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Research report

Cervical stimulation activates A1 and locus coeruleus neurons that project to the paraventricular nucleus of the hypothalamus

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ABSTRACT

In female rats, stimulation of the uterine cervix during mating induces two daily surges of prolactin. Inhibition of hypothalamic dopamine release and stimulation of oxytocin neurons in the paraventricular nucleus (PVN) are required for prolactin secretion. We aim to better understand how stimulation of the uterine cervix is translated into two daily prolactin surges. We hypothesize that noradrenergic neurons in the A1, A2, and locus coeruleus (LC) are responsible for conveying the peripheral stimulus to the PVN. In order to determine whether projections from these neurons to the PVN are activated by cervical stimulation (CS), we injected a retrograde tracer, Fluoro-Gold (FG), into the PVN of ovariectomized rats. Fourteen days after injection, animals were submitted to artificial CS or handling and perfused with a fixative solution. Brains were removed and sectioned from the A1, A2, and LC for c-Fos, tyrosine hydroxylase (TH), and FG triple-labeling using immunohistochemistry. CS increased the percentage of TH/FG+ double-labeled neurons expressing c-Fos in the A1 and LC. CS also increased the percentage of TH+ neurons expressing c-Fos within the A1 and A2, independent of their projections to the PVN. Our data reinforce the significant contributions of the A1 and A2 to carry sensory information during mating, and provide evidence of a functional pathway in which CS activates A1 and LC neurons projecting to the PVN, which is potentially involved in the translation of CS into two daily prolactin surges.

1. Introduction

In female rats, cervical stimulation (CS) induces twice-daily surges of prolactin (PRL) secretion from lactotrophs of the anterior pituitary gland, which are responsible for maintaining the corpus luteum during the first half of gestation (Freeman et al., 2000). The neural pathway by which the stimulus produces this PRL secretion pattern is under investigation. CS transmits a signal from the pelvic nerve to the lumbosacral region of the spinal cord. This signal ascends the anterolateral columns of the spinal cord to the brain stem, where it is conveyed to brain regions *via* noradrenergic bundles (Erskine, 1995; Terkel, 1988). Beyond this, it is not clear how the signal is processed and what specific neural connections stimulate the CS-induced PRL surges.

The tonic inhibition of hypothalamic dopamine (DA) neurons is a well-known mechanism controlling PRL secretion (Ben-Jonathan and Hnasko, 2001). However, the decrease in DA inhibition does not fully account for the magnitude of physiological PRL secretion (De Greef and Neill, 1979). Several factors have been shown to stimulate PRL secretion, many of which are present in the hypothalamic paraventricular nucleus (PVN). Because oxytocin (OT) neurons in the PVN are activated by CS (Arey and Freeman, 1992; Flanagan et al., 1993; Polston et al., 1998; Polston and Erskine, 1995) and OT stimulates PRL secretion *in vitro* and *in vivo* (Egli et al., 2004), we believe that OT is a significant physiological stimulator of PRL secretion. In addition to OT, norepinephrine (NE) is known to stimulate PRL secretion. Intracerebroventricular NE injections increase basal and estradiol (E2)-induced PRL secretion (Negro-Vilar et al., 1979; Vijayan and McCann, 1978), and central inhibition of NE prevents E_2-induced PRL secretion (Carr et al., 1977). More importantly, NE may stimulate PRL secretion by activating PRL-stimulating neurons in the PVN. Bilateral injections of NE in the medial basal hypothalamus (MBH), which encompasses the PVN, increase PRL release (Day et al., 1982). Increased NE release in the MBH

Abbreviations: CVLM, caudal ventrolateral medulla; CS, cervical stimulation; DA, dopamine; FG, Fluoro-Gold; LC, locus coeruleus; MBH, medial basal hypothalamus; NTS, nucleus of the solitary tract; OT, oxytocin; PRL, prolactin; TH, tyrosine-hydroxylase.

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coincides with the E_2-induced PRL surge as well (Jarry et al., 1986). NE stimulates PVN neurons *in vitro* and this response is blocked by noradrenergic receptor antagonists (Daftary et al., 2000). In addition, injections of alpha-1b adrenergic receptor agonist and antagonist in the PVN stimulate and inhibit PRL secretion, respectively (Dodge and Badura, 2004).

NE neurons originating in the A1 located within the caudal ventrolateral medulla (CVLM) and A2 located within the nucleus of the solitary tract (NTS) comprise the ventral noradrenergic bundle (Moore and Bloom, 1979; Ungerstedt, 1971). In response to CS, A1 and A2 neuronal activity increases (Cameron et al., 2004; Yang and Voogt, 2001). Moreover, lesion of the ventral noradrenergic bundle prevents CS-induced PRL secretion (Hansen et al., 1980). NE neurons originating in the A6 locus coeruleus (LC), where the dorsal noradrenergic bundle arises (Moore and Bloom, 1979; Ungerstedt, 1971), also stimulate PRL secretion, given that lesions of the LC disrupt the proestrous (Anselmo-Franci et al., 1997) and E2-induced secretion of PRL (Poletini et al., 2004). Therefore, the A1, A2 and LC noradrenergic nuclei probably play a role in the stimulation of PRL secretion.

