC neurons directly involved in control of chronotropic function (6, 46, 52). Data presented herein demonstrate mechanistically the pivotal role of LCN’s in mediating AF secondary to neural imbalances and that these same neurons are the preferential target for bioelectric therapies to reduce the arrhythmogenic potential. We further demonstrate that these bioelectric interventions exhibit memory to extend the atrial anti-arrhythmic effects well beyond the primary activity phase of VNS therapy.

Methods:

Animal Preparation: All experiments were performed in accordance with the guidelines for animal experimentation described in the Guide for the Care and Use of Laboratory Animals, Eighth Edition, National Academy Press, Washington DC, 2010. The Institutional Animal Care and Use Committee of the East Tennessee State University approved these experiments. Eleven mongrel dogs of either sex, weighing 18.6-26.9 kg, entered this study. Animals were sedated with propofol (3-8 mg/kg, intravenous(i.v.)), followed by endotracheal intubation and mechanical ventilation. General anesthesia was maintained with isoflurane (1-2%, inhalation). Following completion of surgery, anesthesia was changed to α-chloralose (50 mg/kg i.v. bolus), with continuous infusion (8-12 mg/kg/hr i.v.) adjusted to effect throughout the duration of each study. The depth of anesthesia was
assessed throughout the experiments by monitoring corneal reflexes, jaw tone, and 
hemodynamic indices. Body temperature was maintained via a circulating water heating 
pad (Gaymar T/Pump, Gaymar Industries Inc., Orchard Park, NY). At the completion of the 
experiments, animals were humanely euthanized under deep anesthesia and by inducing 
ventricular fibrillation via application of direct current stimulation and removing the heart. 

**Hemodynamic recording:** The left femoral artery was catheterized to record 
arterial blood pressure (Ao BP). The left femoral vein was catheterized to allow for fluid 
replacement, as well as anesthetic and pharmacological agent delivery. The right femoral 
artery was catheterized in order to monitor left ventricular chamber pressure (LVP) via 
placement into the LV chamber of a Mikro-Tip Pressure Transducer Catheter (Millar 
Instruments, Houston, TX). Heart rate was monitored via a Lead II electrocardiogram 
(ECG). Pressures (Ao BP, LVP) and ECG were input to a Cambridge Electronics Design 
(model 1401) data acquisition system for continuous monitoring of hemodynamic status. 

**Vagal stimulation (VNS):** Following a midline incision in the ventral neck, the 
right and left cervical vagi were exposed and bipolar stimulation electrodes (PerrenialFlex, 
Model 304, Cyberonics, Inc.) placed around each nerve. Cervical vagosympathetic trunks 
remained intact throughout each aspect of the protocol. Each lead was connected 
individually to a Grass S88 stimulator via separate PSIU6 constant current isolation units. 
Bradycardia thresholds for each nerve stimulated were identified using 20 Hz, 500μs pulse 
width stimuli, as determined by progressive increases in current intensity until 10% 
bradycardia was evoked. With respect to right-sided VNS, this current was found to be, on 
average, 1.75 mA; for left-sided VNS it was 2.25 mA. VNS was applied to each vagus for 3
min periods (15 Hz; 500µs pulse width) at a current intensity that was 1.2x bradycardia
threshold.

**Mediastinal nerve stimulation (MNS):** Following thoracotomy, an incision was
made in the pericardial sac and a pericardial cradle formed. A bipolar electrode was affixed
to the right atrium 1 cm dorsal to the SA node to record an atrial electrogram. Right-sided
mediastinal nerves were identified visually coursing over the ventral and ventro-lateral
surface of the intrapericardial aspects of the superior vena cava. These mediastinal nerves
represent aggregates of sympathetic and sympathetic efferent axons, as well as inter-
ganglionic projections arising from local circuit neurons contained within the ICNS (11, 23,
27, 70).

