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Control of cardiovascular responses to stress by CRF in the bed nucleus of stria terminalis is mediated by local NMDA/nNOS/sGC/PKG signaling

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ABSTRACT

The aims of the present study were to assess an interaction of corticotropin-releasing factor (CRF) neurotransmission within the bed nucleus of the stria terminalis (BNST) with local nitrergic signaling, as well as to investigate an involvement of activation of local NMDA glutamate receptor and nitric oxide (NO) signaling in control of cardiovascular responses to acute restraint stress by BNST CRF neurotransmission in rats. We observed that CRF microinjection into the BNST increased local NO release during restraint stress. Furthermore, bilateral microinjection of CRF into the BNST enhanced both the arterial pressure and heart rate increases evoked by restraint stress, but without affecting the sympathetically-mediated cutaneous vasoconstriction. The facilitation of both pressor and tachycardiac responses to restraint stress evoked by BNST treatment with CRF were completely inhibited by local pretreatment with either the selective NMDA glutamate receptor antagonist LY235959, the selective neuronal nitric oxide synthase (nNOS) inhibitor *N*ω-Propyl-L-arginine (NPLA), the soluble guanylate cyclase (sGC) inhibitor 1H-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) or the protein kinase G (PKG) inhibitor KT5823. Taken together, these results provide evidence that BNST CRF neurotransmission facilitates local NMDA-mediated glutamatergic neurotransmission and activates nitrergic signaling, and this pathway is involved in control of cardiovascular responses to stress.

1. Introduction

A coordinated and complex set of physiological changes, including cardiovascular and endocrine changes, occurs during aversive stimuli for maintenance of homeostasis (Crestani, 2016; Sterling, 2012; Ulrich-Lai and Herman, 2009). These responses are triggered by overlapping limbic circuits in the central nervous system (Dampney, 2015; Myers, 2017; Ulrich-Lai and Herman, 2009). The bed nucleus of the stria terminalis (BNST) is a limbic structure located in the prosencephalon in which has been recognized as a critical component of neural substrates of responses to aversive stimuli (Crestani et al., 2013; Myers, 2017; Ulrich-Lai and Herman, 2009). Indeed, the BNST is implicated in cardiovascular, neuroendocrine and behavioral responses to stress (Crestani et al., 2013; Davis et al., 2010). However, the local neurochemical mechanisms mediating the BNST control of stress responses are not completely understood.

The corticotropin-releasing factor (CRF) system has emerged as a crucial local neurochemical mechanism involved in BNST-mediated control of both behavioral and physiological responses evoked by aversive threats (Crestani et al., 2013; Daniel and Rainnie, 2016; Davis et al., 2010). Specifically regarding the cardiovascular responses, Nijsen et al. (2001) first reported that BNST treatment with a nonselective CRF receptor antagonist enhanced the heart rate (HR) increase evoked by contextual fear conditioning, thus indicating an inhibitory influence of BNST CRF neurotransmission on cardiac responses to conditioned stressors. Further, results from our group evidenced that microinjection of selective antagonists of either CRF_1 or CRF_2 receptor into the BNST dose-dependently decreased the arterial pressure, HR and the sympathetically-mediated cutaneous vasoconstriction evoked by restraint stress (Oliveira et al., 2015). These results provided evidence of a prominent role of CRF neurotransmission in BNST control of cardiovascular responses to aversive stimuli.

Interaction between CRF and glutamatergic neurotransmissions within the BNST has been described (Silberman and Winder, 2013). This interaction was evidenced by demonstration that CRF application onto BNST *in vitro* increased frequency, but not amplitude, of spontaneous excitatory postsynaptic currents (sEPSC) (Kash et al., 2008; Silberman et al., 2013). Pretreatment with a selective CRF_1 receptor antagonist, but not with a selective CRF_2 receptor antagonist, inhibited the ability of CRF to increase frequency of sEPSC (Kash et al., 2008).

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Taken together, these results indicated that CRF acts presynaptically via CRF_1 receptor to increase glutamatergic neurotransmission within the BNST (Silberman and Winder, 2013).

Activation of *N*-methyl-D-aspartate (NMDA) glutamate receptor in post-synaptic neurons increases Ca^{2+} influx, which in turn activates the neuronal isoform of the enzyme nitric oxide synthase (nNOS), thus resulting in nitric oxide (NO) synthesis (Garthwaite, 2008; Prast and Philippu, 2001). Despite reports of presence of neurons capable of synthesizing NO within the BNST (Guimaraes et al., 2005; Vincent and Kimura, 1992), as well as identification of CRF action facilitating glutamatergic neurotransmission in this structure (Silberman and Winder, 2013), an interaction between CRF and nitrergic neurotransmission has never been reported. Furthermore, a possible role of CRF-glutamate interaction in mediating BNST control of responses during aversive threats has never been evaluated.

Neurons synthesizing NO within the BNST are activated by aversive stimuli (Guimaraes et al., 2005), and systemic treatment with nNOS inhibitors decreases stress-evoked activation of BNST neurons (Silva et al., 2012), thus indicating a recruitment of BNST nitrergic neurotransmission during aversive threats. Furthermore, a recent study from our group reported that BNST treatment with the selective NMDA glutamate receptor LY235959 decreased the HR increase to restraint stress (Adami et al., 2017), an effect similar to that observed following BNST treatment with a CRF_1 receptor antagonist (Oliveira et al., 2015). Based on these pieces of evidence, this study aimed to evaluate the hypothesis that the facilitatory influence of BNST CRF neurotransmission in cardiovascular responses evoked by an acute session of restraint stress in rats is mediated by activation of local NMDA glutamate receptor and nNOS, as well as of signaling mechanisms related to NO effects such as soluble guanylate cyclase (sGC) and protein kinase G (PKG) (Garthwaite, 2008; Hofmann et al., 2006).

2. Materials and methods

2.1. Animals

Ninety-seven male Wistar rats weighting about 250 g (60-days-old) were used. Thirteen of these animals were excluded of the study because the microinjection sites reached structures surrounding the BNST. All animals were supplied by the breeding facility of the UNESP (Botucatu, SP, Brazil), and were housed in collective plastic cages (4 animals/cage) in a temperature-controlled room at 24 °C in the Animal Facility of the Laboratory of Pharmacology/School of Pharmaceutical Sciences-UNESP. They were kept under a 12:12 h light-dark cycle (lights on between 7:00 a.m. and 7:00 p.m.) with free access to water and standard rat chow. Housing conditions and experimental procedures were approved by local Ethical Committee for Use of Animals (School of Pharmaceutical Science/UNESP) (approval # 34/2015), which complies with Brazilian and international guidelines for animal use and welfare.

