

Paraventricular oxytocin neurons are involved in neural modulation of breathing

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Mack, S. O., P. Kc, M. Wu, B. R. Coleman, F. P. Tolentino-Silva, and M. A. Haxhiu. Paraventricular oxytocin neurons are involved in neural modulation of breathing. *J Appl Physiol* 92: 826–834, 2002. First published October 5, 2001; 10.1152/japphysiol.00839.2001.—In this study, we determined the projections of oxytocin-containing neurons of the paraventricular nucleus (PVN) to phrenic nuclei and to the rostral ventrolateral medullary (RVLM) region, which is known to be involved in respiratory rhythm generation. Studies were also designed to determine oxytocin-receptor expression within the RVLM and the physiological effects of their activation on respiratory drive and arterial blood pressure. Oxytocin immunohistochemistry combined with cholera toxin B, a retrograde tracer, showed that a subpopulation of oxytocin-containing parvocellular neurons in the dorsal and medial ventral regions of the PVN projects to phrenic nuclei. Similarly, a subpopulation of pseudorabies virus-labeled neurons in the PVN coexpressed oxytocin after injection of pseudorabies virus, a transsynaptic retrograde marker, into the costal region of the diaphragm. A subpopulation of oxytocin-expressing neurons was also found to project to the RVLM. Activation of this site by microinjection of oxytocin into the RVLM (0.2 nmol/200 nl) significantly increased diaphragm electromyographic activity and frequency discharge ($P < 0.05$). In addition, oxytocin increased blood pressure and heart rate ($P < 0.05$). These data indicate that oxytocin participates in the regulation of respiratory and cardiovascular activity, partly via projections to the RVLM and phrenic nuclei.

pre-Bötzinger complex; spinal cord; phrenic nuclei; oxytocin receptor; neurokinin-1 receptor; cholera toxin B subunit; pseudorabies virus

THE HYPOTHALAMIC PARAVENTRICULAR nucleus (PVN), located along the third ventricle, can be described as a homeostatic motor cell group with neuroendocrine, metabolic, and cardiorespiratory functions (36, 43). Anatomically, the PVN is a heterogeneous structure, consisting of several distinct subdivisions with differing projection patterns (35, 43). The oxytocin neurons of the magnocellular division of the PVN project mainly to the posterior pituitary (44), which is involved in regulating neuroendocrine functions such as plasma osmolality, parturition, and lactation. Parvocellular

neurons, on the other hand, innervate the median eminence and other sites within the central nervous system (44), including cell groups in the brain stem and spinal cord that are involved in the regulation of cardiovascular and respiratory functions (48).

PVN cells with long descending brain stem and spinal projections are primarily peptidergic neurons, expressing multiple neurotransmitters, including oxytocin and vasopressin (Ref. 15; for review, see Ref. 31). There is no information, however, on projections of oxytocin-containing neurons to respiratory-related brain stem regions or spinal cord motoneurons that innervate respiratory muscles such as the diaphragm.

A number of studies have shown that oxytocin plays an important role in mediating a cardiovascular response to environmental stressors via projections to the rostral ventrolateral medulla (RVLM) (1, 28) and the spinal cord (5). In contrast, almost no information exists on the role of oxytocin in the regulation of respiration.

PVN neurons are activated by many stimuli that affect breathing (7, 23, 26, 28). Furthermore, activation of these neurons is associated with a release of oxytocin and vasopressin (20, 21). Based on these findings, we hypothesized that oxytocin-containing neurons constitute a part of the neural pathways involved in the respiratory response to different stimulatory inputs and provide a possible link between neuroendocrine and neural control of respiration.

MATERIALS AND METHODS

Animals. Experimental protocols were approved by Howard University Institutional Animal Care and Use Committee. All experiments were performed in male Sprague-Dawley rats (Harlan, Indianapolis, IN) according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Neuroanatomic experiments. In the first set of experiments, oxytocin-containing PVN cells that project to phrenic nuclei were identified by neuroanatomic tracings in five male rats (250–350 g). Cholera toxin B subunit (CTB; List Biological Laboratories, Campbell, CA), a retrograde tracer, was unilaterally injected into the ventral horns of the cervical

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spinal cord, extending from C₃ to C₅ (total of ~300 nl of 0.1% solution) in anesthetized animals (pentobarbital sodium, 40 mg/kg) as previously described (48). Injections were made with a glass micropipette (40- to 60- μ m diameter), 0.6 mm from the midline and 1.4 mm from the dorsal surface of the spinal cord (48). Five days after CTB injections, the animals were deeply anesthetized with pentobarbital sodium (50 mg/kg ip) and rapidly perfused transcardially with 0.9% buffered saline followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The perfused brains were removed from the animals and postfixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 24 h at 4°C (immersion fixation). After immersion fixation, the brains were placed in 30% sucrose in 0.1 M PBS at 4°C for 48 h.

Projections from PVN oxytocin-containing neurons to the pre-Bötzing complex were determined by unilateral ($n = 4$) or bilateral microinjection of CTB ($n = 1$), 12.30 mm caudal to bregma, 2.2 mm lateral to midline, and 0.8 mm dorsal to the ventral surface of the ventrolateral medulla oblongata. Five days after injection, animals were deeply anesthetized and perfused, as described above; the brains were removed and processed immunohistochemically for CTB and oxytocin labeling.

Transverse sections of the whole brain of each animal were cut at 40 μ m using a Bright OTF cryostat (Hacker Instruments and Industries). The immunohistochemical procedures used were previously described (16, 48) for staining tissue sections of the diencephalon. Briefly, free-floating sections were washed in PBS containing 0.3% Triton-X, and then a 1-in-5 series of tissue sections was exposed for 30 min to a PBS-Triton solution containing 1% BSA to block nonspecific binding sites. After a further wash, the tissue was placed overnight at 4°C in a primary polyclonal antibody solution (1:40,000 dilution of goat anti-CTB in PBS-Triton; List Biological Laboratories) for 48 h at 4°C. The sections were washed twice in PBS, and they were incubated for 2 h in a 1:200 solution of donkey anti-goat IgG conjugated with Alexa Fluoro 594 (Texas red; Molecular Probes, Eugene, OR). The sections were washed with PBS and incubated for 16 h at 4°C in a solution containing a 1:7,500 dilution of a rabbit polyclonal oxytocin antibody (Chemicon International). After washing, a 1:200 dilution of secondary antibody (goat anti-rabbit IgG), conjugated with Alexa Fluoro 488 FITC (Molecular Probes), was applied to tissue sections for 2 h. The sections were washed and mounted on gelatin/alum-coated glass slides. A drop of Vecta Shield (Vector Laboratories) was applied to air-dried sections, and the slides were coverslipped.

