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Research Report

Hypothalamic supraoptic but not paraventricular nucleus is involved in cardiovascular responses to carbachol microinjected into the bed nucleus of stria terminalis of unanesthetized rats

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ABSTRACT

Microinjection of the cholinergic agonist carbachol into the bed nucleus of the stria terminalis (BST) has been reported to cause pressor response in unanesthetized rats, which was shown to be mediated by an acute release of vasopressin into the systemic circulation and followed by baroreflex-mediated bradycardia. In the present study, we tested the possible involvement of the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei in the pressor response evoked by carbachol microinjection into the BST of unanesthetized rats. For this, cardiovascular responses following carbachol (1 nmol/100 nL) microinjection into the BST were studied before and after PVN or SON pretreatment, either ipsilateral or contralateral in relation to BST microinjection site, with the nonselective neurotransmission blocker cobalt chloride ($CoCl_2$, 1 mM/100 nL). Carbachol microinjection into the BST evoked pressor response. Moreover, BST treatment with carbachol significantly increased plasma vasopressin levels, thus confirming previous evidences that carbachol microinjection into the BST evokes pressor response due to vasopressin release into the circulation. SON pretreatment with $CoCl_2$, either ipsilateral or contralateral in relation to BST microinjection site, inhibited the pressor response to carbachol microinjection into the BST. However, $CoCl_2$ microinjection into the ipsilateral or contralateral PVN did not affect carbachol-evoked pressor response. In conclusion, our results suggest that pressor response to carbachol microinjection into the BST is mediated by SON magnocellular neurons, without significant involvement of those in the PVN. The results also indicate that responses to carbachol microinjection into the BST are mediated by a neural pathway that depends on the activation of both ipsilateral and contralateral SON.

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Abbreviations: aCSF, Artificial cerebrospinal fluid; CBH, Carbachol; BST, Bed nucleus of stria terminalis; $CoCl_2$, Cobalt chloride; HR, Heart rate; MAP, Mean arterial pressure; PAP, Pulsatile arterial pressure; PVN, Paraventricular nucleus of the hypothalamus; SON, Supraoptic nucleus of the hypothalamus

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1. Introduction

The bed nucleus of the stria terminalis (BST) is a structure of the limbic system that is involved in behavioral, neuroendocrine and autonomic responses (Alves et al., 2007; Crestani et al., 2007, 2008a; Dunn and Williams, 1995). Moreover, it has been proposed that the BST is an important center for the regulation of cardiovascular activity (Crestani et al., 2009a; Gelsema et al., 1987; Ulrich-Lai and Herman, 2009). The electric stimulation of the BST has been reported to evoke pressor as well as depressor responses in anesthetized rats (Dunn and Williams, 1995). Cardiovascular responses was also observed after chemical stimulation of the BST using either glutamate, D,L-homocysteic acid or noradrenaline (Ciriello and Janssen, 1993; Crestani et al., 2007; Gelsema et al., 1987, 1993; Hatam and Nasimi, 2007). In addition, there is evidence that the BST tonically modulates cardiac baroreflex activity (Alves et al., 2009; Crestani et al., 2006, 2008b; Li and Dampney, 1994; McKitrick et al., 1992).

Cholinergic synaptic terminals as well as muscarinic and nicotinic cholinergic receptors have been identified in the BST (Clarke et al., 1985; Ruggiero et al., 1990; Wamsley et al., 1984), thus providing evidence of a cholinergic neurotransmission in the BST. We have previously reported that microinjection of carbachol, a cholinergic agonist, into the BST of unanesthetized rats caused an increase in arterial pressure that was followed by a baroreflex-mediated reduction of the heart rate (HR) (Alves et al., 2007). These responses were inhibited by systemic pretreatment with a V_1-vasopressinergic receptor antagonist (Alves et al., 2007), thus suggesting a mediation by acute vasopressin release into the systemic circulation. Moreover, cardiovascular responses to carbachol microinjection into the BST were mediated by activation of local M_2-cholinergic receptors (Alves et al., 2007). These results suggested the existence of a cholinergic mechanism in the BST that integrates cardiovascular and neuroendocrine control and could take part in fluid balance adjustments. However, the neural pathway involved in cardiovascular responses to carbachol microinjection into the BST is yet unknown.

Vasopressin, also known as antidiuretic hormone, is a nonapeptide with a potent vasoconstrictor action (Altura and Altura, 1984; Barer, 1961). This peptide is synthesized by magnocellular neurons located in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus and stored in the posterior hypophysis to further release into the systemic circulation (Swaab et al., 1975). Because cardiovascular responses following carbachol microinjection into the BST were shown to be mediated by an acute release of vasopressin into the systemic circulation (Alves et al., 2007), the magnocellular neurons in the SON and/or PVN must be involved in their mediation. Considering this, it is relevant to study which hypothalamic magnocellular nucleus mediates the cardiovascular response evoked by carbachol microinjection into the BST.

Taking that into consideration, we evaluated the hypothesis that PVN and/or SON neurons are part of the neural pathway related to cardiovascular responses following carbachol microinjection into the BST of unanesthetized rats. For this, we investigated cardiovascular responses evoked by carbachol microinjection into the BST before and after PVN or SON pretreatment, either ipsilateral or contralateral in relation to BST microinjection site, with the nonselective neurotransmission blocker cobalt chloride ($CoCl_2$).

2. Results

2.1. *Cardiovascular responses to aCSF or carbachol microinjection into the bed nucleus of the stria terminalis of unanesthetized rats*

Microinjection of aCSF into the BST (n=5) did not affect either MAP (99±2 vs. 98±3 mm Hg, t=0.2, $P>0.05$) or HR (379±11 vs. 352±9 bpm, t=1.3, $P>0.05$) baseline values. However, microinjection of carbachol into the BST caused significant pressor and bradycardiac responses in unanesthetized rats (Fig. 1).

Photomicrography of a coronal brain section showing a representative microinjection site into the BST is presented in Fig. 2. Diagrammatic representation of the BST indicating microinjection sites into the BST of all animals used in the present study is also shown in Fig. 2.

2.2. *Effect of aCSF or carbachol microinjection into the bed nucleus of the stria terminalis in plasma vasopressin levels*

Microinjection of carbachol (n=6) into the BST significantly increased plasma vasopressin content (aCSF: 2.3±0.5 pg/mL vs. carbachol: 21.3±3.6 pg/mL, t=5, $P<0.005$), when compared to the control group that received vehicle (aCSF) injection into the BST (n=6).

2.3. *Effect of supraoptic nucleus pretreatment with aCSF or $CoCl_2$ in cardiovascular responses to carbachol microinjection into the bed nucleus of the stria terminalis*

2.3.1. *Ipsilateral supraoptic nucleus*

Microinjection of aCSF into the ipsilateral SON (n=7) did not affect either MAP (98±2 vs. 101±3 mm Hg, t=0.5, $P>0.05$) or HR (352±7 vs. 367±11 bpm, t=1.5, $P>0.05$) baseline values. Pretreatment of the ipsilateral SON with aCSF also did not affect the pressor (43±2 vs. 38±2 mm Hg, t=2.3, $P>0.05$) and bradycardiac (−67±7 vs. −64±8 bpm, t=0.2, $P>0.05$) response to carbachol microinjection into the BST (Fig. 1A).

Microinjection of $CoCl_2$ into the ipsilateral SON (n=7) did not affect either MAP (102±2 vs. 100±2 mm Hg, t=0.6, $P>0.05$) or HR (351±6 vs. 356±8 bpm, t=0.7, $P>0.05$) baseline values. However, ipsilateral SON pretreatment with $CoCl_2$ significantly reduced the pressor (44±2 vs. 6±1 mm Hg, t=16, $P<0.0001$) and bradycardiac (−74±6 vs. −12±1 bpm, t=10, $P<0.0001$) response to carbachol microinjection into the BST (Fig. 1A). Time-course analysis indicated a significant effect of SON pretreatment with $CoCl_2$ in carbachol cardiovascular effects (ΔMAP: $F_{(1,456)}=468$, $P<0.0001$ and ΔHR: $F_{(1,456)}=111$, $P<0.0001$), a significant effect over time (ΔMAP: $F_{(37,456)}=23$, $P<0.0001$ and ΔHR: $F_{(37,456)}=11$, $P<0.0001$), and an interaction between treatment and time (ΔMAP: $F_{(37,456)}=20$, $P<0.0001$ and ΔHR: $F_{(37,456)}=4$, $P<0.0001$) (Fig. 1B).