2.2. Surgical preparation

At least one week after the arrival of the animals in the laboratory, and five days before the experiment, the animals were anesthetized with tribromoethanol (250 mg/kg, i.p.), scalp was anesthetized with 2% lidocaine, and the skull was exposed. Then, using a stereotaxic apparatus (Stoelting, Wood Dale, Illinois, USA), stainless-steel cannulas (26G, 12 mm-long) were bilaterally implanted into the BNST. Stereotaxic coordinates were: antero-posterior = +7.8 mm from interaural; lateral = +4.0 mm from the medial suture, ventral = −5.8 mm from the skull, with a lateral inclination of 23° (Paxinos and Watson, 1997). Dental cement was used to fix cannulas to the skull. After surgery, the rats were treated with a poly-antibiotic containing streptomycins and penicillins to prevent infection (560 mg/ml/kg, i.m.), and the non-steroidal anti-inflammatory flunixin meglumine for post-operation analgesia (0.5 mg/ml/kg, s.c.).

One day before the trial, rats were again anesthetized with tribromoethanol (250 mg/kg, i.p.) and a polyethylene cannula (a 4 cm segment of PE-10 bound to a 13 cm segment of PE-50) (Clay Adams, Parsippany, NJ, USA) was implanted into the abdominal aorta via the femoral artery for cardiovascular recording. The catheter was tunneled under the skin and exteriorized on the animal's dorsum. After surgery, the non-steroidal anti-inflammatory flunixin meglumine was administered for post-operation analgesia (0.5 mg/ml/kg, s.c.). The animals were kept in individual cages during the post-operative period and cardiovascular recording.

2.3. Blood pressure and heart rate recording

The cannula inserted into the femoral artery was connected to a pressure transducer (DPT100, Utah Medical Products Inc., Midvale, UT, USA). Pulsatile arterial pressure (PAP) was recorded using an amplifier (Bridge Amp, ML224, ADInstruments, Australia) and an acquisition board (PowerLab 4/30, ML866/P, ADInstruments, NSW, Australia) connected to a personal computer. Mean arterial pressure (MAP) and HR values were obtained from the PAP recording.

2.4. Tail skin temperature measurement

The recording of the tail skin temperature was made using an infrared digital thermographic camera (IRI4010, InfraRed Integrates Systems Ltd, Northampton, UK). The analysis was performed using a software for thermographic analysis, and temperature was represented by color intensity variations (Busnardo et al., 2013; Vianna and Carrive, 2005). For image analysis, the temperature was measured at five points along the animal's tail, and the mean was calculated for each recording (Busnardo et al., 2013; Oliveira et al., 2015).

2.5. Drug microinjection into the BNST

The needles (33G, Small Parts, Miami Lakes, FL, USA) used for microinjection into the BNST were 1 mm longer than the guide cannulas and were connected to a 2 μL syringe (7002-KH, Hamilton Co., Reno, NV, USA) via a PE-10 tubing (Clay Adams, Parsippany, NJ, USA). Intra-cerebral microinjections were performed within a 5 s period, and the needle was left in the guide cannula for 1 min after the microinjection before being removed. Microinjection was performed without restraining the animals, and drugs were administrated in a final volume of 100 nL per side (Crestani et al., 2009; Oliveira et al., 2015). Photomicrograph of a coronal brain section depicting bilateral microinjection sites in the BNST of a representative animal is presented in Fig. S1.

2.6. Restraint stress

The acute restraint stress consisted of introducing the animals into plastic cylindrical tubes (diameter = 6.5 cm, length = 15 cm), which were ventilated by ½ inch holes that comprised approximately 20% of the tube. The animals were maintained for a period of 30 min into the restraint tube (Crestani et al., 2010; Oliveira et al., 2015). Each animal was submitted to only one session of stress in order to avoid habituation.

2.7. Drugs and solutions

Corticotropin-releasing factor (CRF) (TOCRIS, Westwoods Business, Park Ellisville, MO, USA; cat. # 1151), LY235959 (selective NMDA glutamate receptor antagonist) (TOCRIS; cat. # 1019), N^{ω}-Propyl-L-arginine hydrochloride (NPLA) (selective nNOS inhibitor) (TOCRIS; cat. # 1200), tribromoethanol (Sigma-Aldrich, St Louis, Missouri, USA; cat. # T48402) and urethane (Sigma-Aldrich; cat. # U2500) were dissolved in saline (NaCl 0.9%). 1H-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one

(ODQ) (inhibitor of sGC) (TOCRIS, cat. # 0880) and KT5823 (inhibitor of PKG) (TOCRIS, cat. # 1289) were dissolved in a solution of saline containing 20% of DMSO (DMSO 20%). Flunixin meglumine (Banamine, Schering Plough, Cotia, SP, Brazil) and the polyantibiotic preparation of streptomycins and penicillins (Pentabiotico, Fort Dodge, Campinas, SP, Brazil) were used as provided.

2.8. Measurement of nitrogen oxides

The animals were decapitated, and then their brains were quickly removed and frozen in isopentane on dry ice. Following this procedure, the brain was stored at −80 °C until dissection of BNST. For microdissection of BNST, frozen brains were serially sliced at 50 µm in the coronal plane until the BNST region in a cryostat (CM1900, Leica, Wetzlar, Germany) kept at −20 °C. Microdissection of the BNST was performed from the brain fixed in the cryostat using a blunt 14-gauge needle. The coordinate for dissection was approximately from +0.20 mm to −0.80 mm related to bregma, according to Paxinos and Watson (1997). Photomicrograph of a coronal brain section depicting a representative tissue punch of the BNST is presented in Fig. S2.

The quantification of NO_2 and NO_3 (NOx) (spontaneous oxidation products) were utilized as an indirect measurement of NO production (Camargo et al., 2013). For this, the samples were homogenized into lysis buffer (Tris-HCl 20 mM pH 7.6; NaCl 137 mM, 10% glycerol) containing proteases and phosphatases inhibitors. The NOx levels were measured from homogenized samples accordingly to methodology previously described (Camargo et al., 2013). Briefly, for conversion of NO_3 into NO_2, homogenized samples were incubated overnight at 37 °C with solution containing nitrate reductase (#N7265, Sigma-Aldrich) and NADPH (#33461, Sigma-Aldrich) (0.4U/ml and 1 mg/ml, respectably, diluted in KH_2PO_4 buffer 1 M pH 7.4). Then, NO_2 level was determined by adding 15 µL of Griess reagent (1% sulphanilamide with 5% phosphoric acid in distilled water + 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in distilled water, v/v). After 10 min of incubation at room temperature, absorbance was read at 540 nm, and the results were calculated based on a standard curve of sodium nitrite ($NaNO_2$) (Sigma-Aldrich, St. Louis, MO, USA). The total content of protein in each sample was measured using a Bio-Rad® kit (DC® protein assay). Measurements of nitrite were performed in duplicate, and results were expressed as µM NOx/µg protein.

