Stimulation of the hypothalamic paraventricular nucleus modulates cardiorespiratory responses via oxytocinergic innervation of neurons in pre-Bötzingher complex

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Mack SO, Wu M, Kc P, Haxhiu MA. Stimulation of the hypothalamic paraventricular nucleus modulates cardiorespiratory responses via oxytocinergic innervation of neurons in pre-Bötzingher complex. J Appl Physiol 102: 189–199, 2007. First published July 20, 2006; doi:10.1152/japplphysiol.00522.2006.—Previously we reported that oxytocin (OT)-containing neurons of the hypothalamic paraventricular nucleus (PVN) project to the pre-Bötzingher complex (pre-BötC) region and phrenic motoneurons innervating the diaphragm (D). The aim of these studies was to determine pathways involved in PVN stimulation-induced changes in upper airway and chest wall pumping muscle activity. In addition, we determined the role of OT-containing neurons in the PVN in mediating increased respiratory output elicited by PVN stimulation. Neuroanatomical experiments, using pseudorabies virus (PRV) as a transneuronal tracer in C8 spinalaneclotomized animals showed that PVN neurons project to hypoglossal motoneurons innervating the genioglossus (GG) muscle. Furthermore, microinjection of the PVN with bicuculline, a GABA<sub>A</sub> receptor antagonist, significantly increased (P < 0.05) peak electromyographic activity of GG (GG EMG) and of D<sub>EMG</sub>, frequency discharge, and arterial blood pressure (BP) and heart rate. Prior injection of OT antagonist ([d-(CH<sub>2</sub>)<sub>5</sub>,Tyr(Me)<sub>2</sub>,Orn<sub>8</sub>]-vasotocin intracranially or blockade of OT receptors in the pre-BötC region with OT antagonist L-368,899, diminished GGEMG and DEMG responses and blunted the increase in BP and heart rate to PVN stimulation. These data show that PVN stimulation affects central regulatory mechanisms via the pre-BötC region controlling both respiratory and cardiovascular functions. The parallel changes induced by PVN stimulation were mediated mainly through an OT-OT receptor signaling pathway.

transneuronal labeling; pseudorabies virus; diaphragm and genioglossus electromyographic activity; arterial pressure; heart rate

THE PARAVENTRICULAR NUCLEUS (PVN) is a complex structure that initiates endocrine and autonomic responses to stress and behavioral changes (3, 4). It receives inputs from visceral receptors, circulating hormones, and limbic circuits and transmits information to multiple central nervous system (CNS) sites (11, 53), including cell groups regulating respiratory drive (27, 30, 58) and sympathetic outflow (6, 13, 24, 26, 27, 41, 48). Chemical stimulation of the PVN by microinjection of glutamate elevated frequency and peak diaphragmatic discharge (60). Furthermore, microinjection of bicuculline, a γ-aminobutyric acid (GABA<sub>A</sub>) receptor antagonist into the PVN of conscious rats increased minute ventilation, mean arterial pressure (MAP), heart rate, and oxygen consumption (52).

The respiratory changes induced by stimulation of PVN cells could be mediated via multiple pathways including the rostral ventrolateral region of the medulla oblongata (RVLM) where inspiratory rhythm generating neurons [pre-BötC] are located (56). This region receives inputs from the PVN (28, 39) and expresses oxytocin (OT) receptors (39). Furthermore, this ventrolateral medullary area plays an important role in coordinating phrenic and hypoglossal nerve discharge or the activity of the muscles that they innervate (21, 23). Thus the respiratory-related network within the region corresponding to the pre-BötC could be involved in mediating responses of the diaphragm and genioglossus muscle (GG) to stimulation of PVN neurons. However, PVN neurons may affect respiratory drive to upper airway dilating and chest wall pumping muscles via direct projections to motoneurons innervating these muscles. Furthermore, through projections to the nucleus tractus solitarius (11, 50, 51), the PVN could affect respiratory output and sympathetic nerve activity at manifold points (35).

The physiological significance of the pre-BötC region in mediating parallel changes in the activity of inspiratory chest wall pumping and upper airway dilating muscles, elicited by activation of PVN neurons, is not well understood. Moreover, it is not known which specific neurotransmitter relays information from the PVN to the pre-BötC or other sites that could affect breathing and cardiovascular function.

OT is one among numerous neurotransmitters expressed by neurons of the PVN that project to respiratory-related sites in the brain stem and spinal cord (39). This neuropeptide, in addition to its well-known hormonal action, produces neuronal effects in various regions of the CNS, including the RVLM (30), a site controlling respiratory drive and sympathetic nerve activity. Located within this region is the pre-BötC, the site thought to generate inspiratory rhythmic breathing (56). However, it is not known whether the release of OT within this region mediates changes in respiratory drive and cardiovascular function induced by PVN stimulation.

Therefore, in these studies we tested the hypothesis that the effect of PVN stimulation on diaphragm and GG responses and cardiovascular changes is mediated through PVN-OT-containing neurons innervating the pre-BötC region. The data support the assumption that release of OT from the PVN activates OT receptors in the pre-BötC region neurons, inducing a parallel

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increase in respiratory drive to the GG and the diaphragm and stimulating sympathetic outflow, causing an elevation in arterial pressure and heart rate.

