Activation of Oxytocin Neurons Improves Cardiac Function in a Pressure-Overload Model of Heart Failure

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HIGHLIGHTS

- Hypothalamic OXT neurons were chronically activated using a chemogenetic approach in an animal model of HF.
- Synaptic release of OXT onto parasympathetic autonomic targets was reduced in animals with HF but restored with daily treatment consisting of activation of OXT neurons.
- Long-term daily OXT neuron activation increased parasympathetic activity to the heart and reduced mortality, cardiac inflammation, and fibrosis and improved critical longitudinal in vivo indices of cardiac function.
- The benefits in cardiac function and autonomic balance in HF closely tracked the study-designed differences in initiation of OXT neuron activation in different groups.
SUMMARY

This work shows long-term restoration of the hypothalamic oxytocin (OXT) network preserves OXT release, reduces mortality, cardiac inflammation, fibrosis, and improves autonomic tone and cardiac function in a model of heart failure. Intranasal administration of OXT in patients mimics the short-term changes seen in animals by increasing parasympathetic—and decreasing sympathetic—cardiac activity. This work provides the essential translational foundation to determine if approaches that mimic paraventricular nucleus (PVN) OXT neuron activation, such as safe, noninvasive, and well-tolerated intranasal administration of OXT, can be beneficial in patients with heart failure. (J Am Coll Cardiol Basic Trans Science 2020;5:484–97) © 2020 The Authors.

The potential benefit of long-term OXT network activation in protracted cardiovascular diseases has not been tested. Heart failure (HF) affects nearly 23 million people worldwide, and prevalence is projected to increase 46% in the next 15 years (5). Approximately 50% of patients diagnosed with HF die within 5 years, necessitating the development of new treatments (5). A hallmark of HF is elevated cardiac sympathetic activity and parasympathetic withdrawal (6,7), an imbalance that contributes to ventricular dysfunction, structural remodeling, and electrical instability (8). In the initial stages of HF, parasympathetic tone decreases as early as 3 days after the development of cardiac dysfunction, typically preceding increases in sympathetic activity (9,10).

Here, using OXT-sensitive sniffer cells, we test the hypothesis that endogenous release of OXT from hypothalamic PVN neurons onto brainstem autonomic targets is blunted in an animal model of HF. Furthermore, we examine if chronic chemogenetic activation of PVN OXT neurons restores OXT release and if novel treatment paradigms of restoring OXT neuron activity, initiated at different intervals during the disease-progression timeline, reduces mortality, cardiac inflammation and fibrosis, and longitudinal indices of cardiac dysfunction compared with untreated animals with HF.

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METHODS

ETHICAL APPROVAL. All animal procedures were completed in agreement with the George Washington University institutional guidelines and in compliance with 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.

SURGICAL PROCEDURE FOR TRANSASCENDING AORTIC CONstriction. Pressure overload induced left-ventricular (LV) hypertrophy was initiated in male Sprague-Dawley rats using a minimally invasive transascending aortic constriction (TAC) procedure, similar to our previous study (11). Rats at 1 week of age were anesthetized by hypothermia and underwent TAC surgery. A 0.5-cm incision was made at the level of the chest, the chest was opened, and the thymus was retracted to reveal the aorta. A 4-0 silk suture was passed around the ascending aorta, and— with a 25-gauge needle temporarily placed adjacent to the aorta—the suture was tied around both the aorta and needle (TAC and TAC + OXT groups). The needle was then removed, leaving the constricting suture around the aorta. Buprenorphine was applied as an analgesic. Successful constriction was confirmed with high aortic-velocity post constriction, using high-resolution echocardiography as well as upon examination of the aorta after each animal was killed.

