Published in final edited form as:

Respir Physiol Neurobiol. 2006 December ; 154(3): 319–330.

Phenotypic traits of the hypothalamic PVN cells innervating airway-related vagal preganglionic neurons

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Abstract

The paraventricular nucleus of the hypothalamus (PVN) integrates multiple inputs via projections from arginine vasopressin (AVP)- and oxytocin (OXT)-containing neurons to the brain stem and spinal cord as well as regulates respiratory and cardiovascular stress-related responses, which also affect airway function. In the present study, we used immunocytochemistry and the retrograde transneuronal tracer, Bartha strain of pseudorabies virus expressing green fluorescent protein (PRV-GFP), to localize AVP- and OXT-producing neurons that project to airway-related vagal preganglionic neurons (AVPNs) innervating intrapulmonary airways. PRV-GFP was microinjected into the upper right lung lobe, and after 4 days survival, hypothalamic tissue sections were processed for co-expression of PRV-GFP and AVP or PRV-GFP and OXT. In addition, in a separate group of five rats, Phaseolus vulgaris leucoagglutinin (PHAL), an anterograde tracer, was injected unilaterally into the PVN and cholera toxin β subunit was microinjected into the tracheal wall. Analysis of five successfully infected animals showed that 14% of PRV-GFP labeled neurons express AVP traits and 18% of transneuronally-labeled neurons contain OXT. Furthermore, the identified AVPNs innervating extrathoracic trachea receive axon terminals of the PVN neurons. The results indicate that AVP- and OXT-producing PVN cells, via direct projections to the AVPNs, could modulate cholinergic outflow to the airways, as a part of overall changes in response to stress.

Keywords

Hypothalamic paraventricular nucleus (PVN); Arginine vasopressin-containing neurons (AVP); Oxytocin-expressing cells (OXT); Transneuronal labeling; Pseudorabies virus expressing green fluorescent protein (PRV-GFP); Phaseolus vulgaris leucoagglutinin (PHAL); Anterograde tracer; Rat

1. Introduction

The neurons of the hypothalamic paraventricular nucleus (PVN), as an integrating site (Schlenker, 2005), receive a variety of afferent inputs and send numerous signals to multiple central nervous system (CNS) cell groups, including those involved in the control of the

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sympathetic activity (Jansen et al., 1995), regulation of cholinergic outflow to the visceral organs (Haxhiu et al., 1993; Loewy and Haxhiu, 1993), and in governing respiratory drive (Yeh et al., 1997; Schlenker et al., 2001; Kc et al., 2002; Mack et al., 2002). Furthermore, PVN neurons participate in mediation of autonomic responses to stress (Benarroch, 2005; Coote, 2005), and are activated following allergen exposure in a murine model of asthma (Costa-Pinto et al., 2005).

The amygdaloid neurons, in particular those located within the medial nucleus (MeA), which project to the PVN (Tanaka et al., 1997), are activated by emotional stress (Ebner et al., 2004). These neurons, in turn through direct projections (Hadziefendic and Haxhiu, 1999) and/or via modulating the PVN outflow to AVPNs, could lead to worsening of bronchial asthma.

Anatomical studies from our laboratories showed that PVN neurons containing arginine vasopressin (AVP) and/or oxytocin (OXT) project to the brain stem and spinal cord cells subserving respiratory and circulatory homeostasis (Kc et al., 2002; Mack et al., 2002). These projections emanate from the same sites, previously shown to innervate airway-related vagal preganglionic neurons (AVPNs) (Haxhiu et al., 1993; Hadziefendic and Haxhiu, 1999). However, the presence of direct projections from PVN neurons containing AVP or OXT to AVPNs has not been investigated. Therefore, the aim of this study was to test our hypothesis that a subpopulation of hypothalamic PVN neurons containing AVP and/or OXT project to AVPNs regulating cholinergic outflow to the airways. The present findings support our hypothesis showing that a sub-population of PVN neurons encoding AVP or OXT innervates AVPNs.

2. Materials and methods

2.1. Animals

Experimental protocols in the present studies were approved by Case Western Reserve University and Howard University Institutional Animal Care and Use Committee. Experiments were performed in male Sprague–Dawley rats of 300–375 g body weight (Harlan, Indianapolis, IN) according to the Public Health Service on Humane Care and Use of Laboratory Animals (NIH Publication 80-23).