The purpose of this study was to determine if CS activates the A1, A2, and LC neurons that project to the PVN, constituting a pathway by which CS induces twice-daily PRL surges. Although projections of brainstem noradrenergic neurons to the PVN are well known, this study aimed to determine if these projections are functionally involved in conveying CS to the PVN, *i.e.* activated in response to CS. The retrograde tracer, Fluoro-Gold (FG), was injected into the PVN and used to delineate the connectivity of neural circuits involved in CS-induced PRL surges. Sections of the A1, A2, and LC were triple-labeled using immunohistochemistry for c-Fos (a marker of neuronal activation), FG (a marker for neurons projecting to the PVN), and tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis (a marker for noradrenergic neurons).

2. Experimental design

Three days following ovariectomy, rats were unilaterally injected with FG into the PVN. Ten days later, rats were submitted to two sessions of artificial cervical stimulation (CS; $n = 5$) or handling (H; $n = 9$), one at 1700 h and another in the following morning at 0900 h and perfused with a fixative solution 90 min later. Manipulation consisted of handling the rats in the same position and for the same duration as cervically stimulated rats, but without CS. Brains were removed and sectioned from the PVN, for injection placement. The A1, A2, and LC were sectioned and triple-labeled for c-Fos, FG, and TH using immunohistochemistry.

3. Materials and methods

3.1. Animals

Adult female Sprague Dawley rats (200–250 g; Charles River, Raleigh, NC) were housed in standard rat cages under a 12-h light, 12-h dark cycle (lights on at 0600 h), with water and rat chow available *ad libitum*. All rats were bilaterally ovariectomized to eliminate the effects of ovarian steroids. This surgery was performed under isoflurane/oxygen gas mixture; isoflurane was set at 2.5–3% and the oxygen flow to 2.0 L/min. The Florida State University Animal Care and Use Committee approved all animal procedures.

3.2. FG injection

Rats were anesthetized with ketamine (80 mg/kg body weight (bw), i.p.; Ketaset; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (10 mg/kg bw, i.p.; Anased; Lloyd Laboratories, Shenandoah, IA) and placed in a stereotaxic apparatus (David Kopf, Tujunga, CA). A midline incision exposed the skull and a small opening in the skull was made with a dental drill. A 32-gauge stainless-steel needle (LASI Needle; Hamilton, Reno, NV) was lowered into right PVN using the following coordinates: anterior–posterior (AP): −1.7 mm posterior to bregma, medial–lateral (ML): 0.3 mm right from midline and dorsoventral: 7.6 mm below dura (Paxinos and Watson, 2007). Sixty nanoliters of 4% the FG (Flurochrome, LLC; Denver, CO) diluted in 0.9% NaCl, was injected for the duration of 1 min with a 10 μL syringe coupled to an infusion pump. The needle was left in place for an additional 10 min for complete diffusion. Fourteen days after injection rats were subjected to CS and perfusion (described below).

3.3. Cervical stimulation

The uterine cervix was stimulated with an electrode constructed from a Teflon rod (diameter, 5 mm), with two platinum wires protruding from the tip. Each rat was stimulated twice, the first time at 1700 h and the second on the following morning at 0900 h. Stimulations were applied as three consecutive trains of electric current of 10 s duration (rectangular pulses, 1 ms of 25 V at 200 Hz). This procedure has been shown to yield the highest success rate in initiating two daily PRL surges that are characteristic of mated rats (Gorospe and Freeman, 1981).

3.4. Tissue preparation

Rats were deeply anesthetized with 83 mg/kg bw of pentobarbital sodium solution (Nembutal, Abbott Laboratories, Chicago, IL) and were transcardially perfused with 40 mL of sterile saline containing heparin (5 IU/mL), followed by 200 mL of 4% paraformaldehyde (Sigma–Aldrich, St. Louis, MO) in 0.1 M PBS (pH = 7.2). After perfusion, brains were removed, postfixed in 4% paraformaldehyde for 2 h, and cryoprotected in 20% sucrose in 0.1 M PBS at 4 °C for at least 24 h. Four series of coronal sections of 30 μm were cut on a sliding microtome (Richard-Allan Scientific, Kalamazoo, MI) for the following brain regions: PVN, LC and A1/A2 (Paxinos and Watson, 2007). Sections of A1 and A2 were obtained caudally to the obex in order to evaluate the noradrenergic neurons as opposed to the adjacent adrenergic neurons of C1 and C2 (Tucker et al., 1987). The obex has been described to mark the transition between noradrenergic and adrenergic neurons (Tucker et al., 1987).