Each nerve was stimulated individually using detailed, published techniques (11, 23).
Briefly, trains of five electrical stimuli (0.3-1.2 mA, 1 ms duration, 5 ms pulse interval) were
delivered during individual atrial refractory periods to identified mediastinal sites for up to 20
seconds. Electrical stimuli were delivered to a mediastinal nerve via a roving bipolar probe
electrode. Active nerve sites were identified by the immediate induction of atrial
tachyarrhythmias (including atrial fibrillation) when first exposed to focal electrical stimuli.
Each active mediastinal nerve site so identified was marked with India ink for repeated
stimulation. By these means, 2-4 active nerve sites were identified in each animal. Contact
between the bipolar electrodes and tissue was discontinued immediately after the onset of
the atrial tachyarrhythmia in order to limit their durations (11, 56).

**Neuronal Recording:** Extracellular activity generated intrinsic cardiac neurons *in situ*
was recorded using a multichannel linear microelectrode array (MicroProbes Inc.,
Gaithersburg, MD) that consisted of 16 platinum/iridium electrodes (25 µm diameter
electrode with an exposed tip of 2 mm; impedance 0.3-0.5 MΩ at 1 kHz). The linear microelectrode array was embedded in the right atrial fat that contained the right atrial ganglionated plexus (RAGP), as described previously (13). The connecting wires of the multichannel electrode, along with ground and reference wires, were attached to a 16-channel microelectrode amplifier with a headstage preamplifier (A-M systems, Inc., model 3600; Carlsborg, WA). For each channel, filters were set to 300-3K Hz and gain to 5K. Another electrode was sewn to the atrial myocardium close to the RAGP to provide a reference right atrial electrogram (RAE) which was utilized to determine atrial rate, duration and characterization of atrial arrhythmias, along with a timing index for subsequent identification of atrial electrical artifacts in IC neural recording data. The 16 microelectrode array signals, along with recorded cardiovascular indices (ECG, right atrium electrogram and hemodynamic data), were digitized via a Cambridge Electronics Design (model 1401) data acquisition system for off-line analysis. The sampling frequency for neuronal data was 5.26 kHz; it was six time lower (0.877 kHz) for all other recorded signals.

**Identification of neuronal activity:** The extracellular activity generated by individual neuronal somata located within the RAGP was recorded. Identification of the activity generated by individual neurons via the 16 channel electrodes was performed off line using Spike2 software program (Cambridge Electronic Design) in two steps: (1) artifact identification and blanking; and (2) spike detection, waveform classification and validation with principal component analysis as defined previously (13). Using these procedures, consistent waveforms derived from individual somata (not axons of passage) can be identified in situ for up to 8-10 hour periods (13, 50, 69). Of the 11 animals, recordings with
sufficient signal to noise were obtained in 8 animals. As such, the remaining three were excluded from all subsequent analysis.

**Statistical analysis of evoked changes in IC neuronal activity:** Using statistical approaches based on a Skellam distribution (63), the significance of changes in firing rates recorded before and during each intervention was computed post-hoc for all identified IC neurons. We classified the behavior of identified neurons according to their activity characteristics in response to the following interventions: (1) touching the ventral LV and then RV (conus vs sinus); (2) 20 sec descending aorta occlusion; (3) 20 sec inferior vena cava (IVC) occlusion; (4) stimulation (1 Hz for 1 min) of right vs left cervical vago-sympathetic trunk (RCV; LCV); and (5) stimulation (1 Hz for 1 min) of right vs left stellate ganglia (RSS; LSS). By these means, each neuron was classified according to how it responded to each of those interventions by its change in firing rate, each serving at its own control. When a neuron responded solely to one or more of the afferent stressors (interventions 1-3 above), it was classified as an afferent LCN. Efferent LCNs were identified as those responding indirectly (variable latency) to one or more of the efferent (vagal vs sympathetic motor; interventions 4 and 5 above) inputs. IC neurons that respond with a fixed latency to efferent inputs were classified at efferent IC neurons (7). IC neurons that responded indirectly to both afferent and efferent stressor were classified as convergent LCN (13). Identified neurons that did not respond to any of these stressors were classified as exhibiting unknown function.