2.2. Neuroanatomical experiments

2.2.1. Retrogradely labeled PVN neurons—In the first set of experiments, vasopressin-containing PVN cells that project to AVPNs innervating intrapulmonary airways were identified by a viral transneuronal tracing technique, combined with immunocytochemistry. The rats were anesthetized (sodium pentobarbital, 40 mg/kg, i.p.), and a weakened strain of pseudorabies virus expressing green fluorescent protein (PRV-GFP), was injected into the upper right lobe of the lung, at 10 sites using (100 nl/site) of PRV-GFP (3×10^7 plaque forming units per ml), as previously described (Hadziefendic and Haxhiu, 1999). Four days after PRV-GFP injections, the rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and rapidly perfused transcardially with 0.9% buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The perfused brains were removed from the animals, post-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and then cryoprotected in 30% sucrose in 0.1 M phosphate buffer to maintain peptide immunoreactivity and tissue morphology (Watson et al., 1986). Immunocytochemical procedures, for staining tissue sections were reported in previous studies (Kc et al., 2002; Mack et al., 2002). After identification of successful transport of the tracer, characterized by infection of AVPNs, in five infected rats, a series of transverse sections of the diencephalon were cut at 40 μ m, using a Bright OTF Cryostat (Hacker Instruments Inc., Fairfield, NJ). Briefly, free-floating sections were washed in PBS containing 0.3% Triton-X. A one-in-five series of tissue

sections were exposed for 30 min to a PBS-Triton solution containing 1% bovine serum albumin (BSA) to block non-specific binding sites. After a further wash in PBS, the sections were placed overnight at 4 °C in a solution containing a 1:1000 dilution of a polyclonal vasopressin antibody (Chemicon International Inc., Temecula, CA). Sections were then washed in PBS and incubated in a 1:200 dilution of secondary antibody (goat anti-rabbit IgG), conjugated with Alexa Fluor 568 (Texas Red; Molecular Probes Inc., Eugene, OR). Subsequently sections were washed and mounted on gelatin/alum-coated glass slides. Vecta Shield (Vector Laboratories Inc., Burlingame, CA) was applied to the air-dried sections and slides were cover-slipped.

In the second series of experiments, in separate hypothalamic coronal sections of the PRV-GFP infected rats, we determined whether PVN neurons projecting to the AVPNs express OXT phenotypic traits. The only difference between the vasopressin and oxytocin immunolabeling studies was the use of a primary antibody. Oxytocin immunolabeling studies used a rabbit polyclonal antibody (Chemicon International Inc., Temecula, CA) at a 1:7500 dilution.

2.2.2. Anterogradely labeled PVN axons within the rostral nucleus ambiguus (rNA) region—Axon terminals of PVN cells ending in close proximity to AVPNs were identified by an anterograde tracing technique. The rats ($n = 5$) were anesthetized (sodium pentobarbital, 40 mg/kg, i.p.), and a *Phaseolus vulgaris* leucoagglutinin (PHAL; 1 μ l of 4% solution; Vector Laboratories Inc., Burlingame, CA) was incrementally microinjected into the PVN where OXT and AVP are located. Ten days post-PHAL injection, rats were re-anesthetized (sodium pentobarbital, 40 mg/kg, i.p.) and cholera toxin β subunit (CTb; List Biological Laboratories Inc., Campbell, CA) was microinjected into the extrathoracic trachea. Five days after the CTb injection, the rats were deeply anesthetized, perfused, brains were removed, cryoprotected, and sectioned. In these experiments, we first completed the PHAL staining protocol followed by the CTb labeling procedure. Immunocytochemical procedures for staining tissue section are similar to the ones described above, except for the antibodies used. Briefly tissues were incubated in rabbit anti-PHAL (Vector Laboratories Inc., Burlingame, CA) at a dilution of 1:500 for 24 h and then stained with donkey anti-rabbit secondary antibody conjugated with Alexa Fluor 488 (FITC; Molecular Probes Inc., Eugene, OR). Sequentially, the sections were exposed to goat anti-CTb (1:20,000; List Biological Laboratories Inc., Campbell, CA) for 36 h followed by incubation in donkey anti-goat secondary antibody conjugated with Alexa Fluor 568 (Texas Red; Molecular Probes Inc., Eugene, OR).