2.9. Experimental protocols

Rats were brought to the experimental room in their own cage. Animals were allowed at least 60 min to adapt to experimental room conditions, such as sound and illumination, before starting the experiment. The experimental room was temperature controlled (24 °C) and acoustically isolated from other rooms.

2.9.1. Effect of CRF microinjection into the BNST on local NOx levels during acute restraint stress

This protocol aimed to investigate whether CRF activates NMDA/nNOS pathway within BNST during acute restraint stress, thus resulting in increased formation of local NO. For this, animals were treated with either vehicle (saline, 100 nL/side) or CRF (0.07 nmol/100 nL/side), and 5 min later were submitted to a 30 min session of restraint stress. Immediately after restraint, the animals were euthanized and the brain was obtained for evaluation of NOx levels in the BNST.

2.9.2. Involvement of local NMDA/nNOS/sGC/PKG signaling in control of cardiovascular responses to restraint stress by CRF into the BNST

The aim of this protocol was to investigate the involvement of local NMDA glutamate receptor, nNOS, sGC and PKG in control of cardiovascular responses to acute restraint stress by CRF into the BNST. For this, independent sets of rats were pretreated into the BNST with either the selective NMDA receptor antagonist LY235959 (0.5 nmol/100 nL/side), the selective nNOS inhibitor NPLA (0.2 nmol/100 nL/side), the sGC inhibitor ODQ (0.5 nmol/100 nL/side), the selective PKG inhibitor KT5823 (0.01 nmol/100 nL/side) or vehicle (saline or DMSO 20%, 100 nL/side) (Alves et al., 2009; Busnardo et al., 2010a; Hott et al., 2017). Five minutes later the animals received either vehicle (saline, 100 nL/side) or CRF (0.07 nmol/100 nL/side) into the BNST (Oliveira et al., 2015).

Five minutes after the second pharmacological treatment of the BNST, animals in all experimental groups underwent a 30-min session of restraint stress. Blood pressure and heart rate recordings started at least 30 min before the onset of the stress session, and were performed throughout the restraint stress period. Tail skin temperature was measured at 10, 5 and 0 min before the restraint, and every 5 min during the stress session.

2.10. Histological determination of the microinjection sites

At the end of each experiment, animals were anesthetized with urethane (1.2 g/kg, i.p.), and 1% Evan's blue dye was microinjected into the brain at the same volume of drug injection (i.e., 100 nL/side) as a marker of microinjection site. Then, the brains were removed and post-fixed in 10% formalin solution for at least 48 h at 4 °C. Afterwards, serial 40 µm thick sections of the BNST region were cut using a cryostat (CM1900, Leica, Wetzlar, Germany). The sites of injection were analyzed according to Paxinos and Watson (1997).

2.11. Statistical analysis

Data were expressed by mean ± standard error of the mean (SEM). NOx levels in the BNST were compared using Student's *t*-test. The basal values of MAP, HR and tail skin temperature were compared using one-way ANOVA. Restraint-evoked cardiovascular changes were obtained for each measure by calculating the difference between the values recorded during the restraint stress and the baseline value obtained by the mean of points recorded across the 10 min before the restraint onset. The time-course curves of MAP, HR and tail skin temperature changes were analyzed using two-way ANOVA, with treatment as main factor and time as repeated measurement. For identification of specific differences between the experimental groups (*post hoc* analysis), the mean of all points across the restraint stress period in the time-course curves (i.e., from time 0–30) was calculated, and these values were compared across the groups using one-way ANOVA followed by Bonferroni's *post hoc* test. Results of statistical tests with $P < 0.05$ were considered significant.

3. Results

3.1. Effect of CRF microinjection into the BNST on local NOx levels during acute restraint stress

Bilateral microinjection of CRF (0.07 nmol/100 nL/side, n = 8) into the BNST increased the local NOx levels following exposure to acute restraint stress ($t = 2.2$, $P < 0.04$), when compared with vehicle-treated animals (100 nL/side, n = 7) (Fig. 1).

3.2. Involvement of local NMDA/nNOS/sGC/PKG signaling in control of cardiovascular responses to restraint stress by BNST CRF neurotransmission

NMDA receptor – Bilateral microinjections of the selective NMDA glutamate receptor antagonist LY235959 (0.5nmol/100 nL/side) and/or CRF (0.07nmol/100 nL/side) into the BNST did not affect baseline values of either MAP, HR or tail skin temperature (Table 1) However, acute restraint stress caused a sustained increase on both MAP (time: $F_{(19,399)} = 12$, $P < 0.0001$) and HR (time: $F_{(19,399)} = 8$, $P < 0.0001$), and decreased the tail skin temperature (time: $F_{(8,160)} = 12$, $P < 0.0001$) (Fig. 2). Besides, analysis indicated an effect of BNST

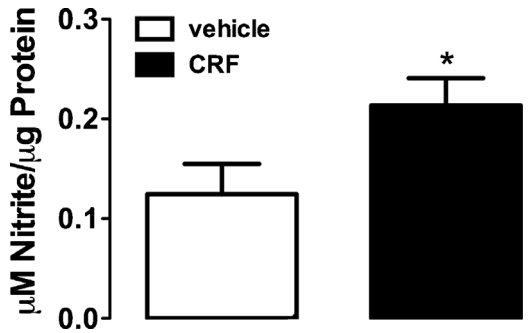

Fig. 1. (Top) Levels of NOx in the bed nucleus of the stria terminalis (BNST) following exposure to a 30-min session of restraint stress in animals that received bilateral microinjection of vehicle (saline, 100 nL/side, n = 7) or CRF (0.07 nmol/100 nL/side, n = 8) into the BNST. Columns represent the mean and bars the S.E.M. *P < 0.05, Student's *t*-test.

Table 1
Basal parameters of mean arterial pressure (MAP), heart rate (HR) and tail skin temperature (T) after pharmacological treatments of the BNST.