In addition to CTB experiments, in the present investigation, as in previous studies (14, 16, 47), a viral transneuronal labeling method was used that employs a weakened strain of pseudorabies virus (PRV), a neurotropic herpes virus, to produce controlled infections that spread in a hierarchical manner within anatomically connected sets of neurons. Rats ($n = 7$) were anesthetized with pentobarbital sodium, and PRV was injected into the costal region of the diaphragm at 10 sites with 100 nl/site of PRV (Bartha strain, 3.55×10^7 plaque-forming units/ml). Four days after PRV injections, rats were deeply anesthetized, perfused, and prepared for immunohistochemistry as described above. In these experiments, pig anti-PRV antibody (1:60,000) was substituted for CTB. The secondary antibody, goat anti-pig IgG, was conjugated with Alexa 594.

The oxytocin receptor-specific antibody used in this study was a goat polyclonal antibody (Research Diagnostics, Flanders, NJ). The antibody was made against amino acids mapped at the carboxy terminus of the human oxytocin

receptor (22), and the specificity of this antibody has been documented with rat brain tissue as well as human and mouse tissues. We determined whether these receptors are expressed in the pre-Bötzing complex within the RVLM by immunohistochemistry. The pre-Bötzing complex is a respiratory-related region thought to be involved in rhythm generation (37), which has been defined anatomically by the expression of neurokinin-1 (NK₁) receptors (13).

In the present study, immunostaining procedures for oxytocin receptors and NK₁ receptors were performed sequentially rather than simultaneously, because, in our laboratory's previous studies, we found that this approach provides better specificity and lower background staining (10). Briefly, the free-floating sections were washed in PBS containing 0.3% Triton-X and then transferred for 30 min to a PBS-Triton solution containing 1% BSA to block nonspecific binding sites. After a further wash, the sections were incubated for 48 h at 4°C in a PBS-Triton solution containing goat anti-oxytocin (1:40 dilution). The sections were rinsed and incubated for 2 h with a 1:200 dilution of donkey anti-goat IgG conjugated with Alexa Fluoro 594. Briefly, in the second step, the sections were washed in PBS containing 0.3% Triton-X and then incubated with the NK₁-receptor polyclonal antibody (1:500 dilution; Novus Biologicals, Littleton, CO). The sections were washed in PBS-Triton and then incubated with a 1:200 dilution of goat anti-rabbit secondary antibody conjugated to Alexa 488. Finally, the sections were rinsed in PBS, mounted, and coverslipped using Vecta Shield antifade mounting medium.

Control experiments for each experiment were done to determine whether the primary or the secondary antibodies produced false-positive results. In these experiments, sections were stained with all possible combinations of primary and secondary antibodies in which a single immunoprobe was omitted. Omission of primary or secondary antibodies resulted in the absence of labeling, demonstrating that no false-positive results were obtained with these reagents.

Slides were viewed with a fluorescence microscope (Olympus AX70, Olympus America) equipped with the adequate filter systems to observe the Texas red and green FITC fluorescence. Colocalization of CTB and oxytocin or PRV and oxytocin was identified by alternating between filters to view Texas red and FITC fluorescence and by analyzing merged images of the exact same sites for CTB and oxytocin or PRV and oxytocin, as shown in Figs. 2–4. The contrasting immunoprecipitates were readily distinguishable. Immunoprecipitates indicating oxytocin immunoreactivity and CTB or PRV traits in the PVN were confined to the cytoplasm. Cells containing CTB or PRV were counted only in PVN regions expressing oxytocin immunoreactivity. Subnuclei in the PVN were determined according to classifications previously described for the rat (15) and outlined in a rat brain atlas (32).

Physiological studies. Saline or oxytocin dissolved in saline was microinjected in the RVLM, as previously described (46). Briefly, 10 Sprague-Dawley rats (300–350 g) were anesthetized with urethane, and the femoral artery and vein were catheterized for recording blood pressure (BP) and heart rate (HR) and for administering fluid and anesthetic as needed. After bilateral vagotomy, the rats were placed in a stereotaxic apparatus in a prone position. Bregma and the midline served as stereotaxic zeroes for rostrocaudal and lateral coordinates, which were 12.30 mm caudal to bregma, 2.2 mm lateral to midline, and 0.8 mm dorsal to the ventral surface of the RVLM. Dorsolateral positioning was achieved by slowly lowering the micropipette to the targeted site. Unilateral microinjections with glass micropipettes containing saline or 0.2 nmol of oxytocin (Sigma Chemical) in 200 nl were deliv-

ered in the RVLM using a pneumatic pressure system (model PPS-2; Medical Systems, Greenvale, NY). Diaphragm electromyographic activity (D_{EMG}) was recorded via bipolar electrodes placed in the costal diaphragm. Animals were ventilated with O_2 at a pump rate that provided 30% of total ventilatory activity obtained with 7% CO_2 in O_2 .

Micropipettes were removed at the end of the experiment, filled with Evans blue dye, and reinserted at the same injection site; and the dye was microinjected to localize the initial injection site. Animals were perfused; and the brain stems were removed, sectioned coronally (50- μm thickness), mounted, and stained with 1% neutral red for visualizing injection sites.

D_{EMG} was used to determine amplitude as well as inspiratory and expiratory durations. The amplitude of inspiratory burst, inspiratory time (T_I), and expiratory time (T_E) were analyzed; and respiratory frequency (f ; breaths/min) and minute D_{EMG} ($D_{EMG} \times f$) were calculated. Respiratory response was quantified by averaging these parameters for the control period for 10–15 consecutive breaths and 10–15 breaths at peak response after microinjection of the saline vehicle or oxytocin. BP and HR were measured in the control period and when peak changes occurred after oxytocin administration. Average values (means \pm SD) for the analyzed variables were compared using the Student *t*-test, with statistical significance set at $P < 0.05$.

RESULTS

Neuroanatomic experiments. Immunoreactivity for CTB was detected in neurons of the PVN 5 days after CTB injection into phrenic nuclei. A schematic diagram of the PVN region with its subdivisions and neurons expressing CTB in the PVN are shown in Fig. 1. CTB-labeled neurons were distributed bilaterally in the PVN, with CTB expression being predominant in the PVN ipsilateral to the site of CTB injection for each animal. Analysis of CTB-containing neurons on the predominant side showed that 64% were parvocellular, whereas the remainder were localized in the magnocellular lateral region. Within parvocellular subnuclei, 54% of the CTB-containing cells were localized in the dorsal region, and the rest (46%) were mainly found within the medial ventral area.

Neurons in the PVN expressing CTB and oxytocin are shown in Fig. 2. Immunostaining for CTB and oxytocin was uniformly distributed in the perikaryon of these cells. A subpopulation of PVN neurons, 56 of 946 CTB-positive cells (6%) coexpressed oxytocin, whereas 56 of 1,039 (5%) oxytocin-containing neurons demonstrated colocalization with CTB immunoreactive cells. Most of the colocalized cells were parvocellular with sporadic colocalization in the magnocellular lateral region.

