Neurotransmission to Parasympathetic Cardiac Vagal Neurons in the Brainstem is Altered With Left Ventricular Hypertrophy Induced Heart Failure

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Running Title: Heart Failure Alters Synaptic Input in Cardiac Vagal Neurons

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Abstract

Hypertension, cardiac hypertrophy and heart failure (HF) are widespread and debilitating cardiovascular diseases that affect nearly 23 million people worldwide. A distinctive hallmark of these cardiovascular diseases is autonomic imbalance, with increased sympathetic activity and decreased parasympathetic vagal tone. Recent device-based approaches, such as implantable vagal stimulators that stimulate a multitude of visceral sensory and motor fibers in the vagus nerve, are being evaluated as new therapeutic approaches for these and other diseases. However little is known about how parasympathetic activity to the heart is altered with these diseases and this lack of knowledge is an obstacle in the goal of devising selective interventions that can target and selectively restore parasympathetic activity to the heart. To identify the changes that occur within the brainstem to diminish the parasympathetic cardiac activity left ventricular hypertrophy was elicited in rats by aortic pressure overload using a transaortic constriction approach. Cardiac vagal neurons (CVNs) in the brainstem that generate parasympathetic activity to the heart were identified with a retrograde tracer and studied using patch clamp electrophysiological recordings in-vitro. Animals with left cardiac hypertrophy had diminished excitation of CVNs which was mediated both by an augmented frequency of spontaneous inhibitory GABAergic neurotransmission, (with no alteration of inhibitory glycineric activity), as well as diminished amplitude and frequency of excitatory neurotransmission to CVNs. Opportunities to alter these network pathways and neurotransmitter receptors provide future targets of intervention in the goal to restore parasympathetic activity and autonomic balance to the heart in cardiac hypertrophy and other cardiovascular diseases.

keywords: Heart failure, cardiac hypertrophy, parasympathetic, autonomic, vagal
New and Noteworthy

A common hallmark of cardiovascular diseases is autonomic imbalance. Left ventricular hypertrophy (LVH) altered both excitatory and inhibitory neurotransmission to cardiac vagal neurons (CVNs) that generate parasympathetic activity to the heart. These preferentially altered network pathways and neurotransmitter receptors provide future targets to restore parasympathetic activity in these diseases.
Introduction

Hypertension, cardiac hypertrophy and heart failure (HF) are widespread and debilitating cardiovascular diseases that affects nearly 23 million people worldwide with approximately 2 million new patients diagnosed annually (15). Cardiac rhythm disturbances lead to sudden cardiac death in 40-50% of advanced heart failure patients with a 1 year mortality rate greater than 50% (10). A distinctive hallmark of cardiac hypertrophy, heart failure and accompanying cardiac conduction abnormalities is autonomic imbalance, particularly increased sympathetic activity and decreased parasympathetic tone.

Parasympathetic cholinergic activity to the heart plays a major role in cardiac function, and is often cardioprotective, suppressing the endogenous high rate of firing of pacemaker cells in the sinoatrial node and maintaining heart rate at normal levels. Cardioinhibitory parasympathetic activity to the heart arises from the preganglionic cardiac vagal neurons (CVNs) located in the nucleus ambiguus (NA), dorsal motor nucleus of the vagus (DMNX), and intermediate zone of the medulla oblongata (4, 5, 34). Vagal efferent axons from these cell bodies terminate upon the postganglionic intracardiac ganglia neurons located near the sinoatrial and atrioventricular nodes of the heart (2). Resting heart rates, and changes in response to challenges, are mediated to a large extent by alterations in parasympathetic vagal outflow originating from CVNs in the brainstem. CVNs exhibit tonic firing activity that is cardiac pulse synchronous, and also inhibited during each inspiration (14, 23, 28, 33).