**MATERIAL AND METHODS**

Experiments were performed on adult male Sprague-Dawley rats (250–350 g) housed in the Howard University College of Medicine Veterinary Services Facility. The rats were given food and water ad libitum and were exposed to a normal 12:12-h light-dark cycle. All experimental procedures were approved by the Howard University Institutional Animal Care and Use Committee and are in accordance with National Institutes of Health guidelines for the care and use of laboratory animals.

**Neuroanatomical Studies: Transneuronal Retrograde Labeling Experiments**

Previously we showed that OT-containing cells project to both the pre-BoötC and phrenic motoneurons. In the present studies, we determined whether PVN neurons innervate hypoglossal motor cells. Pseudorabies virus (PRV), a transneuronal tracer, was injected into the GG of C8 spinalectomized animals. This model was used to avoid viral transmission via sympathetic pathways as described previously (19, 20). Briefly, Sprague-Dawley rats were anesthetized with pentobarbital sodium (40 mg/kg ip). Spinalectomy at the C8 level was performed and the pudendal nerves were dissected and sectioned to prevent urine retention. The GG of the tongue was exposed and injected unilaterally with eight to ten 10 nl injections of PRV (Bartha strain, 3.55×10⁶ plaque-forming units/ml). The rats were carefully monitored and given sterile saline (1 ml/100 g body wt) subcutaneously every 12 h.

Five days after PRV injections, rats were anesthetized with pentobarbital sodium and perfused intracardially with 0.9% saline, which was followed by perfusion with 4% paraformaldehyde. Brains were removed, postfixed in 4% paraformaldehyde, and transferred to a 30% sucrose solution. Brains were cut at 50 μm in the transverse plane on a freezing microtome. A one-in-five series of brain stem sections, extending from 10 mm caudal to bregma to the decussation of the pyramids, was incubated in a 1:60,000 dilution of pig anti-PRV (Bartha strain, 3.55×10⁷ plaque-forming units/ml). The rats were carefully monitored and given sterile saline (1 ml/100 g body wt) subcutaneously every 12 h.

Five days after PRV injections, rats were anesthetized with pentobarbital sodium and perfused intracardially with 0.9% saline, which was followed by perfusion with 4% paraformaldehyde. Brains were removed, postfixed in 4% paraformaldehyde, and transferred to a 30% sucrose solution. Brains were cut at 50 μm in the transverse plane on a freezing microtome. A one-in-five series of brain stem sections, extending from 10 mm caudal to bregma to the decussation of the pyramids, was incubated in a 1:60,000 dilution of pig anti-PRV (Bartha strain, 3.55×10⁷ plaque-forming units/ml). The rats were carefully monitored and given sterile saline (1 ml/100 g body wt) subcutaneously every 12 h.

Physiological Studies

**General preparation.** For physiological studies, animals were anesthetized with an intraperitoneal injection of urethane (1.5 g/kg) and supplemental doses of urethane (0.1–0.3 g/kg iv) were given during surgery as required on the basis of nociceptive reflex responses and increases in arterial blood pressure. The rats were instrumented with catheters in the carotid artery for recording arterial pressure and monitoring arterial blood pH, PCO₂, and PO₂ (Radiometer ABL5, Radiometer, Westlake, OH) and in the jugular vein for administration of anesthetic and fluids. The arterial catheter was attached to a calibrated pressure transducer connected to a blood pressure preamplifier (Buxco Research Systems, Wilmington, NC).

**Electromyographic recordings.** Electromyographic recordings were obtained as described previously (39). Briefly, a midline incision was made on the ventral surface of the neck for bilateral vagotomy in the cervical region and for tracheotomy. The rats were intubated, placed in a prone position, and mechanically ventilated with 100% oxygen at 10 ml/kg body wt using a rodent ventilator (Harvard Apparatus, Holliston, MA). Core body temperature was monitored with a rectal probe and maintained between ~37 and 38°C with a temperature-controlled heating pad (FST, San Francisco, CA). Bipolar electrodes, which consisted of two Teflon-coated stainless steel wires with bared tips, were inserted into the GG and into the right side of the costal region of the diaphragm (D) for recording electromyographic activity (GGEMG, DEMG). The rats were then placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) with blunt ear bars, and the dorsal brain regions overlying the right sides of the hypothalamic PVN and the pre-BoötC were reached through small craniotomies. The stereotaxic coordinates for the PVN were 1.6–2.1 mm caudal to bregma, 0.3–0.4 mm lateral to the midline, and 7.8 mm ventral to dura. Stereotaxic coordinates for the pre-BoötC within the ventral respiratory group were 12.3–12.9 mm caudal to bregma, 1.8–2.1 mm from midline, and 8.0–10.0 mm ventral to dura as previously reported (45).

As we described previously (39), DEMG and GGEMG signals were amplified, band-pass filtered, rectified, and integrated to give moving average traces. These signals along with analog outputs from the blood pressure amplifier and the gas analyzer (ADInstruments, Colorado Springs, CO), which measured end-tidal CO₂, were sent to a PowerLab data-acquisition system (ADInstruments) and visualized on the computer with Chart software (ADInstruments). At the beginning of experiments, arterial blood pH was adjusted to ~7.35–7.45 with administration of NaHCO₃ as required and PCO₂ was kept within the ~34–38 mmHg range by adjusting the rate of the ventilator.