Groups	MAP (mmHg)	HR (bpm)	T(°C)	n
VEH + VEH	111 ± 2	337 ± 22	27.8 ± 1	6
LY + VEH	104 ± 3	383 ± 21	28.9 ± 2	6
VEH + CRF	106 ± 4	363 ± 9	26.6 ± 1	6
LY + CRF	119 ± 4	394 ± 19	26.9 ± 1	6
	$F_{(3,23)} = 2.9$	$F_{(3,23)} = 1.8$	$F_{(3,23)} = 0.7$	
	$P > 0.05$	$P > 0.05$	$P > 0.05$	
VEH + VEH	111 ± 2	337 ± 22	27.8 ± 1	6
NPLA + VEH	107 ± 5	375 ± 9	28.7 ± 1	6
VEH + CRF	106 ± 4	363 ± 9	26.5 ± 1	6
NPLA + CRF	107 ± 3	361 ± 28	26.7 ± 1	6
	$F_{(3,23)} = 0.3$	$F_{(3,23)} = 0.7$	$F_{(3,23)} = 1.0$	
	$P > 0.05$	$P > 0.05$	$P > 0.05$	
DMSO + SAL	114 ± 4	379 ± 15	29.1 ± 1	6
ODQ + SAL	111 ± 3	365 ± 35	27.0 ± 0.4	6
DMSO + CRF	105 ± 3	366 ± 15	28.9 ± 0.3	6
ODQ + CRF	109 ± 2	387 ± 15	27.8 ± 0.4	6
	$F_{(3,23)} = 1.5$	$F_{(3,23)} = 0.2$	$F_{(3,23)} = 2.7$	
	$P > 0.05$	$P > 0.05$	$P > 0.05$	
DMSO + SAL	114 ± 4	379 ± 15	29.1 ± 1	6
KT + SAL	108 ± 4	400 ± 20	29.6 ± 0.6	5
DMSO + CRF	105 ± 3	366 ± 15	28.8 ± 0.4	6
KT + CRF	105 ± 2	372 ± 16	28.8 ± 0.5	5
	$F_{(3,20)} = 1.6$	$F_{(3,20)} = 0.4$	$F_{(3,20)} = 0.3$	
	$P > 0.05$	$P > 0.05$	$P > 0.05$	

pharmacological treatments on MAP ($F_{(3,20)} = 8$, $P < 0.0001$) and HR ($F_{(3,20)} = 12$, $P < 0.0001$) responses to restraint stress, but without affecting the drop on tail skin temperature ($F_{(3,20)} = 0.6$, $P < 0.05$) (Fig. 2). Nevertheless, analysis did not indicate a significant stress x time interaction for either MAP ($F_{(57,399)} = 0.6$, $P > 0.05$), HR ($F_{(57,399)} = 0.5$, $P > 0.05$) or tail skin temperature ($F_{(24,160)} = 0.2$, $P > 0.05$). *Post hoc* analysis revealed that CRF (veh + CRF group) enhanced the increase of both MAP ($P < 0.01$) and HR ($P < 0.05$). CRF effects on both MAP ($P > 0.05$) and HR ($P > 0.05$) responses to restraint stress were inhibited in animals pretreated with LY235959 into the BNST (Fig. 2). Fig. 2 presents diagrammatic representations showing microinjection sites into the BNST of all animals used in this protocol.

nNOS – Bilateral microinjections of the selective nNOS inhibitor NPLA (0.2 nmol/100 nL/side) and/or CRF (0.07 nmol/100 nL/side) into the BNST did not affect baseline values of either MAP, HR or tail skin temperature (Table 1). However, acute restraint stress caused a sustained increase on both MAP (time: $F_{(19,399)} = 34$, $P < 0.0001$) and HR (time: $F_{(19,399)} = 14$, $P < 0.0001$), and evoked a drop on tail skin temperature (time: $F_{(8,160)} = 9$, $P < 0.0001$) (Fig. 3). Besides, analysis indicated an effect of pharmacological treatments on restraint-evoked increase of MAP ($F_{(3,20)} = 11$, $P < 0.0001$) and HR ($F_{(3,20)} = 15$, $P < 0.001$), but without affecting the drop in skin temperature ($F_{(3,20)} = 0.9$, $P > 0.05$) (Fig. 3). Analysis of MAP ($F_{(57,399)} = 1$, $P > 0.05$), HR ($F_{(57,399)} = 0.9$, $P > 0.05$) and tail skin temperature ($F_{(24,160)} = 0.3$, $P > 0.05$) did not indicate a significant stress x time interaction. *Post hoc* analysis revealed that CRF (veh + CRF group) enhanced the increase of both MAP ($P < 0.006$) and HR ($P < 0.02$). CRF effects on both pressor ($P > 0.05$) and tachycardiac ($P > 0.05$) responses to restraint were inhibited in animals pretreated with NPLA into the BNST (Fig. 3). Fig. 3 presents diagrammatic representations showing microinjection sites into the BNST of all animals used in this protocol.

sGC – Bilateral microinjections of the selective sGC inhibitor ODQ (0.5nmol/100 nL/side) and/or CRF (0.07nmol/100 nL/side) into the BNST did not affect baseline values of either MAP, HR or tail skin temperature (Table 1). However, restraint stress caused an sustained increase on both MAP (time: $F_{(19,399)} = 15$, $P < 0.0001$) and HR (time: $F_{(19,399)} = 3$, $P < 0.0001$), and decreased the tail skin temperature (time: $F_{(8,160)} = 30$, $P < 0.0001$) (Fig. 4). Besides, analysis of MAP ($F_{(3,20)} = 26$, $P < 0.0001$) and HR ($F_{(3,20)} = 4$, $P < 0.007$), but not of tail skin temperature ($F_{(3,20)} = 0.6$, $P > 0.05$), indicated an effect of pharmacological treatments of the BNST (Fig. 4). Nevertheless, analysis did not indicate a significant stress x time interaction for MAP ($F_{(57,399)} = 0.7$, $P > 0.05$), HR ($F_{(57,399)} = 0.2$, $P > 0.05$) and tail skin temperature ($F_{(24,160)} = 0.4$, $P > 0.05$). *Post hoc* analysis revealed that CRF (veh + CRF group) enhanced the restraint-evoked increase of MAP ($P < 0.05$) (Fig. 4). The effect of CRF increasing restraint-evoked pressor response was inhibited when animals were pretreated with ODQ ($P > 0.05$) (Fig. 4). Fig. 4 presents diagrammatic representations showing microinjection sites into the BNST of all animals used in this protocol.