While parasympathetic activity to the heart is absent or diminished in many cardiovascular diseases, including hypertension, cardiac hypertrophy and heart failure, augmentation of diminished cardiac vagal activity prevents arrhythmias, decreases risk of sudden death, and protects against ischemia/reperfusion injury (7, 12, 20, 24, 25, 31, 36, 37, 39,
However there are few, if any, selective methods to increase parasympathetic activity to the heart in patients. Recent device-based approaches, such as implantable vagal stimulators that stimulate a multitude of visceral sensory and motor fibers in the vagus nerve, are being evaluated as new therapeutic approaches for these and other diseases. However little is known about how parasympathetic activity to the heart is altered with these diseases and this lack of knowledge is an obstacle in the goal of devising selective interventions that can target and selectively restore parasympathetic activity to the heart. To provide this foundation and identify the changes that occur within the brainstem to diminish parasympathetic cardiac activity left ventricular hypertrophy was elicited in rats by aortic pressure overload using a transaortic constriction approach and the neurobiology of cardiac vagal neurons was studied with patch clamp electrophysiological approaches. The goal of this study was to identify the changes within the brainstem that are likely responsible for diminishing parasympathetic activity to the heart, and by doing so provide future targets of intervention to restore parasympathetic activity and autonomic balance to the heart in cardiac hypertrophy and other cardiovascular diseases.

Methods

Ethical approval

All animal procedures carried out were in accordance with The George Washington University Institutional Guidelines and in compliance with the recommendations of the panel of Euthanasia of the American Veterinary medical association and the NIH publication (85-23, revised 1996) “Guide for the care and use of laboratory animals’. The minimal number of animals was used and care was taken to reduce any possible discomfort.

Aortic banding to Induce Heart Failure
Left ventricular hypertrophy secondary to pressure overload was produced in male Sprague-Dawley rats using a minimally invasive transaortic constriction (TAC) approach previously established for mice (21, 35). Animals at 4-5 weeks of age were subjected to either TAC or a sham aortic banding surgery. A 1-1.5 cm skin incision was made at the level of the suprasternal notch and the thymus was retracted to reveal the aortic arch. A 4-0 silk suture was passed around the aortic arch between the origin of the right innominate and left common carotid arteries and, with a 20 gauge needle temporarily placed adjacent to the aorta, the suture was tied around the aorta and needle. The needle was then removed which produced a chronic partial aortic constriction. Successful aortic banding was confirmed by increased flow through the right carotid artery at the surgical session and upon examination of the aorta after the animal was sacrificed. Sham animals underwent the same surgical procedure except the suture was not tied.

After 6-8 weeks animals were euthanized and the degree of left ventricular hypertrophy was measured. Hearts were rapidly excised and retrogradely perfused with isotonic media containing heparin to wash out the blood. Fat, vessels, and connective tissue were trimmed from the base. Hearts were sliced into four cross-sections and these sections of tissue were photographed and analyzed using the software ImageJ (NIH). The "free wall" measurement was defined as the distance from the closest point on the inside of the left ventricle to the edge of the slice opposite the right ventricle. The "septum" measurement was defined as the distance from the closest point on the inside of the left ventricle to the closest point on the inside of the right ventricle. The weight of the whole heart and left ventricle were recorded. Heart dimensions and weight were divided by animal weight to normalize for variations in the size of the animals.

**Labeling of CVNs**
Animals in which electrophysiological recordings from CVNs were obtained underwent an additional surgery when the animals were at postnatal days 2-5 (Sprague-Dawley, Hilltop Laboratory animals Inc, Scottdale, PA, USA). Animals were anesthetized using hypothermia by cooling to approximately 4°C. A right thoracotomy was performed and retrograde tracer X-Rhoda-mine-5-(and-6)-isothiocyanate (Invitrogen, USA) was then injected into the fat pads at the base of the heart to retrogradely label CVNs (26). The animals were then allowed to recover until they were 4-5 weeks of age and then underwent either sham or TAC surgery.