**Experimental Protocols**

**Stimulation of PVN with GABA₄ receptor antagonist.** In the first series of experiments (n = 10) on the basis of preliminary dose-response studies, bicuculline [−]methiodide (Sigma-Aldrich, St. Louis, MO) was microinjected into the PVN unilaterally (50 nl of a 1 mM solution) with a Hamilton syringe connected to a UMP2 pressure pump with microprocessor (World Precision Instruments, Sarasota, FL). Control studies were conducted by injecting the same volume (50 nl) of physiological saline into the regions of interest. In these studies we avoided use of the excitatory amino acid glutamate because of the narrow window between its stimulating and depolarizing blockade effects on the neurons of interest (33).

**Midcollicular decerebration.** To determine whether the effects of bicuculline are mediated through suprapontine neural structures rather than via resorbed, circulating bicuculline, the drug was microinjected into the PVN (n = 5), following midcollicular decerebration. Briefly, small parietal craniotomies were made 1–2 mm rostral to the interaural line on the left and right sides. A thin blunt spatula was inserted horizontally on each side and then swept back across the depth, preserving the large blood vessels. A 1-h stabilization period was allowed before resuming the experiment by unilateral microinjection of a dose of bicuculline that was twofold higher than the original concentration. Decerebration was verified by visual inspection of the brain after fixation. Only animals with complete decerebration were included in the study.

**Intracisternal injection of OT receptor antagonist.** To define whether OT mediates the effects of PVN stimulation, OT receptors were blocked by intracisternal injection of an OT antagonist. For this, the head of the animal was fixed into a stereotaxic instrument in a nose-down position and the skin and muscle overlying the occipital bone were retracted. A small part of the occipital bone was removed to visualize the fourth ventricle via the atlantooccipital membrane. OT receptor blocker, [−-(CH₂)₅,Tyr(Me)₂,Orn⁵]-vasotocin (AVT; Sigma-Aldrich Chemical) was dissolved in artificial cerebrospinal fluid and slowly injected into the fourth ventricle (n = 7 rats) in a fixed volume of 2 μl of a 0.1 mM solution of the drug. We employed AVT because it is a potent peptide OT receptor blocker and is widely used in pharmacological and physiological experiments (8, 9, 44). Control experiments with intracisternal injection of 2 μl of CSF were carried out in two rats.
**Microinjection of OT receptor antagonist in the pre-Bo"tC region.**

Inasmuch as drugs administered intracere tally could affect multiple sites, we defined the degree of involvement of the pre-Bo"tC network in response to activation of OT signaling pathways by microinjecting OT receptor antagonist into the right RVLM region that contains the pre-Bo"tC. For these microinjection studies, we used a non-peptide OT antagonist, L-368,899 (Sigma-Aldrich). This compound is more selective for the OT receptor and has minimal or no effect on vasopressin receptors (46). In preliminary studies, we tested the effects of prior microinjection of the OT receptor antagonist L-368,899 on respiratory and cardiovascular changes elicited by OT injected into the pre-Bo"tC region. The results showed that L-368,899 diminished the effects of 50 nl of 1 mM OT solution microinjected into the pre-Bo"tC region, but failed to block changes induced by microinjection of vasopressin into the same area, such as an increase in mean arterial blood pressure and diaphragm activity. This indicated to us a high level of its specificity for OT receptors. L-368,899 (0.1 mM, 20 nl) was microinjected into the region of the pre-Bo"tC and responses were recorded (n = 6). After reaching a steady-state condition, 4–7 min, the PVN was stimulated ipsilaterally with the same dose of bicuculline as in the absence of the OT receptor antagonist.

**Microinjection of GABA_A receptor agonist in the pre-Bo"tC region.**

We conducted complementary experiments in determining the overall role of the pre-Bo"tC region in mediating respiratory and cardiovascular effects of PVN activation. Muscimol, a specific GABA_A receptor agonist (50 nl of a 10 mM solution), was unilaterally microinjected into the right pre-Bo"tC region, using the coordinates given above. Muscimol decreased respiratory discharge of both the D and GG muscles and decreased arterial pressure. Only muscimol injections were considered to be in the pre-Bo"tC region when muscimol administration reduced inspiratory drive. To avoid the effects of decreased respiratory drive on responses of the studied variables to PVN stimulation, peak activity of the diaphragm was brought close to its baseline level prior to injection of muscimol. This was accomplished by reducing the rate of the ventilator, consequently increasing its baseline level prior to injection of muscimol. This was achieved by 10.22±0.32.246 on April 29, 2017 http://jap.physiology.org/ Downloaded from  

**RESULTS**

**Neuroanatomical Studies: Transneuronal Retrograde Labeling Experiments**

Five days after PRV injections into the GG of C8 spinalec tromized animals, the majority of PRV-infected cells was observed ipsilaterally within the ventral and ventrolateral portions of hypoglossal motoneurons at 14 mm caudal to bregma and immediately caudal to the area postrema. Within the PVN, labeled cells were also localized mainly ipsilaterally, in the dorsal parvocellular subnucleus shown in Fig. 1 and to a lesser extent within the medial parvocellular, ventral parvocellular, and magnocellular subnuclei.