The primary objective of this study was to assess the efficacy of pre-emptive VNS to alter the IC neural network response to MNS and thereby impact the atrial arrhythmogenic potential. Repeated measure ANOVA was used to assess the effect of different factors on
neuronal activity. The 3 way ANOVA test was performed on RCV and LCV separately. It involved two within-subject factors (effect of MNS vs baseline, and pre- vs post-VNS response) and one between-subjects factor (neuron type). Huynh-Fedlt correction was applied to correct the violation of sphericity assumption. When significance was achieved overall for ANOVA (p<0.05), post hoc test and all other paired sample comparisons were done by paired t-test.

In each animal, a synchrony index (SI) was also calculated (44) in order to evaluate synchrony of activity generated among different populations of IC neurons. This index was estimated during: i) baseline states as compared to ii) during episodes of neurally-induced atrial arrhythmias. The potential of VNS to alter IC synchrony was likewise assessed. There was a limitation of analysis imposed by the limited number of action potentials generated per neuron especially when suppressed during VNS therapy; as such, synchrony analysis was not performed in those instances. The synchrony of activities displayed by different populations of identified neurons, as defined by Agmon (1), was performed by assessing the activity generated by pairs of identified neurons in each animal. In order to calculate such a synchrony index (SI), one neuron was defined as the reference and the other as the target neuron.

Different SI values were obtained that depended on which neuron was considered reference, thus making the SI a non-symmetric measure. As such, calculation of this SI index required the identification of coincidences of activities among differing neurons when reference and target neurons both generate activity within a time window of selected duration $\tau$. We had previously defined the optimal value for $\tau$ with respect to intrinsic cardiac neuronal activities to be 40 ms (44). Given that some coincidences may be random in
nature, the coincidence count was also estimated in surrogate data obtained by applying a random jitter to the reference spikes in each time window of duration $4\tau$ (1). In order to obtain normalized SI values the mean coincidence count in surrogate data was subtracted from the actual coincidence count identified. Thereafter, the resultant was divided by the number of reference spikes. Surrogate data also served to calculate a $p$-value so that we could assess statistical significance of these data. When the number of neuron pairs demonstrating significant synchrony was so identified ($p < 0.01$ and SI > 0.01), a chi-square test was performed to assign statistical significance to changes occurring in the number of synchronized pairs for each neuronal subtype combination studied (65).

**AF characteristics:** Atrial electrograms were recorded from the ventral right atrial free wall and referenced to a Wilson Central terminal. From these atrial electrograms, the following response characteristics were determined during the atrial tachyarrhythmia: i) latency (defined as the interval from the first applied stimulus to tachyarrhythmia initiation); ii) duration of the AF (defined as time from onset to self-termination of AF); and iii) dominant frequency of atrial activity during induced AF episode. When AF was not initiated by MNS, AF duration was by definition set to zero. The duration of AF episodes recorded before and after VNS were compared by reference to the duration of each, as obtained from one or more AF episodes induced before and after full recovery from the VNS protocols. The effects of VNS therapy was separated into 4 categories, using MNS as the constant defined stressor: i) AF prevention (AF initiation failed); ii) AF mitigation (AF duration reduced by 20% or more); iii) AF prolongation (AF duration increased by at least 20%) and iv) having no effect. Results were considered to be not significant (no effect) when occurring within the 20% range.
Time dependence of VNS effect: Kaplan-Meier survival analysis was performed to estimate how long the effect of VNS lasted as represented by the varied number (up to 7) of successive AF initiation attempts (at 5 or 10 min intervals after the first, if needed). When a mediastinal nerve stimulus evoked an AF episode as long as the reference (control state) episode, sequential MNS trials were terminated. Accordingly, VNS efficacy at time \( t \) was defined as the percentage of experiments for which the latest unsuccessful AF attempt (if any) occurred after time \( t \). A second survival curve was also created based on the percentage of experiments in which the latest mitigated AF episode (if any) occurred after time \( t \) in order to determine how long VNS effectiveness lasted.