3.5. Immunohistochemistry

For analysis of triple-labeling of c-Fos, FG and TH, sections from the A1, A2 and LC were rinsed 5 times for 10 min each in 0.01 M PBS (pH = 7.35) containing 0.1% Triton-X 100 (Sigma–Aldrich) to remove cryoprotectant. To reduce binding of residual aldehydes and ketones, tissues were incubated in 1% NaBH4 (Sigma–Aldrich) for 10 min and 3% H_2O_2 (from a 30% H_2O_2 solution, VWR International) for an additional 10 min, separated by washes. Nonspecific binding was blocked with 10% normal goat serum. All primary and secondary antibodies were diluted in 0.01 M PBS (pH = 7.35) containing 0.4% Triton-X 100 and 2% normal-goat serum. Primary antibodies were incubated for 40 h at 4 °C and secondary antibodies for 2 h at room temperature. The primary antibodies used and dilutions: rabbit anti-c-Fos 1:10,000, raised against a synthetic peptide, SGFNADYEASSSRC corresponding to amino acids 4–17 of human c-Fos (AB-5, EMD; Gibbstown, New Jersey); rabbit anti-FG 1:15,000 (AB-153; Chemicon, Temecula, CA, EUA); and mouse anti-TH 1:80,000, raised against a TH purified from PC12 cells (MAB 308; Chemicon). The anti-TH antibody has been well characterized in protocols that include both omission of the primary antibody and pre-adsorption of the antibody with the antigen, in either case, no staining was observed (McDonald et al., 2000; Rinaman et al., 1995). The anti-c-Fos antibody was originally designated AB-2 (Oncogene Sciences), and both AB-2 and AB-5 derived from the same sources of rabbit serum (number #4191) had been previously tested and proven to have specificity against the N-terminal c-Fos protein and to not recognize other immediate-early gene products or FOS-related antigens (Hoffman et al., 1990; Kobelt et al., 2004; Le et al., 1997; Olson et al., 1993; Wang et al., 1995; Wu et al., 2011). Secondary antibodies used and dilutions: biotinylated goat anti-rabbit IgG 1:600 (Vector, Burlingame, CA), goat anti-rabbit Alexa Fluor 488 1:2000 (Invitrogen), and goat anti-mouse Alexa Fluor 555 1:2000 (Invitrogen). For c-Fos staining, following primary antibody incubation, sections were incubated with the biotinylated anti-rabbit goat IgG, and avidin–biotin complex solution at 1:100 for 1 h (Elite ABC Kit, Vector). A solution of nickel sulphate (25 mg/mL), 3,3′-diaminobenzidine-HCl (DAB, 0.2 mg/mL) and 0.03% H_2O_2 (Ni-DAB) was used as the chromogen. Sections were then processed for FG and TH immunofluorescence. Sections were mounted on SuperFrost slides (Fisher Scientific) and underwent a dehydration process. Cover slips were applied using Krystalon™ (EMD; Gibbstown, New Jersey). The immunohistochemistry reactions were also performed in sections omitting the primary antibodies and no cytoplasm stains were detected with the fluorescent secondary antibodies or nuclear stain with the biotinylated anti-rabbit after revelation with avidin–biotin complex solution (data not shown) in those sections. In addition, the immunohistochemistry reactions were performed in ovariectomized rats that did not receive FG injection and no FG staining was observed (Fig. 3F). Images were taken with a Leica DMLB microscope coupled to a digital camera (SPOT-Real Time monochrome, Diagnostic Instruments, Inc., MI, USA). Image acquisition and analysis were made with Metamorph software (Universal Imaging, Downingtown, PA). Each section was acquired 3 times using filter GFP (excitation 450–490 nm and emission 500–550 nm) for green Alexa Fluor 488, filter Y3 (excitation 530–560 nm and emission 573–647 nm) for red Alexa Fluor 555, and bright field. The images were overlaid and the number of TH-, FG/TH- and c-Fos/FG/TH-ir neurons ipsilateral to the FG-injection site (right side of the brain) was quantified in each immunohistochemistry section by an experimenter unaware of the treatment group. For analysis

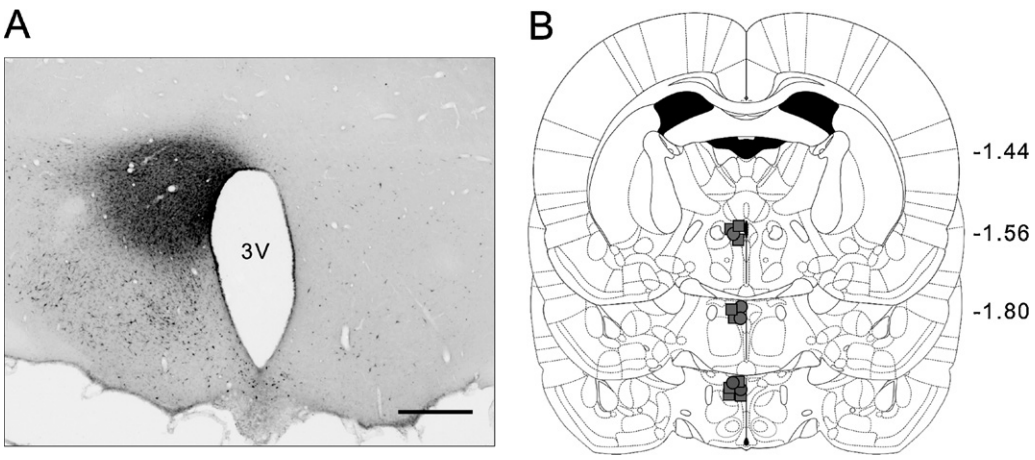

Fig. 1. (A) Representative photomicrography of the paraventricular nucleus immunostained with Fluoro-Gold, showing the site of a successful injection; 3V (3rd ventricle), scale bar = 500 μm. (B) Diagrammatic representation of coronal brain sections showing the location of Fluoro-Gold injection sites inside the paraventricular nucleus (−1.44 to −1.80 mm posterior to bregma) according to the atlas of Paxinos and Watson (2007). Circles and squares indicate the injection sites in CS and non-CS animals, respectively.

of injection placement, sections from the PVN were incubated with rabbit anti-FG antibody at 1:25,000 dilution and visualized using Ni-DAB procedure as described above for c-Fos staining. To verify that the triple labeling is observed from the same neuron, some sections were analyzed on a Zeiss LMS 510 Meta confocal microscope (Carl Zeiss, Jena, Germany) by means of an oil-immersion objective (63×). Argon (Ar2) laser was used to excite FG-labeled neurons at 488 nm wavelength and helium neon (HeNe1) laser at 543 nm wavelength was applied to excite TH-labeled neurons. The emission was filtered at 500–530 and 560 nm for FG and TH, respectively, using sequential scanning mode. The diameter of the pinhole aperture was 200 μm for each channel, resulting in 1.5 μm optical thicknesses. The c-Fos brightfield image was obtained using differential interference contrast optics and combined with the fluorescent images using Zeiss LSM Image Browser software (Carl Zeiss) and Adobe Photoshop (Adobe Systems). The brightfield image was inverted and pseudo-colored to blue for illustration purpose (Fig. 3D).