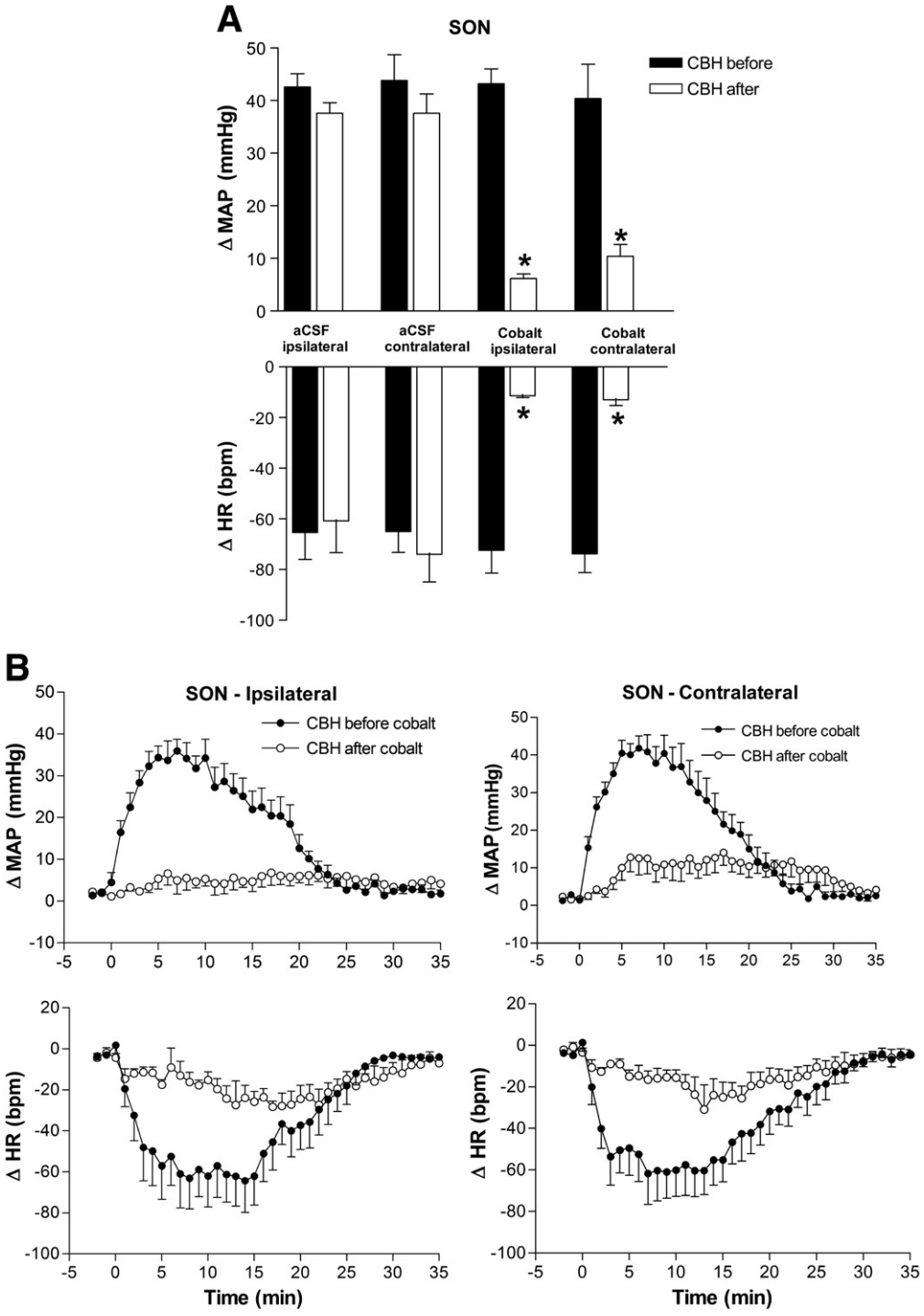

Fig. 1 – A) Changes in MAP (ΔMAP) and HR (ΔHR) in response to carbachol (CBH; 1 nmol/100 nL) microinjection into the BST before (filled columns) and after (open columns) pretreatment of ipsilateral or contralateral SON, in relation to BST microinjection site, with vehicle (aCSF) or $CoCl_2$ (cobalt). Columns represent the mean and bars the SEM. *P<0.05, paired *Student*'s *t*-test. B) Time-course of the effect of CBH microinjection into the BST on MAP and HR before (filled circles) or after (open circles) pretreatment of the ipsilateral SON (left) or contralateral SON (right) with $CoCl_2$. CBH injections were made at time 0. Circles represent the mean and bar the SEM. Data were analyzed using two-way ANOVA.

2.3.2. Contralateral supraoptic nucleus

Microinjection of aCSF into the contralateral SON (n=6) did not affect either MAP (100±3 vs. 102±4 mm Hg, t=0.5, P>0.05) or HR (353±11 vs. 372±6 bpm, t=1.6, P>0.05) baseline values. Pretreatment of the contralateral SON with aCSF also did not affect both the pressor (44±4 vs. 37±3 mm Hg, t=2.2, P>0.05) and bradycardiac (−67±8 vs. −74±8 bpm, t=0.5, P>0.05) response to carbachol microinjection into the BST (Fig. 1A).

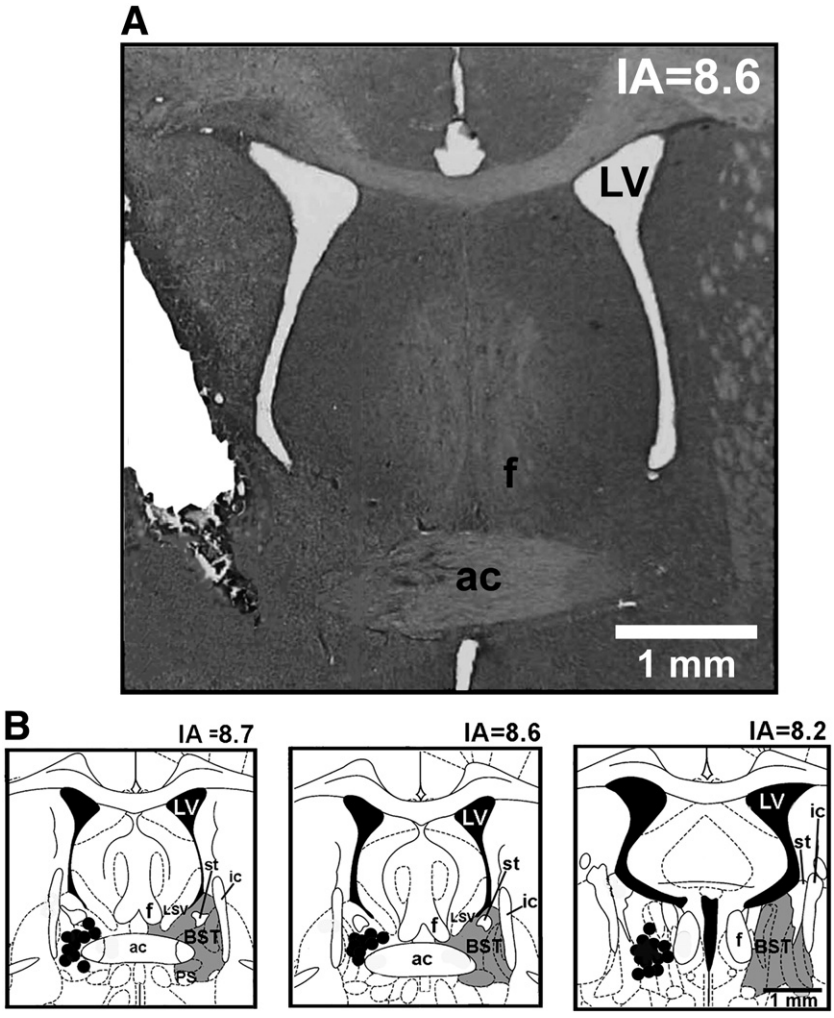

Fig. 2 – A) Photomicrograph of a coronal brain section showing a microinjection site in the BST of one representative animal. B) Diagrammatic representation, modified from the rat brain atlas of Paxinos and Watson (1997), indicating carbachol and aCSF microinjection sites into the BST. ac, anterior commissure; f, fornix; IA, interaural coordinates (mm); ic, internal capsule; LSV, lateral septal ventricle; LV, lateral ventricle; PS, parastrial nuclei; and st, stria terminalis.

