Neurotransmission to parasympathetic cardiac vagal neurons in the brain stem is altered with left ventricular hypertrophy-induced heart failure

Edmund Cauley,1* Xin Wang,1* Jhansi Dyavanapalli,1 Ke Sun,1 Kara Garrott,2 Sarah Kuzmiak-Glancy,2 Matthew W. Kay,2 and David Mendelowitz1

1Department of Pharmacology and Physiology, The George Washington University, Washington, District of Columbia; and 2Department of Biomedical Engineering, The George Washington University, Washington, District of Columbia

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Cauley E, Wang X, Dyavanapalli J, Sun K, Garrott K, Kuzmiak-Glancy S, Kay MW, Mendelowitz D. Neurotransmission to parasympathetic cardiac vagal neurons in the brain stem is altered with left ventricular hypertrophy-induced heart failure. Am J Physiol Heart Circ Physiol 309: H1281–H1287, 2015. First published September 14, 2015; doi:10.1152/ajpheart.00445.2015.—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 brain stem to diminish 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 brain stem 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 glycinergic activity) as well as a 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.

HYPERTENSION, cardiac hypertrophy, and heart failure (HF) are widespread and debilitating cardiovascular diseases that affects nearly 23 million people worldwide with ~2 million new patients diagnosed annually (15). Cardiac rhythm disturbances lead to sudden cardiac death in 40–50% of advanced HF patients with a 1-yr mortality rate of >50% (10). A distinctive hallmark of cardiac hypertrophy, HF, and the 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 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 on 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 brain stem. 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 HF, augmentation of diminished cardiac vagal activity prevents arrhythmias, decreases the risk of sudden death, and protects against ischemia-reperfusion injury (7, 12, 20, 24, 25, 31, 36, 37, 39, 41). 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 brain stem to diminish parasympathetic cardiac activity, left ventricular (LV) hypertrophy (LVH) was elicited in rats by aortic pressure overload using a transaortic constriction (TAC) approach, and the neurobiology of CVNs was studied with patch-clamp electrophysiological approaches. The goal of the present study was to identify the changes within the brain stem that are likely responsible for diminishing parasympathetic

* E. Cauley and X. Wang contributed equally to this work.

Address for reprint requests and other correspondence: D. Mendelowitz, Dept. of Pharmacology and Physiology, The George Washington Univ., 2300 Eye St. NW, Washington, DC 20037 (e-mail: dmendel@gwu.edu).

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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.

METHODO

Ethical approval. All animal procedures carried out were in accordance with The George Washington University institutional guidelines and in compliance with recommendations of the panel of Euthanasia of the American Veterinary Medical Association and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). The minimal number of animals was used, and care was taken to reduce any possible discomfort.

Aortic banding to induce HF. LVH secondary to pressure overload was produced in male Sprague-Dawley rats using a minimally invasive TAC approach previously established for mice (21, 35). Animals at 4–5 wk of age were subjected to either TAC or sham aortic banding surgery. A 1- to 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 euthanized. Sham-operated animals underwent the same surgical procedure except that the suture was not tied.

After 6–8 wk, animals were euthanized, and the degree of LVH 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 ImageJ software (NIH). The “free wall” measurement was defined as the distance from the closest point on the inside of the LV 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 LV to the closest point on the inside of the right ventricle. Weights of the whole heart and LV 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 animals were at postnatal days 2–5 (Sprague-Dawley, Hilltop Laboratory animals, Scottsdale, PA). Animals were anesthetized using hypothermia by cooling to ~4°C. A right thoracotomy was performed, and retrograde tracer X-rhoda mine-5-(and-6)-isothiocyanate (Invitrogen) was then injected into the fat pads at the base of the heart to retrogradely label CVNs (26). Animals were then allowed to recover until they were 4–5 wk of age and then underwent either sham or TAC surgery.

In vitro brain stem slice preparation. We adopted the methodology from Ye and colleagues (45) to obtain viable brain stem slices from 10- to 12-wk-old animals. According to this method, glycerol base artificial cerebrospinal fluid (aCSF) was used for cardiac perfusion and brain stem slicing. Glycerol-based aCSF contained (in mM) 252 glycerol, 1.6 KCl, 1.2 NaH2PO4, 1.2 MgCl2, 2.4 CaCl2, 26 NaHCO3, and 11 glucose. Rats were anesthetized 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 onto a stage using 2% low-melt agarose, and placed in a vibrotome containing glycerol aCSF. Brain stem slices (330-μm thickness), containing either the DMNX or NA, were obtained and briefly placed in a solution of the 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% O2-5% CO2 (pH 7.4) at 34°C for 15 min. NMDG-based aCSF was used to help slices recover and to maintain viable brain stem slices for electrophysiological recordings (47). Slices were then mounted in a recording chamber constantly perfused with normal aCSF of the following composition (in mM): 125 NaCl, 3 KCl, 2 CaCl2, 26 NaHCO3, 5 glucose,