Parvocellular subnuclei of the PVN contained retrogradely labeled neurons after microinjection of CTB in the RVLM. In addition, a few CTB-containing cells were localized in the magnocellular lateral region. Figure 3 shows immunoreactive oxytocin cells and CTB-labeled neurons. Because the distribution of CTB-labeled neurons within the PVN was similar for bilateral and unilateral microinjections in the RVLM, the data were combined. A total of 23 of 707 (3%) CTB immunoreactive cells were colocalized with oxytocin-containing neurons.

PRV was injected into the diaphragm, and, after a survival time of 4 days, PRV-infected parikarya were detected bilaterally in the PVN. PRV-positive cells found in the dorsal and medial ventral parvocellular subnuclei of the PVN were almost twice as abundant as PRV-positive magnocellular lateral neurons. A subpopulation of PRV-infected cells, 3% of 133, coexpressed oxytocin. Similarly, 3% of 135 oxytocin-containing neurons were colocalized with PRV-labeled cells (Fig. 4).

The pre-Bötzinger complex within the RVLM was the site of microinjection of oxytocin (Fig. 5A). This site was identified, as previously described (13), by the abundant expression of NK₁ receptors on cell membranes and in cell processes (Fig. 5B). Neurons expressing oxytocin receptors were also localized in this region (Fig. 5C). Small clusters of cells within the pre-Bötzinger complex were positive for the oxytocin receptor, which appeared as beadedlike structures on cell membranes and in neuronal cell processes.

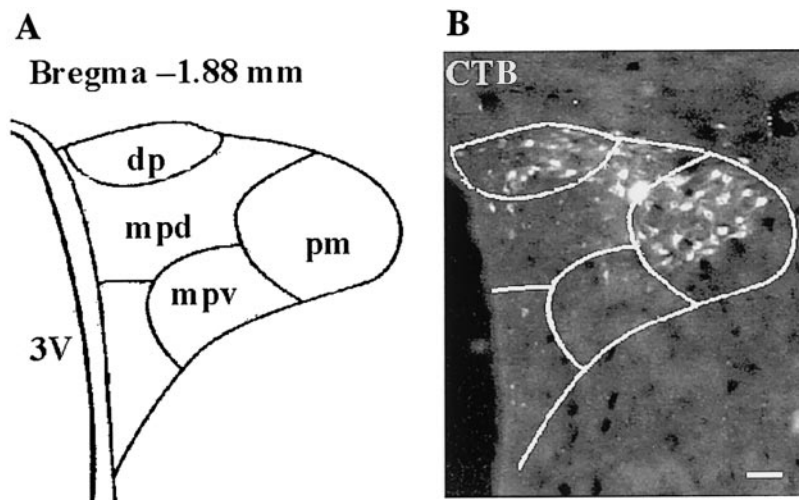


Fig. 1. Schematic drawing showing subdivisions of the hypothalamic paraventricular nucleus (PVN; A) and fluorescent photomicrograph of retrogradely labeled neurons in the PVN (B) after unilateral injection of cholera toxin B subunit (CTB) in the ventral horns of the spinal cord (C_3 – C_4). 3V, 3rd ventricle; dp, dorsal parvocellular; mpd, medial parvocellular dorsal; mpv, medial parvocellular ventral; pm, posterior magnocellular. Scale bar = 100 μm .

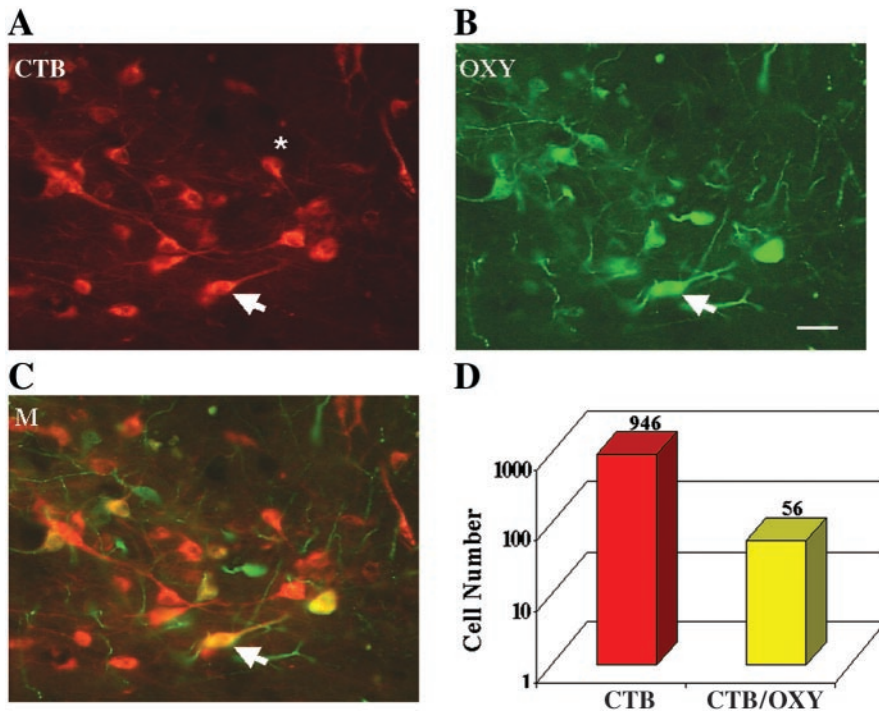


Fig. 2. Fluorescent photomicrographs showing retrograde tracer CTB that was microinjected into phrenic motoneurons (A), immunohistochemical dual labeling of PVN cells for oxytocin (OXY; B), and the merged (M) image of CTB and OXY traits (C). Arrows indicate a representative neuron expressing both CTB and OXY. The asterisk indicates a CTB-positive neuron that does not express OXY. Scale bar = 50 μ m. D: quantitative analysis of the no. of cells in the PVN that were positive for CTB alone and the no. of neurons that expressed both CTB and OXY ($n = 5$).

Physiological studies. Microinjection of saline (200 nl) had insignificant ($P > 0.05$), transient effects on the parameters that were studied. For example, D_{EMG} on average increased only by 2%, whereas f and minute D_{EMG} decreased by 8 and 6%, respectively ($n = 6$ rats). Mean arterial BP was comparable for the control and saline vehicle, and HR increased by 2.4%. However, mi-

croinjection of oxytocin caused a significant increase (74%) in D_{EMG} amplitude (Fig. 6A). There was also a 25% increase in f , which can be attributed to shortening of T_i and T_E , which decreased by 18 and 35%, respectively. The T_E decreased from a mean of 1.06 ± 0.08 to 0.69 ± 0.04 s ($P < 0.05$; Fig. 6C), whereas T_i decreased from 0.66 ± 0.07 to 0.54 ± 0.03 s ($P < 0.05$; Fig. 6D).