Microinjection of $CoCl_2$ into the contralateral SON (n=6) did not affect either MAP (101±3 vs. 100±4 mm Hg, t=0.1, $P>0.05$) or HR (362±9 vs. 359±10 bpm, t=0.3, $P>0.05$) baseline values. However, contralateral SON pretreatment with $CoCl_2$ significantly reduced the pressor (42±5 vs. 9±2 mm Hg, t=5, $P<0.005$) and bradycardiac (−74±6 vs. −13±2 bpm, t=10, $P<0.0001$) response to carbachol microinjection into the BST (Fig. 1A). Time-course analysis indicated a significant effect of SON pretreatment with $CoCl_2$ in carbachol cardiovascular effects (ΔMAP: $F_{(1,380)}=215$, $P<0.0001$ and ΔHR: $F_{(1,380)}=141$, $P<0.0001$), a significant effect over time (ΔMAP: $F_{(37,380)}=16$, $P<0.0001$ and ΔHR: $F_{(37,380)}=8$, $P<0.0001$), and an interaction between treatment and time (ΔMAP: $F_{(37,380)}=11$, $P<0.0001$ and ΔHR: $F_{(37,380)}=3$, $P<0.0001$) (Fig. 1B). Cardiovascular responses to carbachol microinjection into the BST of animals that received $CoCl_2$ in the ipsilateral or contralateral SON were not significantly different (MAP: t=2, $P>0.05$; HR: t=1, $P>0.05$) (Fig. 1).

Representative recordings showing the cardiovascular responses to carbachol microinjection into the BST before and after ipsilateral or contralateral SON pretreatment with $CoCl_2$ is presented in Fig. 3. Moreover, photomicrography of coronal brain section showing the microinjection site in the ipsilateral and contralateral SON of representative animals are presented in Figs. 4 and 5, respectively. Diagrammatic representation showing microinjection sites of $CoCl_2$ and aCSF in the ipsilateral and contralateral SON is also shown in Figs. 4 and 5, respectively.

2.4. *Effect of paraventricular nucleus pretreatment with aCSF or $CoCl_2$ in cardiovascular responses to carbachol microinjection into the bed nucleus of the stria terminalis*

2.4.1. *Ipsilateral paraventricular nucleus*

Microinjection of aCSF into the ipsilateral PVN (n=7) did not affect either MAP (99±3 vs. 102±2 mm Hg, t=0.6, $P>0.05$) or HR (357±7 vs. 364±10 bpm, t=0.5, $P>0.05$) baseline values. Ipsilateral PVN treatment with aCSF also did not affect the pressor (43±3 vs. 40±2 mm Hg, t=0.7, $P>0.05$) and bradycardiac (−78±6 vs. −73±5 bpm, t=0.8, $P>0.05$) response following carbachol microinjection into the BST (Fig. 6A).

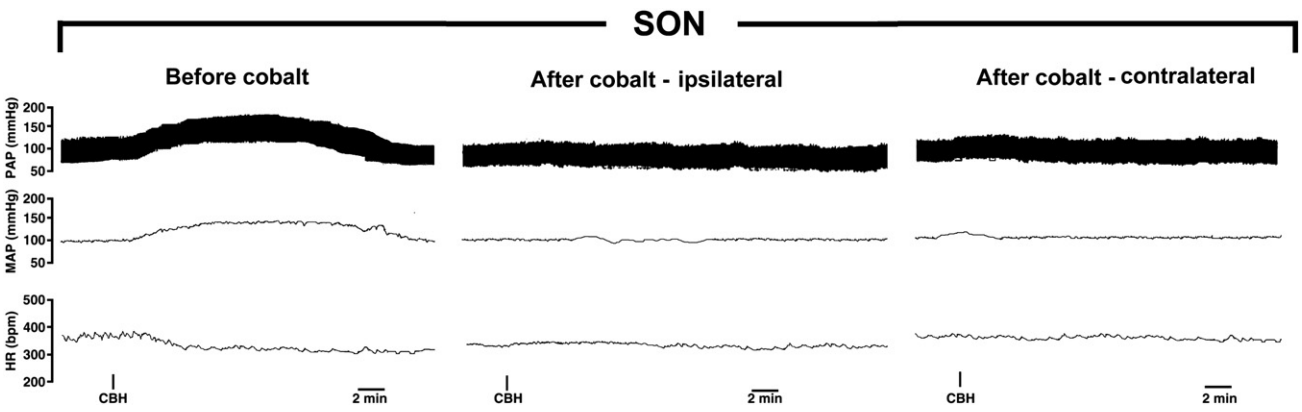

Fig. 3 – Typical recordings showing pulsatile arterial pressure (PAP), mean arterial pressure (MAP) and heart rate (HR) changes in response to carbachol (CBH; 1 nmol/100 nL) microinjection into the BST of unanesthetized rats before (before cobalt) and after (after cobalt) pretreatment of ipsilateral or contralateral SON, in relation to BST microinjection site, with $CoCl_2$. The panels represent segments of recording in different animals.

Microinjection of $CoCl_2$ into the ipsilateral PVN (n=7) did not affect either MAP (99±3 vs. 100±3 mm Hg, t=0.8, $P>0.05$) or HR (366±9 vs. 374±9 bpm, t=0.5, $P>0.05$) baseline values. Moreover, ipsilateral PVN pretreatment with $CoCl_2$ did not affect the pressor (41±3 vs. 38±2 mm Hg, t=0.9, $P>0.05$) and bradycardiac (−76±8 vs. −73±6 bpm, t=0.3, $P>0.05$) response to carbachol microinjection into the BST (Fig. 6A). Time-course analysis did not show a significant effect of PVN pretreatment with $CoCl_2$ in carbachol cardiovascular responses (ΔMAP: $F_{(1,456)}=2$, $P>0.05$; ΔHR: $F_{(1,456)}=2$, $P>0.05$), but indicated a significant effect over time on the MAP ($F_{(37,456)}=45$, $P<0.0001$) and HR ($F_{(37,456)}=18$, $P<0.0001$) (Fig. 6B).

2.4.2. *Contralateral paraventricular nucleus*

Microinjection of aCSF into the contralateral PVN (n=6) did not affect either MAP (101±3 vs. 98±2 mm Hg, t=0.5, $P>0.05$) or HR (353±11 vs. 361±7 bpm, t=0.5, $P>0.05$) baseline values. Contralateral PVN treatment with aCSF also did not affect the pressor (43±4 vs. 39±3 mm Hg, t=0.8, $P>0.05$) and bradycardiac (−76±9 vs. −68±6 bpm, t=0.8, $P>0.05$) response to carbachol microinjection into the BST (Fig. 6A).

Microinjection of $CoCl_2$ into the contralateral PVN (n=6) did not affect either MAP (99±3 vs. 104±4 mm Hg, t=1.5, $P>0.05$) or HR (359±9 vs. 372±12 bpm, t=0.9, $P>0.05$) baseline values. Moreover, contralateral PVN pretreatment with $CoCl_2$ did not affect the pressor (42±4 vs. 41±2 mm Hg, t=0.1, $P>0.05$) and bradycardiac (−70±9 vs. −65±8 bpm, t=0.5, $P>0.05$) response to carbachol microinjection into the BST (Fig. 6A). Time-course analysis did not show a significant effect of contralateral PVN pretreatment with $CoCl_2$ in carbachol cardiovascular responses (ΔMAP: $F_{(1,380)}=2$, $P>0.05$; ΔHR: $F_{(1,380)}=0.2$, $P>0.05$) (Fig. 6B), but indicated a

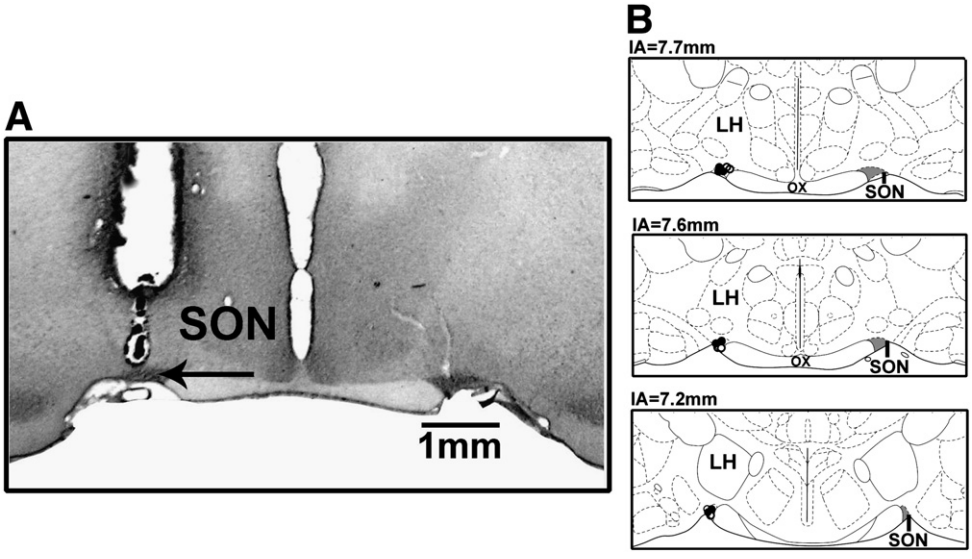

Fig. 4 – A) Photomicrograph of a coronal brain section showing a microinjection site in the ipsilateral SON, in relation to BST microinjection site, of one representative animal. B) Diagrammatic representation, modified from the rat brain atlas of Paxinos and Watson (1997), indicating aCSF (open circles) and $CoCl_2$ (filled black circles) microinjection sites in the ipsilateral SON. IA, interaural coordinates (mm); LH, lateral hypothalamus; and ox, optic chiasm.