**In vitro brainstem slice preparation**

We adopted the methodology from Ye and colleagues (45) to obtain viable brainstem slices from 10-12 week old animals. According to this method, glycerol base artificial cerebrospinal fluid (aCSF) was used for cardiac perfusion and brainstem slicing. Glycerol-based aCSF contained (in mM): 252 glycerol, 1.6 KCl, 1.2 NaH₂PO₄, 1.2 MgCl, 2.4 CaCl₂, 26 NaHCO₃, and 11 glucose. Rats were anaesthetized using isoflurane and placed on ice. Glycerol aCSF (4°C, pH-7.4, bubbled with 95% O₂-5%CO₂) was perfused transcardially at a speed of ~ 10 ml/min after which the brain was quickly removed, glued on to a stage using 2% low melt agarose and placed in a vibrotome containing glycerol aCSF. Brainstem slices (330µm thickness), containing either DMNX or NA, were obtained and briefly placed in a solution with following composition (in mM): 110 N-methyl-d-glucamine (NMDG), 2.5 KCl, 1.2 NaH₂PO₄, 25 NaHCO₃, 25 glucose, 110 HCl, 0.5 CaCl₂, and 10 MgSO₄ equilibrated with 95% O₂ and 5% CO₂ (pH 7.4) at 34°C for 15 min. NMDG based aCSF was used to help slices recover and to maintain viable brainstem slices for electrophysiological recordings (47). The slices were then mounted in a recording chamber constantly perfused with a normal aCSF with following composition (in mM): 125 NaCl, 3 KCl, 2 CaCl₂, 26 NaHCO₃, 5 glucose and 5 HEPES; oxygenated with 95% O₂–5% CO₂ (pH-7.4) and allowed to recover for at least 30 minutes before an experiment was performed.
Electrophysiological recordings

CVNs in NA and DMNX were identified by the presence of fluorescent tracer rhodamine and imaged using differential interference contrast optics and infrared illumination. Whole cell voltage clamp recordings from CVNs were done using Axopatch 200B and pClamp 8 software (Axon Instruments, Union city, USA), at a holding voltage of -80 mV at room temperature. The patch pipettes (2.5–5 MΩ) were filled with a solution consisting (in mM) of KCl (150), MgCl₂ (4), EGTA (10), Na-ATP (2) and HEPES (10) or K-gluconic acid (150), HEPES (10), EGTA (10), MgCl₂ (1) and CaCl₂ (1) at a pH of 7.3 for recording inhibitory or excitatory events respectively. GABAergic inhibitory post synaptic currents (IPSCs) were isolated by application of a solution containing strychnine (1µM, glycine receptor antagonist), 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 50µM, non-NMDA receptor antagonist) and D-2-amino-5-phosphonovalerate (AP5, 50µM, NMDA receptor antagonist). Glycinergic IPSCs were isolated by including gabazine (25µM, GABA-A receptor antagonist), CNQX, and AP5 in the perfusate. The perfusate included gabazine and strychnine (25µM and 1µM, respectively) to isolate glutamatergic excitatory postsynaptic currents. Gabazine, strychnine, CNQX, and AP5 were obtained from Sigma Aldrich (St. Louis, MO, USA).

Data analysis

Synaptosoft software (version 6.0.3; Synaptosoft, Decatur, GA) was used to analyze the synaptic events recorded from CVNs. Threshold value was set to the root mean square of noise levels multiplied by 5. The data were presented as mean ± SEM. Students unpaired t-test was used to compare statistical significance between sham and cardiac hypertrophy groups using Graphpad Prism 5 software (La Jolla, CA, USA). Data with p<0.05 was considered significant; in the figures, * denotes p<0.05, ** denotes p<0.01, *** denotes p<0.001.
Results

Transaortic constriction (TAC) resulted in left ventricular hypertrophy (LVH), consistent with previous reports in rats (44). Hearts from TAC animals were significantly heavier than hearts from control animals and had thicker left ventricles, (Figure 1A). Animal weight did not differ between TAC and control (Figure 1B), but the ratios of LV free wall thickness to body weight, LV weight to body weight, and heart weight to body weight were all significantly higher in TAC animals compared to control (Figure 1C).