**Effect of PVN Stimulation on Diaphragm and GG Activities**

In 4 of 10 animals in this study, saline vehicle was injected into the PVN prior to injection of bicuculline. Ventilatory measurements following saline administration did not change dramatically from baseline values. D_{EMG} decreased by 1.7%, whereas inspiratory and expiratory times and frequency only increased slightly (P > 0.05) by 3.9, 2.3, and 3.5%, respectively. G{GEMG}, blood pressure, and heart rate insignificantly decreased (P > 0.05), on average by <2%.

In contrast, activation of the PVN by microinjection of bicuculline increased D_{EMG} and GG_{EMG} peak activity frequency discharge and breathing rate. Typically, the response peaked within 3–5 min from the start of injection and was followed by a decline back to preinjection levels within 10–20 min. Microinjection sites that elicited changes in respiratory drive and arterial pressure are shown in Fig. 2. Integrated tracings for D_{EMG} discharges and average results for all studied variables are shown in Fig. 3. Rats microinjected unilaterally with bicuculline showed an increase (P < 0.05) in peak D_{EMG} activity of 109% from 0.49 ± 0.01 to 0.94 ± 0.13 units. Inspiratory time did not change significantly (P > 0.05). However, expiratory duration was shortened (P < 0.05) by 22.8 ± 6.8% from a mean of 1.13 ± 0.07 to 0.85 ± 0.07s, which led to a 28.8% increase (P < 0.05) in frequency (Fig. 3); thus minute D_{EMG} changed dramatically by 172% from 1.2–1.4% to reach the expected level of respiratory drive. When a steady state was achieved, ipsilateral microinjection of bicuculline into the PVN was performed and the studied variables were recorded.

**Histology**

At the end of each experiment, rats were overdosed with urethane and perfused intracardially with 0.9% saline, which was followed by 4% paraformaldehyde. Brains were removed, postfixed in 4% paraformaldehyde, and transferred into a 30% sucrose solution prior to cryosectioning with Leica cryostat (Leica Microsystems, Bannockburn, IL). Sections were mounted onto glass slides to identify injection sites.

**Data Analysis**

The baseline values for D_{EMG} and GG_{EMG} activities were analyzed from moving average signals and were quantified in arbitrary units within a 3- to 5-min period for 10 consecutive breaths immediately before microinjection of bicuculline into the PVN. To determine changes in tonic and phasic electrical activity of the GG evoked by microinjection of bicuculline into the PVN, peak response amplitudes were also determined for 10 consecutive breaths. Mean arterial blood pressure and heart rate were measured for the corresponding baseline and peak response periods. Mean peak D_{EMG} and GG_{EMG} activity, respiratory frequency (f), diaphragm minute activity (D_{EMG} × f), GG minute activity (GG_{EMG} × f), and inspiratory and expiratory times were quantified and calculated. Baseline and peak response amplitudes were determined similarly for midcollicular decerebration, OT blocker, and muscimol experiments.

Measured and calculated cardiorespiratory variables were analyzed as appropriate with a paired Student’s t-test or a one-way repeated-measures ANOVA, followed by Tukey’s post hoc test for comparisons between drugs (Sigmastat, SYSTAT Software, Chicago, IL). Summary data in the text and figures are expressed as means ± SE. Differences were considered statistically significant at P < 0.05.
slightly, but significantly \((P < 0.05)\), by 5.6% from 395 ± 11 to 417 ± 12 beats/min.

In experiments when injection sites were located in the nearby regions, 500–700 \(\mu\)m dorsal or lateral to the PVN borders, the same amount of bicuculline had no detectable effect on diaphragm and GG activity. These data are not shown in Fig. 2.

**Effect of PVN Stimulation on Diaphragm and GG Activity After Midcollicular Decerebration**

In decerebrated rats, PVN stimulation with bicuculline had no effect \((P > 0.05)\) on minute \(D_{\text{EMG}}\) activity (before vs. after bicuculline: 20.5 ± 3.6 vs. 20.2 ± 3.6), minute \(G_{GEMG}\) activity (16.5 ± 3.6 vs. 23.8 ± 11.0), arterial blood pressure (115 ± 4.0 vs. 112 ± 4.0 mmHg), or heart rate (426 ± 36 vs. 432 ± 34 beats/min). These findings indicate that changes observed in nondecerebrated rats were due to activation of PVN neurons and not due to spillover of bicuculline into the cerebrospinal fluid.

**Effect of PVN Stimulation on Diaphragm and GG Activities After Intracisternal Injection of OT Receptor Antagonist**

Data for intracisternal injection of OT blocker AVT, in a concentration (2 \(\mu\)l of a 0.1 mM solution of the drug) that blocks the stimulatory effects of 1 mM OT, were analyzed only in rats in which anatomical studies confirmed that microinjections of bicuculline were performed in the PVN as described above for experiments in normal and decerebrated rats. In these animals, administration of AVT elicited a slight decrease in \(D_{\text{EMG}}\), but a slight increase in \(G_{GEMG}\), arterial blood pressure, and heart rate. However, AVT diminished the effects of PVN stimulation on these variables. In Table 1, baseline variables prior to intervention, the steady-state condition after administering AVT intracisternally, and stimulation of the PVN with bicuculline are presented.