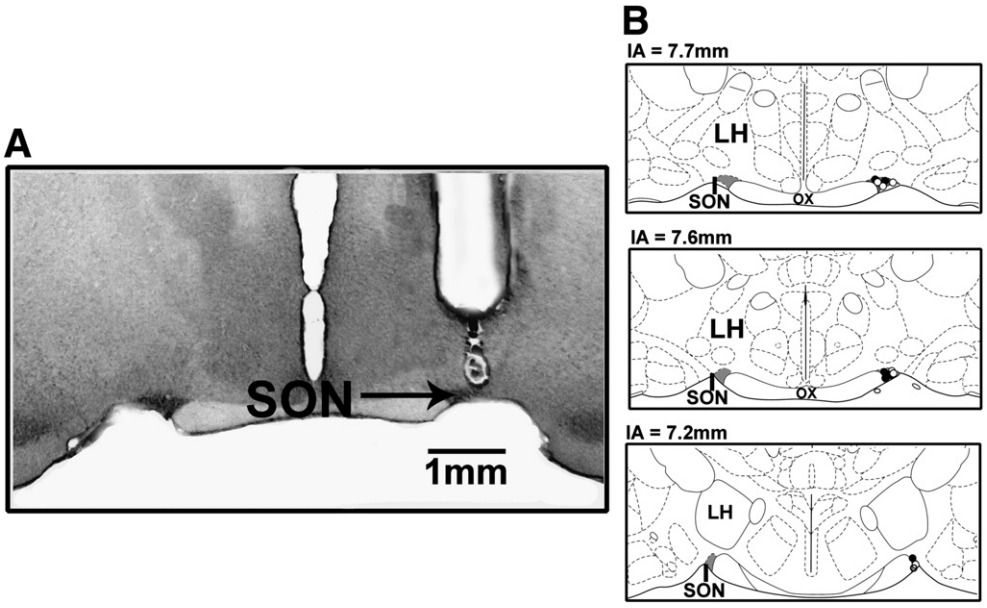

Fig. 5 – A) Photomicrograph of a coronal brain section showing a microinjection site in the contralateral SON, in relation to BST microinjection site, of one representative animal. B) Diagrammatic representation, modified from the rat brain atlas of Paxinos and Watson (1997), indicating aCSF (open circles) and $CoCl_2$ (filled black circles) microinjection sites in the contralateral SON. IA, interaural coordinates (mm); LH, lateral hypothalamus; and ox, optic chiasm.

significant effect over time on the MAP ($F_{(37,380)}=44$, $P<0.0001$) and HR ($F_{(37,380)}=11$, $P<0.0001$).

Photomicrography of coronal brain section showing the microinjection site in the ipsilateral and contralateral PVN of representative animals is presented in Figs. 7 and 8, respectively. Diagrammatic representation showing microinjection sites of $CoCl_2$ and aCSF in the ipsilateral and contralateral PVN is also shown in Figs. 7 and 8, respectively.

3. Discussion

The BST is localized in the rostral prosencephalon, and is associated with autonomic and neuroendocrine functions (Dunn, 1987; Dunn and Williams, 1995; Ulrich-Lai and Herman, 2009). Cholinergic synaptic terminals were indentified in the BST (Ruggiero et al., 1990). Moreover, binding studies described the presence of muscarinic and nicotinic receptors in the BST (Clarke et al., 1985; Wamsley et al., 1984). Electrophysiological studies reported that BST neurons showed an increase in firing rate in response to local administration of acetylcholine through activation of local muscarinic cholinergic receptors (Casada and Dafny, 1993a, 1993b). We have previously reported that microinjection of carbachol, a cholinergic agonist, into the BST of unanesthetized rats evoked a pressor response that was followed by a baroreflex-mediated bradycardia (Alves et al., 2007). These cardiovascular effects were blocked after local pretreatment with an M_2-muscarinic receptor antagonist as well as after systemic pretreatment with a V_1-vasopressinergic receptor antagonist (Alves et al., 2007), thus suggesting that activation of M_2-muscarinic receptor activation within BST evokes cardiovascular changes that are mediated by acute vasopressin release into the circulation. In the present study, we show results indicating that carbachol microinjection into the BST increases circulating vasopressin levels, thus confirming previous evidence that carbachol microinjection into the BST evokes pressor response due to vasopressin release.

Vasopressin is a potent vasoconstrictor agent (Altura and Altura, 1984; Barer, 1961). This nonapeptide is synthesized by magnocellular neurons of the PVN and SON (Swaab et al., 1975). Each neuron gives rise to a single axon into the posterior pituitary gland, where its neurosecretory endings release vasopressin (Swaab et al., 1975). Because the capillaries within the pituitary gland do not have a blood-brain barrier, vasopressin released in close proximity to the capillaries easily enters the bloodstream (Leng et al., 1999). To verify if SON and/or PVN synapses mediate the pressor response to the carbachol microinjection into the BST, we pretreated both nuclei with the nonselective neurotransmission blocker $CoCl_2$. The use of $CoCl_2$ is a common approach to investigate a possible involvement of specific brain areas in a functional neural pathway. The technique is based on the administration of circumscribed microinjections of compounds that reversibly block neuronal activity over a given period of time. The microinjection of $CoCl_2$ into discrete brain areas has been used for the functional inactivation of synapses (Crestani et al., 2006, 2009b; Giancola et al., 1993; Scopinho et al., 2008). The $CoCl_2$ reduces presynaptic Ca^{+2} influx, leading to an inhibition of neurotransmitter release and a consequent synaptic blockade (Kretz, 1984), without influence on passage fibers. The inhibition caused by this compound, when microinjected in volumes and concentrations that were similar to those presently used, was reported to spread over an area up to 1 mm^2 (Lomber, 1999).

Pretreatment of the PVN with $CoCl_2$, either injected ipsilateral or contralateral in relation to BST microinjection