SELECTIVE EXPRESSION AND ACTIVATION OF ChR2 AND DREADDs IN PVN OXT NEURONS. Selective expression of both channelrhodopsin (ChR2) and an excitatory chemogenetic receptor for chronically activating PVN OXT neurons, designer receptors exclusively activated by designer drugs (DREADDs), was accomplished using 3 viral vectors in combination with the Cre-Lox system (NIH, Bethesda, MD). Expression of the enzyme Cre recombinase was exclusively driven by the OXT promoter (rAAV1-OXT-Cre), stereotactically co-injected into the PVN of 1-week old pups with both floxed excitatory ChR2 (AAV1-EF1a-DIO-hChR2) and floxed excitatory DREADDs (AAV2-hSyn-DIO-hM3D(Gq), (30 to 50 nl of each virus selectively microinjected over a 20-min period), as previously described (12).

ACTIVATION OF PVN OXT NEURONS IN VIVO. PVN OXT neurons expressing DREADDs were exclusively activated by clozapine-N-oxide (CNO), 1 mg/kg intraperitoneally (IP). Our previous work has demonstrated that injections of CNO increases the firing of PVN OXT neurons for at least 1 h (12).

IN VIVO ASSESSMENTS OF CHANGES IN AUTONOMIC TONE AND CARdIAC FUNCTION. Sprague-Dawley rats at 2 weeks post-TAC/Sham surgery were anesthetized (isoflurane) and implanted with a telemetry device (DSI wireless transmitters, ETA-F10 [Data Sciences International, St. Paul, Minnesota]) with electrocardiographic (ECG) leads inserted subcutaneously to measure heart rate (HR). The following protocol was used to measure peak effort capacity, defined as the time that animals are able to run on the treadmill and the speed at time of exhaustion. Animals began with an initial warm-up period of 5 min at 6 cm/s with 1 min of recuperation. The treadmill speed then quickly ramped up to 12 cm/s and increased by 6 cm/s every 3 min until exhaustion. Heart rate recovery (HRR) was assessed by subtracting the instantaneous HR following 20 s of recovery from that at peak exercise (Naughton protocol).

Echocardiographic measurements were obtained using a VisualSonics high-resolution small-animal system (Vevo 3100 Imaging System, FUJIFILM VisualSonics Inc, Toronto, Ontario, Canada) that continuously acquired the ECG, respiratory waveform, and body temperature. Imaging was performed at frame rates up to 1KHz, using 25MHz linear array transducer to obtain 2-dimensional (2D) pulse and continuous-wave Doppler and Motion-mode (M-mode) imaging. A 2D parasternal long-axis view of the LV was used to obtain LV outflow tract diameter and cardiac functional measures including percent LV ejection fraction (EF %). M-mode images were obtained from the parasternal short-axis view of the LV, at the level of papillary muscles to measure LV structural variables including LV diastolic posterior-wall diameter (LVPWD), interventricular septal diameter (IVSD), LV diameter in diastole (LVDd) and systole (LVDs), and LV systolic posterior-wall (LVPWs) thickness. These parameters were used to calculate percent of fractional shortening (FS). Pulse-wave Doppler imaging in an apical 4-chamber view was used to obtain inflow velocities through mitral valves for assessing diastolic function, including early diastolic mitral inflow (E-wave), late diastolic mitral inflow (A-wave), and early-to-late diastolic mitral inflow ratio (E/A ratio). Continuous-wave Doppler modality was used to measure functional variables, such as velocity and pressure of blood flow in the ascending and descending aorta, and HR and cardiac output.

EX VIVO ASSESSMENTS OF CARDIAC FUNCTION. At 16 weeks post-Sham/TAC, rats were anesthetized with an IP injection of pentobarbital (50 mg/kg) and isoflurane inhalation anesthesia. Following cessation of pain reflexes, hearts were rapidly excised and Langendorff perfused via the aorta at constant pressure (65 mm Hg) and temperature (37°C [98.6°F]) with a
Krebs-Henseleit solution containing (in mM) 118 NaCl, 4.7 KCl, 1.25 CaCl₂, 0.57 MgSO₄, 1.17KH₂PO₄, 25 NaHCO₃, and 6.0 glucose. Perfusate was oxygenated with 95% O₂-5% CO₂. After a stabilization period of 10 min, a size-5 balloon (Harvard Apparatus, Holliston, MA) was inserted into the LV to measure isovolumic LV-developed pressure (LVDP), as we have previously described (13,14). Diastolic pressure was set to 7 mm Hg, and LVDP was computed as the difference between systolic and diastolic pressures. HR and LVDP were measured for at least 15 min during sinus rhythm. After each study, contractility and relaxation were calculated as the maximum and minimum values of the first derivative of the LVDP waveform, respectively, to assess inotropy and lusitropy.