Results

Functional response characteristics of identified right atrial neurons. A total of 89 neurons were identified in the 8 animals studied (11.1±3.5 neurons per dog). The response characteristics of individual neurons differed with respect to the stressor tested which could be reflected as either an increase or decrease in activity (Figure 1). Of the 89 identified right atrial neurons (those that generated spontaneous activity), 65 neurons were functionally classified as being i) afferent (\( n = 15; \ 17\% \)), ii) efferent (\( n= 20; \ 22\% \)) or iii) convergent local circuit neurons (\( n = 30; \ 34\% \)). The rest (\( n=24; \ 27\% \)) did not respond to any of these imposed stressors; as such, their function was labeled as being unknown.

Effects of right-sided mediastinal nerve stimulation on cardioneural activity. Figure 2 illustrates a representative atrial arrhythmic response elicited by brief periods of MNS stimulation prior to (panel A, control) and following pre-emptive VNS (panel B, post-VNS). In the control state, MNS on average induced transient periods of AF with a latency
to onset of 2.68±2.32 sec, a duration of 11.1±1.2 sec and a dominant frequency of 7.1±1 Hz during AF. Note that bradycardia usually preceded the onset of AT/AF (Figure 2A) and that this onset transient bradycardia was maintained following VNS (Figure 2B). In this same animal, VNS pre-treatment prevented the tachy-arrhythmias induced by MNS (Fig 2B) even when applied for up to 20 seconds. The hemodynamic response to VNS is summarized in Table 1. The evoked changes in chronotropic and left ventricular inotropic function, with suppression during the active VNS phase followed by a rebound phase (1 minute duration) following stimulation, is consistent with the 1.2x threshold intensity utilized herein. By onset of MNS stressors post-VNS, hemodynamics had returned to baseline values (data not shown).

MNS stimulation triggered changes in IC activity leading to atrial arrhythmias, with residual effects continuing even post conversion to sinus rhythm. In the representative animal depicted in figure 3, in the control state bursting of activity was elicited among 9 identified right atrial neurons by mediastinal nerve stimulation (panel 3A MNS: sham RCV). Neural activity enhancement occurred immediately before the induction of the transient atrial arrhythmia (c.f., AF: atrial fibrillation). Activity persisted in 5 of these 9 neurons for a brief period of time even after spontaneous conversion to sinus rhythm. Average neuronal activity recorded among all classified IC neurons across all animals was 0.11±0.29 Hz in control states, increasing to 0.51±1.30 Hz (p < 0.001) during the MNS induced atrial tachyarrhythmia. From sub-set analysis, IC activity increased preferentially among convergent LCNs (0.13±0.3 to 0.88±1.73 Hz, p<0.001) in response to MNS, afferent LCNs responding to a lesser degree (0.07±0.3 to 0.14±0.43 Hz, p<0.032). No changes were identified in identified efferent LCN populations (0.11±0.3 to 0.21±0.74 Hz, p<0.24).
Effects of ipsilateral vagus nerve stimulation on right-sided atrial neuronal activity and the potential for neurally induced atrial arrhythmias.

Pre-emptive right-sided VNS mitigated IC neural responses to MNS (Figs 3B and 4A). It blunted or prevented the potential for neurally-induced AF by 75% (Fig 4B) with no significant changes in onset latency or dominant frequency in residual arrhythmias. Prior to VNS, MNS increased the activity among both afferent and convergent LCN sub-populations (Fig 4A, solid black lines). Following pre-emptive right-sided VNS, basal activity was differentially decreased among efferent LCNs (0.16±0.4 vs 0.06±0.19 Hz; p<0.01). Post-VNS, MNS-induced excitation of convergent LCNs was blunted (0.91±1.73 vs 0.26±0.73 Hz; p<0.002), being totally eliminated among afferent LCN populations (Fig 4A).

Effects of contralateral vagus nerve stimulation on right-sided atrial neuronal activity and the potential for neurally induced atrial arrhythmias.