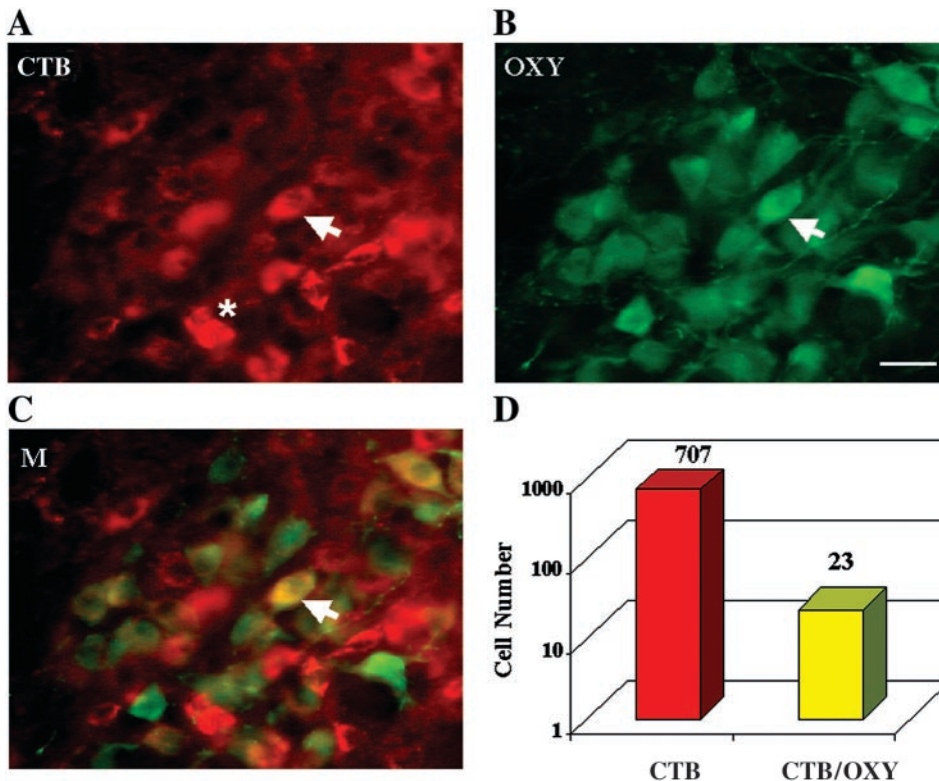


Fig. 3. Fluorescent photomicrographs showing CTB after bilateral microinjection of CTB into the pre-Bötzinger complex (A), immunohistochemical dual labeling of neurons in the PVN expressing OXY (B), and the M image of CTB and OXY traits (C). Arrows show a representative neuron expressing both CTB and OXY. The asterisk indicates a CTB-positive cell that does not express OXY. Scale bar = 50 μ m. D: analysis of the no. of cells in the PVN that expressed CTB alone and the no. of cells that were positive for CTB and OXY ($n = 5$).

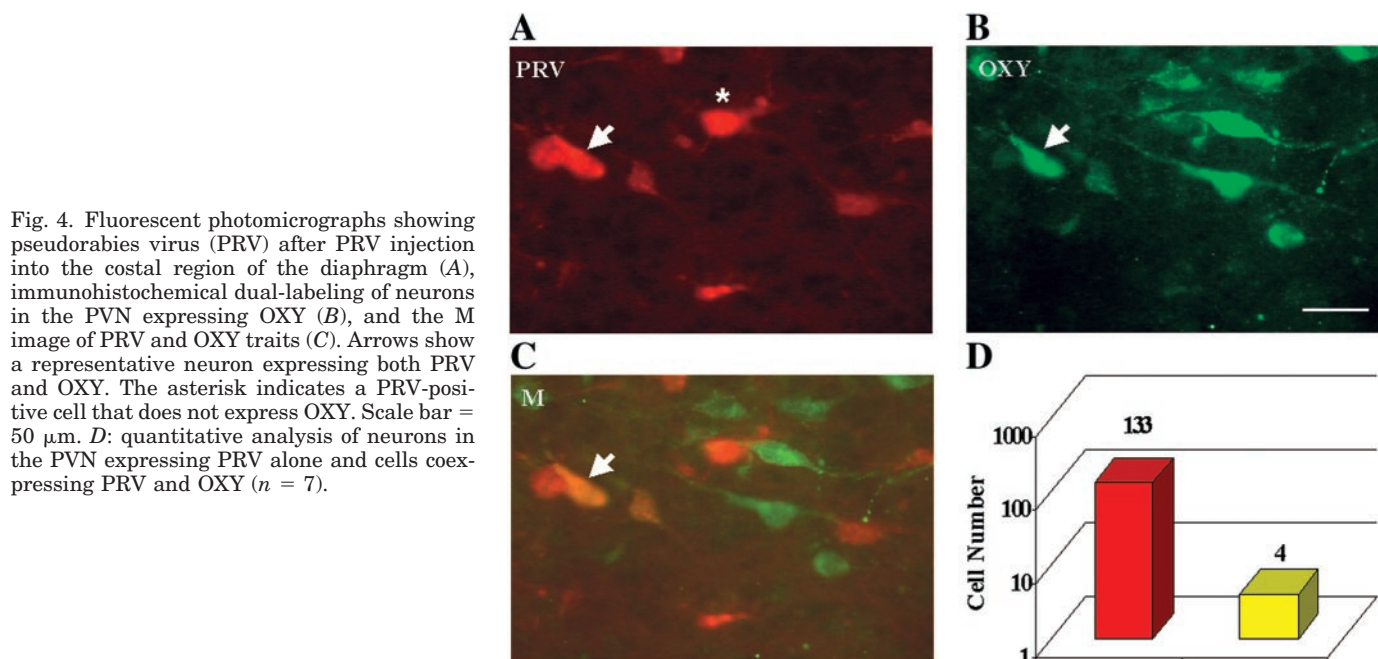


Fig. 4. Fluorescent photomicrographs showing pseudorabies virus (PRV) after PRV injection into the costal region of the diaphragm (A), immunohistochemical dual-labeling of neurons in the PVN expressing OXY (B), and the M image of PRV and OXY traits (C). Arrows show a representative neuron expressing both PRV and OXY. The asterisk indicates a PRV-positive cell that does not express OXY. Scale bar = 50 μ m. D: quantitative analysis of neurons in the PVN expressing PRV alone and cells coexpressing PRV and OXY ($n = 7$).

Microinjection of oxytocin also caused an increase in arterial pressure and HR. Mean arterial pressure increased by 35% from 96.6 ± 22 to 130.6 ± 39 mmHg ($P < 0.05$), and HR was elevated from 423 ± 32 to 435 ± 31 beats/min ($P < 0.05$). This 3% change in HR reached statistical significance but probably has no physiological importance.

