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ORIGINAL RESEARCH

Brain Pathways Involved in the Modulatory Effects of Noradrenaline in Lateral Septal Area on Cardiovascular Responses

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Abstract We have previously reported that stimulation of alpha-1 adrenoceptors by noradrenaline (NA) injected into the lateral septal area (LSA) of anaesthetized rats causes pressor and bradycardic responses that are mediated by acute vasopressin release into the circulation through activation of the paraventricular nucleus (PVN). Although the PVN is the final structure of this pathway, the LSA has no direct connections with the PVN, suggesting that other structures may connect these areas. To address this issue, the present study employed c-Fos immunohistochemistry to investigate changes caused by NA microinjection into the LSA in neuronal activation in brain structures related to systemic vasopressin release. NA microinjected in the LSA caused pressor and bradycardic responses, which were blocked by intraseptal administration of α-1 adrenoceptor antagonist (WB4101, 10 nmol/200 nL) or systemic V-1 receptor antagonist (dTyr(CH2)5(Me)AVP, 50 μg/kg). NA also increased c-Fos immunoreactivity in the prelimbic cortex (PL), infralimbic cortex (IL), dorsomedial periaqueductal gray (dmPAG), bed nucleus of the stria terminalis (BNST), PVN, and medial amygdala (MeA). No differences in the diagonal band of Broca, cingulate cortex, and dorsolateral periaqueductal gray (dlPAG) were found. Systemic administration of the vasopressin receptor antagonist dTyr AVP (CH2)5(Me) did not change the increase in c-Fos expression induced by intra-septal NA. This latter effect, however, was prevented by local injection of the alpha-1 adrenoceptor antagonist WB4101. These results suggest that areas such as the PL, IL, dmPAG, BNST, MeA, and PVN could be part of a circuit responsible for vasopressin release after activation of alpha-1 adrenoceptors in the LSA.

Keywords Lateral septum · Cardiovascular regulation · c-Fos · Vasopressin · Rat

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Introduction

Arterial blood pressure is regulated by a short and long-term feedback control system which relies on autonomic nerves and circulating hormones as their effector mechanisms. The central regulation of the cardiovascular system involves bulbar centers, such as the nucleus of the solitary tract (NTS), the rostral ventrolateral medulla (RVLM), the caudal ventrolateral medulla (CVLM), and nucleus ambiguous (Spyer 1994). Supramedullary structures, such as the periaqueductal gray (PAG), the cuneiform nucleus, the hypothalamus, the central nucleus of amygdala, specific regions of the cerebral cortex, and the lateral septal area (LSA) also participate in the control of the cardiovascular system, usually reflecting behavior changes (Dampney 1994; Loewy 1991; Loewy and McKellar 1980). In particular, the LSA is a limbic structure well described to be involved in the modulation of autonomic and behavioral processes (Covian 1966; Reis et al. 2010; Scopinho et al. 2006), and chemical stimulation of this structure usually evokes cardiovascular responses (Gelsema and Calaresu 1987; Pirola et al. 1987).

We have previously reported that alpha-1 adrenoceptors stimulation by microinjection of noradrenaline (NA) into the LSA of anaesthetized rats causes pressor and bradycardiac responses that are mediated by acute vasopressin release into the circulation (Scopinho et al. 2006), suggesting that noradrenergic mechanisms in this area could regulate the cardiovascular system. The responses to NA were blocked by the nonspecific synaptic blocker cobalt chloride ($CoCl_2$) or the selective non-*N*-methyl-D-aspartate (NMDA) antagonist NBQX microinjected into the paraventricular nucleus (PVN), suggesting that this region is the final structure in the pressor pathway activated by NA microinjection into the LSA (Scopinho et al. 2008). However, there is no evidence of direct connections between these areas (Tavares et al. 2005), suggesting the involvement of other intermediate structures.

The LSA connects with several key regions in the control of motivational and autonomic responses, such as the hippocampus, lateral and medial hypothalamus, periaqueductal gray (PAG), amygdala, bed nucleus of stria terminalis (BNST), medial prefrontal cortex (MPFC), diagonal band of Broca (DBB), and the medial septal area (Risold and Swanson 1997; Sheehan et al. 2004). Previous studies by our group showed that stimulation of BNST, MPFC, DBB, PAG, and medial amygdaloid nucleus (MeA) directly or indirectly activates the hypothalamic nuclei responsible for the release of vasopressin, causing pressor and bradycardic responses in rats (Crestani et al. 2007; Fernandes et al. 2003; Fortaleza et al. 2011; Pelosi and Correa 2005; Tavares and de Aguiar Correa 2003). These responses are similar to those evoked by NA in the LSA, suggesting the involvement of these areas in central mediated release of vasopressin into the systemic circulation.

To further investigate this issue, the present study uses c-Fos immunohistochemistry to indentify if these areas are activated after local injection of NA in the LSA. Our hypothesis is that they are part of a brain circuit engaged by NA in the LSA and responsible for controlling the central-mediated release of vasopressin in the systemic circulation.