In contrast to ipsilateral VNS, left-sided vagus stimulation exerted no significant change in basal IC neuronal activity (Fig 5A). However, as with right-sided VNS, LCV differentially mitigated the MNS-induced increase in convergent LCN activity (0.84±1.74 vs 0.34±0.49 Hz; p=0.057). In contradistinction to ipsilateral mediated effects, though blunted, the neural activity in convergent neurons still increased significantly above baseline during MNS following the LCV VNS. The potential for MNS-induced AF was prevented or blunted in 40% by LCV VNS and without effect in 27% of cases. Pre-emptive left-sided VNS enhanced AF induced from 33% of right-sided MNS sites evaluated (Figure 5B).

IC network characteristics: neuronal synchrony. The MNS-induced increases in IC activity are reflective of common shared inputs and/or IC network interconnections mediated by LCNs (7). Figure 6 evaluates this short-term interactive potential by
determining synchrony among the specific pairs of IC neurons identified within the RAGP during baseline conditions, as well as during MNS induced changes i) prior to (top panel) and following pre-emptive right-sided VNS (bottom panel). In the sham (unstimulated) treatment state, note that while there was minimal coherence of activity among the various sub-populations of IC neurons identified, in response to MNS there was a preferential increase in IC synchrony among convergent LCNs, as well as between convergent and efferent LCN sub-populations. Following right-sided VNS, while there was a differential increase in synchrony during baseline states among convergent LCNs (Fig 6, bottom panel); any MNS-induced change in IC synchrony was extinguished.

**IC network characteristics: memory.** The efficacy of VNS therapy in terms of shortening/preventing MNS-induced arrhythmias (post-VNS) was assessed via Kaplan-Meier survival analysis (Fig. 7). Following right-sided VNS, anti-arrhythmic effects against repeated MNS-induced arrhythmias was attenuated for 20 min after VNS therapy (top panel); it was extinguished by ~40 min post VNS (Fig. 7A; fitting exponential function resulted in a time constant of 26±2 min [95% confidence interval]). While the overall anti-arrhythmic efficacy of contralateral VNS was reduced (Fig 7, bottom panel), the time constants derived from RCV vs LCV responses were not significantly different (logrank test). For corresponding MNS-induced changes in IC activity, the pre-VNS induced change in convergent activity (0.11 to 1.57 Hz, p=0.023) was suppressed immediately after VNS (0.04 to 0.38 Hz, p=0.17), and recovered ~30 min post VNS (0.07 to 1.28 Hz, p=0.016). Following recovery, characteristics of MNS-induced AF (latency, duration and dominant frequency) were similar to sham VNS control (data not shown).


Discussion

The major findings of this study are: 1) enhanced activity on convergent LCN’s underlies neurally-induced AF; 2) VNS therapy attenuates AF via its effects on select intrinsic cardiac neuronal populations, namely convergent LCN’s; 3) Disruptive neural inputs to the ICNS increases coherence of activity among IC neurons and pre-emptive VNS prevents such effects; 4) ipsilateral VNS imparts a greater impact on IC neural function and the ability to stabilize the ICNS against neural imbalance; and 5) the anti-arrhythmic effects imparted by VNS has memory.

ICN modulation of cardiac function

The ICNS is composed of heterogeneous populations of neurons loosely organized in multiple ganglionated plexi located within atrial and ventricular tissues (7, 13, 75). These IC neurons can be functionally stratified by their in situ behavior based on their responses to different stressors according to whether they belong to either afferent, efferent or convergent LCN sub-types (7, 13). Structure is intricately intertwined to function (13, 16, 50). The convergent LCNs are responsible for primary reflex integration within the ICNS (7), coordinating atrial and ventricular tissues via its efferent outputs. With respect to central autonomic efferent preganglionic axons, they project directly onto intrinsic cardiac efferent post-ganglionic (intrinsic cardiac parasympathetic and sympathetic) neurons as well as convergent LCNs (13, 46, 52). These IC network interactions are critical to mediating sympathetic/parasympathetic cardiomotor outflow to control regional cardiac function (46, 51).
ICN processing and atrial arrhythmias