**Effect of PVN Stimulation on Diaphragm and GG Activities After Microinjection of OT Receptor Antagonist in the pre-\(\text{Bo} \text{ötC}\) Region**

In this series of experiments, we microinjected L-368,899, an OT receptor antagonist, into the pre-\(\text{Bo} \text{ötC}\) region. As shown in Table 2, blockade of OT receptors tended to decrease respiratory drive, significantly lowering minute \(G_{GEMG}\) activity and arterial pressure \((P < 0.05)\). Furthermore, there was a decrease in \(D_{\text{EMG}}\) and reduced \(f\), but the OT antagonist abolished changes \((P > 0.05)\) in \(D_{\text{EMG}}\) activity, minute \(D_{\text{EMG}}\) before vs. after bicuculline (19.8 ± 3.7 vs. 18.3 ± 3.7), minute \(G_{GEMG}\) activity (20.7 ± 2.2 vs. 19.7 ± 3.2), arterial blood pressure (92 ± 6.0 vs. 93 ± 7.0 mmHg), and heart rate (422 ± 7.0 vs. 417 ± 5.0 beats/min) elicited by PVN stimulation. These findings indicate that changes in the studied variables induced by stimulation of PVN neurons are mediated mainly by a release of OT within the pre-\(\text{Bo} \text{ötC}\) region and activation of OT receptors.
Effect of the PVN Stimulation on Diaphragm and GG Activities After Microinjection of GABA<sub>A</sub> Receptor Agonist in the pre-Bo¨tC Region

To define the degree of involvement of the pre-Bo¨tC region in mediating respiratory drive to the GG and D and in eliciting cardiovascular responses to PVN stimulation, muscimol was administered unilaterally into the pre-Bo¨tC region to diminish the responses of pre-Bo¨tC neurons to afferent inputs from the PVN. Muscimol decreased peak D and GG activity as well as arterial blood pressure, which was brought close to preinjection levels by adjusting the ventilatory rate and, consequently, increasing end-tidal PCO<sub>2</sub> on average by 1.2–1.4%. Under these conditions, PVN stimulation by microinjection of bicuculline following administration of muscimol, a GABA<sub>A</sub> receptor agonist, in the pre-Bo¨tC region, had no effect (P > 0.05) on DEMG, GEMG, or duration of the respiratory cycle (Table 3). Furthermore, arterial pressure and heart rate only changed slightly (P > 0.05) by 3.7 and 1%, respectively, in response to PVN stimulation, suggesting that the pre-Bo¨tC region is involved in mediation of both respiratory and cardiovascular changes induced by PVN stimulation.

DISCUSSION

The results of the present study show that an increase in inspiratory activity of the diaphragm and GG and elevation of arterial blood pressure and heart rate elicited by stimulation of the PVN are mediated mainly through OT projections to the pre-Bo¨tC region and activation of OT receptors within this area. In addition, the data confirmed previous findings showing that activation of PVN neurons elicited an increase in respiratory drive and arterial blood pressure in anesthetized (60) and in non-sedated, normotensive rats (52).

The present findings are discussed by focusing on 1) neuroanatomical studies, 2) comparison of current results with previous findings related to the involvement of PVN OT-containing neurons in autonomic control, and 3) the functional significance of the results.

Transneuronal Labeling Studies

PRV, as a transneuronal tracer, is used in a number of studies (16, 19, 20, 27, 29, 36, 54, 55). Following microinjection of PRV, the virus is retrogradely transported from the GG to first-order neurons (hypoglossal motoneurons), where it replicates and passes transneuronally to second-order neurons, including those projecting to hypoglossal cells that innervate the GG. In spinelecomized rats 5 days after PRV injections into the GG, labeled cells were found mainly ipsilaterally at the level and immediately caudal to the obex in the ventral and ventrolateral subdivisions of the hypoglossal nucleus. A few scattered PRV-positive cells were observed intermingled with
unlabeled hypoglossal motoneurons in the more dorsomedial and dorsolateral subregions.

Following injection of PRV into the GG, second-order neurons were observed within the PVN dorsal and medial ventral subregions known to project to respiratory-related sites in the medulla oblongata and spinal cord and to express OT or vasopressin traits (28, 39). To better address the question of dual function of PVN neurons in autonomic and motor control, future studies may include simultaneously injecting two different viral strains of a transynaptic tracer, each strain expressing a unique reporter gene as used in determining the sympathomotor circuitry involved in mediating stress responses (29).

If replication time is prolonged through central polysynaptic circuits, the virus can also label higher order cells (17, 18, 34). Hence, we cannot exclude the possibility that some of these cells could be third-order neurons. Furthermore, as with other viruses, some neurons do not express receptors for the virus, or PRV infection can produce neuronal death of receptive neurons, thereby reducing the number of labeled cells. Also there is the potential for non-specific extracellular spread of PRV from compromised infected neurons, leading to lateral, non-specific labeling. In the present studies, cytolytic processes of infected cells were reduced using the attenuated strain of Bartha PRV. As shown in this study, this strain expresses...
involved in central regulation of respiration (28, 39, 60),
sympathetic outflow (26, 27), and cholinergic output to
visceral organs (19, 20, 36). Furthermore, OT-containing PVN neurons
innervating the brain stem-spinal cord-respiratory-related sites
(39) arise from the dorsal and medial ventral PVN subnuclei
that project to hypoglossal motoneurons and vasopressor neu-
rons of the RVLM (27).