Fig. 1. Transaortic constriction (TAC)-induced changes in heart morphology and weight. A: representative hearts and left ventricular (LV) cross sections 8 wk after sham (top, 8 animals) or TAC (bottom, 10 animals) surgery. B: body weight (i) and heart weight (ii) of sham and TAC rats. The horizontal black line represents the median, and the box defines the interquartile range. C: LV free wall thickness normalized to whole body weight (i) and heart weight and LV weight normalized to whole body weight (ii) in sham and TAC rats. In C, values are means ± SE. *Significantly different than sham animals.
and 5 HEPES and oxygenated with 95% O₂-5% CO₂ (pH 7.4) and allowed to recover for at least 30 min before an experiment was performed.

**Electrophysiological recordings.** CVNs in the NA and DMNX were identified by the presence of the 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) at a holding voltage of −80 mV at room temperature. Patch pipettes (2.5–5 MΩ) were filled with solution consisting of (in mM) 150 KCl, 4 MgCl₂, 10 EGTA, 2 Na-ATP, and 10 HEPES or 150 K-gluconic acid, 10 HEPES, 10 EGTA, 1 MgCl₂ and 1 CaCl₂ at pH 7.3 for recording inhibitory or excitatory events, respectively.

GABAergic inhibitory postsynaptic currents (IPSCs) were isolated by the application of solution containing strychnine (1 μM, a glycine receptor antagonist), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 50 μM, a non-NMDA receptor antagonist), and d-2-amino-5-phosphonovalerate (AP5; 50 μM, a NMDA receptor antagonist). Glycinergic IPSCs were isolated by including gabazine (25 μM, a GABA-A receptor antagonist), CNQX, and AP5 in the perfusate. The perfusate included gabazine and strychnine (25 and 1 μM, respectively) to isolate glutamatergic excitatory postsynaptic currents (EPSCs). Gabazine, strychnine, CNQX, and AP5 were obtained from Sigma-Aldrich (St. Louis, MO).

**Data analysis.** Synaptosoft software (version 6.0.3, Synaptosoft, Decatur, GA) was used to analyze synaptic events recorded from CVNs. The threshold value was set to the root mean square of noise levels multiplied by 5. Data are presented as means ± SE. Student’s unpaired t-test was used to compare the statistical significance between sham and cardiac hypertrophy groups using Graphpad Prism 5 software (La Jolla, CA). P values of <0.05 were considered significant. In the figures, *P < 0.05, **P < 0.01, and ***P < 0.001.

**RESULTS**

TAC resulted in LVH, consistent with a previous report in rats (44). Hearts from TAC animals were significantly heavier than hearts from control animals and had thicker LVs (Fig. 1A). Animal weight did not differ between TAC and control animals (Fig. 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 with control animals (Fig. 1C).

**Actions of LVH on excitatory glutamatergic neurotransmission to CVNs.** Spontaneous 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 and amplitude of EPSCs in NA CVNs: 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 with 1.1 ± 0.3 Hz in NA CVNs, P < 0.001).

Animals with LVH had a significantly diminished frequency of EPSCs compared with sham animals in CVNs from both the
NA and DMNX. In DMNX CVNs, the EPSC frequency was significantly diminished from $3.4 \pm 0.3$ to $0.8 \pm 0.1$ Hz ($P < 0.01$). In CVNs from the NA, the EPSC frequency was blunted from $1.1 \pm 0.3$ to $0.4 \pm 0.07$ Hz ($P < 0.05$; Fig. 2). Animals with LVH also had significantly decreased amplitudes of EPSCs in CVNs in the DMNX from $32.3 \pm 1.1$ to $24.3 \pm 1.7$ 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 IPSCs in CVNs from the NA had significantly lower amplitudes (28 $\pm$ 2 pA in the NA and 47 $\pm$ 5 pA in the DMNX, $P < 0.001$) but a higher frequency of GABAergic IPSCs (7.3 $\pm$ 1.3 Hz in the NA and 2.7 $\pm$ 0.5 Hz in DMNX, $P < 0.01$) than CVNs from the DMNX. Spontaneous glycine IPSCs were not significantly different, in amplitude or frequency, in CVNs from the NA and DMNX in sham animals (Fig. 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 a significantly augmented frequency of inhibitory events (from $6.0 \pm 0.7$ Hz in sham animals to $8.4 \pm 1.1$ Hz in LVH animals, $P < 0.05$; Fig. 4). To identify whether these changes were due to alterations in GABAergic or glycine IPSCs were isolated for study in additional experiments in LVH animals. LVH animals had no significant difference compared with sham animals in the frequency of glycine IPSCs in either NA or DMNX CVNs. Whereas LVH animals had no significant change in the amplitude, LVH animals had a significantly ($P < 0.05$) elevated frequency of GABAergic inhibitory events (from $2.7 \pm 0.5$ Hz in sham animals to $5.3 \pm 1.5$ Hz in LVH animals) in CVNs in the DMNX (Fig. 4).