Asymmetric neural inputs to the IC network increase the potential for AT/AF (11, 18). Stochastic processing within that network underlies the instability that can occur within the ICNS to initiate arrhythmias (37, 38). The resultant “hyper-stochasticity” displayed among its convergent LCNs in response to MNS appears to be fundamental to any enhancement of an arrhythmia potential (23). Our study shows, that any such enhancement of activity among IC LCNs is associated with increases in their coherence to effect local efferent neuronal outflows (27, 46). Such coherence or lack thereof, is ultimately dependent on intra-ganglionic interconnections (34, 69). Our data indicates that IC network interactions can be targeted therapeutically to modify atrial arrhythmia induction.

VNS therapy not only impacts excitability among select populations of intrinsic cardiac neurons, but also the coherence of function displayed among its varied neuronal populations (36). Prior to VNS, MNS increased functional connectivity within convergent to convergent neuron pairs and between convergent and efferent IC neuron populations. These data suggests that excessive inputs can cascade through the local neural networks with the potential to overwhelm local feedback mechanisms leading to excessive efferent outputs to disparate regions of the heart. This neural signature can be tempered by VNS, primarily via its suppression of convergent IC neural activity. By dampening intrinsic cardiac neural circuits the potential for atrial arrhythmias is reduced.

Unilateral VNS can exert bilateral influences on IC neural function (13, 50) and on control of regional cardiac function (5, 42, 72). Previous studies have demonstrated that aggregates of the intrinsic ganglionic plexus neurons exert preferential spheres of influence on cardiac indices, manifested by their direct and indirect projections to cardiomyocytes (6,
With respect to the RAGP, although it exerts preferential control of sinoatrial nodal pacemaker activity, some of its neurons also influence distant atrial and ventricular electrical and contractile indices (6, 74). Medullary derived parasympathetic efferent preganglionic neurons likewise have spheres of influence (22, 28), reflecting their projections onto specific populations of intrinsic cardiac neurons as well as their interactions mediated by inter-ganglionic projections (7, 28, 45, 54). Our data shows that ipsilateral VNS exerts substantially greater anti-arrhythmic effects when targeting right atrial neuronal networks than contralateral preganglionic projections to such ganglia (Fig. 7). Presumably, this reflects insufficient preganglionic efferent innervation of respective (contralateral vs ipsilateral) aggregates of IC neurons (51, 53, 54). This anatomical-functional heterogeneity likely underlies any increased AF potential that right-sided ICNS neural imbalance elicits in the presence of left-sided VNS therapy.

VNS and memory

Regardless of VNS site of delivery, its anti-arrhythmic effects exhibit memory. For this study, three minutes of VNS conferred protection for up to 26 minutes. First and foremost, memory is neural and not myocyte dependent (4, 10, 12). It likely involves in the short-term local release of neuromodulators and plasticity within local neural network processing (30, 32, 37, 39, 48) and in the longer-term changes in synaptic efficacy (14, 29). While the precise structure/function mechanisms underlying short- to longer-term effects of VNS on neural function and the nerve/myocyte interface remain poorly defined, future studies should consider potential contributions by muscarinic (11, 56, 64), angiotensin (30, 41), and adrenergic (31, 56) receptor mechanisms.
Limitations

Among the limitations of this study are i) the effects of anesthesia on cardiovascular reflexes initiated by stimulating the cervical vagosympathetic nerve trunk and its associated afferent projections (5, 72); ii) the use of a single stimulation protocol for VNS; iii) the small sampling size of the neurons identified within the intrinsic cardiac nervous system as compared to the complexity of the protocol employed; and iv) neural imbalances evaluated against a normal atrial electrophysiological substrate. It is to be expected that the addition of an altered atrial electrical substrate, as with chronic rapid pace, the neural-myocyte interface will remodel and the efficacy of bioelectric therapies impacted (18, 68). The stimulation protocol utilized herein was chosen to engage central (afferent-mediated) and peripheral (efferent-mediated) aspects of the cardiac nervous system (5), but at a level where the local circuit neurons of the intrinsic cardiac nervous still exert dominant control (36). Because of the dispersed nature of the intrinsic cardiac nervous system (75), even with microarray technology, the yield of recorded IC neurons is low (13, 50). However, functional characteristics of the recorded neurons remain stable in time (13, 23, 50). Finally, long-term VNS has documented efficacy in management of arrhythmias (35, 60) and contractile dysfunction (15, 19, 49) in states of progressive cardiac pathology.