Materials and Methods

Animal Preparation

Male Wistar rats weighing 220–270 g were used. Animals were kept in the Animal Care Unit of the Department of Pharmacology of the School of Medicine of Ribeirão Preto, University of São Paulo. Rats were housed individually in plastic cages in a 23–25 °C temperature-controlled room, with free access to water and commercial food. The Institution's Animal Ethics Committee approved the housing conditions and experimental protocols (057/2009). Rats were anesthetized with tribromoethanol, 250 mg/kg i.p. After local anesthesia with 2% lidocaine, the skull was surgically exposed and stainless-steel guide cannulae (0.6-mm o.d.) were implanted 1 mm above the injection sites using a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). Stereotaxic coordinates for implantation of cannulae were selected from the rat brain atlas (Paxinos and Watson 1997). The coordinates used were AP = +8.3 mm, L = +0.5 mm, and V = −4.1 mm for cannulae implantation in the LSA. All cannulae were implanted unilaterally. Cannulae were fixed to the skull with dental cement (Simplex, DFL, Rio de Janeiro, Brazil) and one metal screw. A tight-fitting trocater was kept inside the guide cannulae to prevent occlusion. Animals were treated with an antibiotic combination of penicillin and streptomycin (Pentabiótico, Fort Dodge Ltda, Brazil; 0.1 ml/kg), and flunixin meglumine (2.5 mg/kg s.c.) was used as a postoperative analgesic and anti-inflammatory agent. Two days later, the animals were anesthetized with tribromoethanol and a polyethylene catheter was implanted into the femoral artery for blood pressure and heart rate recording. The arterial catheter consisted of a segment of PE-10 tubing (4–5 cm) heat-bonded to a longer segment of PE-50 tubing (12 cm). The catheter was filled with 0.3 % heparin (stock solution 5,000 U/ml) in sterile saline (150 mM NaCl). The PE-10 segment was introduced into the femoral artery until the tip reached the aorta. The catheter was secured in position with thread, and the PE-50 segment was passed under the skin to be extruded on the dorsum of the animals. Flunixin meglumine (2.5 mg/kg s.c.) was used as a postoperative analgesic and anti-inflammatory treatment. After surgery, the animals were allowed to recover for 24 h.

Measurement of Cardiovascular Responses

The animals were kept in individual cages in the Animal Care Unit, which were transported to the experimental room. Animals were allowed 15 min to adapt to the conditions of the experimental room, such as sound and illumination, before starting blood pressure and heart rate recording. The experimental room was acoustically isolated and had constant background noise caused by an air exhauster. At last another 15-min period was allowed before the experiments were initiated, and care was taken to start the injection whenever a stable blood pressure and, especially, a stable heart rate were observed. The injection needle was slowly introduced into the guide cannulae without touching or restraining the animals. Pulsatile arterial pressure (PAP) of freely moving animals was recorded using an HP-7754A preamplifier (Hewlett

Packard, Palo Alto, CA, USA) and an acquisition board (MP100A; Biopac Systems Inc., Goleta, CA, USA) connected to a computer.

Drugs

Noradrenaline-HCl (21 nmol/200 nL; Sigma, St Louis, MO, USA) and WB4101 (10 nml/200 nL; α1-adrenergic antagonist; TOCRIS, Westwoods Business Park Ellisville, MO, USA) were dissolved in sterile artificial cerebrospinal fluid (aCSF composition: NaCl 100 mM; Na3PO4 2 mM; KCl 2.5 mM; MgCl2 1 mM; NaHCO3 27 mM; CaCl2 2.5 mM; pH 7.4). tribromoethanol (Sigma, St Louis, MO, USA), urethane (Sigma, St Louis, MO, USA) and dTyr (CH2)5(Me) AVP (50 mg/Kg; V1-vasopressin receptor antagonist, Peninsula, Belmont, CA, USA) were dissolved in saline (0.9 %). flunixine meglumine (Banamine®, Schering Plough, Brazil) and poly-antibiotic preparation of streptomycins and penicillins (Pentabiotico®, Fort Dodge, Brazil) were used as provided. All doses of the drugs used were based in those employed in a previous study by Scopinho et al. 2006.

Histological Procedure

At the end of the experiments, the rats were anesthetized with urethane (1.25 g/kg, i.p.) and 200 nL of filtered 1 % Evan's blue dye was injected into the brain as a marker of the injection site. The chest was surgically opened, the descending aorta occluded, the right atrium severed, and the brain perfused with 10 % formalin through the left ventricle. The brains were post-fixed for 24 h at 4 °C, and 40-μm sections were cut with a cryostat (CM 1900; Leica, Wetzlar, Germany). Brain sections were stained with 0.5 % cresyl violet for light microscopy analysis. The injection sites were verified in serial sections.

Immunohistochemistry

Two hours after the experimental procedures the animals were anaesthetized with urethane and perfused transcardially with saline followed by 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4). Brains were removed and post-fixed over 2 h in PFA and stored for at least 30 h in 30 % sucrose for cryoprotection. Coronal sections (40 μm) were obtained in duplicate in a cryostat. After this, the sections were processed for c-Fos immunohistochemistry as previously described (Aguiar and Guimaraes 2009; Beijamini and Guimaraes 2006). Briefly, tissue sections were washed and incubated overnight at room temperature with rabbit IgG (1/1,000 into TBS 0.01 M, sc 52, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), which was raised against an amino-acid sequence of the N-terminal region of the peptide an specifically recognizes c-Fos. After incubation in the primary antiserum, the tissue sections were washed in TBS and sequentially incubated with a biotinilated goat anti-rabbit IgG (1:1,000 into TBS 0.01 M). Sections were then processed by the avidin–biotin immunoperoxidase method (Vectastain ABC kit, Vector Lab, Burlingame, CA, USA). c-Fos immunoreactivity (FLI) was revealed by the addition of the chromogen diaminobenzidin (Sigma, into TBS, H_2O_2 0.02 % and niquel sulfate 1 %) and visualized as a black reaction product inside the neuronal nuclei.