**Fig. 3.** GABAergic and glycine inhibitory postsynaptic currents (IPSCs) 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 and 13 NA neurons from 5 animals). Glycine IPSCs were not different, in amplitude or frequency, when CVNs were compared from the NA and DMNX (9 DMNX neurons from 5 animals and 6 NA neurons from 5 animals). Representative traces are shown at the top; average results are shown at the bottom. **$P < 0.01$ and ***$P < 0.001$ when DMNX CVNs were compared with NA CVNs.
DISCUSSION

In the present study, we identified two major changes that occur in the function of parasympathetic cardiac vagal neurons in animals with LVH secondary to pressure overload. As other studies have shown, this animal model elicits progressive hypertrophy 2 wk after aortic banding; within 6 wk, there are increases in the lung-to-body weight ratio (11), an index of HF, and this model progresses to end-stage HF with hypertrophy, dilation, and systolic dysfunction within 20 wk (11, 32). The two key findings of the present study are that within 6–8 wk after aortic banding, there is decreased excitation of CVNs by 1) an elevated frequency of inhibitory GABAergic neurotransmission to CVNs in the DMNX and 2) a 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 on CVNs are altered. While we cannot rule out postsynaptic changes in CVN membrane properties in LVH animals, our results indicate 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 coeruleus, whereas the other originates from inspiratory neurons in the brain stem. As previously shown, CVNs are inhibited during each inspiratory burst, and this cardiorespiratory network function is the likely substrate for respiratory sinus arrhythmia as the heart rate increases with each inspiration (28). The second major source of inhibitory activity to CVNs originates in the locus coeruleus, 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 brain stem autonomic and attentiveness circuitry (38). This network interaction is dependent on activation of α1-receptors that mediate increases in both GABAergic and glycinergic neurotransmission, whereas 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: three of the four loci are in close apposition to CVNs in the NA (200 μm medial, 400 μm medial, and 200 μm ventral to CVNs in the NA), and the fourth locus is in the nucleus of the solitary tract (NTS) region close to CVNs in the DMNX (13). These four populations of GABAergic neurons were retained in the brain stem slice preparation used in this study from inspiratory neurons in the brain stem. As previously shown, CVNs are inhibited during each inspiratory burst, and this cardiorespiratory network function is the likely substrate for respiratory sinus arrhythmia as the heart rate increases with each inspiration (28). The second major source of inhibitory activity to CVNs originates in the locus coeruleus, 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 brain stem autonomic and attentiveness circuitry (38). This network interaction is dependent on activation of α1-receptors that mediate increases in both GABAergic and glycinergic neurotransmission, whereas β1-receptor activation increases glycinergic, but not GABAergic, neurotransmission to CVNs upon locus coeruleus photoactivation (38).
and are the probable source of the GABAergic neurons that directly project to CVNs that are facilitated in LVH animals.

There are two known major sources of excitatory input to CVNs: one originates from neurons in the 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 brain stem parasympathetic activity, there has been considerable advances in understanding how LVH and HF augment 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 compared with control rats (42). Other work has shown changes in sympathetic neurons in the rostral ventrolateral medulla with upregulation of ANG II type 1 receptors, G protein-coupled receptor kinase 5, 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 (43). The increased activity of PVN presynaptic neurons with HF may be due to many causes, including diminished endothelial nitric oxide synthase expression and nitric oxide synthase-derived nitric oxide availability in the PVN (3), enhanced expression of chemokines (such as chemokine stromal cell-derived factor 1) (40), and proinflammatory cytokines and ANG II type 1 receptors in the PVN in HF animals (46) as well as a reduction in the frequency of spontaneous IPSCs in rostral ventrolateral medulla-projecting PVN neurons (19).

How LVH augments the inhibitory pathways to CVNs, either originating from the preceding locus coeruleus or inhibitory neurons or by 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 LVH, HF, hypertension, and other cardiovascular diseases with an imbalance in autonomic activity.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**

HEART FAILURE ALTERS SYNAPTIC INPUT IN CARDIAC VAGAL NEURONS