DISCUSSION

In the present report, we have presented new findings showing that a subpopulation of PVN oxytocin-containing cells innervate respiratory-related phrenic motoneurons and neurons located in the pre-Bötzinger

complex within the RVLM. Although the majority of these neurons were found in parvocellular subdivisions of the PVN, a small group was identified within the magnocellular division of the PVN, suggesting their possible role in coordination of neuroendocrine, cardiovascular, and respiratory responses to different behavioral changes. Hence, previous assumptions that magnocellular oxytocin-containing neurons are exclusively involved in endocrine regulation need to be revised.

It has been shown previously that parvocellular neurons project to autonomic brain stem nuclei or spinal cord sites (43), including those regions that are involved in cardiovascular control (1, 4). However, mag-

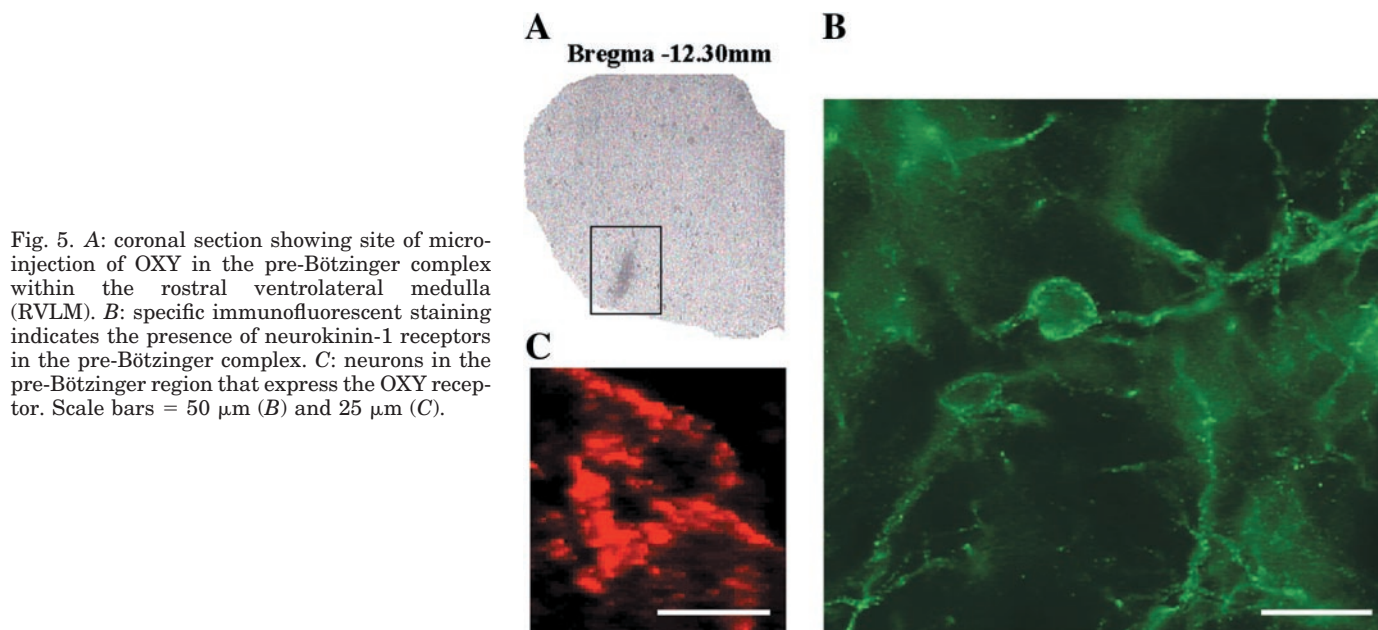


Fig. 5. A: coronal section showing site of microinjection of OXY in the pre-Bötzinger complex within the rostral ventrolateral medulla (RVLM). B: specific immunofluorescent staining indicates the presence of neurokinin-1 receptors in the pre-Bötzinger complex. C: neurons in the pre-Bötzinger region that express the OXY receptor. Scale bars = 50 μ m (B) and 25 μ m (C).

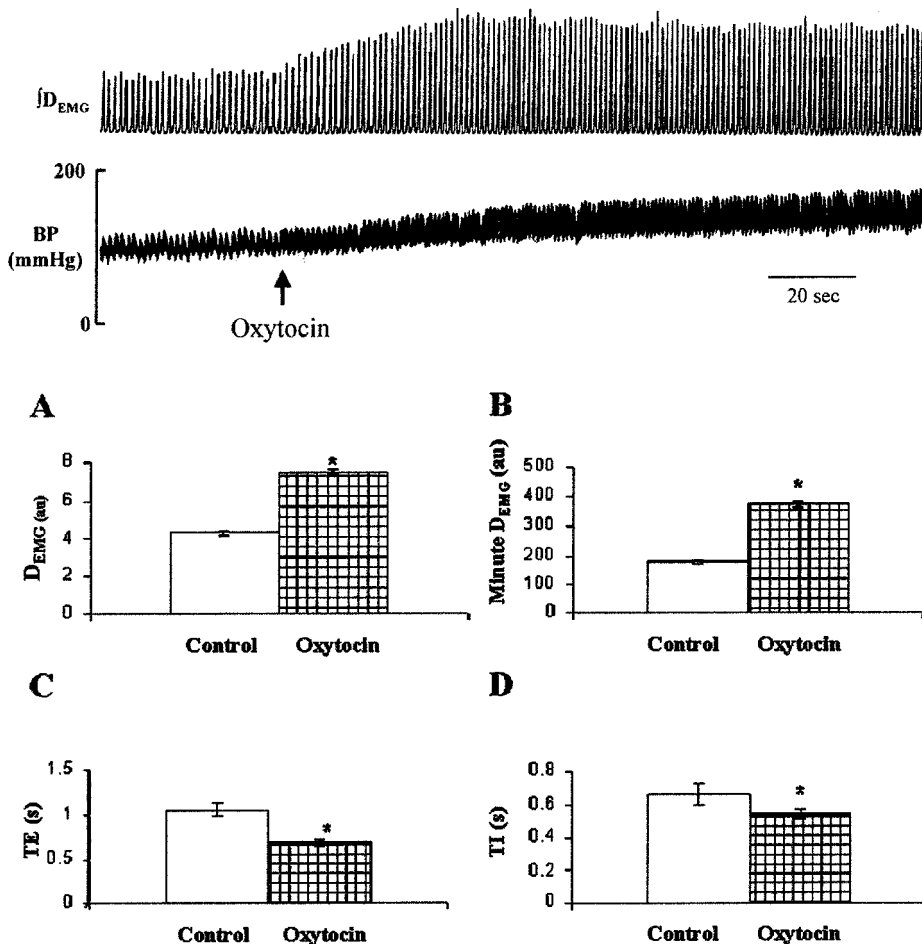


Fig. 6. *Top*: representative recording of integrated diaphragm electromyographic activity (fD_{EMG}) and arterial blood pressure (BP) showing response to unilateral microinjection of OXY (0.2 nmol/site, 200 nl) in the pre-Bötzinger complex within the RVLM. Arrow indicates time of injection. *Bottom*: means \pm SD of changes in peak D_{EMG} (A), minute D_{EMG} (B), expiratory time (TE; C), and inspiratory time (TI; D) duration before (open bars) and after (hatched bars) microinjection ($n = 10$ animals). au, Arbitrary units. *Statistical difference, $P < 0.05$.

