Central-Peripheral Neural Network Interactions Evoked by Vagus Nerve Stimulation:

Functional Consequences on Control of Cardiac Function

Running title: Central-Peripheral Neural Network Interactions Evoked by VNS

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ABSTRACT

Aim: Using vagus nerve stimulation (VNS), the aim of this study was to determine the contribution of vagal afferents on efferent control of cardiac function. **Methods and Results:** In anesthetized canines, the right and left cervical vagosympathetic trunks were stimulated in the intact state, following ipsilateral or contralateral vagus nerve transection (VNTx), and then following bilateral VNTx. Stimulations were performed at currents from 0.25-4.0 mA, frequencies from 2-30 Hz, and a 500 μs pulse width. Right or left VNS evoked significantly greater current- and frequency-dependent suppression of chronotropic, inotropic and lusitropic function subsequent to sequential VNTx. Bradycardia threshold was defined as the current first required for a 5% decrease in heart rate. The threshold for the right vs left vagus induced bradycardia in the intact state (2.91±0.18 mA and 3.47±0.20 mA, respectively) decreased significantly with right VNTx (1.69±0.17 mA for right and 3.04±0.27 mA for left), decreasing further following bilateral VNTx (1.29±0.16 mA for right and 1.74±0.19 mA for left). Similar effects were observed following left VNTx. The thresholds for afferent-mediated effects on cardiac parameters were 0.62±0.04 mA and 0.65±0.06 mA with right and left VNS, respectively, and reflected primarily as augmentation. Afferent-mediated tachycardias were maintained following beta-blockade, but eliminated by VNTx. **Conclusions:** The increased effectiveness and decrease in bradycardia threshold with sequential VNTx suggests that (1) vagal afferents inhibit centrally-mediated parasympathetic efferent outflow and (2) the ipsilateral and contralateral vagi exert a substantial buffering capacity. The intact threshold reflects the interaction between multiple levels of the cardiac neural hierarchy.
New and Noteworthy:

Vagus nerve stimulation (VNS)-evoked changes in cardiac function reflect the dynamic interplay between direct activation of descending efferents against afferent-induced decreases in central parasympathetic drive to the heart. With increasing current, VNS first activates afferent fibers followed by descending parasympathetic efferent fibers, interactions that maintain cardiac stability.

KEYWORDS: vagus nerve stimulation, autonomic nervous system, parasympathetic, afferent, intrinsic cardiac nervous system
INTRODUCTION

Cardiac control is a manifestation of a neural hierarchy that may be considered in three levels (9, 21, 33). Level 1 comprises the spinal cord and medulla as modulated by higher centers (1, 16, 27, 38). Level 2 comprises extracardiac-intrathoracic neurons including the stellate, middle cervical and mediastinal ganglia (4, 6, 8). Level 3 involves the intrinsic cardiac nervous system (9). The peripheral levels (2 and 3) form cardio-centric control loops, while the central nervous system (Level 1) engages neural mechanisms for both cardiac and peripheral vasculature regulation (33, 60). Acting together, these hierarchical populations coordinate and regulate regional cardiac electrical, mechanical and metabolic indices throughout each cardiac cycle (7, 9, 12). Endogenous or exogenous stresses have the potential to impact multiple levels of this hierarchy (19, 21, 32, 60). It is through the understanding of such hierarchical control and how it adapts to acute and chronic stress that rationale, mechanistic-based approaches can be devised to target the cardiac neural hierarchy in order to manage cardiovascular pathology (14, 19, 21).

The vagus nerve is a complex neural structure, containing descending efferent parasympathetic fibers and ascending afferent fibers. Efferent parasympathetic fibers modulate several cardiac indices including chronotropy, dromotropy, inotropy and lusitropy (46, 48). The majority of fibers (approximately 80%) contained within the vagus are afferent (sensory) in nature (13, 42). Thus, the vagus nerve is an important pathway that carries sensory information from visceral organs, including the heart, to the central nervous system. There is also structural and functional data suggesting that the cervical vagus trunk contains a small population of sympathetic fibers (41, 47).

For any bioelectronic approach for therapeutic neuromodulation one must consider both direct and reactive (reflex) responses (14). The vagus can be stimulated in many different ways, at a number of different levels, and for multiple pathologies. As such, the anatomic characteristics of the nerves being stimulated (afferent/efferent) and the functional impact of
stimulation parameters (current, frequency, pulse width, waveform and duty cycle) must be
considered (13, 14). Ultimately, these factors influence both off-target adverse effects, and more
importantly, the acute and chronic efficacy of the applied therapy. In most clinical applications
for cardiovascular pathologies, electrical vagus nerve stimulation (VNS) is imposed unilaterally
to either the right or left cervical vagosympathetic trunk (17, 18, 43).

While preclinical and clinical studies have yielded encouraging results for VNS safety and
efficacy for cardiac therapeutics (14, 50), there is a major information deficit in understanding
how VNS impacts central and peripheral aspects of the cardiac nervous system to exert its
influence on cardiac control. The objectives of this study were therefore: 1) to investigate the
functional role of VNS-evoked changes in afferent vs. efferent activation on integrated efferent
control of cardiac function and 2) the potential for hierarchical interactions within the cardiac
nervous system to impact VNS-evoked responses.