3.6. Data analysis

Statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). Data are shown as mean ± SEM. Data were analyzed using Student's unpaired *t*-test, following square-root transformation because data failed equal variances and normality tests. Differences were considered significant at $P \leq 0.05$.

4. Results

The immunostaining image in Fig. 1A shows an example of coronal diffusion of FG following an accurate injection into the PVN. Anterior–posterior analysis of FG injections indicates that FG staining originated at the following distances from bregma: −1.08 mm (3 animals), −1.2 mm (4 animals), −1.32 mm (6 animals), and −1.44 mm (one animal). The mean ± SEM of the distance between the start and end of FG staining was 0.74 ± 0.03 mm. Thus, FG diffusion covered a region that is within the anterior–posterior extension of the PVN (Paxinos and Watson, 2007). In Fig. 1B, the FG injection site for each animal is represented as circles or squares for CS and handling (H) groups, respectively, in a diagram displaying positions from bregma. Analysis of the diffusion of FG and injection site did not indicate differences between groups.

Fig. 2 displays experimental immunohistochemistry examples of triple-labeled neurons in the LC and A1. C-Fos staining, used to determine activation of neurons, was restricted to the nucleus of the cell. FG and TH were seen in the cytoplasm of the cells as green and red staining, respectively. CS did not alter either the mean number of TH-immunoreactive (ir) neurons per region or the percentage of TH neurons that project to the PVN in the A1, A2 and LC. Thus, data on the distribution of TH-ir retrograde labeled neurons are shown as the average of all rats studied, regardless of the experimental group (Table 1). Of the total number of retrograde labeled neurons 76.3 ± 10.3% and 89.2 ± 6.4% were TH-ir for CS and H groups in the CVLM, and 44.4 ± 5.2% and 35.7 ± 17.9% were TH-ir for CS and H groups in the NTS (Table 2). CS did not have an effect on these percentages or on the number of FG-ir neurons in both CVLM and NTS regions. More importantly CS did not alter the expression of c-Fos in FG-ir neurons of these brain regions (Table 2). This analysis was not performed in the LC area because the great majority of LC cells are immunoreactive to both the catecholamine-convert enzymes (Grzanna and Molliver, 1980) and norepinephrine transporters (Lorang et al., 1994), thus TH negative neurons that project to the PVN (FG-ir neurons) from this region are most likely not LC cells.

Fig. 3 displays a representative image from LC neurons taken with a confocal microscope, showing that TH-FG double labeling is from the same neuron. The superposition of the TH-FG confocal image with the c-Fos brightfield image, obtained through differential interference contrast, indicates that triple labeling is not from an overlying neuron (Fig. 3A–D).

Fig. 4 shows the percentage of double-labeled (c-Fos/TH-ir) and triple-labeled (c-Fos/TH-FG-ir) neurons in the A1, A2 and LC of ovariectomized rats 90 min after the last session of CS or H. CS increased the percentage of double-labeled neurons (c-Fos/TH-ir) within the A1 and A2 ($P < 0.05$), independent of their projections to the PVN, but it did not have the same effect within LC (Fig. 4A, C and E). Moreover, CS increased the percentage of TH-FG-ir neurons expressing c-Fos in both LC and A1 areas ($P < 0.05$), indicating activation of noradrenergic inputs to PVN (Fig. 4B and D). However, CS did not have the same effects in the A2 (Fig. 4F).

The LC consists of three major divisions (Grzanna and Molliver, 1980) that were considered during data analysis. Because no differences were found between these divisions (data not shown), these data were grouped and presented in totality in Fig. 4A and B.

5. Discussion

This study provides anatomical and functional data on the projections of brain stem noradrenergic neurons to the PVN, suggesting a neural pathway through which CS may alter neuronal activity in this forebrain area. In A1 and A2, CS stimulates TH neurons independently of their projections to the PVN. In the A1 and LC, CS activates the population of TH neurons that project to the PVN. These data reinforce the contribution of noradrenergic neurons within the CVLM and NST to carry sensory information to forebrain regions during mating. Also, our data reveal a population of TH neurons from A1 and LC that constitute a neural pathway by which CS may activate PVN neurons.