nocellular neurons were considered to innervate the posterior pituitary gland. Hence, our findings indicate that a subpopulation of magnocellular neurons may provide monosynaptic inputs to the RVLM pre-Bötzinger region and to the spinal cord. It is conceivable that this finding could be the result of nonspecific labeling produced by some diffusion of retrograde tracer into the region that contains nonrespiratory related cells or the uptake of tracer by descending fibers of magnocellular neurons that innervate spinal cord cells. The results of an experiment in which FluoroGold was used as a retrograde tracer suggested that labeling could be due to a transport of the marker through fibers of passage (6). However, this is an unlikely occurrence after CTB injections (48). It has been shown that latex microspheres, a different retrograde tracer, injected into the white matter do not cause labeling of specific cell groups in the medulla oblongata as it does when injected into the intermediolateral cell column (34). Furthermore, results with PRV, a highly specific transneuronal marker (25), indicate that the PVN neurons project to phrenic motoneurons (16). Recently, it has been shown that PVN parvocellular neurons innervate motoneurons that control the activity of the posterior cricoarytenoid muscle, an abductor of the vocal cords (47), and vagal motoneurons that innervate the tracheobronchial system (14, 16). These findings

suggest that PVN neurons play a central integrative role and coordinate the activity of different sets of respiratory-related muscles and airway geometry.

The present results indicate that oxytocin, in response to a variety of stimuli, such as suckling, parturition, or certain kinds of stress, could be released not only from the posterior pituitary into the systemic circulation but also at different sites along the neural axis, including inspiration-related medullary neurons and phrenic motoneurons. The effects of released oxytocin are mediated through oxytocin receptors, which we found to be present in the pre-Bötzinger complex.

The results of the present study showed that neurons within the pre-Bötzinger complex, which receive descending inputs from PVN oxytocin-containing neurons, express oxytocin receptors. These results extend previous findings demonstrating localization of oxytocin receptors within the central nervous system (49). The encoded oxytocin receptor is a polypeptide with seven transmembrane domains, and it belongs to the G class of protein-coupled receptors that are linked to phospholipase C β -isoforms. How the oxytocin-oxytocin-receptor signaling pathway affects respiratory drive is not known. However, it has been shown that activation of the receptor leads to generation of inositol triphosphate, which triggers Ca^{2+} release from intracellular stores, and 1,2-diacylglycerol, which stimu-

lates protein kinase C. By binding to calmodulin, Ca^{2+} activates a Ca^{2+} /calmodulin-sensitive adenylyl cyclase, resulting in protein kinase A activation and suppression of the glycine-evoked current. This leads to increased cell excitability and to cell depolarization (for review, see Ref. 12).

Physiological experiments showed that activation of oxytocin receptors by microinjection of oxytocin (pmol concentration) into the pre-Bötzinger region, which was identified anatomically by the expression of NK_1 receptors (13), increased respiratory drive because of an increase in tidal volume activity and f discharge. It has been shown that injection of peptidergic drugs such as NK_1 and μ -opioid-receptor agonists into the pre-Bötzinger region also modulate f (13). In addition, oxytocin microinjection increased arterial BP and HR. These effects were similar to changes observed by chemical stimulation of the hypothalamic PVN (48), indicating that oxytocin of neuronal origin can affect respiratory drive and sympathetic outflow by acting on neurons located within the RVLM. However, this does not exclude the contribution of PVN-brain stem and PVN-spinal cord pathways. In the isolated spinal cord of newborn rats, oxytocin induced depolarization of spinal motoneurons, an effect that is partially reduced, but not suppressed, by the addition of tetrodotoxin or by switching to a low- Ca^{2+} -high- Mg^{2+} perfusion solution (40). These findings suggest that the peptide acts by decreasing K^+ conductance.

In addition to direct effects of oxytocin on the membrane ionic permeability in selected neuronal populations of respiratory-related neurons, oxytocin can influence neurotransmission via presynaptic mechanisms. It is expected that oxytocin-containing terminals form axosomatic, axodendritic, and axoaxonic synapses as in other brain and spinal cord regions (19). Oxytocin acting presynaptically will enhance glutamate release from these terminals and facilitate glutamatergic synaptic transmission by presynaptic mechanisms.

Activation of oxytocin neurons after PVN stimulation may affect respiration through various brain regions that receive oxytocinergic projections (for a bibliography, see Refs. 3, 8, 38), such as neurons within the solitary tract nucleus (NTS). Vasopressin and oxytocin, acting on NTS neurons, cause a reversible, concentration-dependent increase in firing rate (18, 33). Hence, oxytocin-sensitive NTS cells, which receive afferent inputs from visceral receptors, including peripheral chemoreceptors (11), may be partly involved in respiratory changes induced by PVN stimulation.

Stimulation of the PVN also elicits a release of oxytocin into the blood and cerebrospinal fluid (20). Circulating oxytocin does not readily cross the blood-brain barrier. However, it may indirectly influence breathing through activation of neurons that are outside of the blood-brain barrier, including those of the area postrema, which, when activated, cause an increase in respiratory drive (2). Electrical or chemical stimulation of the caudal compact portion of the area postrema provokes excitatory effects on the inspiratory motor output, without apparent changes in arterial BP (39).

Further studies are needed to define the role of these pathways in regulation of breathing by endogenously released oxytocin.

The PVN neurons are activated by many stimuli that also affect breathing. It has been shown that about one-third of PVN neurons are excited by increased CO_2 (7), and these activated neurons express c-Fos (17). Hypoxia also induces an increase in c-Fos expression in PVN neurons (26), and prolonged hypoxia induces the release of oxytocin and vasopressin (21). During exercise, muscle contractions activate a large number of oxytocin-containing neurons (23). All of these stimuli increase breathing activity and sympathetic outflow. Taken together, these results indicate that oxytocin-containing neurons may constitute a part of the neural pathways involved in respiratory responses to changes in chemical drive and could participate in reflexly increased respiration during static and dynamic muscle contraction.