METHODS

Mongrel dogs (n=26, male or female, weighing 22.2 ± 0.5 kg) were used in this study. All
experiments were performed in accordance with the guidelines set forth by the National
Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by
the East Tennessee State University Institutional Animal Care and Use Committee.

Animal preparation

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). Depth of anesthesia was assessed by monitoring corneal reflexes,
jaw tone, and hemodynamic indices. Left femoral venous access was obtained for maintenance
fluid and drug administration. Right femoral arterial access was obtained for monitoring aortic
pressure. A pressure transducer catheter (Mikro-Tip, Millar Instruments, Houston, TX, USA) was
inserted into the left ventricle (LV) chamber via the left femoral artery. Lateral incisions of the
neck were made bilaterally to expose the cervical vagosympathetic nerve trunks. At the
closure of the surgery, general anesthesia was switched to α-chloralose (50 mg/kg i.v. bolus
followed by 8-12 mg/kg/hr continuous i.v. infusion). Acid-base status was evaluated hourly (Irma
TruePoint, ITC, Edison, NJ, USA); respiratory rate and tidal volume were adjusted and/or
sodium bicarbonate was infused as necessary to maintain blood gas homeostasis. At the
completion of the experiments, animals were humanely euthanized under deep anesthesia and
by inducing ventricular fibrillation via direct current stimulation.

**Vagus nerve stimulation**

Bipolar stimulating helical cuff electrodes (PerenniaFlex Model 304, Cyberonics Inc.,
Houston, TX, USA) were placed around the right and left cervical vagosympathetic trunks with
the anodes positioned cephalad to the cathode. A stimulator with photoelectric constant current
isolation unit (S88 and PSIU6, Grass Technologies, Warwick, RI, USA) was used to deliver
square pulses to these electrodes. Bradycardia threshold was defined as the current required to
first produce a 5% decrease in HR at a frequency of 10 Hz and a pulse width of 500 μs in the
intact state. Tachycardia threshold was defined as the current required to first produce a 5%
increase in HR at a frequency of 10 Hz and a pulse width of 500 μs in the intact state. The
effects of VNS on chronotropic, LV inotropic and LV lusitropic function were evaluated over a
range of currents (0.25-4.0 mA), with 10 Hz frequency and 500 μs pulse width. The effects of
VNS on these cardiac indices were also evaluated over a range of frequencies (2, 5, 10, 15, 20
and 30 Hz), delivered at 1.2 times the bradycardia threshold determined in the intact state at 10
Hz and with a 500 μs pulse width. VNS was performed for 14 s followed by a 66 s off phase.
This time period was sufficient for cardiac indices to return to baseline values with no
degradation in the responses to VNS over the duration of the experiments. Following
stimulations in the intact state, the vagus nerve was transected central to the stimulating
electrode, allowing for the stimulation of only efferent vagal fibers in subsequent parts of the
protocol.
Hemodynamic assessment

A pressure transducer catheter (Mikro-Tip, Millar Instruments) was inserted into the LV chamber and connected to a control unit (PCU-200, Millar Instruments). A lead II electrocardiogram was recorded via needle electrodes and amplified by a pre-amplifier (P511, Grass Technologies). Hemodynamic data were acquired with a data acquisition system (Power1401, Cambridge Electronic Design, Cambridge, UK) and analyzed off-line with Spike2 (Cambridge Electronic Design). Derived indices included HR, aortic blood pressure (BP), LV end-systolic pressure (LVSP), maximum rate of change in LV pressure (LV dP/dt maximum), and minimum rate of change in LV pressure (LV dP/dt minimum). Off-line analysis was used to determine the average response for each of the parameters at baseline and during the 14 s VNS on-phase.

Experimental protocol

Animals were divided into 3 experimental groups. In Group 1 (n=10), the right and left vagus nerves were individually stimulated in the following three states: (1) intact; (2) following right vagus nerve transection (VNTx); and then (3) following left VNTx (bilateral VNTx). In Group 2 (n=10), the right and left vagus were individually stimulated in the following three states: (1) intact; (2) following left VNTx; and then (3) following right VNTx (bilateral VNTx). In Group 3 (n=6), the right and left vagus nerves were stimulated individually in the following four states: (1) intact; (2) intact with timolol, a non-selective beta blocker (1 mg/kg i.v. bolus with 0.5 mg/kg i.v. bolus every 90 min as needed); (3) following bilateral VNTx with timolol; and then (4) following bilateral VNTx with timolol and atropine (1 mg/kg i.v. bolus).

Statistics

Data are presented as mean ± standard error. A repeated measures mixed analysis of variance model was used for comparisons of mean current and frequency curves generated in the different states. Repeated measures analysis of variance model with Tukey multiple
comparison was used for analysis of threshold. P<0.05 was considered to be statistically significant. Statistical analyses were performed with JMP Pro v11.2 (SAS Institute Inc., Cary, NC, USA).