The identification method of stained cells was similar to that described in previous works (Aguiar and Guimaraes 2009; Lino-de-Oliveira et al. 2001). The number of FLI was counted with the help of a computerized image analysis system (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA). The system was calibrated to ignore background staining. Darker objects with areas between 10 and 80 μm^2 were identified as Fos-positive neurons. An observer blind to group assignment performed the analysis. For each group one section from each animal was evaluated. All stained cells in the whole area of each brain region of interested were recorded. For bilateral structures, the mean value between of the structure was calculated. The areas of the analyzed regions were calculated and the results expressed as the number of positive cells/0.1 mm^2. Neuroanatomical sites were identified with the help of the Paxinos and Watson's atlas (Paxinos and Watson 1997). The anterior–posterior (AP) localization from bregma of the analyzed regions were as follows: cingulate cortex 1 (Cg 1, AP: 2.7 mm), infralimbic cortex (IL, AP: 2.7 mm), prelimbic cortex (PL, AP: 2.7 mm), diagonal band of Broca (DBB, AP: 0.84 mm), bed nucleus of stria terminalis (BNST, AP: −0.2 mm), paraventricular hypothalamic nucleus (PVN, AP: −1.8 mm), medial amygdaloid nucleus (MeA, AP: −2.76 mm), dorsomedial periaqueductal gray (dmPAG, AP: −6.6 mm), and dorsolateral periaqueductal gray (dlPAG, AP: −6.6 mm).

Statistical Analysis

Two-way ANOVA (treatment vs. time) followed by Bonferroni's post hoc test was used to compare effects of the treatments on cardiovascular MAP and HR changes observed after microinjection of NA into the LSA. Basal values of MAP and HR were compared by paired Student's *t* test. The immunohistochemistry data were analyzed by One-way ANOVA followed by Newman and Keuls post hoc test. The significance level was set at $P < 0.05$ in GraphPad Prism software.

Results

Effect of Intra-LSA Pretreatment with aCSF or WB4101 on Cardiovascular Responses to the Microinjection of NA into the LSA of Unanesthetized Rats

aCSF

Pretreatment with aCSF ($n = 5$) in the LSA did not affect neither the pressor (26.8 ± 1.9 vs. 26 ± 1.8 mm Hg, $t = 2.1$, $P > 0.05$) nor the bradycardiac (45.4 ± 8.3 vs. 43 ± 1.9 bpm, $t = 0.3$, $P > 0.05$) responses to NA microinjected into the LSA.

WB4101

Intraseptal injection of WB4101 (10 nmol/200 nL) did not affect baseline MAP (96.9 ± 2.4 vs. 94.5 ± 0.7 mmHg, $t = 0.8$, $P > 0.05$, $n = 5$) or HR (350 ± 3.5 vs. 359.1 ± 4.1 bpm, $t = 2.1$, $P > 0.05$, $n = 5$). However, two-way ANOVA indicated significant effects of WB4101 on NA cardiovascular effects (MAP: $F_{1,88} = 437.3$, $P < 0.0001$; and HR: $F_{1,88} = 111$, $P < 0.0001$); a significant effect over time (MAP: $F_{10,88} = 21.60$, $P < 0.0001$; and HR: $F_{10,88} = 10.4$, $P < 0.0001$) and interaction between treatment and time (MAP: $F_{10,88} = 30.17$, $P < 0.0001$; and HR: $F_{10,88} = 10.9$, $P < 0.0001$; Fig. 1 left)

Diagrammatic representation of the injection sites in the LSA are represented in the Fig. 2.

Effect of Systemic Pretreatment with ACSF or dTyr(CH2)5(Me)AVP on Cardiovascular Responses to the Microinjection of NA into the LSA of Unanesthetized Rats

aCSF

I.v. pretreatment with aCSF ($n = 5$) did not affect neither the pressor (27.2 ± 1.2 vs. 25.6 ± 2 mm Hg, $t = 0.9$, $P > 0.05$) nor the bradycardiac (43.8 ± 5 vs. 43.6 ± 5.1 bpm, $t = 0.04$, $P > 0.05$) responses to NA microinjected into the LSA.

dTyr(CH2)5(Me)AVP

Pretreatment with dTyr(CH2)5(Me)AVP, i.v., (50 μg/kg) did not affect baseline MAP (100.4 ± 3.9 vs. 100.7 ± 3.2 mmHg, $t = 0.7$, $P > 0.05$, $n = 5$) or baseline HR (345.9 ± 2.7vs. 349.1 ± 3.3 bpm, $t = 1.3$, $P > 0.05$, $n = 5$). However, two-way ANOVA indicated significant effects of dTyr(CH 2) 5 (Me)AVP on NA cardiovascular effects (MAP: $F_{1,88} = 283.6$, $P < 0.0001$; and HR: $F_{1,88} = 95.62$, $P < 0.0001$); a significant effect over time (MAP: $F_{10,88} = 14.1$, $P < 0.0001$; and HR: $F_{10,88} = 9.5$, $P < 0.0001$) and interaction between treatment and time (MAP: $F_{10,88} = 18.9$, $P < 0.0001$; and HR: $F_{10,88} = 7.6$, $P < 0.0001$; Fig. 1 right).

c-Fos Immunoreactivity (FLI) Following NA or aCSF Microinjected into the LSA of Rats Before and After Local Pretreatment with WB4101 or dTyr(CH2)5(Me)AVP Injected Systemically

FLI was detected in several areas after NA microinjection into the LSA (Table 1). Intra-LSA NA induced a significant increase in FLI in the PVN ($F_{(5,29)} = 104.3$, $P < 0.0001$), BNST ($F_{(5,29)} = 1644$, $P < 0.0001$), dmPAG ($F_{(5,29)} = 733.9$, $P < 0.0001$), PL ($F_{(5,29)} = 3718$, $P < 0.0001$), IL ($F_{(5,29)} = 3236$, $P < 0.0001$) and MeA ($F_{(5,29)} = 1210$, $P < 0.0001$). In the DBB ($F_{(5,29)} = 0.2$, $P > 0.05$), dlPAG ($F_{(5,29)} = 1.1$, $P > 0.05$) and Cg1 ($F_{(5,29)} = 1.0$, $P > 0.05$) there were no differences between animals that received NA or vehicle (Fig. 3).