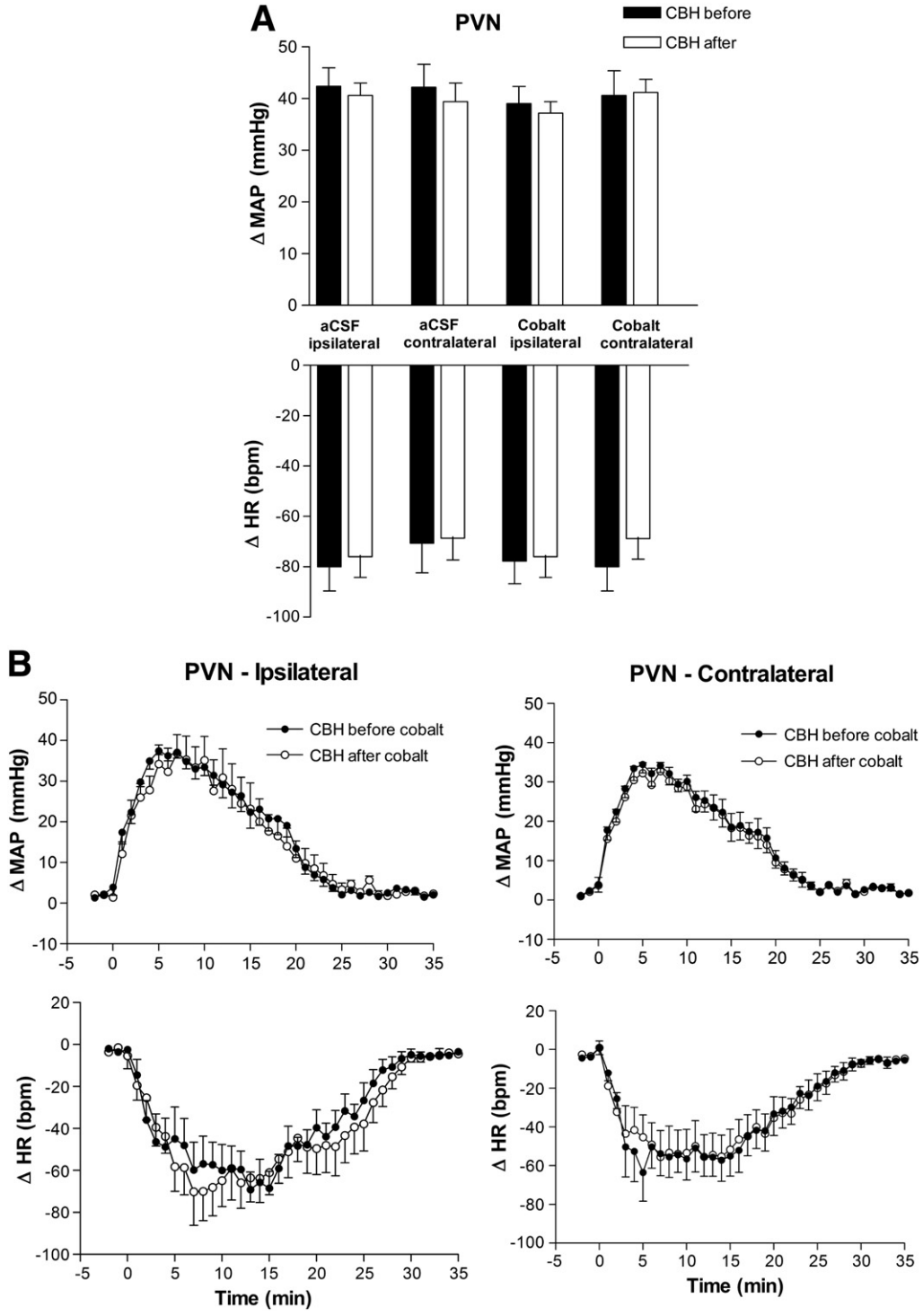

Fig. 6 – A) Changes in MAP (ΔMAP) and HR (ΔHR) in response to carbachol (CBH; 1 nmol/100 nL) microinjection into the BST before (filled columns) and after (open columns) pretreatment of the ipsilateral and contralateral PVN, in relation to BST microinjection site, with vehicle (aCSF) or $CoCl_2$ (cobalt). Columns represent the mean and bars the SEM. *P<0.05, paired *Student's* t-test. B) Time-course of the effect of CBH microinjection into the BST on MAP and HR before (filled circles) or after (open circles) pretreatment of the ipsilateral or contralateral PVN with $CoCl_2$. CBH injections were made at time 0. Circles represent the mean and bar the SEM. Data were analyzed using two-way ANOVA.

site, did not affect the cardiovascular response to the microinjection carbachol into the BST. The absence of effect was not due to an insufficient dose of $CoCl_2$, because in previous studies it has been reported that the microinjection of such dose into the PVN was effective to inhibit vasopressin-mediated pressor responses observed after the injection of

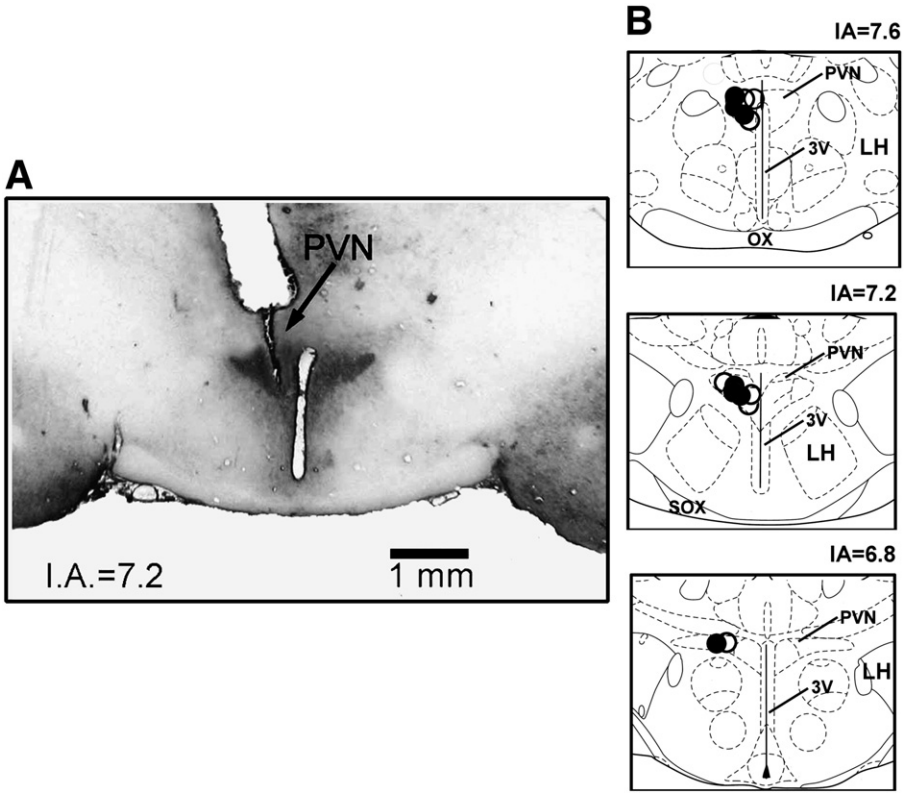

Fig. 7 – A) Photomicrograph of a coronal brain section showing a microinjection site in the ipsilateral PVN, in relation to BST microinjection site, of one representative animal. B) Diagrammatic representation modified from the rat brain atlas of Paxinos and Watson (1997), indicating aCSF (open circles) and $CoCl_2$ (filled black circles) microinjection sites in the ipsilateral PVN. 3V, third ventricle; IA, interaural coordinates (mm); LH, lateral hypothalamus; ox, optic chiasm; and sox, supraoptic decussation.

noradrenaline into either the BST or the lateral septal area (Crestani et al., 2009b; Scopinho et al., 2008). Pretreatment of either the ipsilateral or the contralateral SON with $CoCl_2$ blocked the pressor and bradycardiac responses caused by the microinjection carbachol into the BST. Together, these results suggest that synapses in the SON, but not in the PVN, mediate the cardiovascular responses to the microinjection of carbachol into the BST.

There is neuroanatomic evidence that the BST projects to the SON and that such projections are mainly ipsilateral (Dong and Swanson, 2003, 2004, 2006). The existence of bilateral neural connections between the two SON was suggested by electrophysiological and in vivo studies, thus supporting our results that both SON are involved in the mediation of the cardiovascular response to the microinjection of carbachol into the BST. Takano et al. (1990) reported that one-third of the vasopressin-containing neurons tested in the SON were excited by electric stimulation of the contralateral SON. In the same study, those authors reported that vasopressin neurons tested in the SON were not antidromically activated by a contralateral SON stimulation, thus suggesting that neural connections between the bilateral SON are mainly polysynaptic. It was also reported that antidiuretic effect associated with noradrenaline microinjection into the SON was inhibited either by a lesion of the contralateral SON or its pretreatment with adrenoceptor antagonists (Tsushima et al., 1996), indicating the existence of bilateral adrenergic neural connections between supraoptic nuclei. Because the pressor response to the microinjection of carbachol into the BST was inhibited by the blockade of either the ipsilateral or the contralateral SON, it is possible that carbachol administration into the BST activates a pathway from the BST to the ipsilateral SON, in relation to BST microinjection site, which would stimulate neuron(s) that project to contralateral SON, thus suggesting that carbachol responses would depend on a bilateral SON cross-talking. Therefore, activation of vasopressinergic neurons in the contralateral SON in relation to BST stimulation site would mediate pressor response to carbachol administration into the BST. A schematic representation sketching the mechanism by which carbachol microinjection into the BST evokes a vasopressin-mediated pressor response is presented in Fig. 9.