PKG – Bilateral microinjections of the selective PKG inhibitor KT5823 (0.01 nmol/100 nL/side) and/or CRF (0.07 nmol/100 nL/side) into the BNST did not affect baseline values of either MAP, HR or tail skin temperature (Table 1). Nevertheless, restraint stress caused a sustained increase on both MAP (time: $F_{(19,342)} = 13$, $P < 0.0001$) and HR (time: $F_{(19,342)} = 7$, $P < 0.0001$), and decreased the tail skin temperature (time: $F_{(8,144)} = 28$, $P < 0.0001$) (Fig. 5). Besides, analysis of MAP ($F_{(3,18)} = 20$, $P < 0.0001$), HR ($F_{(3,18)} = 7$, $P < 0.0002$) and tail skin temperature ($F_{(3,18)} = 18$, $P < 0.0001$) indicated effect of BNST pharmacological treatments (Fig. 5). However, analysis did not indicate a significant stress x time interaction for MAP ($F_{(57,342)} = 0.9$, $P > 0.05$), HR ($F_{(57,342)} = 0.4$, $P > 0.05$) and tail skin temperature ($F_{(24,144)} = 0.6$, $P > 0.05$). *Post hoc* analysis revealed that CRF (veh + CRF group) increased the restraint-evoked increase of MAP ($P < 0.05$) and HR ($P < 0.05$). CRF effects on restraint-evoked pressor ($P > 0.05$) and tachycardiac ($P > 0.05$) responses were inhibited in animals pretreated with KT5823 into the BNST (Fig. 5). Furthermore, the drop on tail skin temperature evoked by restraint was decreased in animals subjected to combined treatment with KT5823 plus CRF (KT + CRF group) ($P < 0.04$) (Fig. 5). Fig. 5 presents diagrammatic representations showing microinjection sites into the BNST of all animals used in the present study.

Microinjection of the antagonist/inhibitors (i.e., LY235959, NPLA, ODQ or KT5328) and/or CRF into structures surrounding the BNST did not affect the restraint-evoked cardiovascular responses (data not shown).

4. Discussion

The present study provides the first evidence of an interaction between CRF and nitrergic neurotransmissions within the BNST. Furthermore, this study first describes that the modulation of cardiovascular responses to stress by CRF within the BNST is mediated by activation of local glutamatergic and nitrergic neurotransmissions.

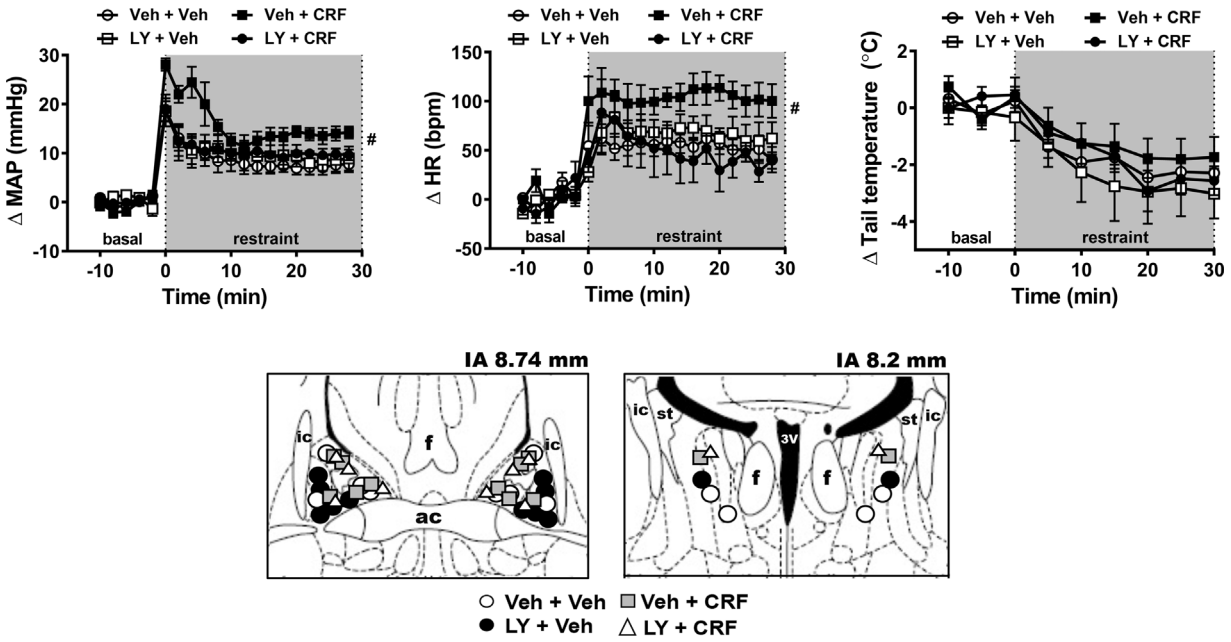

Fig. 2. (Top) Time-course curves of changes on mean arterial pressure (ΔMAP), heart rate (ΔHR) and tail skin temperature (Δ tail temperature) evoked by acute restraint stress in animals treated bilaterally into the BNST with vehicle (Veh) (saline, 100 nL/side) or the selective NMDA glutamate receptor antagonist LY235959 (LY) (0.5 nmol/100 nL/side), followed by a second microinjection of vehicle (Veh) (saline, 100 nL/side) or CRF (0.07 nmol/100 nL/side) (n = 6/group). The onset of stress was at t = 0. Circles represent the mean and the bars the SEM. # P < 0.05 over the whole restraint period compared to veh + veh group, ANOVA followed by Bonferroni's *post hoc* test. **(Bottom)** Diagrammatic representation based on the rat brain atlas of Paxinos and Watson (1997) indicating the microinjection sites into the BNST of all animals used for evaluation of the involvement of local NMDA glutamate receptor on modulation of restraint-evoked cardiovascular responses by CRF within the BNST. 3V: third ventricle; ac: anterior commissure; f: fornix; IA: interaural coordinate; ic: internal capsule; st: stria terminalis.

Indeed, we observed that CRF microinjection into the BNST increased local NO formation during restraint stress, as evidenced by increased local NOx levels. Furthermore, local BNST pretreatment with a selective NMDA receptor antagonist, as well as with inhibitors of either the nNOS, sGC or PKG, inhibited the enhance of restraint-evoked pressor and tachycardiac responses observed following microinjection of CRF into the BNST.