Intra-LSA microinjection of WB4101 (10 nmol/200 nL) significantly attenuated the increased in FLI induced by NA intra-LSA in PVN (Newman–Keuls $P < 0.001$), BNST (Newman–Keuls $P < 0.001$), dmPAG (Newman–Keuls $P < 0.001$), PL (Newman–Keuls $P < 0.001$), IL (Newman–Keuls $P < 0.001$) and MeA (Newman–Keuls $P < 0.001$), when compared with the group that received aCSF (Fig. 3).

dTyr (CH2)5(Me) AVP (50 microg/kg) i.v. treatment did not attenuate the increase in FLI induced by intra-LSA NA in the PVN (Newman–Keuls $P > 0.05$), BNST (Newman–Keuls $P > 0.05$), dmPAG (Newman–Keuls $P > 0.05$), PL (Newman–Keuls $P > 0.05$), IL (Newman–Keuls $P > 0.05$) and MeA (Newman–Keuls $P > 0.05$) (Fig. 3).

Photomicrographs showing c-Fos immunohistochemistry (black arrow) in some structures related above are present in Fig. 4.

Discussion

In the present study, we showed that NA microinjected into the LSA, in addition to evoke pressor and bradycardic responses dependent on vasopressin, is also able to increase c-Fos expression in brain regions that can facilitate the release of this hormone.

c-Fos expression increased in the bed nucleus of stria terminalis (BNST), dorsomedial periaqueductal gray (dmPAG), prelimbic (PL) and infralimbic (IL) cortices, medial amygdaloid nucleus (MeA), and paraventricular nucleus of hypothalamus (PVN), suggesting that these

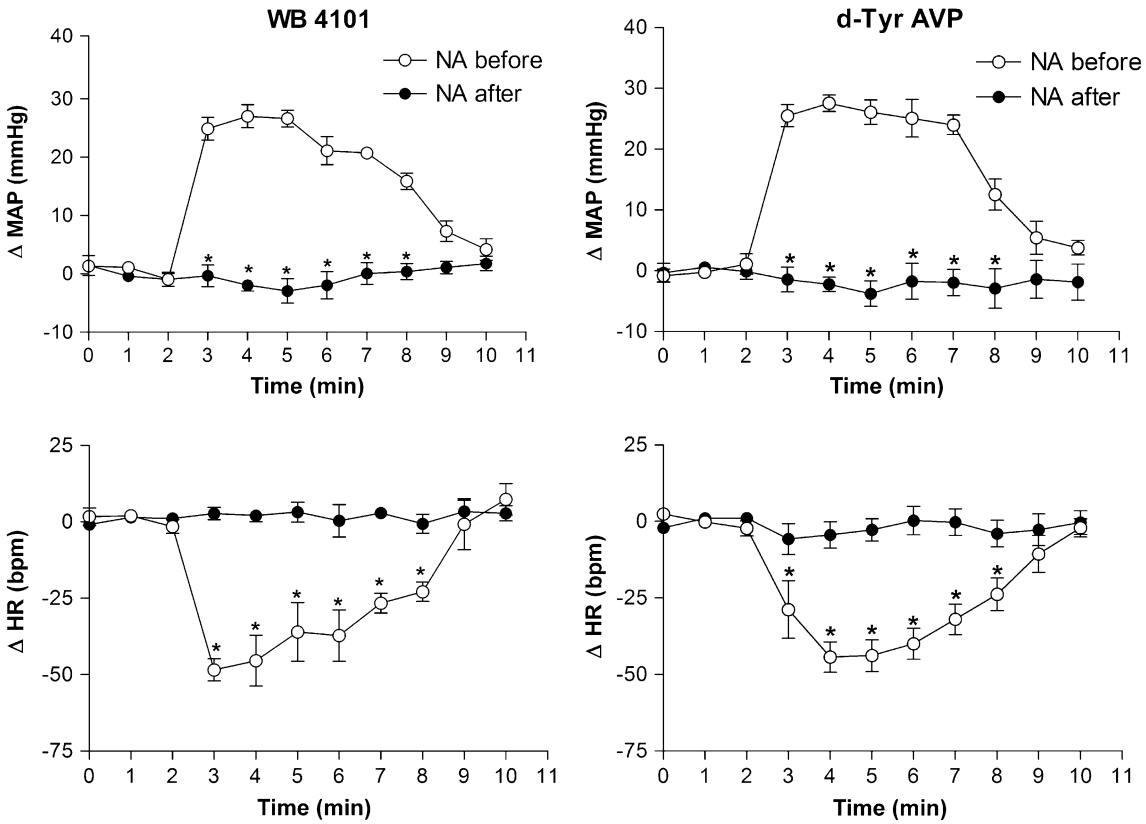

Fig. 1 Left-time course of the effect of NA (21 nmol/200 nL) microinjected into the LSA on MAP and HR before and after local pretreatment with WB4101 (10 nmol/200 nL, $n = 5$). Right-time course of the effect of NA (21 nmol/200 nL) microinjected into the LSA on MAP and HR before and after i.v. pretreatment with dTyr(CH2)5(Me)AVP (d-Tyr AVP, 50 l g/kg, $n = 5$). NA injections were made at time 2. Points represent the mean and bars the SEM; two-way ANOVA followed by Bonferroni's post hoc test