The pathway for the neural connection between bilateral SON is not totally understood. Moos and Richard (1989) concluded that the supraventricular gray commissura is important for interconnection of oxytocin-containing neurons in the SON, because synchronization of oxytocin-containing neurons in the bilateral SON disappeared after an inter hemisphere sectioning (including the supraventricular gray commissura and the corpus callosum), but persisted after a superficial interhemisphere sectioning that was limited to the corpus callosum. Therefore, the supraventricular gray commissura is a possible pathway for interconnections between bilateral SON vasopressin-containing neurons. Also, other

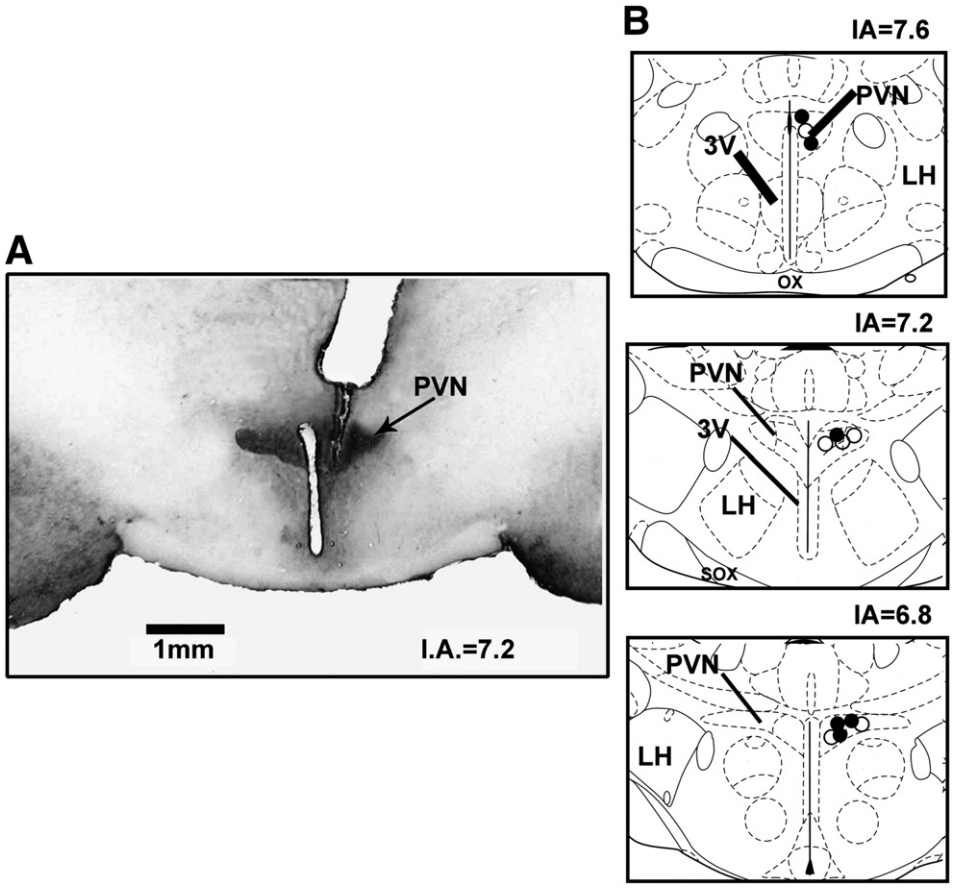

Fig. 8 – A) Photomicrograph of a coronal brain section showing a microinjection site in the contralateral PVN, in relation to BST microinjection site, of one representative animal. B) Diagrammatic representation, modified from the rat brain atlas of Paxinos and Watson (1997), indicating aCSF (open circles) and $CoCl_2$ (filled black circles) microinjection sites in the contralateral PVN. 3V, third ventricle; IA, interaural coordinates (mm); LH, lateral hypothalamus; ox, optic chiasm; and sox, supraoptic decussation.

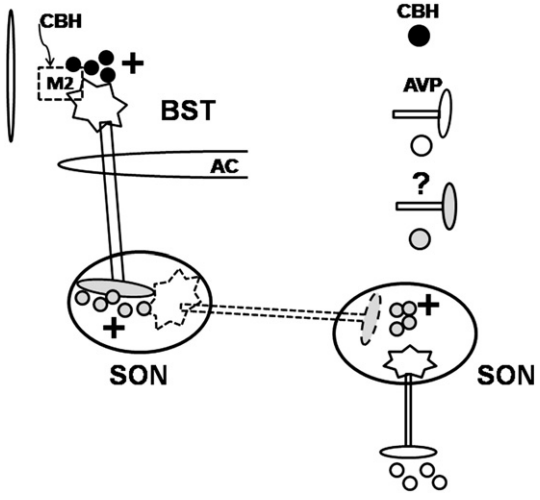

Fig. 9 – Schematic representation illustrating the proposed mechanism by which carbachol (CBH) microinjection into the BST evokes vasopressin release. ac — anterior commissure, AVP — vasopressin, CBH — carbachol, and M2 — subtype M_2 of cholinergic muscarinic receptor.

connections between bilateral supraoptic nuclei, through the medulla oblongata and pons, have been suggested to exist (Tsushima et al., 1996), thus indicating alternative pathways for a bilateral SON cross-talking.

It has been reported that the microinjection of noradrenaline into the BST also evoked pressor response that was mediated by an acute release of vasopressin into the circulation (Crestani et al., 2007). This response was blocked by PVN pretreatment with $CoCl_2$ and not affected by SON blockade (Crestani et al., 2009b), thus suggesting a major involvement of PVN magnocellular neurons without any significant involvement of neurons in the SON. The present study led to the interesting observation that BST noradrenergic and cholinergic neurotransmissions modulate vasopressin release into the circulation through different neural pathways. This modulation of the vasopressin release by BST noradrenergic and cholinergic neurotransmission through specific neural pathway may have a physiological importance, since BST neurons can stimulate vasopressin release through local cholinergic receptor despite of changes in pathway related to local noradrenergic neurotransmission, or vice versa.

There is evidence pointing to the BST as a relay in the neural circuitry connecting limbic structures that are known to modulate neuroendocrine responses, such as the medial

and central amygdala, hippocampus and medial prefrontal cortex (Choi et al., 2007; Feldman et al., 1995; Herman and Cullinan, 1997; Herman et al., 2005; Ulrich-Lai and Herman, 2009). This idea is reinforced by results showing that BST lesions inhibit amygdala and hippocampus-related neuroendocrine responses (Feldman et al., 1990; Zhu et al., 2001). Based on this, the present results suggest that BST cholinergic neurotransmission could be an important system in the neural circuitry of neuroendocrine regulation involved in the integration to vasopressin systemic release by SON.

In summary, the present results indicate that cardiovascular responses following carbachol microinjection into the BST are mediated by SON magnocellular neurons, without significant involvement of those in the PVN. The results also indicate that responses to the carbachol microinjection into the BST are mediated by a pathway involving a bilateral SON cross-talking, possibly through ipsilateral projections from the BST to the SON and activation of vasopressinergic neurons in the contralateral SON.

4. Experimental procedures

4.1. *Animals*

Sixty-four male Wistar rats weighing 230–270 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 kept under a 12 h:12 h light–dark cycle (lights on between 06:00 am and 6:00 pm) and had free access to water and standard laboratory food. Housing conditions and experimental procedures were approved by the University of São Paulo Animal Ethical Committee, which complies with the Guiding Principles of Research Involving Animals and Human Beings of the American Physiological Society.

4.2. *Surgical preparation*

Four days before the experiment rats were anesthetized with tribromoethanol (250 mg/kg, i.p.). After scalp anesthesia with 2% lidocaine, the skull was exposed and stainless steel guide cannulae (26 G) were implanted in the BST and in the SON or PVN, using a stereotaxic apparatus (Stoelting, Wood Dale, Illinois, USA). Cannulae were positioned 1 mm above injection sites. Stereotaxic coordinates for cannula implantation in the BST, PVN or SON were selected according to the rat brain atlas of Paxinos and Watson (1997). Cannula was implanted unilaterally in the BST and stereotaxic coordinates were: anteroposterior: +8.6 mm from the interaural, lateral: 4.0 mm from the medial suture, ventral: −5.8 mm from the skull, with a lateral inclination of 23°. Cannulae were implanted in the ipsilateral or contralateral PVN, in relation to BST cannula, and stereotaxic coordinates were: anteroposterior: +7.2 mm from the interaural, lateral: 2 mm from the medial suture, ventral: −6.9 mm from the skull, with a lateral inclination of 12°. Cannulae were implanted in the ipsilateral or contralateral SON, in relation to BST cannula, and stereotaxic coordinates were: anteroposterior: +6.9 mm from the interaural, lateral: 1.8 mm from the medial suture, ventral: −8.1 mm from the skull. Cannulae were fixed to the skull with dental cement and one metal screw. After surgery, the animals received a poly-antibiotic veterinarian preparation of streptomycins and penicillins (i.m., 0.27 mg/kg, Pentabiotico®; Fort Dodge, Campinas, SP, Brazil), to prevent infection, and the nonsteroidal anti-inflammatory flunixine meglumine (i.m., 0.025 mg/kg, Banamine®; Schering Plough, Cotia, SP, Brazil), for post-operative analgesia.

One day before the experiment, animals were anesthetized with tribromoethanol (250 mg/kg, i.p.) and a catheter was inserted into the abdominal aorta through the femoral artery for arterial pressure and HR recording. Catheters consisted of a 4 cm piece of PE-10 heat-bound to a 13 cm piece of PE-50 (Clay Adams, Parsippany, NJ, USA). The catheters were tunneled under the skin and exteriorized on the animal's dorsum. After surgery, animals were kept in individual cages, which were later used for transport to the experimental room. The nonsteroidal anti-inflammatory flunixine meglumine (i.m., 0.025 mg/kg, Banamine®; Schering Plough, Cotia, SP, Brazil) was administered for postoperative analgesia.