RESULTS

VNS impacted multiple indices of cardiac electrical and mechanical function. Figure 1 shows a typical example of the response to right VNS at intensity sufficient to impose acute changes in cardiovascular function. Note the VNS-evoked decrease in heart rate, systemic (BP) and left ventricular pressure (LVP) as well as LV dp/dt maximum and LV dp/dt minimum. At VNS offset, all the indices recovered rapidly to baselines values with a potential to transiently overshoot baseline values, especially at higher current and frequency levels.

Central-peripheral neural network interactions with vagus nerve stimulation: effects on current intensity

Figure 2 shows percent changes in chronotropy (Figure 2A and 2B), as well as LV inotropy and lusitropy (Figure 2C-2F), in response to 10 Hz right-sided VNS across different currents in the intact state as well as following right (ipsilateral, left panels) or left (contralateral, right panels) VNTx, and then following bilateral VNTx. Figure 3 shows the corresponding heart rate levels at baseline and in response to right-sided VNS following first ipsilateral (panel A) or contralateral (panel B) VNTx and then followed by bilateral VNTx. In the intact state, right VNS produce tachycardia at lower currents (starting at ~0.25-0.50 mA) and bradycardia at higher currents (Figure 2A and 2B; Figure 3A and 3B). The augmentor effects on HR produced at lower currents were eliminated following ipsilateral but not contralateral VNTx (Figure 2A and 2B; Figure 3A and 3B). Right-sided VNS following either ipsilateral or contralateral VNTx resulted in greater reductions in HR, LV dp/dt maximum and LV dp/dt minimum compared to the intact state. Right-sided VNS following bilateral VNTx resulted in further reduction in these
indices, comparing bilateral VNTx vs. intact (p<0.002) or bilateral VNTx vs. unilateral VNTx (p<0.0001). This result was manifest when evaluating either the relative (Figure 2) or absolute levels (Figure 3) of evoked responses.

Figure 4 shows the percent changes in chronotropy (Figure 4A and 4B) as well as LV inotropy and lusitropy (Figure 4C-4F) in response to 10 Hz left-sided VNS across current intensities ranging from 0.25 to 4.0 mA in the intact state, following left (ipsilateral, left panels) or right-sided (contralateral, right panels) VNTx, and then following bilateral VNTx. The augmenting effects on HR produced at lower currents (starting at approximately 0.5 mA) were eliminated following ipsilateral, but not contralateral, VNTx (p<0.004, ipsilateral vs. intact) (Figure 4A and 4B). In the intact state, left VNS resulted in negligible changes in LV dp/dt maximum and minimum, even at currents up to 4.0 mA (Figure 4C-4F). Following ipsilateral, but not contralateral VNTx, left VNS reduced HR, LV dp/dt maximum and LV dp/dt minimum compared to the intact state (p<0.004). The suppressing effects of left VNS were further enhanced following bilateral VNTx, comparing bilateral VNTx vs. intact (p<0.004) or bilateral VNTx vs. unilateral VNTx (p<0.0001).

Central-peripheral neural network interactions with vagus nerve stimulation: effects on threshold

Bradycardia threshold was defined as the current required to first produce a 5% decrease in mean HR during the 14s of VNS. VNS was delivered at 10Hz and pulse width of 500 μs. In the right-sided protocol in the intact state, threshold for the right vagus nerve was 2.91±0.18 mA and for the left vagus nerve it was 3.47±0.20 mA (Figure 5A). Following right VNTx, the thresholds for right and left vagus decreased (1.69±0.17 mA, p<0.001 for right; 3.04±0.27 mA, p<0.04 for left). Thresholds decreased further following bilateral VNTx (1.29±0.16 mA for right and 1.74±0.19 mA for left, p<0.001 vs. intact and p<0.002 vs. right VNTx). Figure 5B displays the percent change in bradycardia threshold for right and left VNS following right and bilateral VNTx compared to the intact state. A similar pattern was observed in the left-sided protocol.
Threshold for the right vagus nerve was 3.03±0.24 mA and for the left vagus nerve was 2.99±0.15 mA in the intact state (Figure 5C). The thresholds for the right and left vagus decreased following left VNTx (2.56±0.25 mA, p<0.001 for right; 1.81±0.22 mA, p<0.001 for left). The thresholds decreased further following bilateral VNTx (1.64±0.21 mA for right and 1.32±0.22 mA for left, p<0.001 vs. intact and p<0.002 vs. left VNTx). Figure 5D displays the percent change in bradycardia threshold for right and left VNS following left and bilateral VNTx compared to the intact state. Taken together, the bradycardia threshold following bilateral VNTx was approximately 50% lower than that established in the intact state for either right or left vagus stimulation.

Tachycardia threshold was defined as the current required to first produce a 5% increase in mean HR during the 14s of VNS. VNS was delivered at 10Hz and pulse width of 500 μs. Across all animals in the intact state, the tachycardia threshold was 0.62±0.04 mA for right VNS and 0.65±0.06 for left VNS. The potential for VNS to augment chronotropic function at the lower ranges of current was maintained following contralateral VNTx (Figures 2B, 3B and 4B) but eliminated by ipsilateral VNTx (Figures 2A, 3A, and 4A). VNS-evoked changes in HR occurred against a significant background of parasympathetic central drive as evidenced by elevations in baseline HR (approximately 60 beats/min) intact vs. bilateral VNTx (approximately 110 beats/min) states (Table 1 and Figure 3).