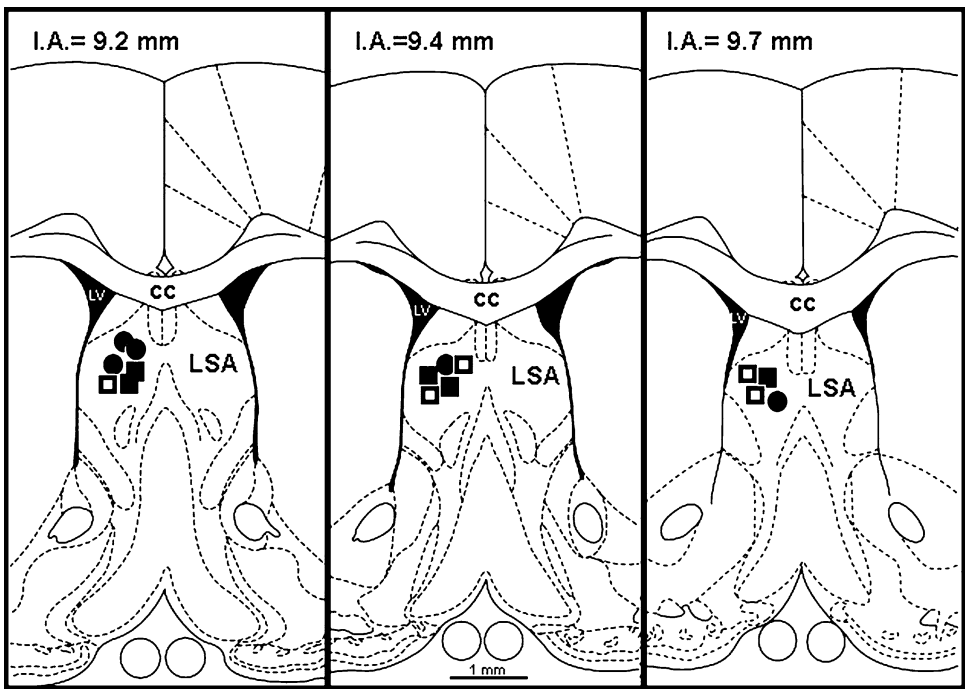

Fig. 2 Diagrammatic representation indicating the microinjection sites of NA (*black circles*), aCSF (*black squares*), or WB4101 (*white squares*) in the LSA. Coordinates based on the atlas of Paxinos and Watson (1997). *I.A.* interaural coordinate; *LSA* lateral septal area; *CC* corpo calosum; and *LV* lateral ventricle

Table 1 Fos-like immunoreactivity in brain regions of rats after microinjections into the LSA of WB4101 (WB, 10 nml/200 nL) or systemic injection of dTyr (CH2)5(Me) AVP (Dtyr, 50 mg/Kg) followed by intra-LSA microinjection of aCSF or Noradrenaline (NA, 21 nmol/200 nL), $n = 5$ per group

Structure	aCSF	NA	Dtyr + aCSF	Dtyr + NA	WB + aCSF	WB + NA
PVN	5.98 ± 1.45	91.54 ± 6.72*	2.42 ± 0.71#	85.50 ± 7.20*	3.59 ± 1.43#	9.54 ± 2.16#
BNST	1.45 ± 0.47	108.3 ± 2.45*	2.33 ± 0.73#	111.0 ± 1.65*	3.43 ± 0.93#	2.99 ± 0.87#
dmPAG	5.78 ± 0.90	110.7 ± 2.60*	3.87 ± 1.36#	111.9 ± 2.14*	4.47 ± 1.55#	6.01 ± 2.84#
PL	1.58 ± 0.44	107.0 ± 1.54*	2.87 ± 0.62#	105.4 ± 0.84*	1.74 ± 0.81#	1.72 ± 0.57#
IL	1.40 ± 0.56	107.6 ± 1.61*	2.43 ± 0.61#	108.3 ± 1.32*	2.25 ± 0.53#	1.59 ± 0.45#
MeA	1.71 ± 0.63	106.6 ± 1.82*	3.79 ± 1.03#	108.4 ± 2.87*	2.14 ± 0.79#	2.18 ± 0.95#
DBB	5.04 ± 0.75	4.69 ± 1.43	4.13 ± 0.87	4.77 ± 1.23	6.07 ± 2.40	4.66 ± 1.18
dlPAG	4.69 ± 0.95	3.57 ± 0.90	4.11 ± 1.21	3.46 ± 0.89	5.52 ± 1.34	6.83 ± 1.69
Cg1	5.34 ± 1.56	4.08 ± 1.15	4.30 ± 1.12	5.11 ± 0.64	8.60 ± 2.81	6.29 ± 1.66

Data are expressed as means ± SEM, $n = 5$/group. *PVN* paraventricular nucleus of hypothalamus, *BNST* bed nucleus of stria terminalis, *dmPAG* dorsomedial periaqueductal gray, *PL* and *IL* prelimbic and infralimbic prefrontal cortex, *MeA* medial amygdaloid nucleus, DBB diagonal band of Broca, *dlPAG* dorsolateral periaqueductal gray, and *Cg1* cingulate cortex area 1