**ANATOMIC MEASUREMENTS AND HISTOLOGY.** Hearts from ex vivo studies were sliced longitudinally, halfway between the anterior and posterior sides. One-half of the heart was preserved in 10% formalin and stained with Masson’s trichrome to visualize collagen deposition.

**WESTERN BLOTTING.** Hearts from ex vivo studies were sliced longitudinally, halfway between the anterior and posterior sides. LV tissue from one-half
FIGURE 2  Long-Term Chemogenetic Activation of PVN Oxytocin Neurons Attenuates Cardiac Dysfunction Seen in Animals With Heart Failure

A  Sham (6)  TAC saline (6)  TAC CNO (5)

B  Early Treatment (6)  Late Treatment (7)

C  SV (microL)

D  % EF

E  CO (mL/min)

F  % FS

Continued on the next page
of the heart were flash frozen in liquid nitrogen and stored at -80°C (-112°F). Samples were thawed and homogenized using the Qproteome Mammalian Protein Prep Kit (QIAGEN, Hilden, Germany) in tubes containing metallic beads. Samples were centrifuged at 14,000 g for 10 min, and protein concentrations were determined by the Pierce BCA Protein Assay (Thermo Fisher Scientific, Waltham, Massachusetts). Laemmli buffer (Bio-Rad, Hercules, California) with 10% 2-mercaptoethanol (Sigma-Aldrich, St. Louis, Missouri) was added to the samples, which were then heated to 98°C (208.4°F). Equal protein concentration was loaded into wells containing 4% to 15% PROTEAN or Criterion TGX Gels (Bio-Rad). The samples were then run between 50V to 100V for up to 2 h to separate proteins by electrophoresis.

After electrophoresis, the samples were then transferred to polyvinylidene fluoride (PVDF) membranes, using the Trans-Blot Turbo Transfer System (Bio-Rad). The membranes to be probed for collagen III (Abcam, Cambridge, United Kingdom) were blocked with 5% milk for 18 h and then incubated for 1 h at room temperature with collagen III, at a concentration of 1:1,000. The membranes to be probed for interleukin(IL)-1β were blocked with 5% bovine serum albumin (BSA) for 2 h. They were then incubated with IL-1β (Cell Signaling Technology, Danvers, Massachusetts) at a concentration of 1:1,000 overnight at 4°C (39.2 °F). All membranes were washed and incubated for 1 h with the horseradish peroxidase (HRP)-conjugated secondary antibodies anti-rabbit 1:6,000 and anti-mouse 1:6,000 (Santa Cruz Biotechnology, Dallas, Texas). The membranes were washed again, and gels were imaged using an Azure Cseries c600 (VWR, Radnor, Pennsylvania) gel imager. The membranes were stripped with a mild stripping buffer containing 0.2 M glycine (Sigma-Aldrich), 3.5 mM sodium dodecyl sulfate (Bio-Rad), and 1% Tween 20 (Sigma-Aldrich). They were then reblocked with 5% milk for 19 h and incubated with glycerol-dehyde-3-phosphate dehydrogenase (GAPDH) antibody (Sigma-Aldrich) 1:6,000 for 1 h. The membranes were washed and incubated for 1 h with the anti-rabbit HRP-conjugated secondary antibody. Band intensities for collagen III and IL-1β were measured using NIH ImageJ or Li-Cor Image Studio (LI-COR Biosciences, Lincoln, Nebraska) and normalized to the corresponding band intensity for GAPDH; collagen III bands were normalized to Sham values as well as GAPDH.