The CRF neuropeptide family includes the CRF and other three peptides designated urocortin 1 (Ucn1), urocortin 2 (Ucn2) and urocortin 3 (Ucn3) (Bale and Vale, 2004; Hauger et al., 2003). Actions of CRF and the related peptides are mediated by activation of two receptors, CRF_1 and CRF_2 receptors, as well as of one binding-protein of

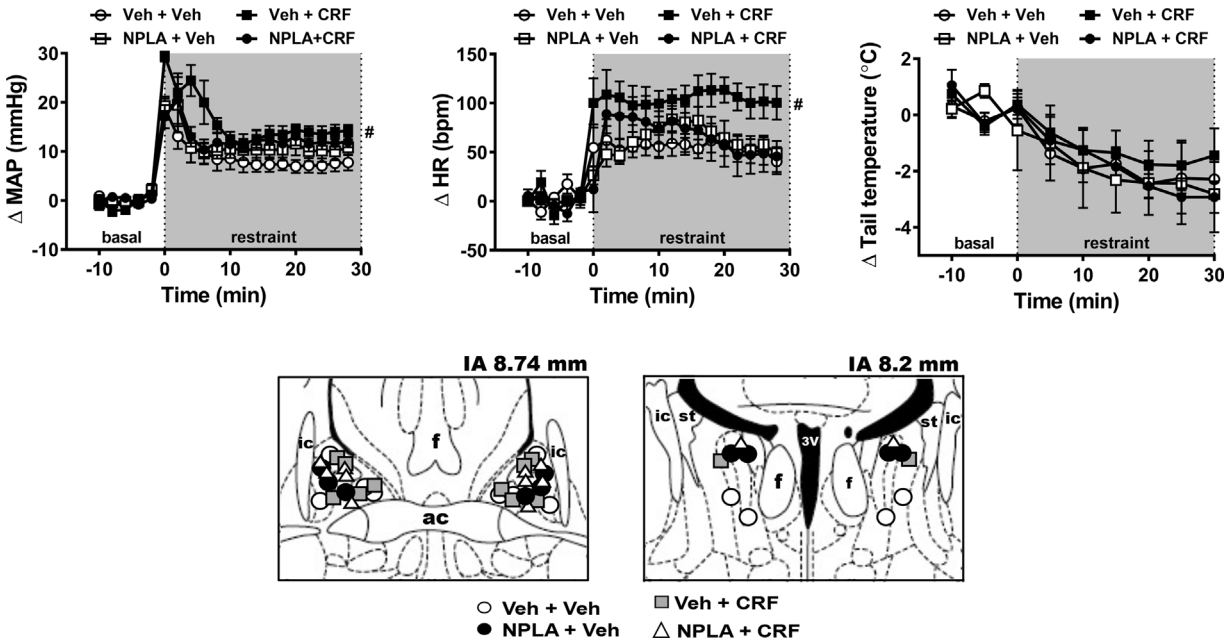

Fig. 3. (Top) Time-course curves of changes on mean arterial pressure (ΔMAP), heart rate (ΔHR) and tail skin temperature (Δ tail temperature) evoked by acute restraint stress in animals treated bilaterally into the BNST with vehicle (Veh) (saline, 100 nL/side) or the selective nNOS inhibitor NPLA (0.2 nmol/100 nL/side), followed by a second microinjection of vehicle (Veh) (saline, 100 nL/side) or CRF (0.07 nmol/100 nL/side) (n = 6/group). The onset of stress was at t = 0. Circles represent the mean and the bars the SEM. # P < 0.05 over the whole restraint period compared to veh + veh group, ANOVA followed by Bonferroni's *post hoc* test. **(Bottom)** Diagrammatic representation based on the rat brain atlas of Paxinos and Watson (1997) indicating the microinjection sites into the BNST of all animals used for evaluation of the involvement of local nNOS activation on modulation of restraint-evoked cardiovascular responses by CRF within the BNST. 3V: third ventricle; ac: anterior commissure; f: fornix; IA: interaural coordinate; ic: internal capsule; st: stria terminalis.

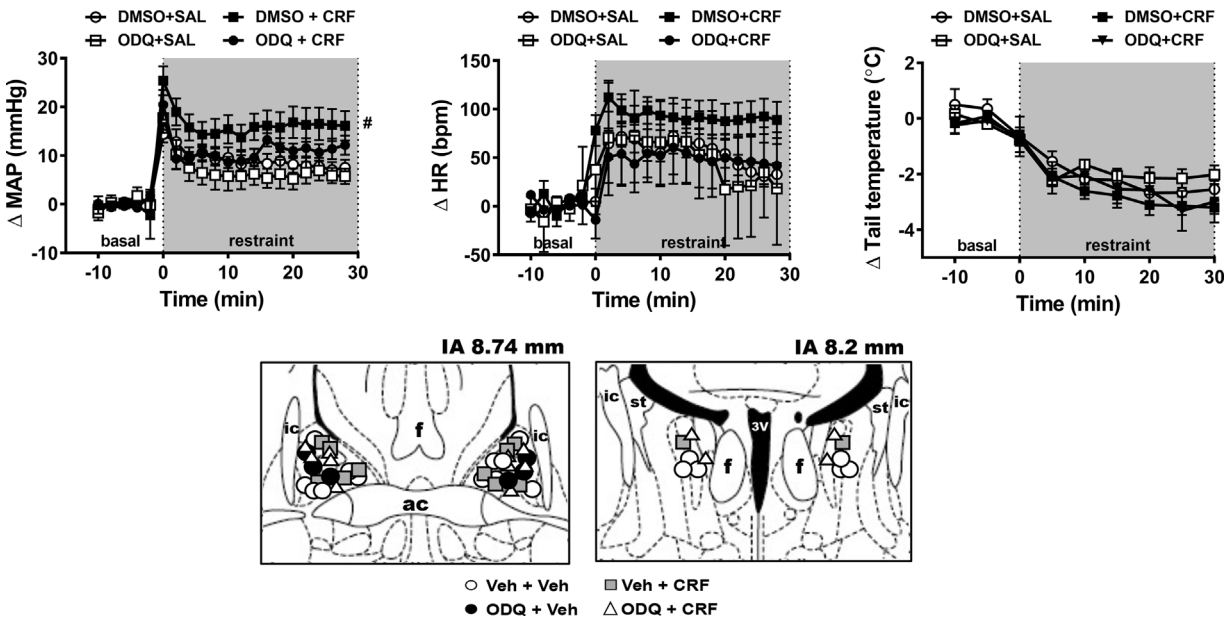

Fig. 4. (Top) Time-course curves of changes on mean arterial pressure (ΔMAP), heart rate (ΔHR) and tail skin temperature (Δ tail temperature) evoked by acute restraint stress in animals treated bilaterally into the BNST with vehicle (DMSO) (saline solution containing 20% of DMSO, 100 nL/side) or the selective sGC inhibitor ODQ (0.2 nmol/100 nL/side), followed by a second microinjection of vehicle (SAL) (saline, 100 nL/side) or CRF (0.07 nmol/100 nL/side) (n = 6/group). The onset of stress was at t = 0. Circles represent the mean and the bars the SEM. # P < 0.05 over the whole restraint period compared to DMSO + SAL group, ANOVA followed by Bonferroni's *post hoc* test. **(Bottom)** Diagrammatic representation based on the rat brain atlas of Paxinos and Watson (1997) indicating the microinjection sites into the BNST of all animals used for evaluation of the involvement of local sGC activation on modulation of restraint-evoked cardiovascular responses by CRF within the BNST. 3V: third ventricle; ac: anterior commissure; f: fornix; IA: interaural coordinate; ic: internal capsule; st: stria terminalis.