* Different from vehicle-treated group (aCSF) group, $P < 0.0001$

\# Different from NA-treated (NA) group, $P < 0.0001$

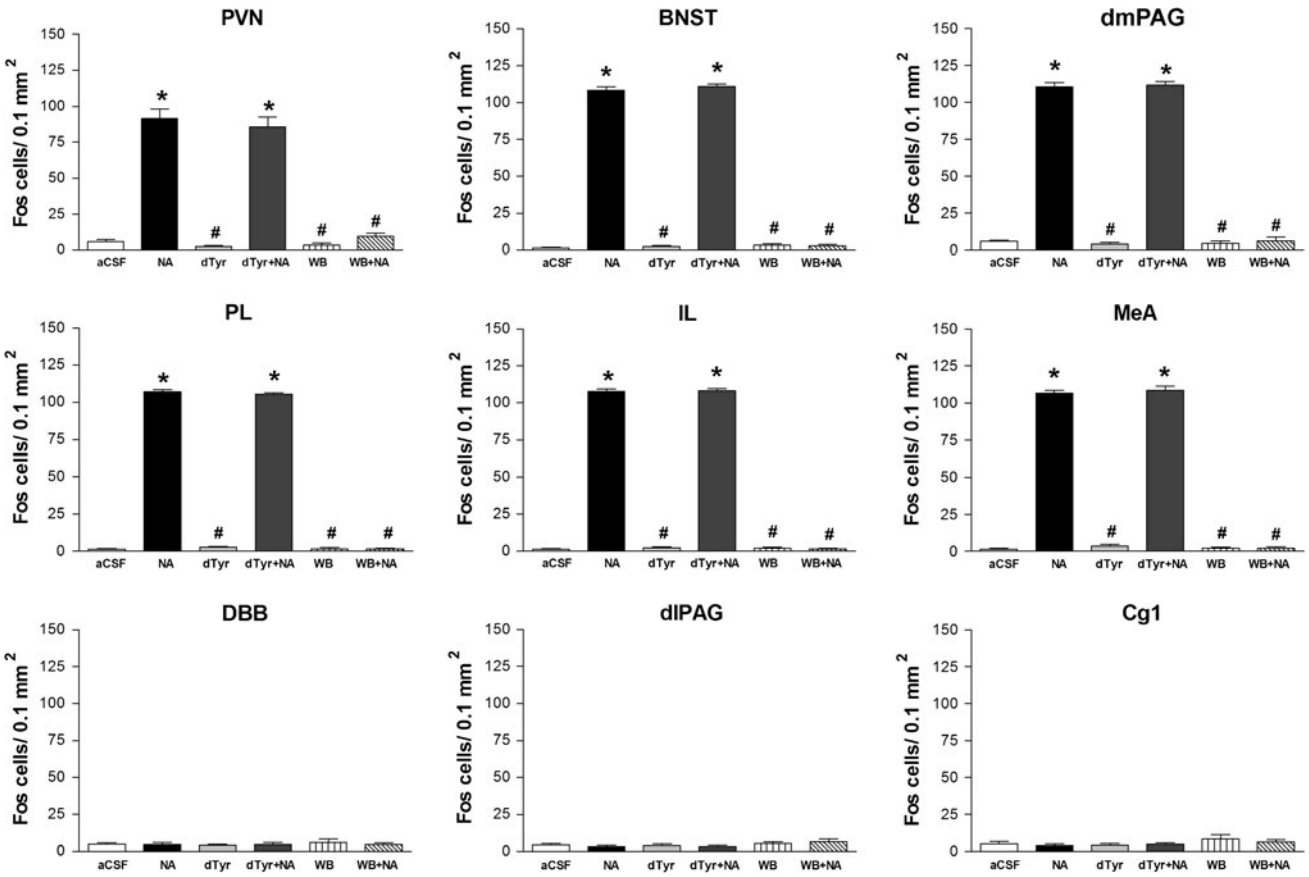

Fig. 3 Effects of microinjection of 200 nL of aCSF or 21 nmol of noradrenaline (NA) into the LSA before and after i.v. pretreatment with dTyr(CH2)5(Me)AVP (d-Tyr AVP, 50 μg/kg) or LSA local pretreatment with 10 nmol of WB4101 on the number of c-Fos positive cells in rats ($n = 5$ per group). Bars represent the mean (±SEM). $^{*}P < 0.05$ compared to vehicle-treated group and $^{\#}P < 0.05$ compared to NA-treated group, ANOVA followed by the Newman–Keuls post test