Ventilatory responses to changes in chemical drive are altered in a variety of genetic disorders, including Prader-Willi syndrome (PWS). Patients with PWS are reported to have sleep-disordered breathing, a daytime hypersomnolence, aberrant control of body temperature, blunted hypercapnic ventilatory response, decreased response to hypoxia (27), and a significantly higher arousal threshold to hypercapnia compared with the control subjects (24). In subjects suffering from PWS, a reduction in the total cell number in the PVN was observed, and the number of oxytocin-expressing neurons was decreased by 54% (42). The decreased number of oxytocin neurons, the putative satiety neurons in the hypothalamic PVN, could be the basis of the insatiable hunger and obesity of patients with PWS (41), as well as the cause of reduced ventilatory responses to hypercapnia and hypoxia.

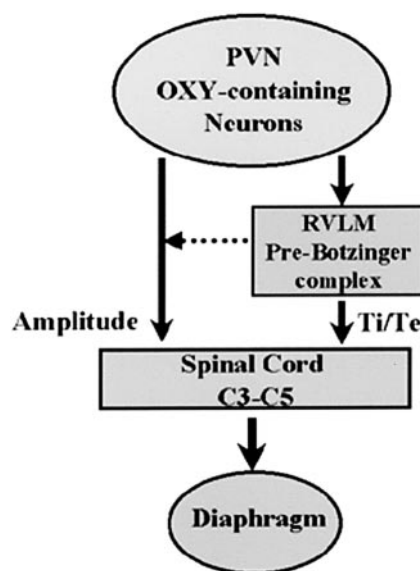


Fig. 7. Schematic presentation of the neural pathways involved in OXY-related regulation of respiratory output. See explanation in the text.

The changes in PWS could be related to leptin deficiency. Recently, it has been shown that the lack of leptin in mice is associated with obesity, CO₂ retention, and a decreased ventilatory response to hypercapnia (30, 45). Hence, in PWS, a relative deficiency in leptin might play a role in the observed breathing disorders. However, it has been shown that PWS is not accompanied by deranged leptin levels (29). Therefore, leptin's role in preserving chemosensitivity during sleep, in providing adequate ventilatory responses to CO₂ in the presence of obesity, and in regulating sympathetic outflow (9) could be linked to the oxytocinergic system of the hypothalamus, as well as other hypothalamic neuromodulators and/or their receptors.

In summary, we provide new information on two neural pathways, PVN-pre-Bötzinger complex and PVN-phrenic nuclei, that use oxytocin as a neurotransmitter to regulate respiratory drive, as schematically presented in Fig. 7. These neural networks provide a possible neural link among metabolism, energy balance, and respiratory drive. Based on the results of the present study and previous findings, theoretically, oxytocin-containing neurons could be considered as the coordinating cells that adjust respiratory drive to behavioral changes, metabolism, and energy balance. Alterations in the function of these neurons may contribute to obesity, development of obesity hypoventilation syndrome, and sleep-related disorders of breathing.

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REFERENCES

1. Badoer E. Hypothalamic paraventricular nucleus and cardiovascular regulation. *Clin Exp Pharmacol Physiol* 28: 95–99, 2001.
2. Bongiani F, Mutolo D, Carfi M, and Pantaleo T. Area postrema glutamate receptors mediate respiratory and gastric responses in the rabbit. *Neuroreport* 9: 2057–2062, 1998.
3. Buijs RM. Intra- and extrahypothalamic vasopressin and oxytocin pathways in the rat. Pathways to the limbic system, medulla oblongata and spinal cord. *Cell Tissue Res* 192: 423–435, 1978.
4. Ciriello J and Calaresu FR. Role of paraventricular and supraoptic nuclei in central cardiovascular regulation in the cat. *Am J Physiol Regulatory Integrative Comp Physiol* 239: R137–R142, 1980.
5. Coote JH. Cardiovascular function of the paraventricular nucleus of the hypothalamus. *Biol Signals* 4: 142–149, 1995.
6. Dado RJ, Burstein R, Cliffer KD, and Giesler GJ Jr. Evidence that Fluoro-Gold can be transported avidly through fibers of passage. *Brain Res* 533: 329–333, 1990.
7. Dillon GH and Waldrop TG. Responses of feline caudal hypothalamic cardiorespiratory neurons to hypoxia and hypercapnia. *Exp Brain Res* 96: 260–272, 1993.
8. Dubois-Dauphin M, Raggenbass M, Widmer H, Tribollet E, and Dreifuss J. Morphological and electrophysiological evidence for postsynaptic localization of functional oxytocin receptors in the rat dorsal motor nucleus of the vagus nerve. *Brain Res* 575: 124–131, 1992.
9. Dunbar JC and Lu H. Leptin-induced increase in sympathetic nervous and cardiovascular tone is mediated by proopiomelanocortin (POMC) products. *Brain Res Bull* 50: 215–221, 1999.
10. Ferguson DG, Haxhiu MA, To AJ, Erokku B, and Dreshaj IA. The alpha3 subtype of the nicotinic acetylcholine receptor is expressed in airway-related neurons of the nucleus tractus solitarius, but is not essential for reflex bronchoconstriction in ferrets. *Neurosci Lett* 287: 141–145, 2000.
11. Finley JC and Katz DM. The central organization of carotid body afferent projections to the brainstem of the rat. *Brain Res* 572: 108–116, 1992.
12. Gimpl G and Fahrenholz F. The oxytocin receptor system: structure, function, and regulation. *Physiol Rev* 81: 629–683, 2001.
13. Gray PA, Rekling JC, Bocchiaro CM, and Feldman JL. Modulation of respiratory frequency by peptidergic input to rhythmogenic neurons in the pre-Bötzinger complex. *Science* 286: 1566–1568, 1999.
14. Hadziefendic S and Haxhiu MA. CNS innervation of vagal preganglionic neurons controlling peripheral airways: a transneuronal labeling study using pseudorabies virus. *J Auton Nerv Syst* 76: 135–145, 1999.
15. Hallbeck M, Larhammar D, and Blomqvist A. Neuropeptide expression in rat paraventricular hypothalamic neurons that project to the spinal cord. *J Comp Neurol* 433: 222–238, 2001.
16. Haxhiu MA, Jansen AS, Cherniack NS, and Loewy AD. CNS innervation of airway-related parasympathetic preganglionic neurons: a transneuronal labeling study using pseudorabies virus. *Brain Res* 618: 115–134, 1993.
17. Haxhiu MA, Yung K, Erokku B, and Cherniack NS. CO₂-induced c-fos expression in the CNS catecholaminergic neurons. *Respir Physiol* 105: 35–45, 1996.
18. Henry JL and Sessle BJ. Vasopressin and oxytocin express excitatory effects on respiratory and respiration-related neurones in the nuclei of the tractus solitarius in the cat. *Brain Res* 491: 150–155, 1989.
19. Jo YH, Stoeckel ME, Freund-Mercier MJ, and Schlichter R. Oxytocin modulates glutamatergic synaptic transmission between cultured neonatal spinal cord dorsal horn neurons. *J Neurosci* 18: 2377–2386, 1998.
20. Jones PM, Robinson IC, and Harris MC. Release of oxytocin into blood and cerebrospinal fluid by electrical stimulation of the hypothalamus or neural lobe in the rat. *Neuroendocrinology* 37: 454–458, 1983.
21. Kelestimur H, Leach RM, Ward JP, and Forsling ML. Vasopressin and oxytocin release during prolonged environmental hypoxia in the rat. *Thorax* 52: 84–88, 1997.
22. Kimura T, Tanizawa O, Mori K, Brownstein MJ, and Okayama H. Structure and expression of a human oxytocin receptor. *Nature* 356: 526–529, 1992.
23. Li J, Hand GA, Potts JT, and Mitchell JH. Identification of hypothalamic vasopressin and oxytocin neurons activated during the exercise pressor reflex in cats. *Brain Res* 752: 45–51, 1997.
24. Livingston FR, Arens R, Bailey SL, Keens TG, and Ward SL. Hypercapnic arousal responses in Prader-Willi syndrome. *Chest* 108: 1627–1631, 1995.
25. Loewy AD. Viruses as transneuronal tracers for defining neural circuits. *Neurosci Biobehav Rev* 22: 679–684, 1998.
26. Luo Y, Kaur C, and Ling EA. Hypobaric hypoxia induces fos and neuronal nitric oxide synthase expression in the paraventricular and supraoptic nucleus in rats. *Neurosci Lett* 296: 145–148, 2000.
27. Menendez AA. Abnormal ventilatory responses in patients with Prader-Willi syndrome. *Eur J Pediatr* 158: 941–942, 1999.
28. Morris M, Callahan MF, Li P, and Lucion AB. Central oxytocin mediates stress-induced tachycardia. *J Neuroendocrinol* 7: 455–459, 1995.
29. Myers SE, Davis A, Whitman BY, Santiago JV, and Landt M. Leptin concentrations in Prader-Willi syndrome before and after growth hormone replacement. *Clin Endocrinol (Oxf)* 52: 101–105, 2000.
30. O'Donnell CP, Tankersley CG, Polotsky VP, Schwartz AR, and Smith PL. Leptin, obesity, and respiratory function. *Respir Physiol* 119: 163–170, 2000.
31. Palkovits M. Interconnections between the neuroendocrine hypothalamus and the central autonomic system. Geoffrey Harris Memorial Lecture, Kitakyushu, Japan, October 1998. *Front Neuroendocrinol* 20: 270–295, 1999.