CRF (Bale and Vale, 2004; Hauger et al., 2003). The peptides of CRF system differently bind to CRF_1 and CRF_2 receptors. For instance, CRF selectively binds to the CRF_1 receptor, Ucn1 has similar affinity to both receptors, whereas Ucn2 and Ucn3 are selective ligands of the CRF_2 receptor (Hauger et al., 2003).

The BNST anterior division is proposed as the critical region involved in control of autonomic activity and cardiovascular function (Crestani et al., 2013; Dong et al., 2001). This idea is supported by neuroanatomical results demonstrating that the anterior division is the preferential region of the BNST connected with hypothalamic and lower brainstem regions involved in control of autonomic activity (Dong et al., 2001; Dong and Swanson, 2004), as well as by functional studies

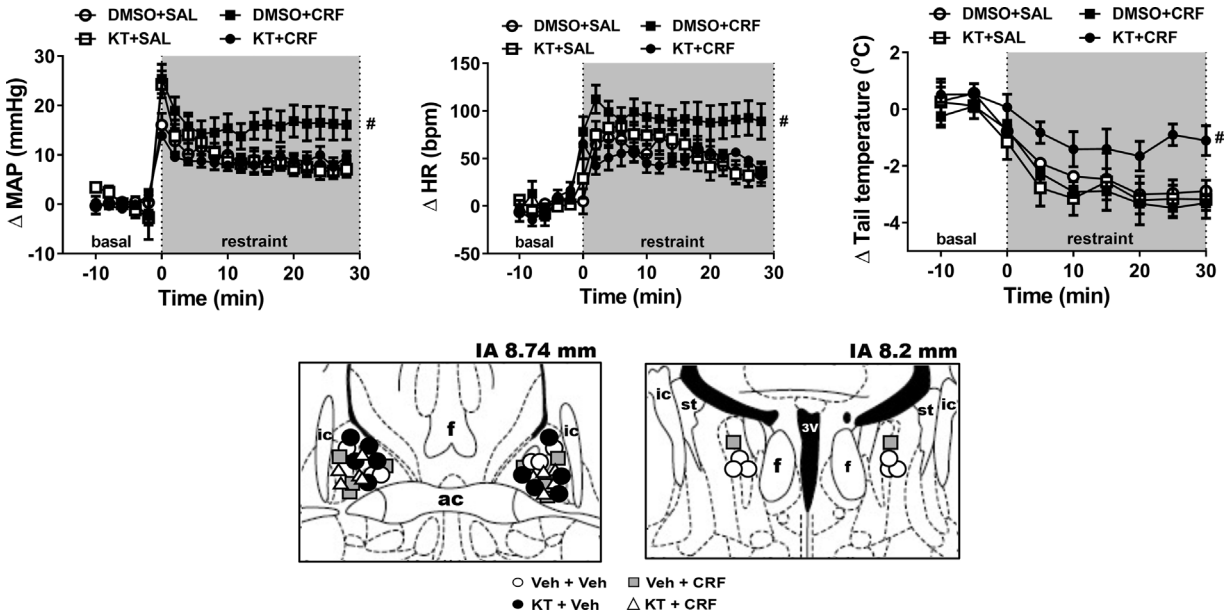

Fig. 5. (Top) Time-course curves of changes on mean arterial pressure (ΔMAP), heart rate (ΔHR) and tail skin temperature (Δ tail temperature) evoked by acute restraint stress in animals treated bilaterally into the BNST with vehicle (DMSO) (saline solution containing 20% of DMSO, 100 nL/side) or the selective PKG inhibitor KT5823 (KT) (0.01nmol/100 nL/side), followed by a second microinjection of vehicle (SAL) (saline, 100 nL/side) or CRF (0.07nmol/100 nL/side) (n = 5–6/group). The onset of stress was at t = 0. Circles represent the mean and the bars the SEM. # P < 0.05 over the whole restraint period compared to DMSO + SAL group, ANOVA followed by Bonferroni's *post hoc* test. **(Bottom)** Diagrammatic representation based on the rat brain atlas of Paxinos and Watson (1997) indicating the microinjection sites into the BNST of all animals used for evaluation of the involvement of local PKG activation on modulation of restraint-evoked cardiovascular responses by CRF within the BNST. 3V: third ventricle; ac: anterior commissure; f: fornix; IA: interaural coordinate; ic: internal capsule; st: stria terminalis.

reporting that largest cardiovascular responses are elicited more often when this region of the BNST is stimulated (Ciriello and Janssen, 1993; Zhang et al., 2009). Accordingly, most of the microinjection sites in the present study reached regions of the BNST anterior division. Indeed, both CRF_1 and CRF_2 receptors were reported to be expressed in nuclei of the BNST anterior division (Dabrowska et al., 2011; Van Pett et al., 2000). Besides, terminals immunoreactive for CRF and Ucn1- and Ucn3-immunoreactive fibers were found within rostral regions of the BNST (Bittencourt et al., 1999; Li et al., 2002). Local neurons immunoreactive for CRF and Ucn3 were also identified within the BNST anterior division (Li et al., 2002; Phelix and Paull, 1990; Swanson et al., 1983). Altogether, these pieces of evidence support present findings indicating a modulation of restraint-evoked cardiovascular responses by CRF microinjection into rostral regions of the BNST.

Previous studies from our group revealed a role of CRF neurotransmission present within the regions of the BNST anterior division in control of cardiovascular responses to restraint stress. For instance, we reported that microinjection of a selective CRF_1 receptor antagonist into rostral regions of the BNST dose-dependently decreased the increase on arterial pressure and HR evoked by acute restraint stress, whereas a selective CRF_2 receptor antagonist decreased the pressor response and the sympathetically-mediated cutaneous vasoconstriction during restraint stress (Oliveira et al., 2015). Conversely, BNST treatment with either the selective CRF_1 receptor agonist CRF or the selective CRF_2 receptor agonist Ucn3 enhanced the restraint-evoked cardiovascular changes (Oliveira et al., 2015). Therefore, enhances of arterial pressure and HR evoked by CRF microinjection into the BNST reported in the present study are in line with results previously reported.