Because of the large number of animals in each group, not all samples could be loaded on the same gel. We therefore ran 2 to 3 gels and included samples from each of the 3 groups (Sham, TAC, and TAC + OXT) on each gel and repeated selected samples on each gel. We then normalized signal intensity between membranes based on the repeated sample expression. This method provides consistent normalization of all samples both within each set and across the minimum number of required gels. Refer to Supplemental Figure 1 for more details.

**Statistical methods.** Data were presented as mean ± standard error mean and analyzed using GraphPad Prism (GraphPad, San Diego, California) statistical software (version 8). Longitudinal data were compared among all groups using mixed-effects model (restricted maximum likelihood method) with Tukey’s postmultiple comparison test. For survival analysis, Kaplan-Meier curves were plotted and compared, using the log-rank test. One-way analysis of variance (ANOVA) was used to compare in vitro heart data statistically from Langendorff and Western-blot studies. A p value of ≤0.05 was considered statistically significant.

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**Figure 2 Continued**

(A) The top panel depicts high-resolution 2D echocardiograms in parasternal longitudinal-axis B mode taken from 16-week post-Sham, TAC, early activation of DREADDs-expressing PVN oxytocin neurons (initiated at 4 weeks post-TAC), and late treatment (starting at 6 weeks post-TAC) animals showing left ventricle (LV) in mid-systole with directional vectors in green. Vertical scale bar represents 3 mm. The middle panel shows 2D echocardiograms in parasternal short-axis M-mode at 16 weeks post-Sham, TAC, early- and late-treatment animals, used to measure the LV posterior-wall thickness and interventricular septal-wall thickness, LV internal diameter, and percent fractional shortening. Vertical and horizontal scale bar represents 3 mm and 0.1 sec, respectively. The bottom panel displays pulsed Doppler measurements of descending aortic flow velocities taken immediately after TAC point in Sham, TAC, and treatment animals. (B) Quantitative longitudinal Doppler descending aortic flow velocities in Sham, TAC saline (expressing DREADDs), TAC CNO (not expressing DREADDs), early- and late-treatment animals taken biweekly from 2 to 16 weeks post-Sham/TAC surgery. The velocities were 3-fold greater in TAC and treatment animals compared with Sham animals. Graphs displaying longitudinal echocardiographic measurements of stroke volume (C), percent ejection fraction (D), cardiac output (CO) (E) from parasternal longitudinal axis B-mode and percent fractional shortening from parasternal short-axis M-mode measured biweekly from 2 to 16 weeks post-Sham, TAC saline (expressing DREADDs), TAC CNO (not expressing DREADDs), and early- and late- treatment animals. Data were analyzed using mixed-effects model with Tukey’s multiple comparison post-test. Sham (n = 6), TAC (n = 6); early treatment (n = 6), late treatment (n = 7); TAC CNO (n = 5), *Sham vs. TAC; †TAC vs. early treatment; ‡TAC vs. late treatment; ‡‡Sham vs. early treatment; ‡‡Sham vs. late treatment; *p < 0.05; †p < 0.01; ‡‡p < 0.001. CNO, dizopamine-N-oxide, DREADDs, designer receptors exclusively activated by designer drugs; PVN, paraventricular nucleus of the hypothalamus; TAC, transascending aortic constriction.
FIGURE 3  Long-Term Activation of PVN Oxytocin Neurons Improves Heart Rate Recovery and Survival Rates in Animals With Heart Failure

A

![Graph A]

B

![Graph B]

C

![Graph C]
RESULTS

To test the hypothesis that synaptic release of OXT from hypothalamic OXT neurons at downstream autonomic targets is reduced in an animal model of HF, we quantified OXT release using engineered sniffer cells sensitive to OXT, in combination with photoactivation of ChR2 selectively expressed in PVN OXT neurons. Chinese hamster ovary (CHO) cells that expressed both OXT receptors and the red fluorescent gene OXT release using engineered