4.3. *Measurement of cardiovascular responses*

On the day of the experiment, the arterial cannulas were connected to a pressure transducer. The pulsatile arterial pressure (PAP) of freely moving animals was recorded using an HP-7754A amplifier (Hewlett Packard, Palo Alto, CA, USA) and an acquisition board (MP100A; Biopac Systems Inc., Goleta, CA, USA) connected to a computer. Mean arterial pressure (MAP) and HR values were derived from PAP recordings and processed on-line.

4.4. *Drug microinjection in the central nervous system*

The needles (33 G; Small Parts, Miami Lakes, FL, USA) used for microinjection into the BST, SON and PVN were 1 mm longer than the guide cannulas and were connected to a 2 μL syringe (7002 KH; Hamilton, Reno, NV, USA) through PE-10 tubing. The needle was carefully introduced into the guide cannula without touching or restraining the animal and drugs were injected in a final volume of 100 nL. After a 20 s period, the needle was removed.

4.5. *Radioimmunoassay*

Blood samples were collected in chilled plastic tubes containing heparin (10 μL of heparin per mL of collected blood). Samples were centrifuged at 4 °C, 3000 rpm for 20 min. Plasma vasopressin levels were measured by specific radioimmunoassay after previous extraction from plasma using acetone and petroleum ether (Glick and Kagan, 1979; Elias et al., 1997). The recovery rates were greater than 87%. The assay sensitivity and intra- and inter-assay coefficients of variation were 0.9 pg/mL, 4.6 and 18.6%, respectively. All samples from a single experiment were assayed in duplicate in the same assay.

4.6. *Drugs*

Carbachol (Sigma, St Louis, MO, USA) and $CoCl_2$ (Sigma) were dissolved in artificial cerebrospinal fluid (aCSF), with the following composition: 100 mM NaCl, 2 mM Na_3PO_4, 2.5 mM

KCl, 1.0 mM $MgCl_2$, 27 mM $NaHCO_3$ and 2.5 mM $CaCl_2$ (pH 7.4). Tribomoethanol (Sigma) and urethane (Sigma) were dissolved in saline (0.9% NaCl). Flunixine meglumine (Banamine®, Schering Plough, Brazil) and poly-antibiotic preparation of streptomycins and penicillins (Pentabiotico®, Fort Dodge, Brazil) were used as provided.

4.7. *Experimental protocols*

On the day of the experiment, animals were transported to the experimental room and were allowed 60 min period to adapt to the experimental room conditions, such as sound and illumination, before starting arterial pressure and HR recording. The experimental room was acoustically isolated and a constant background noise was generated by an air exhauster to minimize sound interference within the experimental room.

4.7.1. *Effect of aCSF or carbachol microinjection into the bed nucleus of the stria terminalis of unanesthetized rats in plasma vasopressin levels*

This experiment aimed to study the effect of carbachol microinjection into the BST of unanesthetized rats on plasma vasopressin levels. For this, two different groups of animals received vehicle (aCSF, 100 nL, n=6) or carbachol (1 nmol/100 nL, n=6) injected into the BST (Alves et al., 2007). Five minutes after BST treatment, animals were decapitated and blood samples were collected to determine of plasma vasopressin levels.

4.7.2. *Effect of supraoptic nucleus pretreatment with aCSF or $CoCl_2$ on cardiovascular responses to carbachol microinjection into the bed nucleus of the stria terminalis*

These experiments aimed to study the involvement of the SON in cardiovascular responses to carbachol microinjection into the BST of unanesthetized rats. For this, animals were divided into two groups, ipsilateral and contralateral SON groups. In the ipsilateral SON group, rats had cannulas implanted unilaterally in the BST and in the ipsilateral SON, in relation to BST cannula, and were subdivided into vehicle (aCSF, 100 nL, n=7) and $CoCl_2$ (1 mM/100 nL, n=7) groups (Busnardo et al., 2007; Crestani et al., 2009a, 2009b; Scopinho et al., 2008). In the contralateral SON group, rats had cannulas implanted unilaterally in the BST and in the contralateral SON and were subdivided into vehicle (aCSF, 100 nL, n=6) and $CoCl_2$ (1 mM/100 nL, n=6) groups (Alves et al., 2007; Busnardo et al., 2007; Crestani et al., 2009a, 2009b). Carbachol (1 nmol/100 nL) was microinjected into the BST on the first day and again 24 h later, at 10 min after aCSF or $CoCl_2$ microinjection into the SON (Alves et al., 2007). Different set of animals received aCSF or $CoCl_2$ into the SON in either ipsilateral or contralateral SON groups.

4.7.3. *Effect of paraventricular nucleus pretreatment with aCSF or $CoCl_2$ in cardiovascular responses to carbachol microinjection into the bed nucleus of the stria terminalis*

These experiments aimed to study the involvement of the PVN in cardiovascular responses following carbachol microinjection into the BST of unanesthetized rats. For this, animals were also divided in two groups, ipsilateral and contralateral PVN groups. In the ipsilateral PVN group, rats had cannulas implanted unilaterally in the BST and in the ipsilateral PVN, in relation to BST cannula, and were subdivided in vehicle (aCSF, 100 nL, n=7) and $CoCl_2$ (1 mM/100 nL, n=7) groups (Alves et al., 2007; Crestani et al., 2009a, 2009b; Scopinho et al., 2008). In the contralateral PVN group, rats had cannulas implanted unilaterally in the BST and in the contralateral PVN and were further subdivided in vehicle (aCSF, 100 nL, n=6) and $CoCl_2$ (1 mM/100 nL, n=6) group (Alves et al., 2007; Crestani et al., 2009a, 2009b; Scopinho et al., 2008). Carbachol (1 nmol/100 nL) was microinjected into the BST on the first day and again 24 h later, at 10 min after aCSF or $CoCl_2$ microinjection into the PVN (Alves et al., 2007). Different set of animals received aCSF or $CoCl_2$ into the PVN in either ipsilateral PVN or contralateral PVN groups.

4.8. *Histological determination of microinjection sites*

At the end of the experiment, animals were anesthetized with urethane (1.25 g/kg i.p.) and 100 nL of 1% Evan's Blue dye was injected into the BST, SON and PVN as an injection site marker. Animals were submitted to intracardiac perfusion with saline (0.9% NaCl) followed by 10% formalin. Brains were removed and 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 1% neutral red for light microscopy analysis. The actual placement of the microinjection needles was determined according to the rat brain atlas of Paxinos and Watson (1997).

4.9. *Statistical analysis*

Data are presented as mean ± SEM. The maximum MAP and HR responses to carbachol microinjection into the BST, MAP and HR basal values and the effect of BST treatment with aCSF or carbachol in plasma vasopressin levels were compared using paired *Student's t*-test. Time-course curves of the MAP and HR changes caused by carbachol microinjection into the BST before and after SON or PVN pharmacological manipulation were compared using two-way ANOVA for repeated measurements (treatment vs. time) with repeated measures on the second factor. Significance was set at $P<0.05$.

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REFERENCES

Altura, B.M., Altura, B.T., 1984. Actions of vasopressin, oxytocin, and synthetic analogs on vascular smooth muscle. Fed. Proc. 43, 80–86.

Alves, F.H., Crestani, C.C., Resstel, L.B., Correa, F.M., 2007. Cardiovascular effects of carbachol microinjected into the bed nucleus of the stria terminalis of the rat brain. Brain Res. 1143, 161–168.

Alves, F.H., Crestani, C.C., Resstel, L.B., Correa, F.M., 2009. Bed nucleus of the stria terminalis N-methyl-D-aspartate receptors and nitric oxide modulate the baroreflex cardiac component in unanesthetized rats. J. Neurosci. Res. 87, 1703–1711.

Barer, G.R., 1961. A comparison of the circulatory effects of angiotensin, vasopressin and adrenaline in the anaesthetized cat. J. Physiol. 156, 49–66.

Busnardo, C., Tavares, R.F., Antunes-Rodrigues, J., Correa, F.M., 2007. Cardiovascular effects of L-glutamate microinjection in the supraoptic nucleus of unanaesthetized rats. Neuropharmacology 52, 1378–1384.

Casada, J.H., Dafny, N., 1993a. Responses of neurons in bed nucleus of the stria terminalis to microiontophoretically applied morphine, norepinephrine and acetylcholine. Neuropharmacology 32, 279–284.

Casada, J.H., Dafny, N., 1993b. Muscarinic receptors mediate the effect of acetylcholine (ACh) on neurons of the bed nucleus of the stria terminalis (BNST). Brain Res. 631, 124–128.