Furthermore, in separate coronal sections of the medulla oblongata of rats injected with CTb into the extrathoracic trachea, we determined whether nerve terminals within the rNA region express AVP or OXT phenotypic traits. In these experiments, we first completed the AVP or OXT immunostaining protocol, followed by the CTb labeling procedure, as described above except for AVP or OXT which was visualized with donkey anti-rabbit secondary antibody conjugated with Alexa Fluor 568, and CTb with donkey anti-goat secondary antibody conjugated with Alexa Fluor 488.

2.2.3. Control experiments—In the present studies, the specificity of primary antibodies used has been documented with human and rodent tissues and in our laboratory in rat brain tissue (Kc et al., 2002; Mack et al., 2002). In addition, for each set of immunocytochemistry labeling studies, control experiments were done to determine whether the primary or the secondary antibodies produced false-positive results. Sections were stained with secondary antibodies in which a primary antibody was omitted. The omission of primary antibodies resulted in the absence of immunolabeling, demonstrating that no false-positive results were obtained with primary or secondary immunoprobe.

2.2.4. Fluorescence and confocal laser microscopy—Immunostained tissue sections were viewed with fluorescence (Olympus AX70, Olympus America Inc., Melville, NY) and laser confocal (Olympus BX61, Olympus America Inc., Melville, NY) microscopes that are equipped with filter systems to observe the Rhodamine (red) and FITC (green) fluorescence. Digital images of the exact same sites for PRV and vasopressin or oxytocin were captured in PVN subregions that contained vasopressin or oxytocin immunoreactivity. For each trait, the intensity of the signal for immunolabeled neurons and the intensity of the background signal were measured using Sigma Scan Pro image analysis software (SPSS, Chicago, IL). Only those cells that had an intensity two-fold above the background and where the entire outline of the same neuron was clearly delineated in individual and merged images were counted. In addition, axon terminals of PVN neurons within the rNA region and their relation to CTb-labeled AVPN neurons were analyzed. Data obtained with confocal microscopy are presented due to higher sensitivity and specificity of this technique in detecting two fluorescent labels in a single neuron within relatively thick section, and a single axonal terminal ending in the proximity of a labeled AVPN.

2.2.5. Data analysis—The PVN neurons expressing only PRV-GFP, neurons containing only AVP or OXT, neurons containing both PRV-GFP and AVP or PRV-GFP and OXT were counted in each rat, and presented as total number of neurons in all studied animals.

3. Results

Five successfully infected rats were carefully analyzed. The presence of PRV-GFP and AVP- or OXT-like immunoreactivity was observed in the cytoplasm. First, a brief analysis of the distribution of PRV-GFP neurons will be made, and then a description of the extent of co-expression of PRV-GFP with AVP or OXT traits will be done.

3.1. Distribution of PRV-GFP labeled neurons within the hypothalamic PVN

Within the PVN, PRV-GFP infected neurons were observed 4 days after injection of the tracer into the lung. As presented in Fig. 1, PRV-GFP expressing cells were distributed bilaterally, with an ipsilateral dominance. The density of labeling varied among rats and between the PVN subnuclei, defined according to the classification previously described (Swanson and Sawchenko, 1980), as shown in a schematic diagram (Fig. 1). However, the majority of PVN neurons containing PRV-GFP were observed in the parvocellular subnucleus, with a fewer number of labeled neurons found in the lateral magnocellular region.

3.2. PRV-GFP labeling of AVP-containing PVN neurons

The PVN neurons expressing both PRV-GFP and AVP are shown in Fig. 2. Immunostaining for AVP was uniformly distributed in the perikaryon of these cells. Double-labeled neurons were characterized by the yellowish perikarya due to the combination of perikarya stained with FITC fluorescence for GFP-containing cells and Rhodamine red for vasopressin-labeled neurons. Analysis of five rats demonstrated that a subpopulation of PVN neurons, 257 out of 1795 PRV-GFP positive cells (14.3%) co-expressed AVP, while 257 out of 1097 (23.4%) AVP-containing neurons demonstrated co-localization with PRV-GFP infected cells.

3.3. PRV-GFP labeling of OXT-containing PVN neurons

Neurons in the PVN expressing PRV-GFP and OXT are shown in Fig. 3. A subpopulation of PVN neurons, 247 out of 1394 PRV-GFP positive cells (17.7%) co-expressed OXT, whereas 247 of 690 (35.8%) OXT-containing neurons demonstrated co-localization with PRV-GFP infected cells.