Central-peripheral neural network interactions with vagus nerve stimulation: effects of frequency

After determining thresholds in the intact state, the effects of VNS on chronotropy, as well as LV inotropy and lusitropy, were evaluated over a range of frequencies (2-30 Hz) while maintaining the same current (1.2x bradycardia threshold determined at 10 Hz) and pulse width (500 μs). Figure 6 shows the percent changes in chronotropy, LV inotropy and LV lusitropy in response to left VNS across a range of frequencies in the intact state, following left VNTx or
right VNTx, and then following bilateral VNTx. In all states, increasing frequency resulted in
greater reductions in HR, LV dp/dt maximum and LV dp/dt minimum. Left VNS following either
ipsilateral or contralateral VNTx resulted in a greater reduction in all measured indices
compared to the intact state (p<0.0001), with the exception of LV dp/dt maximum following
ipsilateral VNTx. There was an even further reduction in these indices with left VNS following
bilateral VNTx compared to the intact state (p<0.0001), as well as compared to ipsilateral
(p<0.0001) or contralateral VNTx (p<0.0001).

Figure 7 shows the analogous percent changes in chronotropic and LV inotropic function in
response to right-sided VNS across different frequencies in the intact state, following left or right
VNTx, and then following bilateral VTNx. In all these states, increasing frequency suppressed
chronotropic and LV inotropic function. Ipsilateral (right-sided) VNTx exerted a predominant shift
in the evoked functional response surface to right-sided VNS; contralateral VNTx resulted in a
significant shift in the response surface only in LV lusitropy (panel F). Following bilateral VNTx,
there was a further enhancement in these negative chronotropic and LV inotropic effects
compared to the intact state (p<0.0001), as well as compared to ipsilateral (p<0.0001) or
contralateral VNTx (p<0.0001).

Central-peripheral neural network interactions with vagus nerve stimulation: effects of
autonomic blockade

Changes in cardiac function mediated by the autonomic nervous system are manifest, in
part, by changes in parasympathetic and/or sympathetic outflows. While timolol exerted minimal
effects on hemodynamic function (table 2) and unilateral VNTx was likewise associated with
minimal changes (table 1), bilateral VNTx was associated with approximately 55% increase in
HR (tables 1 and 2). Figures 8 and 9 summarizes VNS-evoked effects on chronotropic function
(Figures 8A and 9A), LVSP (Figures 8B and 9B), LV contractility and LV relaxation (Figures 8C,
8D, 9C, and 9D) in the control state, following beta-adrenergic blockade (timolol); subsequent
bilateral VNTx (timolol+bilateral VNTx); and following subsequent muscarinic blockage
(timolol+bilateral VNTx+atropine). While timolol by itself exerted no significant effects on right VNS chronotropic or LV inotropic function (Figure 8), it did alter chronotropic, inotropic and lusitropic responses to left VNS (Figure 9). Subsequent VNTx substantially enhanced VNS-induced negative chronotropic and LV inotropic effects, which atropine abolished.

**DISCUSSION**

The aim of the present study was to determine the role of cervical VNS-evoked afferent vs. efferent axon activation on integrated control of regional cardiac functions. The major findings of this study are as follows:

1. VNS-evoked changes in cardiac function, delivered to an intact vagus nerve, reflect the dynamic interplay between direct activation of descending efferents against afferent-induced changes in central drive to the heart.

2. The functional threshold for activation of vagal afferent fibers is lower than that for activation of efferent fibers.

3. Activation of vagal afferents is primarily reflected as withdrawal of central parasympathetic drive.

4. The potential exists for low-level sympathoexcitation as a result of bioelectric activation by vagal afferents.

Structure/function of the cervical vagosympathetic nerves: relationship to the neural hierarchy for cardiac control

The cervical vagosympathetic nerve is a mixed nerve containing both ascending (afferent) and descending (efferent) axons (13, 28). Figure 10 schematically represents the relationship of these axonal projections within the framework of the hierarchy for cardiac control. The axons themselves are ~80% afferent, including both myelinated and unmyelinated fibers (13, 42). Cardiac-related vagal afferent neurons have cell bodies located in the nodose ganglia that project sensory information onto secondary afferent neurons in the nucleus tractus solitarii.
(NTS) located in the medulla (23, 28, 52). NTS secondary neurons project primarily to neuronal somata in the nucleus amibiguus (NA) for control of parasympathetic preganglionic efferent neuronal activity and, via brainstem reticulospinal (BSRF) projections, to the spinal cord intermediolateral (IML) cell column for control of preganglionic sympathetic neuronal activity (1, 23, 29, 60). Sympathetic afferents, arising from the dorsal root ganglia (DRG), likewise input to the NTS via spinoreticular projections to contribute to reflex control of cardiovascular function (1, 20, 60).