FIGURE 3 Continued

(A) Longitudinal weekly measurements of heart rate recovery at 20-s post-treadmill stress test in Sham, TAC saline (expressing DREADDs), TAC CNO (not expressing DREADDs), and early- and late-treatment animals from 4 to 16 weeks post-Sham/TAC surgery. All groups of animals expressing DREADDs in PVN oxytocin neurons were implanted with DSI ETA-F10 telemetry devices at 2 weeks post-TAC to measure electrocardiography and heart rate. Excitatory DREADDs activation was achieved by intraperitoneal injections of CNO (1 mg/kg) starting at 4 weeks post-TAC in the early-treatment group and 6 weeks post-TAC in the late-treatment group until 16 weeks post-TAC; Sham and TAC saline animals received saline injections. Heart rate recovery (HRR) was calculated as the heart rate at peak exercise minus 20-s recovery postexercise. TAC animals displayed significantly lower HRR from 5 to 16 weeks post-TAC compared with Sham animals. Early treatment significantly improved HRR at 7 to 11 and 16 weeks post-TAC compared with TAC animals. Data were analyzed using mixed-effects model with Tukey’s multiple comparison post-test. Sham (n = 7); TAC (n = 9); early treatment (n = 9); late treatment (n = 7); TAC CNO (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001. (B) Graph depicting changes in heart rate (beats per minute) in response to intraperitoneal injections of saline in TAC animals and CNO (1 mg/kg) in treatment animals from 6 to 16 weeks post-TAC/Sham. A significant reduction in heart rate was seen in all treatment animals at all weeks compared with saline-treated animals. Numbers in parentheses represent number of animals. Data were analyzed using mixed-effects model with Tukey’s multiple comparison post-test; *p < 0.05. (C) Kaplan-Meier curves depicting percent survival in Sham, combined TAC saline (expressing DREADDs), and TAC CNO (not expressing DREADDs) early- and late-treatment animals. TAC animals had significantly reduced survival compared with Sham, whereas early and late treatment significantly improved survival rates in animals with heart failure. Data were analyzed using log-rank test of survival analysis; overall significance is represented as **p < 0.01. CNO – clozapine-N-oxide; DREADDs – designer receptors exclusively activated by designer drug; PVN – paraventricular nucleus of the hypothalamus; TAC – transascending aortic constriction.
FIGURE 4  Hearts From TAC Animals With PVN OXT Neuron Activation Had Improved LV Function and Less Collagen III and IL-1β Expression Compared with Untreated TAC Animals

A

B

C

D

E

F

Continued on the next page
of OXT in the DMNX was lowest at 6 weeks post-TAC and cardiac dysfunction and mortality had been established (Figures 2 and 3). We examined longitudinal indices of cardiac function beginning at 2 weeks post-TAC, using high-resolution echocardiography (echo) in Sham, TAC (saline), TAC (CNO), and TAC + PVN OXT-activated animals. As expected, aortic velocity was 3-fold higher in each of the animal groups with TAC (TAC [saline], TAC [CNO], early and late treatments) compared with Sham animals (Figure 2B). Importantly, the degree of aortic narrowing, quantified by the increase in aortic velocity immediately downstream of the constriction, was not different in either of the TAC groups and treatment groups (Figure 2B). Indices of cardiac function—including stroke volume, EF, cardiac output, and FS—indicate preserved function with PVN OXT neuron treatment (early or late) compared with TAC (Figures 2C to 2F).

The benefit in cardiac function closely followed the timed initiation of PVN OXT neuron activation. In animals in which PVN oxytocin neurons were activated at 4 weeks, at the initial stage of disease initiation the benefit in cardiac function was observed beginning at the onset of treatment at 4 weeks post-TAC (Figure 2). Likewise, those animals that had late treatment (PVN OXT neuron-activated at 6 weeks post-TAC) when cardiac dysfunction was established, also showed benefits to cardiac function only beginning when treatment was started. From 8 to 16 weeks post-TAC, there were no significant differences in the improved cardiac function when comparing the 2 treatment groups, and both PVN OXT neuron-activated groups showed significant improvements in cardiac function compared with the untreated TAC group.