32. **Paxinos G and Watson C.** *The Rat Brain in Stereotaxic Coordinates* (4th ed.). Orlando, FL: Academic, 1998, p. 25–27.
33. **Raggenbass M, Tribollet E, Dubois-Dauphin M, and Dreifuss JJ.** Vasopressin receptors of the vasopressor (V1) type in the nucleus of the solitary tract of the rat mediate direct neuronal excitation. *J Neurosci* 9: 3929–3936, 1989.
34. **Sasek CA and Helke CJ.** Enkephalin-immunoreactive neuronal projections from the medulla oblongata to the intermediolateral cell column: relationship to substance P-immunoreactive neurons. *J Comp Neurol* 287: 484–494, 1989.
35. **Sawchenko PE and Swanson LW.** Immunohistochemical identification of neurons in the paraventricular nucleus of the hypothalamus that project to the medulla or to the spinal cord in the rat. *J Comp Neurol* 205: 260–272, 1982.
36. **Schlenker E, Barnes L, Hansen S, and Martin D.** Cardiorespiratory and metabolic responses to injection of bicuculline into the hypothalamic paraventricular nucleus (PVN) of conscious rats. *Brain Res* 895: 33–40, 2001.
37. **Smith JC, Ellenberger HH, Ballanyi K, Richter DW, and Feldman JL.** Pre-Botzinger complex: a brainstem region that may generate respiratory rhythm in mammals. *Science* 254: 726–729, 1991.
38. **Sofroniew MV.** Morphology of vasopressin, and oxytocin neurons and their central and vascular projections. *Prog Brain Res* 60: 101–114, 1983.
39. **Srinivasan M, Bongianini F, Fontana GA, and Pantaleo T.** Respiratory responses to electrical and chemical stimulation of the area postrema in the rabbit. *J Physiol* 463: 409–420, 1993.
40. **Suzue T, Yanaihara N, and Otsuka M.** Actions of vasopressin, gastrin releasing peptide and other peptides on neurons on newborn rat spinal cord in vitro. *Neurosci Lett* 26: 137–142, 1981.
41. **Swaab DF.** Prader-Willi syndrome and the hypothalamus. *Acta Paediatr Suppl* 423: 50–54, 1997.
42. **Swaab DF, Purba JS, and Hofman MA.** Alterations in the hypothalamic paraventricular nucleus and its oxytocin neurons (putative satiety cells) in Prader-Willi syndrome: a study of five cases. *J Clin Endocrinol Metab* 80: 573–579, 1995.
43. **Swanson LW and Kuypers HG.** The paraventricular nucleus of the hypothalamus: cytoarchitectonic subdivisions and organization of projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods. *J Comp Neurol* 194: 555–570, 1980.
44. **Swanson LW and Sawchenko PE.** Paraventricular nucleus: a site for the integration of neuroendocrine and autonomic mechanisms. *Neuroendocrinology* 31: 410–417, 1980.
45. **Tankersley CG, O'Donnell C, Daood MJ, Watchko JF, Mitzner W, Schwartz A, and Smith P.** Leptin attenuates respiratory complications associated with the obese phenotype. *J Appl Physiol* 85: 2261–2269, 1998.
46. **Tolentino-Silva FP, Haxhiu MA, Ernsberger P, Waldbaum S, and Dreshaj IA.** Differential cardiorespiratory control elicited by activation of ventral medullary sites in mice. *J Appl Physiol* 89: 437–444, 2000.
47. **Waldbaum S, Hadziefendic S, Erokku B, Zaidi SI, and Haxhiu MA.** CNS innervation of posterior cricoarytenoid muscles: a transneuronal labeling study. *Respir Physiol* 126: 113–125, 2001.
48. **Yeh ER, Erokku B, LaManna JC, and Haxhiu MA.** The paraventricular nucleus of the hypothalamus influences respiratory timing and activity in the rat. *Neurosci Lett* 232: 63–66, 1997.
49. **Yoshimura R, Kiyama H, Kimura T, Araki T, Maeno H, Tanizawa O, and Tohyama M.** Localization of oxytocin receptor messenger ribonucleic acid in the rat brain. *Endocrinology* 133: 1239–1246, 1993.