Cardiac-related efferent projections contained within each cervical vagosympathetic trunk are predominantly parasympathetic preganglionic axons (46, 47). Parasympathetic preganglionic projections arising bilaterally from NA neurons innervate multiple intrinsic cardiac ganglionic plexuses located within atrial and ventricular tissues, making direct contact with parasympathetic postganglionic neurons (Fig. 10). They also modulate the activity of local circuit neurons (LCNs) therein (9, 12). The LCNs represent the predominant neuronal population (~80%) of the intrinsic cardiac nervous system (ICNS), providing the primary integrating function for local reflex control of cardiac performance (3, 9, 12). The direct parasympathetic projection pathway, pre- to postganglionic neurons, represents approximately 15% of the ICNS (10, 12, 22). The remaining 5% of ICNS neurons are afferent (3, 12). There is anatomical and functional evidence to indicate that the vagosympathetic trunk also contains a small number of sympathetic efferent fibers (41, 47). These sympathetic fibers have been described to originate from sympathetic efferent parasympathetic somata in the superior and middle cervical ganglia (29, 41, 48). Sympathetic projections to the heart can either directly project to cardiac myocyte end-effectors or modulate cardiac function via network sympathetic/parasympathetic interactions mediated with the ICNS (12, 39, 45, 55).

**VNS induced changes in regional cardiac function: hierarchical interactions**
Any bioelectronic approach for neuromodulation needs to be considered in the context of the cardiac nervous system as a whole as there are both direct and reactive (reflex) responses that are evoked (14). Thus, in order to understand the central and peripheral neural network interactions within the cardiac neuronal hierarchy evoked by VNS, we studied the effects of VNS in i) the intact state, ii) following sequential cervical VNTx, and iii) in the presence of autonomic blockade.

*Chronotropic control:* In the intact state, tachycardias evoked at low stimulus intensities (~0.6 mA) transitioned to bradycardias at higher stimulus intensities (~3.0 mA). VNS-induced tachycardias were maintained after contralateral VNTx, but eliminated by ipsilateral, VNTx. Pretreatment with a beta-adrenoceptor blocker did not affect VNS-evoked positive chronotropic effects. The positive chronotropic responses were also manifest at stimulus intensities below that required to evoke bradycardias following bilateral VNTx. Moreover, VNS-evoked changes in HR in the intact state were elicited against a significant background of parasympathetic central drive - as evidenced by elevations in baseline HR in intact (approximately 60 beats/min) vs. bilateral VNTx (approximately 110 beats/min) states (Table 1 and Figure 3). Taken together, these data support the hypothesis that vagal afferent axons are activated at lower VNS current densities and that the primary effect of stimulation of these afferents is transient withdrawal of central parasympathetic drive.

In the present study, we also demonstrate that the VNS bradycardia threshold, specifically the output current required to first produce a minimum defined (5%) decrease in HR, reflects the interdependent interactions between central and peripheral neural network aspects of the cardiac nervous system. This threshold progressively decreased following sequential VNTx, regardless of whether the right or left vagus was transected first. Compared to the intact state, the bradycardia threshold was reduced by approximately 50% when the peripheral ends of the cervical vagosympathetic trunks were stimulated in decentralized preparations.
Efferent outflows from the ICNS to the cardiac tissues reflect the dynamic network interactions between central drive and local afferent feedback as modulated by LCNs. The LCNs subserve intra- and inter-ganglionic coordination thereby acting as primary coordinators of regional function (3, 9, 12). When intrinsic cardiac ganglion function is evaluated *in vitro*, the principal synapse between pre- to postganglionic parasympathetic fibers is primarily obligatory in nature (24-26). Thus, the evoked response to VNS when delivered in the bilateral decentralized state *in vivo* most closely approximates that *in vitro* condition. The differences in VNS-evoked responses between bilateral VNTx, unilateral VNTx and intact conditions reflect the hierarchical organization of the interdependent reflex control circuits whose ultimate function is to stabilize and optimize cardiac electrical, mechanical and metabolic function (12).

There are multiple hypotheses to explain observed decreases in threshold. The first reflects the role of vagal afferent axons in reflexly modulating parasympathetic efferent outflows to the heart. Our data indicate that activated vagal afferents initiate centrally-mediated reflexes that inhibit parasympathetic efferent outflows to the heart. As such, the level of activity on a given parasympathetic postganglionic neuron would reflect not only diminished endogenous central drive, but also inputs arising from direct electrical activation of parasympathetic efferent fibers. Since both vagi project to neurons throughout the ICNS (5, 9), this would have the net effect of shifting the VNS chronotropic response surface/curve to the right. A second hypothesis is that bioelectrical stimulation of the cervical vagosympathetic trunk activates axons projecting to the LCNs in the ICNS that, in turn, inhibit the direct pre- to postganglionic parasympathetic neuron pathways (31). We have previously demonstrated that similar inputs to the ICNS can initiate directionally opposite responses from adjacent intrinsic cardiac neurons that reflect this potential (12). In the context of the current study, this proposed descending direct inhibitory mechanism is unlikely to be functionally important since it should also occur following unilateral VNTx and, as such, response surfaces would not have shifted, as was observed in this study. A third hypothesis involves induced alterations in sympathetic-parasympathetic interactions both within
the ICNS and at the end-effectors (39, 45). Such a mechanism would, at best, represent a minor component, as reflected in the minimal changes observed with VNS in the presence of beta adrenergic blockade. However, the fall in LV and systemic blood pressure following bilateral VNTx may be reflective of a tonic sympathetic component for cardiovascular control mediated by axonal projections contained within the cervical vagosympathetic complex or alterations in integrated reflex control resulting from the loss of afferent inputs. A fourth possibility is that VNTx alters the neural interactions/balance for control of the sinoatrial nodal complex (39). In this regard, a similar mechanism could also occur within other intrinsic cardiac local circuit neuronal populations regulating atrial and/or ventricular contractile function (15, 56). Future experiments will be required to explore these hypotheses.