HRR after peak effort capacity is a common clinical assessment of autonomic balance and risk of adverse cardiovascular events, with a lower HRR associated with depressed parasympathetic activity and increased mortality (17). To assess HRR in each of the 5 groups of animals, ECG wireless transmitters were implanted 2 weeks post-TAC to measure the ECG and HR immediately following—and 20 s after—peak exercise treadmill tests (Naughton protocol). HRR values were lowest in TAC animals and significantly greater in animals in which PVN OXT neurons were activated both early (at 4 weeks post-TAC) and late (6 weeks post-TAC) compared with untreated TAC animals (Figure 3A). We then tested whether the change in HR responses to either daily CNO delivery or long-term activation of PVN OXT neurons were preserved throughout the treatment regimen. As shown in Figure 3B, the increase in parasympathetic activity to the heart, assessed as decreases in HR upon activation of PVN OXT neurons, shown previously to be blocked by muscarinic receptor blockade (16), persisted each week during the entire 16-week duration of this study. We next examined if PVN OXT neuron treatment reduced mortality following TAC-induced HF. There were no fatalities in the Sham group of animals during the course of the 16-week study (Figure 3C). The survival rate in TAC animals 16 weeks post-TAC was 50% and was significantly increased to 66% and 70%, respectively, in the early- and late-treatment animals (Figure 3C).

To elucidate potential intracardiac mechanisms responsible for the improvement in cardiac function following PVN OXT neuron activation, the expression of collagen III and the inflammatory marker, IL-1β, was measured by Western-blot analysis at the end of the study (16 weeks post-TAC). In addition, the isovolumic contractile function of hearts excised from animals was assessed during normal sinus rhythm. As cardiac function in both early- and late-treatment groups were not significantly different from each other at the end of the study, both treatment groups were combined for further analysis.

As expected, IL-1β expression was significantly higher in TAC animals compared with Sham-operated animals. PVN OXT treatment significantly attenuated...
this increase (Figure 4D, Supplemental Figure 1). Collagen III was likewise significantly higher in TAC animals than in Sham animals, and the expression of this fibrotic protein was significantly blunted in PVN OXT-treated animals (Figure 4E, Supplemental Figure 1). Trichrome histological sections suggest increased fibrosis in all disease groups compared with Sham; however right-ventricular wall thinning was more apparent in TAC animals compared with TAC treatment groups (Figure 4F). Cardiac function in excised hearts from these animals was examined using a Langendorff preparation in which the heart was perfused via the aorta at constant pressure (65 mm Hg). A size-5 balloon (Harvard Apparatus) was inserted into the LV to measure isovolumic LVDP. Contractility and relaxation were measured as the maximum and minimum values of the first derivative of the LVDP waveform, respectively, to assess inotropy and lusitropy, as we have previously described (16). TAC significantly impaired LVDP, and PVN OXT treatment attenuated this dysfunction (Figure 4A). Similarly, cardiac contractility and relaxation (Figures 4B and 4C) were significantly compromised in TAC compared with Sham animals; however, Sham and PVN-treated animals did not vary significantly.

**DISCUSSION**

Parasympathetic activity to the heart originates in the brainstem and acts to reduce heart rate (18) and increase coronary flow (19,20). Our previous work has shown CVNs receive powerful excitation from a population of PVN OXT neurons that corelease OXT and glutamate to excite parasympathetic cardiac vagal neurons (CVNs) in the brainstem. The synaptic release of OXT was assessed using sniffer CHO cells that express both OXT receptors and the red fluorescent Ca2+ indicator (R-GECO) that were placed in close proximity to both PVN OXT synapses and their targeted CVNs. Increases in cardiac parasympathetic activity by activation of CVNs excites downstream parasympathetic cardiac ganglia neurons that release acetylcholine and activate muscarinic (M2) receptors in the heart.