Actions of LVH on excitatory glutamatergic neurotransmission to CVNs

Spontaneous excitatory synaptic currents (EPSCs) were different in CVNs from the NA and DMNX. DMNX CVNs had EPSCs with a larger amplitude than CVNs in the NA (amplitude of EPSCs in DMNX CVNs 32.3 ± 1.1 pA, NA CVN amplitude 17.6 ± 2.8 pA, p<0.001), and DMNX CVNs had a higher frequency of EPSCs (3.4 ± 0.3 Hz in DMNX CVNs compared to 1.1 ± 0.3 Hz in NA CVNs, p<0.001).

Animals with LVH had a significantly diminished frequency of EPSCs, compared to sham animals, in CVNs from both the NA and DMNX. In the DMNX CVNs EPSC frequency was significantly diminished from 3.4 ± 0.3 to 0.8 ± 0.1 Hz (p<0.01). In CVNs from the NA EPSC frequency was blunted from 1.1 ± 0.3 to 0.4 ±0.07 Hz (p<0.05), see figure 2. Animals with LVH also had significantly decreased amplitudes of EPSCs in CVNs in the DMNX from 32.3 ± 1.1 pA to 24.3 ± 1.7 pA, but the amplitude of EPSCs in the NA were not different in LVH and sham animals.

Actions of LVH on inhibitory neurotransmission to CVNs
In sham animals spontaneous GABAergic inhibitory post-synaptic currents (IPSCs) in CVNs from the NA had significantly lower amplitudes (28 ± 2 pA in NA, 47 ± 5 pA in DMNX, p<0.001) but a higher frequency of GABAergic IPSCs (7.3 ± 1.3 Hz in NA, 2.7 ± 0.5 Hz in DMNX, p<0.01), than CVNs from the DMNX. Spontaneous glycinergic IPSCs were not significantly different, in amplitude or frequency, in CVNs from the NA and DMNX in sham animals, see figure 3.

LVH animals did not differ from sham animals in the frequency of IPSCs in NA CVNs. However in CVNs in the DMNX LVH animals had significantly augmented frequency of inhibitory events (from 6.0 ± 0.7 Hz in the sham animals to 8.4 ± 1.1 Hz in the LVH animals, p<0.05), see figure 4. To identify whether these changes were due to alterations in GABAergic or glycinergic neurotransmission, or both, glycinergic and GABAergic IPSCs were isolated for study in additional studies in LVH animals. LVH animals had no significant difference compared to sham animals in the frequency of glycinergic IPSCs in either the NA or DMNX CVNs. While LVH animals had no significant change in the amplitude, LVH animals had significantly (p<0.05) elevated frequency of GABAergic inhibitory events (from 2.7 ± 0.5 Hz in the sham animals to 5.3 ± 1.5 Hz in the LVH animals) in CVNs in the DMNX, see figure 4.

**Discussion**

In this study we identified two major changes that occur in the function of parasympathetic cardiac vagal neurons in animals with left ventricular hypertrophy secondary to pressure overload. As other studies have shown, this animal model elicits progressive hypertrophy 2 weeks after aortic banding, within 6 weeks there are increases in lung-to-body weight ratio (11), an index of HF, and this model progresses to end-stage heart failure with hypertrophy, dilation and systolic dysfunction, within 20 weeks (11, 32). The 2 key findings of this study are that within 6-8 weeks after aortic banding there is decreased excitation of CVNs.
by 1) elevated frequency of inhibitory GABAergic neurotransmission to CVNs in the DMNX, and
2) diminished frequency of excitatory neurotransmission to CVNs in both the NA and DMNX.
As LVH animals possessed an opposing alteration in IPSC and EPSC frequencies (increasing
and decreasing, respectively), it is highly likely that in LVH animals the activity of preceding
excitatory and inhibitory neurons and pathways that synapse upon CVNs are altered. While we
cannot rule out postsynaptic changes in CVN membrane properties in the LVH animals, our
results indicate the LVH animals have significantly altered excitatory and inhibitory GABAergic
(but not glycinergic) pathways to CVNs.