**Inotropic and lusitropic control:** Integrated parasympathetic control of atrial, atrioventricular and ventricular function is mediated via parasympathetic preganglionic inputs to the distributed network of interdependent intrinsic cardiac ganglionated plexi (9, 15, 57). While earlier work suggested that vagal restraint on inotropic function is manifest against an enhanced sympathetic background (e.g., accentuated antagonism) (34, 35), later work has demonstrated that activation of the ICNS, either endogenously or exogenously, impacts cardiac contraction and relaxation directly (15, 44, 56). As is evident from the VNS intensity plots depicted herein (Figures 2-4), with intact vagi the magnitude of the evoked inotropic and lusitropic responses are moderate (approximately 5% of baseline). Subsequent to VNTx, responses evoked in ventricular contractility were shifted to the left - to substantially greater levels. This shift is likewise evident when a fixed output current was applied (Figs. 6 and 7), where even at the lowest frequency (2 Hz), the minimal change in contractility evoked in the intact state transitioned to a 15-25% decreases following bilateral VNTx. These data demonstrate that in the intact state the neuronal hierarchy for cardiac control has a substantial buffering capacity that acts reflexly to maintain cardiac stability in the face of endogenous and exogenous stresses, including those induced by VNS itself.
Differential effects of VNS on autonomic efferent outflows

Parasympathetic and sympathetic divisions of the autonomic nervous system cannot be considered in isolation as they function as interdependent components of the cardiac neuronal hierarchy (9, 21, 59). As such, changes in autonomic efferent outflow in response to VNS therapy reflect: 1) direct activation of autonomic efferent axons; 2) central reflex induced changes in efferent activity in response to bioelectrical activation of vagal afferent axons; and 3) reflex induced changes in autonomic function as cardiovascular afferents transduce altered mechanical stress in both cardiac and extracardiac (e.g. arterial baroreceptors) sites accompanying VNS-induced effects on cardiac function (11, 37, 60). Data presented in Figures 8 and 9 demonstrate the minor contribution to cardiac control evoked by sympathetic efferent axons contained within the vagosympathetic complex in response to VNS, as delineated from the minimal changes in VNS-induced effects on regional cardiac indices post-timolol infusion. In contrast, atropine blocked induced changes in chronotropic, inotropic and lusitropic function, even against an enhanced sensitivity imposed by bilateral VNTx.

VNS and the neural fulcrum

When considering stimulation parameters necessary for VNS therapy one must be cognizant of not only the anatomic/functional characteristics of the axons being stimulated therein (afferent/efferent axons), but also the effects of direct vs. reactive changes that result from the whole cardiac neuronal hierarchy attempting to maintain stability of cardiovascular function (14). Since the cardiac hierarchy normally acts as a negative feedback system, perturbing the system in one direction evokes a corresponding reflex response in the opposite direction to maintain stability. The greater the perturbation, the greater the reflex response and the greater the instability of the entire system.

As such, we propose that the optimum therapeutic parameters for cervical VNS therapy are at the point at which afferent and efferent fibers are activated in a balanced manner. That is,
when afferent driven decreases in central parasympathetic drive are counteracted by direct
activation of the cardiac parasympathetic efferent projections to the ICNS and heart - the net
result of which being a null HR response (2, 31). We define this point as the *neural fulcrum*.
VNS performed near this neural fulcrum operates within the normal constraints of the cardiac
neuronal hierarchy, without evoking reactive changes that occur when high currents and/or
frequencies are utilized (31). At this neural fulcrum it should be recognized that both central and
peripheral aspects of the cardiac nervous system become engaged such that the reflex
hierarchy still maintains its capability to respond to stressors.

**Clinical perspectives**

Autonomic imbalance plays an important role in the genesis of cardiac arrhythmias and
progression of heart failure (21, 32, 51, 54). Sympathetic activation and parasympathetic
withdrawal is not only pro-arrhythmic (21), but also accelerates progression of heart failure (19,
30, 58, 60). Furthermore, heart failure patients with poor vagal tone are known to have a worse
prognosis (40). In animal models of chronic heart failure, VNS has been shown to decrease
resting HR, improve left ventricular function and decrease mortality, presumably by preventing
adverse cardiac remodeling (36). Given the promising results from preclinical studies, VNS is
currently be evaluated in multiple clinical trials for reduced ejection fraction heart failure. These
include the CardioFit-HF, NECTAR-HF, and ANTHEM-HF (17, 18, 43). Initials results of these
trials have been positive for CardioFit-HF and ANTHEM-HF, with neutral effects for NECTAR-
HF after the first 6 months of follow-up. One of the key differences between these trials is the
choice of stimulation parameters (current, frequency, pulse width and duty cycle) and, in the
case of CardioFit, a proposed methodology to induce transient vagal afferent block during VNS.
Based on data presented herein, for proposed application of afferent blockade, one should
consider potential deleterious effects on the effective gain of VNS therapy (17). Nevertheless,
what is obvious from these ongoing trials is that VNS is safe and feasible in the setting of
reduced ejection fraction heart failure. Data from randomized, controlled studies are required to
elucidate the impact VNS on morbidity and mortality in patients with chronic heart failure syndrome.