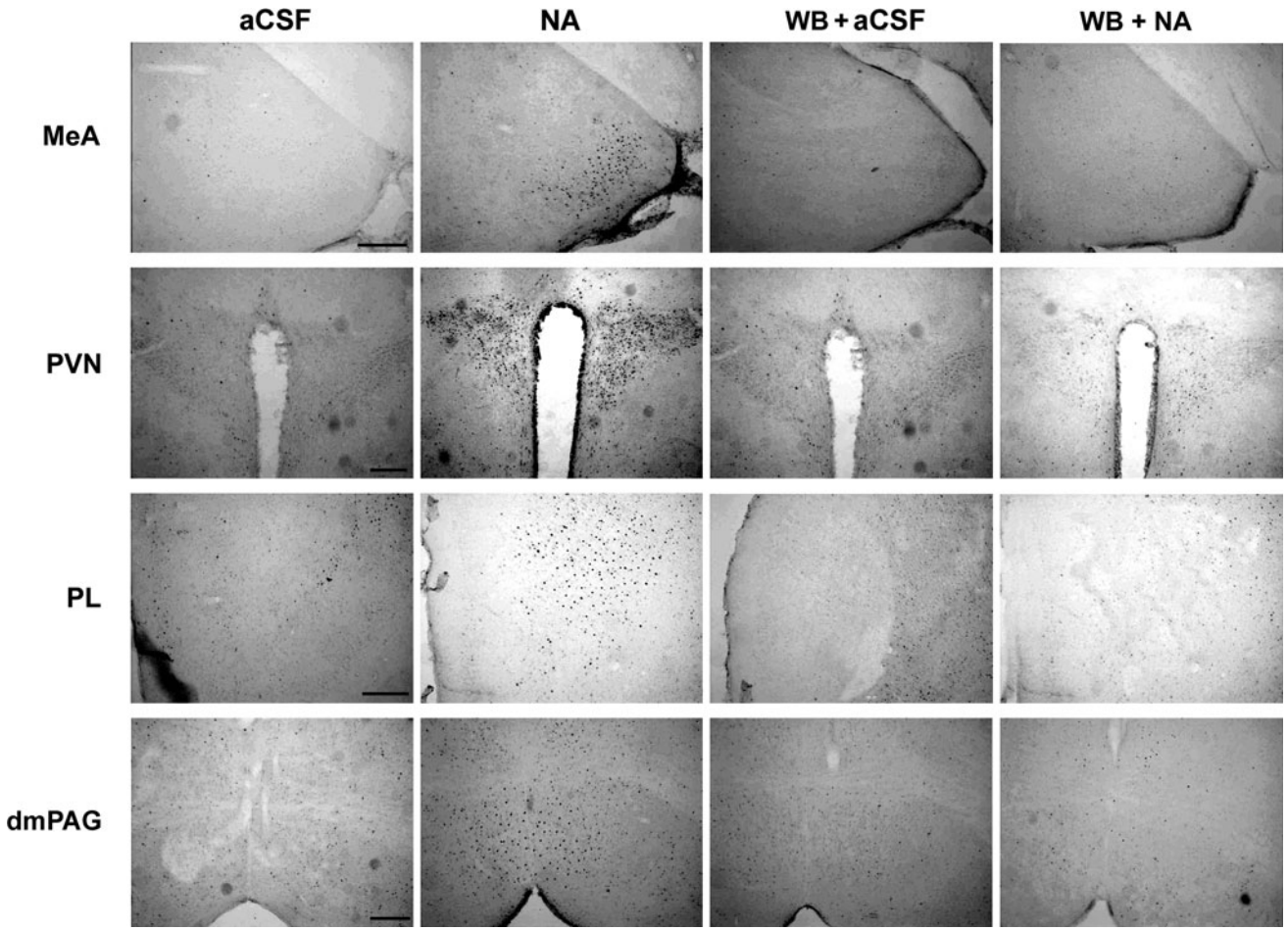

Fig. 4 Photomicrographs showing c-Fos immunohistochemistry (arrowhead) in the medial amygdaloid nucleus (MeA), paraventricular nucleus of hypothalamus (PVN), prelimbic cortex (PL), and dPAG (dorsal periaqueductal gray), of rats that received the following treatments into the LSA: two microinjections of aCSF (Veh + Veh), aCSF and noradrenaline (Veh + NA), WB4101 and aCSF (WB + Veh), and WB4101 and noradrenaline (WB + NA). *Scale bar* 150 μm

structures are activated after administration of NA into the LSA. All these structures are able to cause vasopressin release to periphery after stimulation (Busnardo et al. 2009b; Crestani et al. 2007; Fernandes et al. 2003; Fortaleza et al. 2011; Pelosi and Correa 2005). In contrast, no changes in the pattern of c-Fos expression were found in the diagonal band of Broca (DBB), cingulate cortex 1 (Cg1), and dorsolateral periaqueductal gray (dlPAG). These results suggest areas where there was increased expression of c-Fos protein are recruited after microinjection of NA in LSA.

NA microinjected into the BNST caused pressor and bradycardic response dependent of vasopressin release (Crestani et al. 2007). PVN pretreatment with the selective non-NMDA receptor antagonist NBQX significantly reduced these cardiovascular responses, suggesting that glutamate-mediated neurotransmission through non-NMDA glutamate receptors in the PVN mediates these responses (Crestani et al. 2009b). Moreover, NA microinjection into the dPAG (Pelosi and Correa 2005), PL (Fernandes et al. 2003) and MeA (Fortaleza et al. 2011) also evoked pressor and bradycardiac responses that depend on vasopressin release. The nonselective synaptic blocker cobalt chloride injected into the PVN blocked the cardiovascular responses caused by NA microinjected into the dPAG (Pelosi et al. 2009) and PL (Fernandes et al. 2007) indicating that synapses in PVN mediate the vasopressin-induced pressor response caused by NA microinjection.

Glutamate microinjected into the DBB evoked pressor and bradycardic responses that also is mediated by vasopressin release into the circulation, similar to the effects observed after NA microinjection into the dPAG (Pelosi and Correa 2005), LSA (Scopinho et al. 2006), PL (Fernandes et al. 2003), BNST (Crestani et al. 2007), SON (Busnardo et al. 2009a), or MeA (Fortaleza et al. 2011). The DBB plays an important role in cardiovascular modulation, and it is connected to the brain nuclei involved in

neurovegetative regulation (Cullinan and Zaborszky 1991; Gaykema et al. 1990).

Since the LSA did not present direct connections with the magnocellular nuclei of PVN and SON (Tavares et al. 2005), and activation of this region releases vasopressin through the PVN but not the SON (Scopinho et al. 2008), the structures mentioned above could be potential relays between the LSA and the PVN. Neuroanatomical studies using neuronal tracers show that the LSA is densely interconnected with the amygdala (Sheehan et al. 2004; Volz et al. 1990), PL and IL cortices (Vertes 2004) and BNST (Risold and Swanson 1997).

On the other hand, although the LSA also presents connections with the DBB (Tavares et al. 2005) and Cg1 cortex (Vertes 2004), we did not observ changes in the pattern of c-Fos expression in these structures. Also, despite no direct connections with LSA has been described for the dPAG, only in the dorsomedial subdivision of dPAG (dmPAG) presents increased c-Fos expression after NA injection into the LSA, suggesting that the dmPAG but not dorsolateral subdivions of dPAG (dlPAG) is activated under this situation.