Results obtained using electrophysiological techniques indicated that CRF acts presynaptically via CRF_1 receptor to increase glutamatergic neurotransmission within the BNST anterior division (Silberman and Winder, 2013). It was evidenced by demonstration that CRF application onto BNST *in vitro* increased frequency, but not amplitude, of sEPSC; and this effect was inhibited by pretreatment with a selective CRF_1 receptor antagonist (Kash et al., 2008; Silberman et al., 2013). This CRF-mediated facilitation of glutamatergic neurotransmission within the BNST has been implicated in drug addiction, as evidenced by demonstration that this neurochemical mechanism is recruited following chronic exposure to alcohol and cocaine (Kash et al., 2008; Nobis et al., 2011; Silberman et al., 2013). However, present findings provide the first evidence that CRF-glutamate interaction within the BNST in involved in control of physiological responses during aversive threats. Furthermore, results reported in the present study indicate a novel interaction between CRF and nitrergic signaling within rostral regions of the BNST, which plays a role in the control of stress-evoked cardiovascular responses.

An additional component of BNST CRF neurocircuitry is a noradrenaline-CRF interaction (Silberman and Winder, 2013). In this sense, data from electrophysiological studies indicated that noradrenaline released within the BNST anterior division activates local neurons synthetizing CRF (Silberman et al., 2013), which likely increase local CRF levels (Silberman and Winder, 2013). In turn, the enhanced CRF levels increase BNST glutamatergic activity, thus increasing local excitation (Nobis et al., 2011; Silberman et al., 2013). A role of noradrenergic neurotransmission present in the BNST anterior division in control of restraint-evoked cardiovascular changes was previously reported (Crestani et al., 2013). Indeed, BNST treatment with a selective α_1-adrenoceptor antagonist, but not with selective α_2-adrenoceptor or β-adrenoceptor antagonists, enhanced the tachycardia evoked by restraint stress without affecting the pressor response (Crestani et al., 2009). Despite these pieces of evidence, the report of an inhibitory role of BNST noradrenergic neurotransmission in restraint-evoked cardiovascular changes (Crestani et al., 2013; Crestani et al., 2009) precludes the idea that the facilitatory influence of local CRF neurotransmission in control of cardiovascular responses to stress is related to local noradrenaline release. Therefore, release of CRF and the related peptides within the BNST during restraint stress seems to be mediated by independent mechanisms of local noradrenergic neurotransmission. In this regard, in addition to intrinsic sources (i.e., from local BNST CRF-synthesizing neurons), CRF and the related peptides may also be released from extrinsic source, such as terminals arising from the central nucleus of the amygdala (CeA) and paraventricular nucleus of the hypothalamus (PVN) (Vranjkovic et al., 2017). In this sense, similar to BNST CRF neurotransmission, a facilitatory role of both CeA and PVN in modulation of cardiovascular responses to stress was previously reported (Busnardo et al., 2013; Busnardo et al., 2010b; Sanders et al., 1994), thus supporting the idea that CRF and the related peptides can be released into the BNST from terminals coming from other limbic structures.

Cardiovascular responses during aversive threats are mediated by changes on activity of both sympathetic and parasympathetic nervous system (Crestani, 2016). For instance, increase on HR during aversive threats is abolished following inhibition of cardiac sympathetic activity, while blockade of parasympathetic tone to the heart increases this response (Carrive, 2006; Dos Reis et al., 2014), thus suggesting a coativation of cardiac sympathetic and parasympathetic activity during stress. The pressor response during stress is inhibited following treatment with α_1-adrenoceptor antagonists (Crestani, 2016; Dos Reis et al., 2014), thus indicating that vasoconstriction in splanchnic, renal and cutaneous beds (Blessing, 2003; Schadt and Hasser, 1998; Zhang et al., 1996) contribute to pressor response. Neurons in the anterior division of the BNST project to medullary structures controlling autonomic activity, such as the nucleus of the solitary tract, nucleus ambiguus, and ventrolateral regions (Dong and Swanson, 2004; Gray and Magnuson, 1987). Therefore, the facilitatory role of CRF in the BNST on restraint-evoked tachycardia may be mediated by stimulation of either facilitatory drive to sympathetic premotor neurons or inhibitory pathways to parasympathetic neurons in the medulla, whereas facilitation of pressor response is mediated by a sympathetic facilitation.

A surprising finding was the decreased drop in tail skin temperature response identified in animals subjected to combined treatment with KT5823 plus CRF into the BNST (i.e., KT + CRF group). The mechanism related to this effect is unclear. However, the blockade of the PKG unmasked an effect of CRF decreasing the restraint-evoked cutaneous vasoconstriction. In this sense, although the present findings indicate that CRF control of cardiovascular responses to stress within the BNST is mediated by facilitation of local glutamatergic neurotransmission and activation of nitric oxide signaling, the decreased tail skin temperature response found in KT + CRF group provides evidence that CRF can also interacts with other local neurochemical mechanisms controlling the sympathetically-mediated cutaneous vasoconstriction, which seem to counteract the effect mediated by PKG activation. As stated above, previous studies reported an interaction of CRF neurotransmission with local noradrenergic neurotransmission within the BNST (Silberman and Winder, 2013). However, a role of BNST noradrenergic neurotransmission in control of cutaneous vasoconstriction response to restraint stress has never been evaluated. Besides, we cannot exclude the possibility that this effect is independent of interaction with other neurotransmitters, being able to be mediated by a direct action of CRF in central pathways controlling the sympathetic activity. Anyway, further studies are necessary to clarify the mechanisms involved in this effect.

In summary, results of the present study provide evidence that CRF activates local NMDA-mediated excitation and nitrergic signaling within the BSNT. Furthermore, our data suggest that facilitatory influence of BNST CRF neurotransmission on cardiovascular responses evoked by aversive stimuli are mediated by activation of local NMDA glutamate receptor, nNOS, sGC and PKG.

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Contributors

L.A.O. and C.C.C. conceived and designed this research; L.A.O., L.G.S. and R.B. performed the experiments and analyzed the data; L.A.O., L.G.S. and R.B. and C.C.C. interpreted the results of experiments; L.A.O. prepared the figures and drafted the manuscript; L.A.O. and C.C.C. edited and revised the manuscript; C.C.C. approved the final version of the manuscript.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.psyneuen.2018.01.010.

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