3.4. PVN neurons projecting to AVPNs

Confocal microscopic images showed that anterogradely-labeled fibers were within the rNA region where CTb-labeled AVPNs are located. Furthermore, PHAL-labeled fibers were observed to be in close proximity to the identified AVPNs (Fig. 4). Similarly AVP- and OXT-containing fibers were found within the rNA region, and in the vicinity of labeled AVPNs (Figs. 5 and 6, respectively).

4. Discussion

We performed these experiments to determine whether AVP- and/or OXT-containing neurons of the hypothalamic PVN project to the identified vagal preganglionic neurons generating cholinergic outflow to the airways. Using an attenuated PRV strain (Smith et al., 2000), constructed to express a green fluorescent protein (PRV-GFP), combined with fluorescent immunocytochemistry and confocal microscopy, we showed for the first time that the PVN–AVPNs pathways are comprised of at least two phenotypically distinct cell types, those encoding AVP and/or OXT. Data suggest that these neurotransmitters may participate in mediating important stress-related changes in parasympathetic outflow to the airways. The present findings are discussed by focusing on (1) the specificity of techniques employed, (2) comparison of current results with previous findings related to PVN projections to sympathetic preganglionic neurons, and respiratory network, and (3) the functional significance of obtained results.

4.1. Limitations of methods used

Following microinjection of the PRV-GFP, as with previously used Bartha strain, the virus is retrogradely transported from the lung parenchyma or trachea to the first order neurons (AVPNs), where it replicates and then transneuronally passes to the second order neurons, those projecting to vagal preganglionic neurons innervating airways. If replication time is prolonged, through central polysynaptic circuits, the virus can also label higher order cells. The transsynaptic labeling of neurons in defined central nervous system circuits allow their detailed morphological analysis (Loewy, 1998; Enquist, 2002), characterization of neurotransmitters encoded by labeled cells (Haxhiu et al., 1993), and electrophysiological properties of labeled neurons (Smith et al., 2000; Wang et al., 2001). However, as with other viruses, some neurons do not express receptors for the virus that are used, or the 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 a lateral, non-specific labeling. In the present studies, cytolytic processes of infected cells were reduced by using the attenuated strain, isogenic to Bartha PRV, and reducing survival time. Previous studies showed that this strain expresses neurotropism for PVN neurons innervating the AVPNs (Haxhiu et al., 1993; Perez Fontan and Velloff, 1997; Hadziefendic and Haxhiu, 1999). Furthermore, non-specific spread of the virus should not be considered as a problem in this study. Presented results clearly show that only one of two neurons lying side by side is infected, excluding the possibility of lateral spread. This could be due to restriction of spread by activation of astrocytes, microglia, and reactive gliosis, isolating infected neurons from non-infected ones, thereby preventing non-specific neuronal infection (Loewy, 1998; Enquist, 2002).

PRV-GFP has been successfully used as a transneuronal marker in a number of recent studies (Kerman et al., 2003; Shintani et al., 2003). Difficulties may arise in identifying infected neurons due to the low fluorescence of the marker. In the present studies, the GFP signal in PRV-labeled neurons was strong enough that enhancement using anti-GFP IgG was not needed. Furthermore, subsequent immunocytochemistry for identification of AVP or OXT trait

in infected PVN neurons had no effect on the clarity of the conveyed green fluorescence information.

It is worth to be mentioned that in the C8 spinalectomized animals, as described in the previous studies (Haxhiu et al., 1993; Hadziefendic and Haxhiu, 1999), sympathetic pathways are eliminated as possible route for virus to reach the supraspinal structures. Furthermore, the lungs are poorly innervated by sympathetic efferent fibers. Therefore, we believe that transneuronal labeling of the PVN neurons, following injection of tracer into the lungs, refer to second order neurons, which innervate AVPNs.

In complementary studies, we characterized anterograde projections from the PVN cells to the AVPNs and their phenotypic profile. In these experiments, CTb was used as a retrograde tracer to label neurons that project to extrathoracic trachea. These neurons also project to intrapulmonary airways (Perez Fontan and Velloff, 2001). Therefore, different injections sites of two used retrograde tracers do not present difficulties in interpretation of the results.

We are aware that the results of immunocytochemistry studies employing specific primary antibodies should be interpreted with caution. A number of control experiments are required to avoid possible nonspecific labeling (Saper and Sawchenko, 2003). In our experiments, the specificity of the antibodies used was tested using Western blotting, and these antibodies have been previously characterized and successfully used in immunocytochemistry. In addition to our control experiments, where the primary antibody was omitted, no neuronal labeling above the background was observed within the hypothalamic regions known to express AVP or OXT traits (Kc et al., 2002; Mack et al., 2002). Hence, we consider that the employed primary antibodies specifically labeled AVP and OXT antigen-like molecules.