Perspectives and significance

What is clear from recent studies is that there is asymmetry in neural remodeling with progressive cardiac disease and that this neural process is a major determinant of adverse outcomes including the potential for arrhythmias (2, 18, 20). The adaptations in neuronal remodeling must likewise be evaluated in terms of the alterations in the cardiac
electrophysiological substrate (18, 25). In contradistinction to ablation approaches, a major
advantage of electrical neuromodulation when applied at more rostral sites in the cardiac
neuraxis is that single point therapy can moderate reflex function in the disparate ganglia
within the ICNS (7, 54, 55, 77). As demonstrated herein, this form of bioelectric therapy is
readily reversible, has a rapid therapeutic onset and exhibits memory (induces effects that
outlast application). For the first time, we have also defined the pivotal role of local circuit
neurons in mediating neurally-involved arrhythmias and as the primary target for bioelectric
medicine.

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experiments. EB, JLA and JAA performed experiments. SS, EB, JPL, AV and VJ analyzed
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Conflict of interest: None
**Figure legends**

**Figure 1.** IC neurons classified based on their functional responses to afferent stressors [(touch of right (RV) or left (LV) ventricle; occlusion of descending aorta (AOR) or inferior vena cava (IVC))] vs efferent stressors [(right (RCV) or left (LCV) cervical vagus or stellate ganglia (right, RSS; left, LSS) electrical stimulation] interventions. In response to stressors, IC firing could either increase (green bars) or decrease (red bars), that individual response being stable over time. Grey bars mean a specific test was not done for that neuron (designated N/A). Afferent-related IC neurons were defined as those that responded differentially to at least one of the following stressors: RV, LV, AOR or IVC. Efferent-related IC neurons responded to cervical vagal and/or stellate ganglion stimulation. Convergent IC neurons were modulated by both afferent and efferent inputs. Note that 11 IC neurons (~13%) responded solely to the MNS stressor. Approximately 27% of spontaneously identified IC neurons were unaffected by any stressors tested; as such, they are defined as unknown and are not illustrated in this panel.

**Figure 2.** VNS effects on MNS-induced AF. Atrial electrical activity recorded from a unipolar electrode on the ventral right atrial free wall along with lead II ECG. Bursts of electrical stimuli applied to a caudal right-sided mediastinal nerve during the atrial refractory period (downward arrows) elicited arrhythmias (A) before but not (B) after preemptive right-sided VNS.

**Figure 3.** Representative responses to mediastinal nerve stimulation (A) prior to and (B) following RCV VNS. A right atrial electrogram (RAE, bottom) is displayed with concomitant activities generated by 9 identified IC neurons. Panel A shows the control state where AF was induced by right-sided MNS. Panel B shows response when the same MNS site was stimulated 1 min following 3 min of pre-emptive right-sided VNS. Horizontal solid
arrows delimit time of MNS nerve stimulations. Dashed vertical lines (panel A) indicate
duration of AF induced by MNS. Note that following RCV (panel B), MNS failed to induce
AF - even when applied for a longer time period (20 s). IC activity correspondingly
remained unchanged during and following MNS stimulation.

**Figure 4.** A. Response of IC neurons to MNS before (solid line) vs following pre-emptive right-sided (ipsilateral) bioelectric therapy (Dashed line: RCV VNS). IC neurons
were sub-classified as convergent, afferent or efferent LCN’s (c.f., Fig. 1). Convergent
LCNs were the predominant population of neurons activated by MNS and the primary target
for preemptive RCV neuromodulation therapy. B. Impact of ipsilateral (right sided) VNS
therapy on the atrial arrhythmogenic potential to MNS, classified according to whether it
prevented, blunted or enhanced AF or exerted no effects. * p<0.05 from baseline; # p<0.05
from control (sham VNS).