Selective channelrhodopsin-2 (ChR2) and excitatory designer receptors exclusively activated by designer drugs (DREADDs) expression in hypothalamic paraventricular nucleus (PVN) oxytocin (OXT) neurons was achieved with viral vectors that selectively expressed Cre under an OXT promoter and Cre-dependent vectors expressing either ChR2 or the excitatory hM3D(Gq) DREADDs. PVN OXT neuron activity was increased in vivo by daily injections of the DREADDs agonist, clozapine-N-oxide (CNO). Activation of PVN OXT neuron synaptic endings in vitro was accomplished by photoexcitation of ChR2. PVN OXT neurons corelease OXT and glutamate (Glut) to excite parasympathetic cardiac vagal neurons (CVNs) in the brainstem. The synaptic release of OXT was assessed using sniffer CHO cells that express both OXT receptors and the red fluorescent Ca2+ indicator (R-GECO) that were placed in close proximity to both PVN OXT synapses and their targeted CVNs. Increases in cardiac parasympathetic activity by activation of CVNs excites downstream parasympathetic cardiac ganglia neurons that release acetylcholine and activate muscarinic (M2) receptors in the heart.

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activation of PVN OXT neurons with DREADDs in which OXT release is assessed by sniffer CHO cells surrounding CVNs—and the OXT network that increases parasympathetic activity to the heart—is shown in Figure 5. We have recently demonstrated that, in rats with LV hypertrophy that progresses to HF, CVNs have diminished excitation owing to both an increase in spontaneous inhibitory gamma amino butyric acid (GABA)ergic neurotransmission frequency and a decrease in amplitude and frequency of excitatory glutamatergic neurotransmission to CVNs (11). Taken together, these findings suggested increasing excitatory input to CVNs such as via the oxytocinergic PVN OXT/glutamate pathway—could be a promising approach to maintain cardiac parasympathetic activity, autonomic balance, and cardiac function during HF.

Using sniffer CHO cells as a novel approach to detect OXT, we have shown that photoactivated synaptic release of OXT from ChR2-expressing PVN fibers at brainstem targets (DMNX) where CVNs are localized is blunted in TAC animals but that this release can be restored with DREADDs-mediated selective activation of PVN OXT neurons. In Figure 1, we show that CHO cell responses were significantly blunted at 6 and 10 weeks but not 8 weeks post-TAC. Although we do not know the reason that the decrease at 8 weeks was not significantly different, it is likely due to the experimental design necessity of nonlongitudinal use of different groups of animals at each time point post-TAC. As PVN OXT release in the DMNX was lowest at 6 weeks post-TAC, we examined whether treatment by chronic PVN OXT neuron activation would benefit cardiac function, assessed both in vivo and ex vivo, as well as improve autonomic balance and reduce mortality. We began treatment in 1 group of animals early, at 4 weeks post-TAC, and another group late, at 6 weeks post-TAC, to reflect treatment further in progression of disease at a time when cardiac dysfunction has been established and mortality to the disease has begun.

Our results show that both early and late PVN OXT neuron activation improved mortality, as the survival rate in TAC animals—50%—was significantly improved to 66% and 70%, respectively, in the early- and late-treatment animals. PVN OXT neuron activation significantly mitigated the progression of cardiac dysfunction following TAC. Cardiac function indices, including EF, stroke volume, cardiac output, and FS all showed very similar improvements in animals with PVN OXT neuron activation, and those improvements followed a similar time course. Fibrosis, assessed by expression levels of collagen III, was significantly higher in TAC animals than in Sham animals, and this index of fibrosis was significantly blunted in PVN OXT-treated animals. Increased myocardial fibrosis is indicative of diffusive loss of working myocardium, which would increase wall stiffness while also having a negative impact on contractile function. Reduced ventricular compliance and increased perivascular collagen would also have a negative effect on vasodilatory reserve (23,24). Reduced coronary flow, plus the increased metabolic demands of pressure overload, would motivate ischemia, causing further myocardial damage. This would further increase fibrosis as myocytes were lost from ischemic injury, then replaced by collagen to maintain structural integrity (23), further reducing working myocardial mass. Indeed, in our ex vivo assessments of cardiac function, LVDP was significantly impaired in TAC animals and higher in treated animals. The LV hypertrophy observed in the untreated TAC animals was not reduced by PVN OXT neuron activation in the treated animals, but fibrosis and IL-1β levels were reduced, likely contributing to compensation mechanisms in the treatment groups that maintained a healthier myocardium.