4.2. The present findings

The PVN is a complex structure, receiving multiple information and projecting along the neural axis, including brain stem and spinal cord sites involved in central regulation of respiration (Yeh et al., 1997; Kc et al., 2002; Mack et al., 2002), sympathetic outflow (Hosoya et al., 1995; Jansen et al., 1995), and cholinergic output to visceral organs (Haxhiu et al., 1993; Loewy and Haxhiu, 1993; Hadziefendic and Haxhiu, 1999). Furthermore, it has been shown that AVP-expressing cells (Kc et al., 2002) and OXT-containing PVN neurons (Mack et al., 2002) innervating the brain stem-spinal cord-respiratory-related sites (Kc et al., 2002; Mack et al., 2002) arise from the same PVN subnuclei that project to AVPNs. We assume that some of these AVP- or OXT-containing cells are involved in parallel control of respiratory drive and cholinergic output to the airways, under circumstances where global dynamic changes occur, particularly during emotional stress, as previously suggested (Jansen et al., 1995).

4.3. The functional implications

The findings of the present study raise a number of possibilities that need to be elucidated for the involvement of the PVN in the airway control. Preliminary, unpublished data indicates that OXT increases sensitivity of single unit discharge of AVPNs to bronchoconstrictive stimuli (KC, Wilson and Haxhiu). However, whether endogenously released AVP and/or OXT affect the excitability of AVPNs is an important issue that remains to be studied.

The PVN, a major integrating region in the hypothalamus (Schlenker, 2005), is innervated by the nucleus tractus solitarius (nTS) neurons (Ter Horst et al., 1989) and projects to a variety of brain stem and spinal cord cardiovascular (Buijs, 1978; Nilaver et al., 1980) and respiratory-related sites (Yeh et al., 1997; Kc et al., 2002; Mack et al., 2002;). These loops could be partly involved in mediating chemoreflex pressor responses (Olivan et al., 2001), as well as sympathoexcitatory and respiratory changes induced by stimulation of the carotid body (Reddy

et al., 2005). Stimulation of PVN neurons induces similar physiological changes (Yeh et al., 1997; Schlenker et al., 2001), which are comparable to those observed following microinjection of AVP (Sun and Guyenet, 1989; Kc et al., 2002) or OXT (Mack et al., 2002) into the ventrolateral medulla oblongata. Furthermore, stimulation of carotid bodies by injection of cyanide into the carotid circulation, induces centrally mediated increases in airway smooth muscle tone (Deal et al., 1987). Therefore, the possibility exists that the PVN could partly mediate elevated cholinergic outflow to the airways, using OXT and/or AVP as neurotransmitters.

Previous studies provided morphological and physiological evidence for post-synaptic localization of OXT and AVP receptors in the rat's preganglionic neurons of the dorsal motor nucleus of the vagus nerve (Dubois-Dauphin et al., 1992; Mo et al., 1992). Stimulation of these receptors facilitates the discharge of these neurons (Charpak et al., 1984; Raggenbass et al., 1988; McCann and Rogers, 1990; Mo et al., 1992). Therefore, it can be anticipated that AVP and OXT when released within the rNA could elicit augmentation of cholinergic outflow to the airways by direct effects on AVP or OXT receptors expressed by AVPNs.

The PVN receives direct input from the medial amygdala (Tanaka et al., 1997), a structure known to be involved in the stress response (Ebner et al., 2004). In asthmatic patients, emotional distress is often associated with worsening of airway functions (Rietveld et al., 2000; Aboussafy et al., 2005). Therefore, the possibility exists that a stress induced airway hyperreactive response could be partly mediated by the PVN neurons projecting to the airways. Hence, an increased cholinergic outflow, associated with a bronchospasm, could be a part of the homeostatic changes induced by the release of OXT and/or AVP at synapses or in the vicinity of the AVPNs, acting via synaptic and/or non-synaptic transmission, or through the nTS neurons which are known to receive oxytocinergic inputs (Rinaman, 1998) that could facilitate vagal outflow and the slowdown of the heart rate (Higa et al., 2002).