**Figure 5.** A. Response of IC neurons to MNS before (solid line) vs after pre-emptive
left-sided (contralateral) bioelectric therapy (Dashed line: LCV VNS). IC neurons were sub-classified as convergent, afferent or efferent LCN’s, as defined in fig. 1. Convergent LCNs
were the predominant population activated by MNS as well as the primary target for pre-emptive LCV therapy. B. Impact of contralateral VNS therapy on the atrial arrhythmogenic
potential to MNS. While LCV VNS mitigated the AF potential for 40% of MNS sites tested,
in contradistinction to RCV VNS it enhanced that potential in 1/3 of MNS sites tested. *
p<0.05 from baseline.

**Figure 6.** *MNS-induced changes in IC network synchrony.* The synchronized
activities generated by identified pairs of IC neurons (SI>0.01 and p<0.01) were determined
and classified post-hoc, according to comparing concomitant activities generated by: [A]
afferent LCNs; [E] efferent LCNs; and [C] convergent LCNs. Vertical columns represent the degree of synchrony (number of synchronous pairs) between the 6 combinations of neuron pairings elicited during: i) baseline (black bars) vs ii) during MNS-induced arrhythmias (grey bars). These relationships are depicted in untreated (sham VNS, top panel) states as well as following pre-emptive bioelectric therapy (RCV VNS, bottom panel). Note that MNS-induced differential increases in synchrony between efferent to convergent IC pairs (E:C), as well as convergent to convergent neuronal pairings (C:C) (top panel). While at baseline pre-emptive RCV differentially increased synchrony between convergent LCNs, it eliminated the increase in synchrony across all other neuronal subclass pairings during MNS (bottom panel). * p< 0.02 from baseline; # p<0.01 sham to RCV VNS state.

**Figure 7.** VNS induced anti-arrhythmic effects exhibit memory. (A) Evolution of effects induced by right-sided VNS therapy on the capacity of MNS to induce AF (% efficacy), as a function of time post-therapy. Light gray curve represent the percentage of cases (Kaplan-Meier survival curve) in which AF duration was mitigated (shortened or prevented); dark curve indicates time effects of pre-emptive RCV in preventing MNS-induced AF. (B) Similar data derived with respect to AF potential when left-sided (LCV) therapy was applied pre-emptively.

**Table 1:** Hemodynamic response to pre-emptive VNS. Data reflects mean ± SE for heart rate (HR), left ventricular systolic (LVSP) and diastolic (LVEDP) pressure and first derivative of LV pressure (dt/dt) prior to (baseline), during and for 1 min following VNS. * p<0.01 from baseline; # p<0.01 from VNS.
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A. Sham RCV

B. Pre-emptive RCV

ICN neuronal activity

RAE (mV)

Time (sec)

MNS

AF

RCV Stim. 3 min

MNS
### Table 1. Hemodynamic Response to pre-emptive VNS

<table>
<thead>
<tr>
<th></th>
<th>HR (beat/min)</th>
<th>LVSP</th>
<th>LVEDP</th>
<th>LV +dp/dt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>83.8 ± 4.8</td>
<td>122.1 ± 5.1</td>
<td>2.2 ± 0.6</td>
<td>1913.9 ± 125.5</td>
</tr>
<tr>
<td>VNS</td>
<td>57.5 ± 3.7*</td>
<td>120.3 ± 5.6</td>
<td>2.8 ± 0.6</td>
<td>1935.4 ± 154.2</td>
</tr>
<tr>
<td>Post VNS</td>
<td>95.0 ± 4.4#</td>
<td>127.8 ± 5.5#</td>
<td>2.2 ± 0.8</td>
<td>2308.8 ± 181.9#</td>
</tr>
</tbody>
</table>

* p<0.01 from baseline
# p<0.01 from VNS