In view of the data presented herein, future studies should consider what is “subthreshold” VNS. Multiple studies have recently evaluated the effects of low-level VNS for treatment of atrial arrhythmias and heart failure (49, 53). What is obvious from data presented herein is that central and peripheral elements of the hierarchy for cardiac control become engaged by output current levels well below those current density applications that suppress chronotropism. Thus, these data lead us to propose that: 1) the chronic effects of vagus nerve stimulation therapy rest primarily on the indirect pathways that target intrinsic cardiac LCNs; and 2) that the optimal VNS stimulus parameters are coincident with the neural fulcrum (31). Future studies on the efficacy of VNS therapy for heart failure should focus on optimization of stimulation parameters, while focusing on patient selection and therapeutic transition where indicated in the standard of care. As such, future preclinical and clinical studies should be designed to employ the entire cardiac nervous system in order to achieve long-term therapeutic benefits while minimizing off-target side effects of VNS.

FUNDING

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Author contributions: All authors contributed equally to researching data for this article, writing the manuscript, discussions of content and approved final version before submission.

Figure legends

**Figure 1.** Representative hemodynamic response to right cervical vagus (RCV) bioelectric stimulation in an anesthetized animal with intact vagus nerves. Vagus nerve stimulation (VNS) was delivered at 10 Hz, 500 μs duration and 2.50 mA for 14 s. LVP – left ventricular pressure; LV – left ventricle; dp/dt – first derivative of LV pressure; BP – blood pressure, EKG – lead II electrocardiogram.

**Figure 2.** Evoked changes in chronotropic (panel A,B) and left ventricular inotropic and lusitropic (Panels C-F) function in response to RCV bioelectric stimulation prior to and following sequential cervical vagus transection rostral to stimulating electrode. VNS delivered at 10 Hz, 500 μs pulse width for 14 s. Responses reflect % change from baseline during VNS as a function of stimulus intensity. Left panels show responses in intact state, after the right vagus and then following bilateral cervical vagus transection. Right panels show similar responses, but with the left vagus transected first. Note that all augmenting response to VNS were eliminated when ipsilateral vagi were transected. * p<0.004 vs intact; # p<0.0001 unilateral vs bilateral vagus transection.

**Figure 3.** Baseline and RCV-evoked heart rate levels in intact state and then following sequential unilateral (right: 3A; left: 3B) and bilateral (3A and 3B) cervical vagotomy. Vagus nerves cut rostral to stimulating electrode. Note that all positive chronotropic responses to RCV were eliminated when the right vagus was transected and that negative chronotropic responses to RCV were progressively increased going from unilateral to bilateral vagotomy conditions. * p<.001 vs intact; # p<0.005 unilateral vs bilateral vagus transection.

**Figure 4.** Same as for figure 2, but with data summarized for left cervical vagus stimulation (LCV). Effects of ipsilateral (left) vs bilateral vagus transection are shown to left. Effects of
contralateral vs bilateral vagus transection are shown to right. *p<0.002 vs intact; # p<0.0001 unilateral vs bilateral vagus transection.

**Figure 5.** VNS bradycardia threshold (mA) is reduced by sequential vagal transection. Threshold is defined as 5% decrease in HR. Left panels indicate average current required in intact state, following unilateral transection of the right (panel A) or left (panel C) cervical vagus and following bilateral VNTx. Vagi transected rostral to stimulation point. Panels B and D indicate the percent change from intact controls for baseline threshold following unilateral right (panel B), left (panel D) and bilateral vagus transection. + p<0.04 vs intact; * p<0.001 vs intact; # p<0.002 unilateral vs bilateral vagus transection.

**Figure 6.** Evoked changes in chronotropic (panels A,B), left ventricular inotropic (panels B,E) and lusitropic (panels C,D) function in response left cervical VNS prior to and following sequential cervical vagus transections rostral to stimulating electrode. VNS delivered at 500 μs pulse width for 14 s at an intensity of 1.2x bradycardiac threshold as determined in intact state. Responses reflect % change from baseline during VNS as a function of stimulus frequency. Left panels show responses in intact state, after the left vagus and then following bilateral cervical vagal transection. Right panels show similar responses, but with the right vagus transected first. Across all conditions: * p<0.001 vs intact; # p<0.0001 unilateral vs bilateral vagus transection. + p<0.0001 paired comparison intact vs bilateral decentralization.