The PVN is involved in coordination of respiratory and
cardiovascular responses to a number of stimuli (39, 53),
including those from peripheral chemoreceptors (41, 48). Pre-
viously, it was shown that bilateral microinjection of glutamate
into the PVN in urethane-anesthetized vagotomized and ven-
tilated rats caused an increased in peak D_{DEMG} activity and
D_{DEMG} frequency discharge. These changes were associated
with elevation of arterial blood pressure (52). Subsequently, in
well-designed studies, Schlenker and colleagues (52) reported
that unilateral microinjection of bicuculline, a GABA_A recep-
tor antagonist, into the PVN of conscious rats increased breath-
ing frequency, volume of a breath, mean arterial pressure, heart
rate, and oxygen consumption for up to 10 min following
injection. In the present study we used a GABA_A receptor
antagonist instead of glutamate to stimulate PVN neurons. We
avoided the use of glutamate because its application after a
brief period of excitation time could cause a long-lasting (up to
30 min) decrease in excitability or silencing of discharge,
probably due to a depolarizing block and disturbances in the
ionic composition of the extracellular space (33). Previous
studies have shown that local injections of OT in the PVN elicit
a positive feedback mechanism for the oxytocinergic activation
(31), suggesting that OT injections into the PVN could be used
for studies aimed to examine the physiological role of the PVN
oxytocinergic pathway. We did not use OT administration
because previous studies provided evidence that blockade of
GABA_A receptors expressed by PVN neurons affects both
respiratory and cardiovascular function (52). Using low con-
centrations of GABA_A receptor antagonist bicuculline, we
circumvented possible difficulties and generated the present
results that extend previous data (52, 60) showing that PVN
stimulation also increases GG activity. Although the first series
of experiments clearly demonstrates PVN involvement in reg-
ulation of respiratory drive to different inspiratory muscle
groups and in coordination of breathing activity and cardio-
vascular function, they do not indicate which PVN neurons
mediate these responses, nor the neurotransmitters involved.
Therefore, in the second series of our studies, we focused on
this issue and proved our hypothesis that under well-defined
and reproducible experimental conditions, OT-containing cells

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Physiological Studies

PVN neurons project to a number of CNS sites (30), includ-
ing brain stem and spinal cord nuclei that are known to be
neurotropism for PVN neurons innervating hypoglossal neu-
rons that project to the GG. Furthermore, the non-specific
spread of the virus should not be considered a problem because
its spread is restricted by activation of astrocytes, microglia,
and reactive gliosis, isolating infected neurons from non-
infected ones, thereby preventing non-specific neuronal infec-
tion (17, 18, 34).

It should be mentioned that in the C8 spinalectomized
animals, as described in the previous studies (19, 20), sympa-
thetic pathways are eliminated as a possible route for the virus
to reach supraspinal structures. Therefore, we believe that
transneuronal labeling of the PVN neurons, following injection
of tracer into the GG, are second-order neurons that innervate
hypoglossal motoneurons.

Physiological Studies

PVN neurons project to a number of CNS sites (30), includ-
ing brain stem and spinal cord nuclei that are known to be

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Table 1. Effect of PVN stimulation on cardiorespiratory variables after intracisternal blockade of OT receptors

<table>
<thead>
<tr>
<th>Drug</th>
<th>DD_{DEMG}</th>
<th>Min</th>
<th>TI</th>
<th>f</th>
<th>GG_{DEMG}</th>
<th>Min</th>
<th>BP</th>
<th>HR</th>
</tr>
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<tbody>
<tr>
<td>Baseline</td>
<td>0.38±0.05</td>
<td>18.6±2.6</td>
<td>0.22±0.02</td>
<td>1.01±0.05</td>
<td>49±2</td>
<td>0.60±0.10</td>
<td>30±5</td>
<td>110±6</td>
</tr>
<tr>
<td>AVT</td>
<td>0.37±0.05</td>
<td>18.4±2.8</td>
<td>0.24±0.02</td>
<td>0.99±0.08</td>
<td>50±3</td>
<td>0.62±0.13</td>
<td>31±5</td>
<td>112±7</td>
</tr>
<tr>
<td>Bicuculline</td>
<td>0.38±0.05</td>
<td>19.4±2.9</td>
<td>0.22±0.02</td>
<td>0.99±0.07</td>
<td>51±3</td>
<td>0.49±0.09</td>
<td>25±4</td>
<td>107±5</td>
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</tbody>
</table>

Values are means ± SE; n = 7 animals. DD_{DEMG}, GG_{DEMG}, electromyographic activity of diaphragm and genioglossus, respectively; TI and Ti, inspiratory and expiratory times, respectively; f, frequency of diaphragm discharge; Min, minute activity, PVN, paraventricular nucleus; OT, oxytocin; HR, heart rate; BP, blood pressure; AU, arbitrary units. Differences between baseline and changes induced by AVT were not significant (P < 0.05). Bicuculline administration did not significantly change (P < 0.05) the values recorded post-AVT.
mediate major changes in respiratory drive and cardiovascular function.