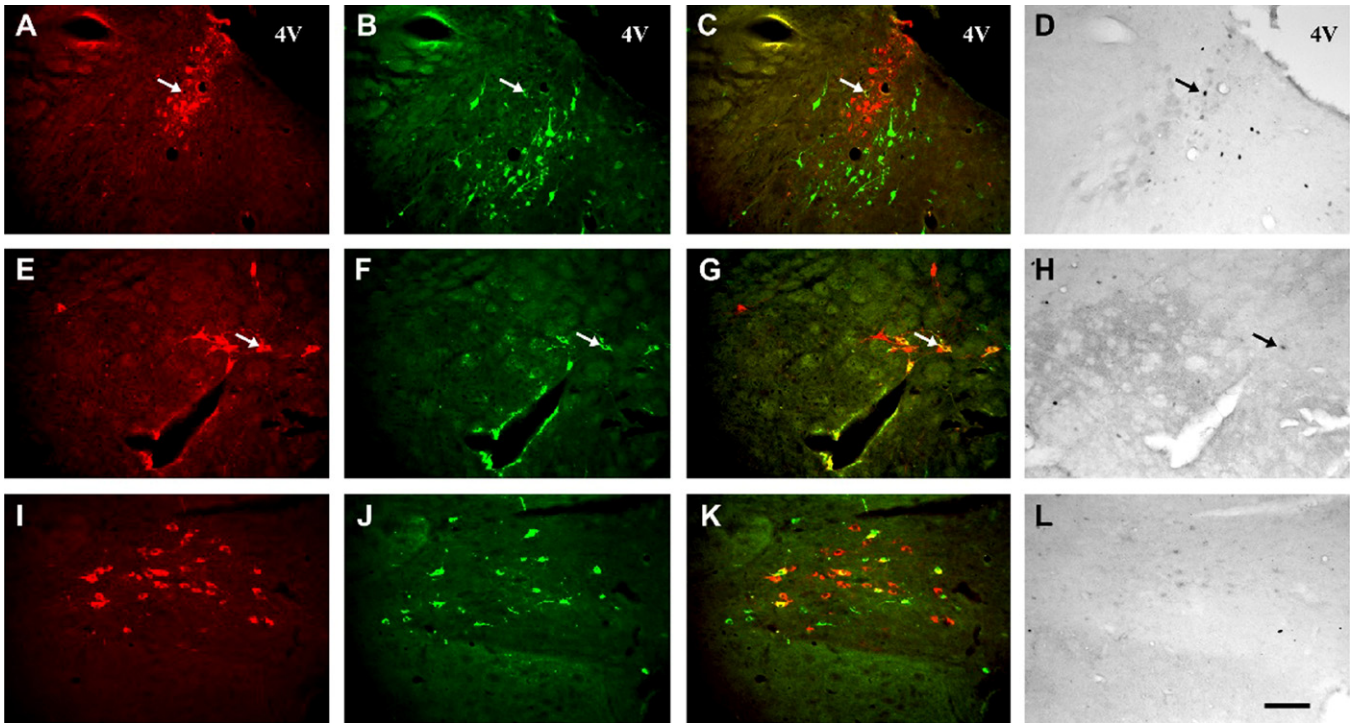

Fig. 2. Representative photomicrographs of the locus coeruleus (A–D), A1 (E–H), and A2 (I–L) after immunohistochemistry with anti-tyrosine-hydroxylase (TH), anti-Fluoro-Gold (FG) and anti-c-Fos antibodies. Red channel images (A, E and I) show TH positive (+) neurons; green channel images (B, F, and J) show FG+ neurons; merged images of TH and FG (C, G and K) show double labeled neurons; bright field images show c-Fos+ neurons (D, H and L), with arrows representing c-Fos staining in the same double-labeled neurons marked in (C) and (G), indicating triple-labeled neurons (c-Fos+/TH+/FG+); 4V (4th ventricle); scale bar = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

In rats, studies have shown that CS derived from parturition (Luckman, 1995) or from male intromissions during mating (Cameron et al., 2004; Yang and Voogt, 2001) increases c-Fos expression in noradrenergic neurons of both the CVML and NTS. In agreement with these studies, our data showed that CS activates TH positive neurons in A1 and A2 regions, independently of their projections to PVN, as determined by the percentage of TH-ir neurons expressing c-Fos. Also, as previously demonstrated (Yang and Voogt, 2001), CS did not affect the expressing of c-Fos in NE neurons of the LC. Although, direct comparisons in the number of activated neurons are difficult because of major differences in the technical procedures, such as the detection of c-Fos, the degree of activation differs considerably among studies. In the present study, the triple-labeling immunohistochemistry may have diminished the detection of c-Fos. In the other hand, the percentage of TH-ir neurons expressing c-Fos (Fig. 4A, C and E) obtained with our triple-labeling protocol nicely agreed with previous data obtained with double-labeling staining of c-Fos/TH-ir neurons (Cameron et al., 2004; Yang and Voogt, 2001), this concordance supports our immunohistochemistry protocol. In terms of the regions from where the A1 and A2 sections originated, a previous study, in which sections were obtained caudally to the obex as we have performed,

Table 1
Distribution of tyrosine hydroxylase (TH)-immunoreactive (ir) neurons retrograde labeled across selected norepinephrine nuclei following Fluoro-Gold (FG) injection in the paraventricular nucleus (PVN).

Brain area	No. TH-ir neurons/region	No. FG/TH-ir neurons/region	% FG/TH-ir neurons/region
A1	64.4 ± 12.5	6.3 ± 1.8	15.1 ± 5.0
A2	125.4 ± 24.1	6.1 ± 1.7	6.7 ± 2.8
LC	339.4 ± 40.0	5.3 ± 2.0	1.5 ± 0.5

Data are shown as mean ± SEM ($n = 14$) of number of TH-ir neurons and TH/FG-ir double-labeled neurons per region, and percentage of TH-ir neurons expressing FG (% FG/TH-ir) in the A1, A2 and locus coeruleus (LC). Cervically stimulated and non-cervically stimulated rats are shown grouped because there were no significant differences between groups. Number of sections analyzed per neuronal group is shown in parentheses.