The sites of action of LVH on preceding neurons and pathways to CVNs are not known, however
there are two known major origins of inhibitory inputs to CVNs, one originates from the locus
coeeruleus, while the other originates from inspiratory neurons in the brainstem. As shown
previously, CVNs are inhibited during each inspiratory burst, and this cardiorespiratory network
function is the likely substrate for respiratory sinus arrhythmia as heart rate increases with each
inspiration (28). The second major source of inhibitory activity to CVNs originates in the locus
coeeruleus, a nucleus involved in inducing cortical arousal and orchestrating changes in
accompanying autonomic system function that compliments increased attention, such as during
stress, excitation and/or exposure to aversive or novel stimuli. Locus coeruleus noradrenergic
neurons depress the activity of cardioinhibitory parasympathetic cardiac vagal neurons by
polysynaptic activation of inhibitory neurotransmission within this brainstem autonomic and
attentiveness circuitry (38). This network interaction is dependent upon activation of α1
receptors that mediate increases in both GABAergic and glycinergic neurotransmission, while β1
receptor activation increases glycinergic, but not GABAergic neurotransmission to CVNs upon
locus coeruleus photoactivation (38).
Other work has shown there are four specific areas that contain GABAergic cells that monosynaptically project to CVNs; 3 of the 4 loci are in close apposition to the CVNs in the NA (200 microns medial, 400 microns medial, 200 microns ventral to the CVNs in the NA) and the fourth locus is in the nucleus of the solitary tract region close to CVNs in the DMNX (13). These 4 populations of GABAergic neurons were retained in the brainstem slice utilized in this study and are the probable source of the GABAergic neurons that directly project to CVNs that are facilitated in LVH animals.

There are 2 known major sources of excitatory input to CVNs, one originates from neurons in the nucleus tractus solitaries (NTS) (27), and the other is an excitatory pathway from neurons in the hypothalamic paraventricular nucleus (PVN) (9, 29, 30). The NTS receives primary information from cardiorespiratory sensory neurons and the excitatory pathway from the NTS to CVNs likely plays an essential role in the chemoreceptor and baroreceptor reflex control of heart rate (1). Retrograde tracing studies have demonstrated direct projections from the PVN to the NA (6, 22). This long range neurotransmission from the PVN is excitatory with the endogenous release of oxytocin facilitating glutamatergic neurotransmission and excitation of CVNs (30). This oxytocin pathway is most likely involved in the slowing of heart rate during periods of low vigilance as activation of oxytocin receptors reduces the adverse cardiovascular consequences of anxiety and stress (8, 16, 17).

While no other work has, to the best of our knowledge, examined how LVH induces blunting of brainstem parasympathetic activity, there has been considerable advances in understanding how LVH and HF augments neurons involved in sympathetic activity. The discharge rates of neurons likely involved in sympathetic activity within the dorsolateral periaqueductal gray neurons are augmented in HF as compared with control rats (42). Other work has shown changes in sympathetic neurons in the rostral ventrolateral medulla with upregulation of AT1R,
GRK5, and NF-κB expression (18), as well as increased firing activity of PVN neurons that were antidromically activated from the sympathetic target in the rostral ventrolateral medulla (RVLM) (43). The increased activity of PVN pre-sympathetic neurons with HF may be due to many causes including diminished endothelial NOS expression and NOS-derived NO availability in the PVN (3), enhanced expression of chemokines (such as chemokine stromal cell-derived factor 1) (40), proinflammatory cytokines and angiotensin II type-1 receptors in the PVN in HF animals (46), as well as a reduction in the frequency of spontaneous inhibitory postsynaptic currents in RVLM-projecting PVN neurons (19).