Physiological experimental design has its own limitations, therefore the results need to be interpreted with caution. Difficulties are mainly related to drug delivery and selectivity of drugs used. For drug delivery, we used pressure ejection. We found this method to be very useful in administering drugs in a controlled volume to discrete brain stem regions. Nonetheless, drug delivery by this method into the PVN may affect cells other than OT-producing neurons. Furthermore, variations in the size of the head could lead to ejection of the drug outside of targeted regions. However, head size in rats of the same age and strain may differ only slightly. Hence, the majority of experimental rats (~85%) was microinjected within the PVN and responded with an increase in respiratory drive following bicuculline administration. Missed injections, or control administrations of used drug, lateral or dorsal to PVN borders (500–700 µm), had no observable effects on recorded variables.

Difficulties could also occur due to low specificity of OT receptor antagonists. Initially, we employed AVT, a potent peptide OT receptor blocker that is widely used in pharmacological and physiological experiments (8, 9, 43). For our pre-Bo¨tC experiments, we used a non-peptide antagonist, L-368,899, because it is more selective for the OT receptor (46). To achieve even greater specificity over pharmacological blockade, RNA interference (RNAi) technology with small interfering RNA (siRNA) can be used to inhibit target gene expression. Exogenous interfering RNAs have been delivered to cells in vivo via viral infection (59), lipofection (4), and electroporation (1). In addition to potential toxicity associated with RNAi, the time required for recovery after surgical intervention and the transient knockdown of target gene expression may not be compatible for acute experiments as performed in the present study.

OT-responsive cells and OT receptors are found along the neural axis (13, 16, 21, 43). Cell signaling generated by OT is mediated via OT receptors, which are typical members of the rhodopsin-type (class I) G protein-coupled receptor (GPCR) family (5). We found that OT receptors are present in the ventrolateral medulla where neurokinin-1 receptor expressing neurons are located. Their activation by exogenous OT mimics respiratory and cardiovascular responses elicited by PVN stimulation (36). However, the changes caused by stimulation of PVN neurons could be mediated through projections to other brain stem sites involved in autonomic control (14, 51, 58), i.e., the nucleus tractus solitarius (8, 24). Therefore, prior blockade of these effector responses by intracisternal administration of the OT receptor antagonist but not vehicle into the fourth ventricle, does not exclude this possibility. However, microinjection of OT antagonist within the pre-Bo¨tC region similarly abolished the observed changes. Similarly, prior administration of specific GABA_A receptor agonist muscimol into the pre-Bo¨tC nearly abolished responses of the GG and diaphragm and cardiovascular changes induced by PVN stimulation. These experiments indicate that neurons within the ventrolateral region of the medulla oblongata where inspiratory rhythm generating cells are found (56), mediate respiratory and cardiovascular effects of stimulation of PVN OT-containing cells. This is accomplished mainly through proprioibular neurons to hypoglossal motor cells regulating GG activity, via bulbospinal neurons to phrenic nuclei controlling diaphragm discharge, and via connectivity with the intermediolateral cell column influencing sympathetic outflow and consequently arterial pressure and heart rate, as presented in Fig. 6.

Our conceptual model as diagrammatically presented in Fig. 6, is supported by findings generated two decades ago showing that excitatory substances administered systemically, intravenicularly, or applied topically on the ventrolateral medulla increase hypoglossal nerve activity largely through a neuronal network located beneath the ventrolateral medullary surface (21, 23). Furthermore, cooling, local anesthesia, or activation of GABA_A receptors beneath the surface of ventrolateral medulla abolish phrenic and preferentially hypoglossal nerve activity and responses to hypercapnic loading (22). Alterations in this network could lead to parallel changes in respiration and

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Table 2. Effect of PVN stimulation on cardiorespiratory variables after blockade of OT receptors in pre-Bo¨tC

<table>
<thead>
<tr>
<th>Drug</th>
<th>D_MG, AU</th>
<th>Min D_MG, AU</th>
<th>T_i, s</th>
<th>T_e, s</th>
<th>f, breaths/min</th>
<th>GG EMG, AU</th>
<th>Min GG EMG, AU</th>
<th>BP, mmHg</th>
<th>HR, beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.46±0.07</td>
<td>24.0±3.7</td>
<td>0.26±0.02</td>
<td>0.91±0.07</td>
<td>53±3</td>
<td>0.49±0.03</td>
<td>25.7±2.1</td>
<td>106±6</td>
<td>426±6</td>
</tr>
<tr>
<td>L-368,899</td>
<td>0.39±0.07</td>
<td>19.8±3.7</td>
<td>0.25±0.02</td>
<td>0.94±0.06</td>
<td>51±3</td>
<td>0.40±0.04</td>
<td>20.7±2.2</td>
<td>92±6*</td>
<td>422±7</td>
</tr>
<tr>
<td>Bicuculline</td>
<td>0.35±0.07</td>
<td>18.3±3.7</td>
<td>0.21±0.04</td>
<td>0.97±0.09</td>
<td>52±3</td>
<td>0.38±0.06</td>
<td>19.7±3.2</td>
<td>93±7</td>
<td>417±5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 animals. *Represents differences (P < 0.05) between baseline and administration of L-368,899 for Min GG EMG and arterial BP. Differences between values post L-368,899 and bicuculline were not significant (P > 0.05), indicating that blockade of OT receptor within the pre-Bo¨tC region inhibited responses elicited by PVN stimulation.