Table 2
Characterization of retrograde-labeled neurons in the caudal ventrolateral medulla (CVLM) and nucleus of the solitary tract (NTS) following Fluoro-Gold (FG) injection in the paraventricular nucleus (PVN).

Brain area	Groups	No. FG-ir neurons/region	% TH/FG-ir neurons/region	% c-Fos/FG-ir neurons/region
CVLM	CS	10.0 ± 3.9	76.3 ± 10.3	6.8 ± 6.0
	H	5.3 ± 2.1	89.2 ± 6.4	12.5 ± 8.0
NTS	CS	16.0 ± 4.5	44.2 ± 5.2	2.1 ± 2.0
	H	13.0 ± 5.0	35.7 ± 17.9	0.0 ± 0.0

Mean ± SEM number of FG-ir neurons per region, percentage of FG-ir neurons expressing TH (% TH/FG-ir), and percentage of FG-ir neurons expressing c-Fos in the CVLM and NTS of cervically stimulated (CS; $n = 5$) and handled (H; $n = 9$) rats. There were no statistically significant differences between groups.

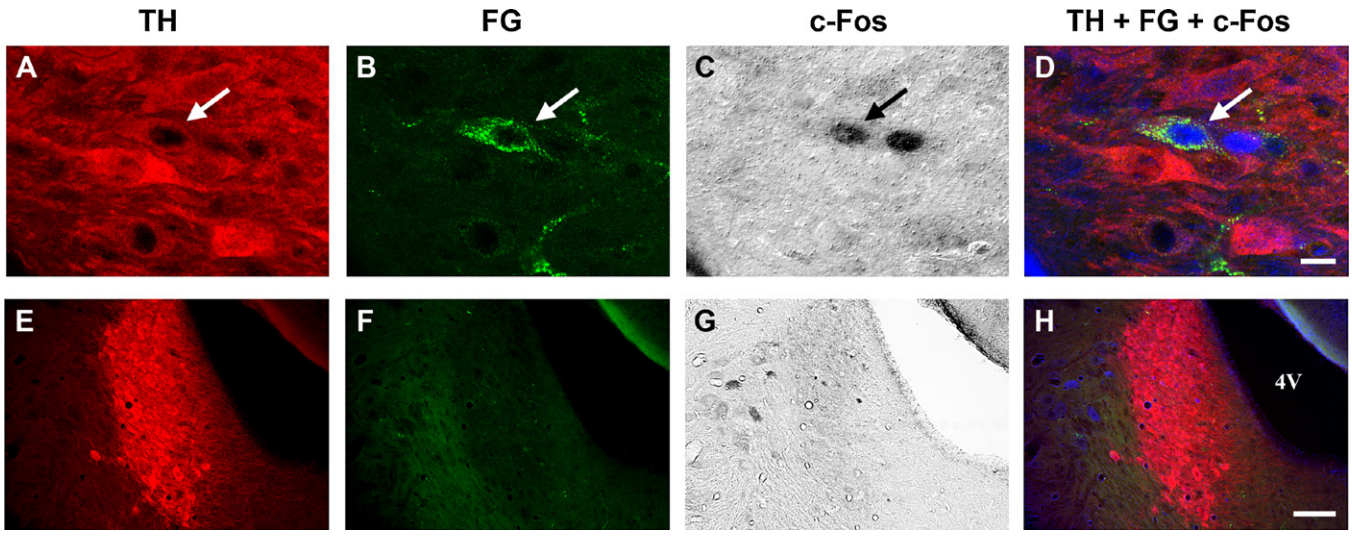

Fig. 3. Representative photomicrographs of locus coeruleus (LC) sections after triple labeling with tyrosine-hydroxylase (TH), Fluoro-Gold (FG) and c-Fos. Top panel shows a high magnification image taken with a confocal microscope. Confocal fluorescence images of single-labeling for TH and FG are shown in (A) and (B), respectively. c-Fos labeled neurons are shown in (C), obtained using DIC (differential interference contrast). The DIC image was inverted and pseudo-colored to blue for illustration purpose in the overlaying images (D), with the arrow pointing to a triple-labeled neuron. Scale bar = 10 μm. Bottom panels display images of a LC from an ovariectomized rat not injected with FG. Single fluorescence images showing TH positive and FG negative staining (E and F). The bright-field image shows c-Fos positive neurons (G), inverted and pseudo-colored in the overlaying image (H). Scale bar = 100 μm.