Varicose fibers were observed in the DBB after microinjection of the neurotracer BDA into the dPAG, indicating the existence of a direct neural connection between these two regions. Moreover, BDA microinjected into the DBB showed the existence of direct efferent connections from the DBB to the magnocellular portion in the PVN (Tavares et al. 2005). DBB pretreatment with cobalt chloride (calcium channel blocking agent) significantly reduced the cardiovascular response to NA microinjection into the dPAG (Pelosi et al. 2009). Because cobalt chloride is a nonspecific blocker of synaptic transmission, it is suggested that the response to NA microinjection into the dPAG is mediated by synaptic neurotransmission within the DBB and is not related to fibers of passage. However, cobalt injection into DBB did not completely block the dPAG-evoked cardiovascular effects. One possible explanation could be the involvement of the contralateral side of the DBB in the cardiovascular response to NA injection into the dPAG. Another possibility could be an additional involvement of dPAG projections to other nuclei than the DBB.

The bradycardiac response was blocked by systemic administration of pentolinium (ganglion blocker), without affecting the pressor response to NA injected into the LSA. This suggests that the bradycardia induced by the latter treatment depends on a vagal baroreflex response consequent to MAP increase (Scopinho et al. 2006). In addition, LSA modulates baroreflex responses as well as all structures mentioned above (Crestani et al. 2006, 2008, 2010; Pelosi et al. 2007; Quagliotto et al. 2008; Resstel et al. 2004; Scopinho et al. 2007). Since activation of the baroreflex is able to activate the structures investigated in this study, the present results could be reflecting a nonspecific response to changes in blood pressure. To discard this possibility, we pretreated animals with a systemic (i.v.) injection of the selective V1-vasopressin receptor antagonist dTyr(CH2)5(Me)AVP. As previously described, the antagonist blocked the pressor and bradycardiac responses caused by NA microinjection into the LSA (Scopinho et al. 2006), but did not affect the increase in c-Fos expression in BNST, dmPAG, PL, IL, MeA, and PVN. We have also reported previously that the alpha1- adrenoceptor antagonist WB4101 blocks pressor and bradycardic responses evoked by NA into the LSA (Scopinho et al. 2006). The present study confirmed these results and showed that this treatment also attenuated the FLI increase in the BNST, dmPAG, PL, IL, MeA, and PVN. Taken together, these results suggest that the activation of alpha-1 adrenoceptors in the LSA can increase neuronal activation of the aforementioned regions.

The LSA is a limbic structure involved in motivational, behavioral, and autonomic processes (Alheid and Heimer 1988; Reis et al. 2010; Sheehan et al. 2004) as well as in cardiovascular control (Correa and Polon 1978; Gelsema and Calaresu 1987; Scopinho et al. 2006, 2007). Noradrenergic nerve terminals have been identified throughout the LSA (Antonopoulos et al. 2004; Lindvall and Stenevi 1978; Risold and Swanson 1997) providing a neuroanatomic substrate for a septal noradrenergic influence. We have previously demonstrated that pressor and bradycardic responses evoked by NA injection in the LSA are due to local α1-adrenoceptors activation and are mediated by vasopressin release into the circulation, suggesting that LSA noradrenergic mechanisms play a role in prosencephalic limbic system and vasopressin release integration. The neurohypophyseal hormone arginine vasopressin (AVP) is involved in several physiological regulatory processes that occur via stimulation of specific V1a, V1b, and V2 receptors (Thibonnier et al. 2002). The role of AVP has been well characterized in the regulation of blood pressure in pathophysiological conditions such as severe hypovolemia and hypotension episodes (Laszlo et al. 1991). However, its contribution to blood pressure homeostasis in normal physiological situations is poorly known (Jackson et al. 1996). Vasopressin is synthesized by magnocellular cells within the supraotic (SON) and PVN nuclei of the hypothalamus and released into circulation by the neurohypophysis. This hormone is a potent stimulator of vascular smooth muscle contraction in vitro, and V1a receptors mediate its vasoconstrictor effect (Jackson et al. 1996). Vasopressin was reported to be released in greater proportion following several kinds of stress, such as hemorrhage, isotonic hypovolemia, and restraint (Kasting 1988).

Microdialysis studies show that stressors such as immobilization increases NA release in a number of limbic forebrain regions, which thought to be involved in a variety of behavioral, cognitive, affective, autonomic, and neuroendocrine responses to stress, including the MeA, BNST, PL and IL cortex, and LS (Cecchi et al. 2002a, b; Garcia et al. 2003; Ma and Morilak 2005; Pardon et al. 2002). Also, the LSA and the structures that showed increased expression of c-Fos after intra-LSA microinjection of NA have been implicated in the mediation of the cardiovascular responses observed during aversive situations (Busnardo et al. 2010; Crestani et al. 2009a; Fortaleza et al. 2009; Kubo et al. 2002; Leman et al. 2003; Resstel et al. 2006).

c-Fos expression occurs in neurons in response to their activation by excitatory synaptic inputs as well as other stimuli (Dragunow and Faull 1989; Morgan and Curran 1991). The aim of this study was to investigate: the areas which will be activated from the microinjection of noradrenaline in the LSA and so in the future and perform functional experiments with the administration of inhibitors or antagonists of synaptic neurotransmission, and thus establish a relationship between the areas in a common pathway. The connectivity of these structures is not presented by our results. We cannot know if these areas have direct or indirect connections among themselves. Only when used in combination with retrograde tracing and/or immunohistochemical identification of neurotransmitters, c-fos expression method can be used to define the connections and transmitter properties of central neurons activated by specific stimuli.

Conclusion

Considering that MeA, BNST, PL, and IL cortex, dmPAG and PVN are activated by LSA stimulus with noradrenaline, and these areas are able to evoke cardiovascular responses depending on vasopressin release, our results suggest that these structures constitute a cardiovascular pathway involved with the vasopressin releases.

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