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Table 3. Effect of PVN stimulation on cardiorespiratory variables after microinjection of GABA_A receptor agonist into pre-Bo¨tC

<table>
<thead>
<tr>
<th>Drug</th>
<th>D_MG, AU</th>
<th>Min D_MG, AU</th>
<th>T_i, s</th>
<th>T_e, s</th>
<th>f, breaths/min</th>
<th>GG EMG, AU</th>
<th>Min GG EMG, AU</th>
<th>BP, mmHg</th>
<th>HR, beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.40±0.09</td>
<td>18.0±5.8</td>
<td>0.33±0.03</td>
<td>1.19±0.11</td>
<td>41±4</td>
<td>0.54±0.09</td>
<td>21.2±3.5</td>
<td>117±6</td>
<td>408±11</td>
</tr>
<tr>
<td>Muscimol</td>
<td>0.35±0.15</td>
<td>17.3±8.8</td>
<td>0.27±0.05</td>
<td>1.19±0.16</td>
<td>45±6</td>
<td>0.31±0.09</td>
<td>13.1±4.7</td>
<td>102±7</td>
<td>398±8</td>
</tr>
<tr>
<td>Bicuculline</td>
<td>0.42±0.17</td>
<td>17.5±7.2</td>
<td>0.40±0.16</td>
<td>1.14±0.16</td>
<td>43±6</td>
<td>0.41±0.14</td>
<td>14.9±3.7</td>
<td>107±12</td>
<td>401±9</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6. Administration of muscimol at the adjusted ventilator rate were slightly lower than baseline values; however, differences were not significant (P > 0.05). Differences between values post-muscimol and bicuculline were not significant (P > 0.05) for all analyzed variables.
cardiovascular function (10). Hence, the pre-BötC region is an integrative site for respiratory and cardiovascular function and is involved in transmitting excitatory information from the PVN to upper airway dilating and chest wall pumping muscles, as well as to bulbospinal neurons regulating sympathetic outflow. Studies elucidating anatomic connections between the pre-BötC region and brain stem neurons regulating sympathetic outflow could provide a definitive explanation for the present results.

The hypoglossal nucleus receives excitatory and inhibitory inputs from the brain stem and suprapontine regions to produce signals to the GG regulating suckling, swallowing, and speech (12, 15, 25, 57). The findings of the present study raise the possibility that PVN innervation of hypoglossal motoneurons could be related to these functions rather than directly controlling respiratory discharge of hypoglossal neurons.

The PVN plays an important role in modulating the sympathoexcitatory component of peripheral, but not central, chemoreflexes (41, 48). Stimulation of the PVN increased arterial pressure and heart rate, which were diminished by prior blockade of OT receptors or muscimol administered into the ventrolateral medulla. The data suggest that vasopressor neurons outside of the ventrolateral pressor region do not play a critical role in mediating a PVN stimulation-induced increase in arterial pressure and heart rate. Furthermore, these results suggest that activation of PVN OT-producing neurons elicit parallel changes in respiratory and cardiovascular functions by a single mechanism that controls these interlinked systems.

**Functional Implications**

Respiration is regulated by both involuntary (neural, endocrine, metabolic, and emotional components) and voluntary controlling mechanisms. Understandably, being a vital and continuous process, breathing is not dependent on endocrine hormones but it is modulated by a number of molecules, including PVN peptides such as OT. Previous studies suggested that OT affects respiratory drive via direct stimulation of the pre-BötC within the ventrolateral medulla oblongata (39). The present findings indicate that through this site, endogenously released OT regulates both respiratory and cardiovascular function.

The parvocellular OT-containing neurons are involved in the regulation of appetite. Although lesions in the PVN in rat brains and OT receptor antagonism cause overeating and obesity (2, 7, 32), central administration of OT and an OT agonist decrease food intake in rats (37, 43) and could be involved in anorexia and the bulimia nervosa syndrome (3). A loss of PVN neurons producing OT or functional abnormalities in OT-OT receptor signaling may cause obesity and increased susceptibility to hypoventilation and obstructive sleep apnea (42). In humans, a decrease in the activity of tongue muscles during sleep, which is exaggerated by obesity, could cause upper airway occlusion (49).

In conclusion, the results of the present study suggest that PVN stimulation affects central regulatory mechanisms controlling both respiratory and cardiovascular functions, resulting in parallel changes mediated mainly by an OT-OT receptor signaling pathway. Hence, drugs that potentiate central OT-dependent mechanisms, via the activation of OT receptors expressed by these neurons, may represent targets for the development of new treatments for respiratory disorders associated with obesity and respiratory depression. For example, subtype-selective agonists directed toward OT receptors may represent a successful therapeutic intervention for some forms of obesity associated with obstructive sleep apneas and CO₂ retention.

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REFERENCES