In asthmatic patients, severe worsening of airway functions is associated with an impaired diuretic capacity and an increase in AVP (Shimura and Arisaka, 1990), which returns to normalcy with resolution of the disorder (Baker et al., 1976). This suggests that an excessive release of AVP could play a role in the worsening of bronchoconstriction in chronic obstructive disorders following water overload. Whether release of AVP through projections to AVPNs contributes to bronchospasm and an asthmatic attack that in turn may further enhance the increase of plasma AVP concentration during the acute illness, needs to be investigated.

In conclusion, the present study has identified two distinct phenotypes of PVN cells projecting to the AVPNs: AVP- and OXT-containing neurons, raising the possibility that they may contribute to the regulation of cholinergic outflow to the airways under distinct physiological or pathological conditions.

Acknowledgments

We thank Ms Lee Watson for secretarial assistance and Ms Loubat Changizi for performing immunostaining. This work was supported by NIH-HL 50527 (to M.A. Haxhiu); National Institute of Neurological Disorders and Stroke and National Center for Research and Resources 1 U54-NS-39407 (to M.A. Haxhiu).

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Fig. 1.

(A) Schematic diagram showing hypothalamic paraventricular (PVN) subnuclei and their targeted sites. Confocal microscopic images showing (B) the distribution of transneuronal retrogradely-labeled neurons following microinjection of PRV-GFP into the upper right lung lobe, (C) OXT- and (D) AVP-containing PVN neurons where the majority of OXT- or AVP-containing cells that project to airway-related preganglionic neurons (AVPNs) are localized. DP: dorsal parvocellular, M: magnocellular, MP: medial parvocellular, VP: ventral parvocellular, 3V: third ventricle (scale bar: 100 μ M).

Fig. 2.

(A) Confocal microscopic images showing transneuronal retrogradely-labeled PVN neurons following microinjection of PRV-GFP into the upper right lung lobe and (B) AVP-labeled PVN neurons visualized with rhodamine in the same field. (C) The merged (M) image showing the AVP-containing PVN neurons infected with PRV-GFP (scale bar: 20 μM). Quantitative analysis of PRV-GFP positive neurons alone, AVP-containing neurons alone, and the number of the double-labeled cells (PRV-GFP and AVP).

Fig. 3.

(A) Confocal microscopic images showing transneuronal retrogradely-labeled PVN neurons following microinjection of PRV-GFP into the upper right lung lobe and (B) OXT-labeled PVN neurons visualized with rhodamine in the same field. (C) The merged (M) image showing the OXT-containing PVN neurons infected with a PRV-GFP (scale bar: 20 μ M). Quantitative analysis of PRV-GFP positive neurons alone, OXT-containing neurons alone, and the number of the double-labeled cells (PRV-GFP and OXT).

Fig. 4.

Confocal microscopic images showing anterogradely-labeled fibers within the rostral nucleus ambiguus (rNA) region where AVPNs are located. (A) PHAL-containing fibers immunolabeled and visualized with FITC. (B) CTb-labeled AVPNs following microinjection of CTb into the extrathoracic trachea visualized with rhodamine. (C) The merged (M) image showing terminals of PHAL in the vicinity of CTb-labeled AVPNs (scale bar: 20 μ m). (D) Schematic representation of a coronal section indicating the location of AVPN within the rNA region. The drawing of a coronal section was derived from the atlas of Paxinos and Watson (1998).

Fig. 5. Confocal microscopic images showing AVP-containing fibers within the rostral nucleus ambiguus (rNA) region where AVPNs are located. (A) CTb-labeled AVPNs following microinjection of CTb into the extrathoracic trachea visualized with FITC and (B) AVP-containing fibers immunolabeled and visualized with rhodamine. (C) The merged (M) image showing terminals of AVP in the vicinity of CTb-labeled AVPNs (scale bar: 20 μ m). (D) Schematic representation of a coronal section indicating the location of AVPNs within the rNA region. The drawing of a coronal section was derived from the atlas of Paxinos and Watson (1998).

Fig. 6.

Confocal microscopic images showing OXT-containing fibers within the rostral nucleus ambiguus (rNA) region where AVPNs are located. (A) CTb-labeled AVPNs following microinjection of CTb into the extrathoracic trachea visualized with FITC and (B) OXT-containing fibers immunolabeled and visualized with rhodamine. (C) The merged (M) image showing terminals of OXT in the vicinity of CTb-labeled AVPNs (scale bar: 20 μ m). (D) Schematic representation of a coronal section indicating the location of AVPN within the rNA region. The drawing of a coronal section was derived from the atlas of Paxinos and Watson (1998).