Choi, D.C., Furay, A.R., Evanson, N.K., Ostrander, M.M., Ulrich-Lai, Y.M., Herman, J.P., 2007. Bed nucleus of the stria terminalis subregions differentially regulate hypothalamic–pituitary–adrenal axis activity: implications for the integration of limbic inputs. J. Neurosci. 27, 2025–2034.

Ciriello, J., Janssen, S.A., 1993. Effect of glutamate stimulation of bed nucleus of the stria terminalis on arterial pressure and heart rate. Am. J. Physiol. 265, H1516–H1522.

Clarke, P.B., Schwartz, R.D., Paul, S.M., Pert, C.B., Pert, A., 1985. Nicotinic binding in rat brain: autoradiographic comparison of [3H]acetylcholine, [3H]nicotine, and [125I]-alpha-bungarotoxin. J. Neurosci. 5, 1307–1315.

Crestani, C.C., Alves, F.H., Resstel, L.B., Correa, F.M., 2006. The bed nucleus of the stria terminalis modulates baroreflex in rats. Neuroreport 17, 1531–1535.

Crestani, C.C., Alves, F.H., Resstel, L.B., Correa, F.M., 2007. Cardiovascular effects of noradrenaline microinjection in the bed nucleus of the stria terminalis of the rat brain. J. Neurosci. Res. 85, 1592–1599.

Crestani, C.C., Alves, F.H., Resstel, L.B., Correa, F.M., 2008a. Both alpha1 and alpha2-adrenoceptors mediate the cardiovascular responses to noradrenaline microinjected into the bed nucleus of the stria terminal of rats. Br. J. Pharmacol. 153, 583–590.

Crestani, C.C., Alves, F.H., Resstel, L.B., Correa, F.M., 2008b. Bed nucleus of the stria terminalis alpha(1)-adrenoceptor modulates baroreflex cardiac component in unanesthetized rats. Brain Res. 1245, 108–115.

Crestani, C.C., Alves, F.H., Tavares, R.F., Correa, F.M., 2009a. Role of the bed nucleus of the stria terminalis in the cardiovascular responses to acute restraint stress in rats. Stress 12, 268–278.

Crestani, C.C., Busnardo, C., Tavares, R.F., Alves, F.H., Correa, F. M., 2009b. Involvement of hypothalamic paraventricular nucleus non-N-methyl-D-aspartate receptors in the pressor response to noradrenaline microinjected into the bed nucleus of the stria terminalis of unanesthetized rats. Eur. J. Neurosci. 29, 2166–2176.

Dong, H.W., Swanson, L.W., 2003. Projections from the rhomboid nucleus of the bed nuclei of the stria terminalis: implications for cerebral hemisphere regulation of ingestive behaviors. J. Comp. Neurol. 463, 434–472.

Dong, H.W., Swanson, L.W., 2004. Organization of axonal projections from the anterolateral area of the bed nuclei of the stria terminalis. J. Comp. Neurol. 468, 277–298.

Dong, H.W., Swanson, L.W., 2006. Projections from bed nuclei of the stria terminalis, dorsomedial nucleus: implications for cerebral hemisphere integration of neuroendocrine, autonomic, and drinking responses. J. Comp. Neurol. 494, 75–107.

Dunn, J.D., 1987. Plasma corticosterone responses to electrical stimulation of the bed nucleus of the stria terminalis. Brain Res. 407, 327–331.

Dunn, J.D., Williams, T.J., 1995. Cardiovascular responses to electrical stimulation of the bed nucleus of the stria terminalis. J. Comp. Neurol. 352, 227–234.

Elias, L.L.K., Antunes-Rodrigues, J., Elias, P.C.L., Moreira, A.C., 1997. Effect of plasma osmolality on pituitary-adrenal responses to corticotropin-releasing hormone and atrial natriuretic peptide changes in central diabetes insipidus. J. Clin. Endocrinol. Metab. 82 (4), 1243–1247.

Feldman, S., Conforti, N., Saphier, D., 1990. The preoptic area and bed nucleus of the stria terminalis are involved in the effects of the amygdala on adrenocortical secretion. Neuroscience 37, 775–779.

Feldman, S., Conforti, N., Weidenfeld, J., 1995. Limbic pathways and hypothalamic neurotransmitters mediating adrenocortical responses to neural stimuli. Neurosci. Biobehav. Rev. 19, 235–240.

Gelsema, A.J., McKitrick, D.J., Calaresu, F.R., 1987. Cardiovascular responses to chemical and electrical stimulation of amygdala in rats. Am. J. Physiol. 253, R712–R718.

Gelsema, A.J., Copeland, N.E., Drolet, G., Bachelard, H., 1993. Cardiovascular effects of neuronal activation of the extended amygdala in rats. Brain Res. 626, 156–166.

Giancola, S.B., Roder, S., Ciriello, J., 1993. Contribution of caudal ventrolateral medulla to the cardiovascular responses elicited by activation of bed nucleus of the stria terminalis. Brain Res. 606, 162–166.

Glick, S.M., Kagan, A., 1979. Vasopressin. In: Jaffe, B.M., Behrman, H.R. (Eds.), Methods of Hormone Radioimunoassay. Academic Press, New York.

Hatam, M., Nasimi, A., 2007. Glutamatergic systems in the bed nucleus of the stria terminalis, effects on cardiovascular system. Exp. Brain Res. 178, 394–401.

Herman, J.P., Cullinan, W.E., 1997. Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. Trends Neurosci. 20, 78–84.

Herman, J.P., Ostrander, M.M., Mueller, N.K., Figueiredo, H., 2005. Limbic system mechanisms of stress regulation: hypothalamo-pituitary–adrenocortical axis. Prog. Neuropsychopharmacol. Biol. Psychiatry 29, 1201–1213.

Kretz, R., 1984. Local cobalt injection: a method to discriminate presynaptic axonal from postsynaptic neuronal activity. J. Neurosci. Methods 11, 129–135.

Leng, G., Brown, C.H., Russell, J.A., 1999. Physiological pathways regulating the activity of magnocellular neurosecretory cells. Prog. Neurobiol. 57, 625–655.

Li, Y.W., Dampney, R.A., 1994. Expression of Fos-like protein in brain following sustained hypertension and hypotension in conscious rabbits. Neuroscience 61, 613–634.

Lomber, S.G., 1999. The advantages and limitations of permanent or reversible deactivation techniques in the assessment of neural function. J. Neurosci. Methods 86, 109–117.

McKitrick, D.J., Krukoff, T.L., Calaresu, F.R., 1992. Expression of c-fos protein in rat brain after electrical stimulation of the aortic depressor nerve. Brain Res. 599, 215–222.

Moos, F., Richard, P., 1989. Paraventricular and supraoptic oxytocin cells in rat are locally regulated by oxytocin and functionally related. J. Physiol. (Lond) 408, 1–18.

Paxinos, G., Watson, C., 1997. The Rat Brain in Steretaxic Coordinates, third edition. Academic Press, Sydney.

Ruggiero, D.A., Giuliano, R., Anwar, M., Stornetta, R., Reis, D.J., 1990. Anatomical substrates of cholinergic–autonomic regulation in the rat. J. Comp. Neurol. 292, 1–53.

Scopinho, A.A., Tavares, R.F., Busnardo, C., Correa, F.M., 2008. Non-N-methyl-D-aspartate glutamate receptors in the

paraventricular nucleus of hypothalamus mediate the pressor response evoked by noradrenaline microinjected into the lateral septal area in rats. J. Neurosci. Res. 86, 3203–3211.

Swaab, D.F., Nijveldt, F., Pool, C.W., 1975. Distribution of oxytocin and vasopressin in the rat supraoptic and paraventricular nucleus. J. Endocrinol. 67, 461–462.

Takano, S., Negoro, H., Honda, K., Higuchi, T., 1990. Electrophysio logical evidence for neural connections between the supraoptic nuclei. Neurosci Lett 111, 122–126.

Tsushima, H., Mori, M., Matsuda, T., 1996. Adrenergic neural connections between the bilateral supraoptic nuclei of the rat hypothalamus. Jpn. J. Pharmacol. 71, 73–79.

Ulrich-Lai, Y.M., Herman, J.P., 2009. Neural regulation of endocrine and autonomic stress responses. Nat. Rev. Neurosci. 10, 397–409.

Wamsley, J.K., Zarbin, M.A., Kuhar, M.J., 1984. Distribution of muscarinic cholinergic high and low affinity agonist binding sites: a light microscopic autoradiographic study. Brain Res. Bull. 12, 233–243.

Zhu, W., Umegaki, H., Suzuki, Y., Miura, H., Iguchi, A., 2001. Involvement of the bed nucleus of the stria terminalis in hippocampal cholinergic system-mediated activation of the hypothalamo-pituitary–adrenocortical axis in rats. Brain Res. 916, 101–106.