How LVH augments the inhibitory pathways to CVNs, either originating from the preceding locus coeruleus or inspiratory neurons, or via the inhibitory GABAergic neurons themselves, is not known. Likewise, LVH may inhibit the excitatory neurotransmission originating from the NTS, PVN or both pathways that provides excitation to CVNs. Potential candidates for selectively restoring parasympathetic activity to the heart include identifying parameters to selectively stimulate parasympathetic cardiac vagal fibers with vagal nerve stimulators, blunting the activity of neurons in the locus coeruleus that inhibit CVNs, and preferentially augmenting the excitatory oxytocin/glutamate pathway from the PVN to CVNs. These sites and neurotransmitter receptors provide future targets of intervention in the goal to restore parasympathetic activity to the heart in left ventricular hypertrophy, heart failure, hypertension and other cardiovascular diseases with imbalanced autonomic activity.
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Figure 1: TAC induced changes in heart morphology and weight. (A) Representative hearts and LV cross sections 8 weeks after sham (upper panels, 8 animals) or TAC (lower panels, 10 animals) surgery. (Bi) Body weight and (Bii) heart weight of sham and TAC rats. The horizontal black line represents the median and the box defines the interquartile range. (Ci) LV free wall thickness normalized to whole body weight and (Cii) heart weight and LV weight normalized to whole body weight in sham (grey) and TAC (black) rats. In C, values are mean ± SEM. *significantly different than sham.

Figure 2: Spontaneous excitatory postsynaptic currents in CVNs. Spontaneous EPSC frequency was diminished in CVNs, in both the NA and DMNX, in LVH (n=8) animals compared to sham (n=5) animals. EPSC amplitude was significantly diminished in DMNX CVNs from LVH animals (n=8) compared to sham animals (n=5). Representative traces are shown in the top traces, average results are shown below. *denotes p<0.05, *** denotes p<0.001 when comparing LVH to sham animals.

Figure 3: GABAergic and glycinergic inhibitory postsynaptic currents in CVNs. Spontaneous GABAergic IPSCs in CVNs from the DMNX were significantly greater in amplitude and lower in frequency than GABAergic IPSCs in CVNs in the NA (8 DMNX neurons from 5 animals, 13 NA neurons from 5 animals). Glycinergic IPSCs were not different, in amplitude or frequency, when comparing CVNs from the NA and DMNX (9 DMNX neurons from 5 animals, 6 NA neurons from 5 animals). Representative traces are shown in the top traces, average results are shown below. **denotes p<0.01, *** denotes p<0.001 when comparing DMNX to NA CVNs.

Figure 4: LVH animals had an exaggerated IPSC frequency in DMNX CVNs (11 neurons from 5 animals) compared to sham animals (12 neurons from 5 animals). Spontaneous IPSCs,
and isolated GABAergic and glycinergic IPSCs are shown in the top 3 rows. Average results are
shown in the bar graphs below. LVH had no effect on glycinergic IPSCs (9 sham and 12 LVH
neurons from 5 LVH and 5 sham animals), but LVH animals had a significantly augmented
GABAergic IPSC frequency 8 sham and 7 LVH neurons from 7 sham animals and 5 LVH
animals). * denotes p< 0.05 when comparing LVH to sham animals.
Sham animals vs. LVH animals

EPSCs in NA CVNs

EPSCs in DMNX CVNs

EPSC frequency (Hz)

NA CVNs | DMNX CVNs

EPSC amplitude (pA)

Sham | LVH

Sham | LVH

* | ***
GABAergic IPSC amplitude (pA)

Glycinergic IPSC amplitude (pA)

GABAergic IPSC frequency (Hz)

Glycinergic IPSC frequency (Hz)
Sham animal LVH animal

IPSCs

GABAergic IPSCs

Glycinergic IPSCs

IPSC frequency (Hz)

Total IPSCs

GABAergic IPSCs

Glycinergic IPSCs

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