c-Fos expression is reported to be approximately 3 times higher due to vaginocervical stimulation in parturient rats (Luckman, 1995). Likewise, our data showed a CS-induced increase of approximately 3 times in c-Fos expression in TH-ir neurons of the A1 and A2. In addition to these technical differences, the discrepancy in the degree of activation may be due to the source of vaginocervical stimulation, since male intromissions (Cameron et al., 2004; Yang and Voogt, 2001) or parturition were used in the previous studies compared to artificial-cervical stimulation used in the present study. It is worth mentioning that in a previous study (Yang and Voogt, 2001), mounting (without intromission) also increased c-Fos expression within the A1 and A2, similar to when the mating stimulus (male intromissions) is used. This suggests that the higher degree of activation of these nuclei may be related to the combination of flank and cervical stimulation but not cervical stimulation alone. In our study, this was the only relevant stimulus. Moreover, the discrepancy may be due to the absence of E_2 and progesterone (P_4) permissive effects, since untreated ovariectomized rats were used in our study. Ovarian steroid hormones are known to increase c-Fos expression in the A2 (Jennes et al., 1992) and modulate activity of LC neurons (Szawka et al., 2009). Both α-estrogen receptor and progesterone receptors in TH-ir cells are modulated by circulating estrogen concentration within the A2 (Haywood et al., 1999). More importantly, mating with intromission increases c-Fos expression in α-estrogen receptor-containing neurons of A1 and A2 (Yang and Voogt, 2001), and ovariectomy significantly reduces the mating-induced c-Fos activation in these neurons (Cameron et al., 2004).

The PVN consists of two major divisions, the magnocellular and the parvicellular. The magnocellular consists of large neurosecretory neurons that project to the posterior lobe. The parvicellular division contains small neurosecretory neurons that project to the median eminence and other brain regions (Sawchenko and Swanson, 1982). Although the primary sources of NE to the PVN (Moore and Bloom, 1979; Petrov et al., 1993; Sawchenko and Swanson, 1982; Sawyer and Clifton, 1980; Ungerstedt, 1971) are the A1, A2 and LC, the NE-innervation to the PVN from each of these nuclei vary substantially. Among the three, A1 has the densest projections, innervating essentially all parts of the parvicellular PVN and sparsely innervating the magnocellular regions (McKellar and Loewy, 1981; Sawchenko and Swanson, 1982). The A2 projections are quite similar, except that they are generally less dense and do not include projections to magnocellular PVN regions. The LC projects primarily to the medial portion of the parvicellular PVN, and the projection density is low (Sawchenko and Swanson, 1982). Accordingly, LC lesions cause only a slight but significant reduction in NE content in the PVN (Szawka et al., 2005). Consistent with these previous studies, we identified relatively similar contributions of each brain region that provides NE innervations to the PVN. The A1 displayed a higher percentage of TH neurons that project to the PVN (15.1 ± 5.0), followed by A2 (6.7 ± 2.8) and LC (1.5 ± 0.5). Using neuronal tracers at different doses and methods of application may affect the uptake mechanism, intracellular transport of tracers, and, eventually, signal detection (Kobbert et al., 2000). It is possible that these technical aspects may have caused an underestimation of NE innervation to PVN, because our data did not show a high number of FG positive neurons within A1, A2 and LC (Table 1). Conceivably, the triple-labeled staining may also have contributed to this outcome. Compared to a previous study, however, we found very similar percentages of FG-labeled neurons expressing TH in the CVLM (approximately 80%) (Cunningham and Sawchenko, 1988; Gaykema et al., 2007; Rinaman et al., 1995), and in the NTS (approximately 40%) (Hermes et al., 2006), which gives support to the efficacy of FG retrograde labeling in the present study.

CS only activated those noradrenergic neurons in the LC that projected to the PVN. In the A1, besides the activation of NE neurons in general, CS also activated the NE neurons projecting to the PVN. This reveals a new population of NE neurons that seem to be activated by mating. In none of the brainstem areas studied was CS able to increase c-Fos expression in the FG neurons (Table 2). Together, these data allow us to suggest a pathway by which CS-induced sensory information reaches PVN through NE neurons from the A1 and LC, but not from A2. Perhaps, in the present study, CS did not activate A2 neurons projecting to the PVN because the A2 neuronal projections lack the specificity, which is present in the A1 and LC projections, respectively. However, the importance of TH neurons that do not project to the PVN cannot be excluded, these neurons may comprise projections that have permissive roles for the onset of pregnancy/pseudopregnancy in intact animals. This possibility