**Figure 7.** Same as for figure 6, but with data derived from right cervical vagus stimulation. Effects of ipsilateral (right) vs bilateral vagus transection are shown to left. Effects of contralateral vs bilateral vagal transection are shown to right. Across all conditions: * p<0.0001 vs intact; # p<0.0001 unilateral vs. bilateral vagus transection. + p<0.0001 paired comparison intact vs bilateral decentralization.

**Figure 8.** Evoked changes in heart (panel A), left ventricular systolic pressure (panel B) and left ventricular dp/dt (positive panel C; negative panel D) in response to RCV (10 Hz) prior to
and following sequential timolol (1 mg/kg), bilateral cervical vagus transection (vagotomy) and atropine (1 mg/kg). Responses reflect % change from baseline during VNS as a function of stimulus current intensity. * p< 0.02 vs. control; # p<0.001 vs. Timolol; + p<0.0001 vs. Timolol+ vagotomy.

**Figure 9.** Same as for Figure 8, but for left cervical vagus stimulation. * p< 0.03 vs control; # p<0.003 vs. Timolol; + p<0.0001 vs. Timolol + vagotomy.

**Figure 10.** Schematic summarizing proposed neural interactions within hierarchy for cardiac control engaged by cervical vagal stimulation. Centrally-mediated changes in preganglionic input to the intrinsic cardiac nervous system (ICNS) evoked at current intensities ~20% of bradycardiac threshold. Dotted lines indicate preganglionic projections. NTS – Nucleus tractus solitaries; NA – Nucleus Ambiguus; BSRF – brainstem reticular formation; IML – intermediolateral cell column, DRG – dorsal root ganglia; MCG – middle cervical ganglia; LCN – local circuit neuron; β – beta adrenergic receptor; M₂ – muscarinic receptor; Gₛ and Gᵢ – g-coupled proteins; AC – adenylate cyclase.
REFERENCES


**Table 1.** Hemodynamic effects of sequential cervical vagal transection

<table>
<thead>
<tr>
<th>State</th>
<th>LV +dp/dt (mmHg/s)</th>
<th>LV –dp/dt (mmHg/s)</th>
<th>LVSP (mmHg)</th>
<th>Aortic BP (mmHg)</th>
<th>Heart rate (beats/min)</th>
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<tr>
<td><strong>Intact</strong></td>
<td>2011 ± 130</td>
<td>-2402 ± 175</td>
<td>152.5 ± 6.3</td>
<td>127.4 ± 4.0</td>
<td>59.3 ± 3.6</td>
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<td>Right Vagus cut</td>
<td>1819 ± 123</td>
<td>-2334 ± 196</td>
<td>138.4 ± 8.2</td>
<td>119.8 ± 6.2</td>
<td>69.8 ± 4.0</td>
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<td>Both Vagi cut</td>
<td>1914 ± 164</td>
<td>-2234 ± 201</td>
<td>121.5 ± 9.4*</td>
<td>111.5 ± 8.9*</td>
<td>112.8 ± 7.7*</td>
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<tr>
<th>State</th>
<th>LV +dp/dt (mmHg/s)</th>
<th>LV –dp/dt (mmHg/s)</th>
<th>LVSP (mmHg)</th>
<th>Aortic BP (mmHg)</th>
<th>Heart rate (beats/min)</th>
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<td>Left Vagus cut</td>
<td>1751 ± 140</td>
<td>-2340 ± 163</td>
<td>150.0 ± 9.2</td>
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<td>69.2 ± 6.5</td>
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<td>128.7 ± 12.3*</td>
<td>119.9 ± 13.2</td>
<td>106.0 ± 5.4*</td>
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* p ≤ 0.05 intact versus unilateral or bilateral cervical vagal transection
# p ≤ 0.05 unilateral versus bilateral cervical vagal transection
Table 2. Effects of beta adrenergic blockade, bilateral cervical transection and muscarinic blockade on baseline hemodynamic function.

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<th>State</th>
<th>LV +dp/dt (mmHg/s)</th>
<th>LV –dp/dt (mmHg/s)</th>
<th>LVSP (mmHg)</th>
<th>Aortic BP (mmHg)</th>
<th>Heart rate (beats/min)</th>
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<tbody>
<tr>
<td>Intact</td>
<td>2045 ± 165</td>
<td>-2700 ± 189</td>
<td>154.6 ± 13.0</td>
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<td>74.8 ± 4.6</td>
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<td>Timolol</td>
<td>1791 ± 52</td>
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<td>143.4 ± 9.2</td>
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<td>67.7 ± 3.5</td>
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<td>Timolol + Vagi cut</td>
<td>2060 ± 108</td>
<td>-2375 ± 101</td>
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<td>143.9 ± 13.8</td>
<td>111.7 ± 6.0* #</td>
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<tr>
<td>Timolol + Vagi cut + atropine</td>
<td>1911 ± 110</td>
<td>-2348 ± 109</td>
<td>142.9 ± 8.1</td>
<td>129.7 ± 7.2</td>
<td>114.2 ± 7.8* #</td>
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* p ≤ 0.05 from intact
# p ≤ 0.05 from Timolol