HRR, an index of parasympathetic activity to the heart and a common clinical assessment of the risk of adverse cardiovascular events (with a lower HRR associated with increased mortality [17]), was lowest in TAC animals and significantly greater in early- and late-treatment animals. Consistent with these findings, HR responses to either daily CNO delivery or long-term activation of PVN OXT neurons were preserved throughout the treatment period as the decreases in HR upon activation of PVN OXT neurons was consistent upon testing each week throughout the entire 16-week duration of this study.

Beneficial outcomes observed with OXT neuron activation could be due to a number of downstream parasympathetic cardioprotective benefits. In addition to evidence that increasing parasympathetic activity to the heart decreases HR and increases coronary flow, PVN OXT neuron activation may have initiated a parasympathetic muscarinic receptor-mediated reduction in sympathetic activity. M2 muscarinic-receptor activation attenuates the production of cyclic adenosine monophosphate (AMP) to reduce the inotropic effects of β-adrenergic receptor activation in the ventricles (25,26). During long-term sympathetic stimulation, M2 activation reduces myocardial stress by lowering cyclic AMP to reduce the cellular hypercontractile state and increase relaxation during diastole, thereby improving myocyte viability (27) and slowing the progression of hypertrophy (28,29).

Although our work used an approach of selectively activating PVN OXT neurons, studies that have
activated downstream targets of PVN OXT neurons report similar increases in parasympathetic activity to the heart. Electrical stimulation of the parvocellular PVN evokes a bradycardia that is suppressed by application of an OXT receptor antagonist into the DMNX (30). In addition, a bradycardia is elicited upon injection of OXT into the DMNX, a response that is blocked by atropine (31). Basal HR varies inversely with dorsal brainstem oxytocin content, with the normal resting HR of normotensive rats associated with higher OXT content than in hypertensive animals (32), suggesting an important role of endogenous OXT release in the autonomic control of HR and the maintenance of a normal HR, autonomic balance, and blood pressure.

Overall, the improvements in cardiac function in HF we observed with PVN OXT neuron activation is consistent with the effects reported for other more invasive and poorly tolerated approaches to increase parasympathetic activity to the heart. Recent device-based approaches—such as implantable vagal stimulators that stimulate a multitude of visceral sensory and motor fibers in the vagus nerve (33)—are being evaluated as new therapeutic approaches for HF (34,35). In animal studies, long-term vagal stimulation improves LV function, reduces infarct size, and decreases mortality (6). Despite initial promising clinical trials, a recent large-scale clinical study, NECTAR-HF (Neural Cardiac Therapy for clinical trials, a recent large-scale clinical study, decreases mortality (8)). Despite initial promising results, a recent large-scale clinical study, NECTAR-HF (Neural Cardiac Therapy for Heart Failure), provided a negative result for chronic vagal nerve stimulation in patients with HF (36).

There are many potential reasons for the lack of effect seen in this clinical study, which includes ineffective and/or nonspecific stimulation parameters (electrical stimulation amplitude and frequency) as well as the inherent disadvantage of activating noncardiac parasympathetic efferent fibers along with the sensory afferent fibers in the vagus nerve with vagal nerve stimulators (37). Our work in targeted activation of OXT neurons in the PVN provides an important step forward to determine if approaches that mimic PVN OXT neuron activation, such as noninvasive and well-tolerated intranasal administration of OXT, can increase parasympathetic activity to the heart in patients with HF, as it does in patients with obstructive sleep apnea (4), with, it is hoped, similar benefits of PVN OXT neuron activation shown in this animal model of HF.

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**KEY WORDS** heart failure, oxytocin, parasympathetic

**APPENDIX** For a supplemental figure, please see the online version of this paper.