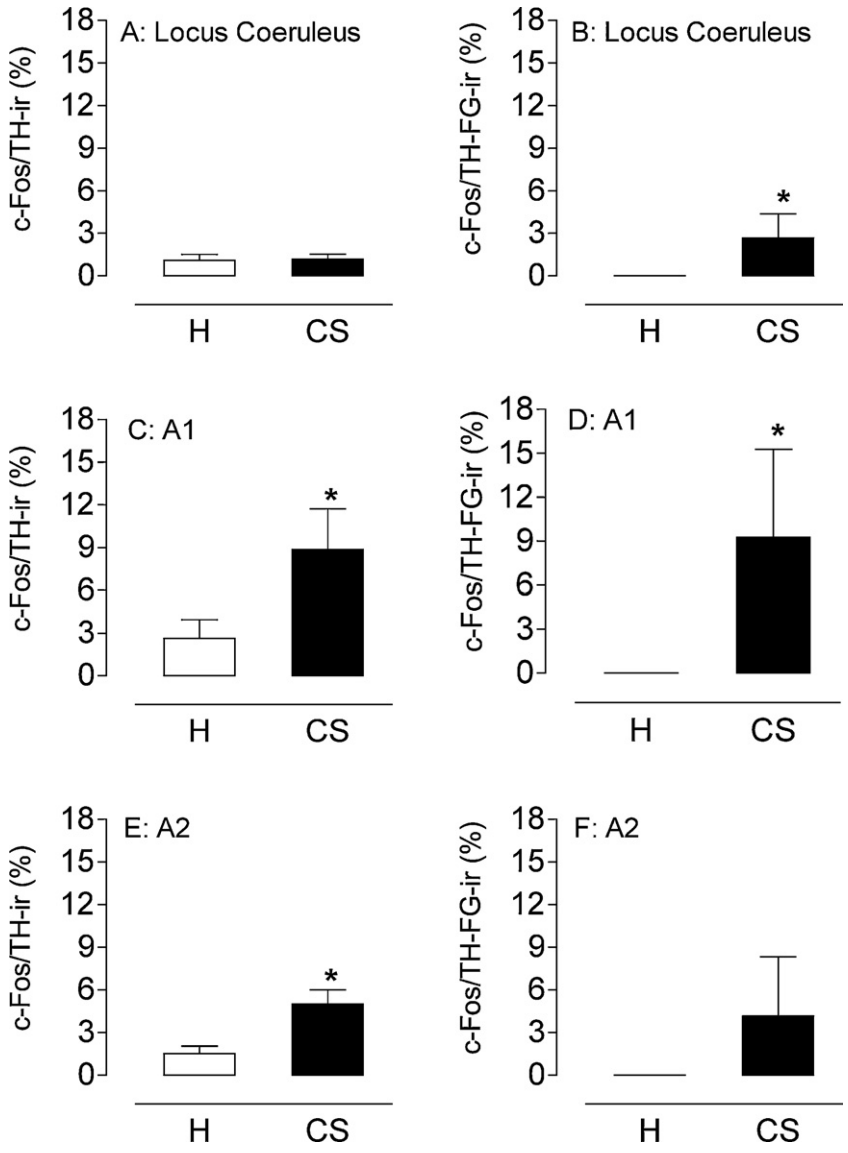

Fig. 4. Activation of noradrenergic neurons after cervical stimulation (CS) in ovariectomized rats. The left panel shows the percentage of noradrenaline-(tyrosine hydroxylase, TH positive) immunoreactive (ir) neurons activated by CS (c-Fos-ir) in the locus coeruleus (A), A1 (C) and A2 (E). The right panel shows the percentage of TH-FG-ir neurons expressing c-Fos in the locus coeruleus (B), A1 (D) and A2 (F). Data shown as mean ± SEM in cervically stimulated ($n = 5$) and handling (H, non-cervically stimulated, $n = 9$) rats. CS induced an increase in the percentage of double-labeled neurons (c-Fos/TH-ir) in the A1 and A2, as well as in the percentage of triple-labeled neurons (c-Fos/TH-FG-ir) in the LC and A1. $^*P \leq 0.05$ determined using Student's unpaired *t*-test.

is supported by findings showing that destruction of these projections decreases the incidence of pseudopregnancy by 50% in mated rats (Northrop et al., 2010).

Several neuronal phenotypes are located in the PVN. Those that have been shown to stimulate PRL secretion include thyrotropin releasing hormone, vasoactive intestinal peptide, vasopressin and OT-releasing neurons (Freeman et al., 2000). We have previously demonstrated that OT stimulates PRL secretion *in vitro* and *in vivo*. Administration of OT to lactotrophs initiates PRL release that is preceded by intracellular Ca^{2+} release, suggesting that OT stimulates PRL secretion *via* a calcium-dependent mechanism (Egli et al., 2004). It was also shown that blocking OT receptors prohibits suckling- and E_2-induced PRL secretion (Kennett et al., 2008). Most notably, a bolus injection of OT initiates CS-like PRL surges (Bertram et al., 2006) and this OT signal is transmitted through the pelvic nerve (Helena et al., 2011). Cervical stimulation activates OT neurons in the PVN blocking peripheral OT receptors prevents CS-induced PRL secretion (McKee et al., 2007). Lastly, OT neurons in the PVN are activated during CS-induced PRL surges (Arey and Freeman, 1992). Together, these data suggest that OT from the PVN plays an important role in PRL regulation.

PRL release seems to be under the control of NE through actions on the PVN (Dodge and Badura, 2004). Thus, we hypothesize that NE may activate OT neurons in the PVN that in turn stimulate PRL secretion. This is likely, as central NE stimulates OT in different physiological conditions in rats and other species (Ginsberg et al., 1994; Vacher et al., 2002). NE seems to stimulate OT secretion induced by hemorrhage (Rodovalho et al., 2006), angiotensin II, and conditioned fear (Zhu and Onaka, 2002). When injected intracerebroventricularly, NE activates OT neurons in the PVN (Ji et al., 1998) and stimulates OT secretion during late gestation (Lipschitz et al., 2004). Suckling induces an increase in OT and NE in the PVN, and blocking NE receptors prevents the increase in OT (Bealer and Crowley, 1998). Importantly, A1 and A2 neurons synapse onto cell bodies of OT neurons in the PVN (Michaloudi et al., 1997), and excitation of A2 noradrenergic neurons increases the activity of the OT

neurons (Day et al., 1984). This suggests that noradrenergic neurons projecting to the PVN, identified in the present study, are likely to synapse on OT neurons.

In conclusion, the present study reveals a neural pathway involved in conveying the CS signal to the autonomic and neuroendocrine systems in the brain, resulting in PRL secretion. CS selectively activates the A1 and LC noradrenergic neurons projecting to the PVN in ovariectomized rats, a pathway likely involved in the initiation of twice-daily surges of PRL secretion.

Conflicts of interest

There are no conflicts of interest.

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