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RESEARCH PAPER

Dual role of nitrergic neurotransmission in the bed nucleus of the stria terminalis in controlling cardiovascular responses to emotional stress in rats

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BACKGROUND AND PURPOSE

The aim of the present study was to assess the interaction of nitrergic neurotransmission within the bed nucleus of the stria terminalis (BNST) with local glutamatergic and noradrenergic neurotransmission in the control of cardiovascular responses to acute restraint stress in rats.

EXPERIMENTAL APPROACH

Interaction with local noradrenergic neurotransmission was evaluated using local pretreatment with the selective α_1-adrenoceptor antagonist WB4101 before microinjection of the NO donor NOC-9 into the BNST. Interaction with glutamatergic neurotransmission was assessed by pretreating the BNST with a selective inhibitor of neuronal NOS (nNOS), *N*ω-propyl-L-arginine (NPLA) before local microinjection of NMDA. The effect of intra-BNST NPLA microinjection in animals locally pretreated with WB4101 was also evaluated.

KEY RESULTS

NOC-9 reduced the heart rate (HR) and blood pressure increases evoked by restraint stress. These effects of NOC-9 on HR, but not in blood pressure, was inhibited by pretreatment of BNST with WB4101. NMDA enhanced the restraint-evoked HR increase, and this effect was abolished following BNST pretreatment with NPLA. Administration of NPLA to the BNST of animals pretreated locally with WB4101 decreased the HR and blood pressure increases induced by restraint.

CONCLUSION AND IMPLICATIONS

These results indicate that inhibitory control of stress-evoked cardiovascular responses by nitrergic signalling in the BNST is mediated by a facilitation of local noradrenergic neurotransmission. The present data also provide evidence of an involvement of local nNOS in facilitatory control of tachycardia during stress by NMDA receptors within the BNST.

Abbreviations

BNST, bed nucleus of the stria terminalis; HR, heart rate; MAP, mean arterial pressure; nNOS, neuronal isoform of the NOS enzyme; NPLA, N$^{\omega}$propyl-L-arginine (N5-[imino(propylamino)methyl]-L-ornithine hydrochloride); NOC-9, 6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-hexanamine; sGC, soluble guanylate cyclase; WB4101, 2-[(2,6-dimethoxyphenoxyethyl)aminomethyl]-1,4-benzodioxane hydrochloride

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Introduction

The physiological and behavioural responses evoked by aversive situations are essential for species survival and adaptation (Crestani, 2016; Sterling, 2012; Ulrich-Lai and Herman, 2009). The control of such responses is performed by overlapping circuits in the CNS (Dampney, 2015; Myers, 2017). The bed nucleus of the stria terminalis (BNST) is a structure situated in the rostral prosencephalon, which has been linked to the central network that regulates stress responses (Crestani *et al.*, 2013; Myers, 2017; Ulrich-Lai and Herman, 2009). In particular, control of the cardiovascular responses induced by stress involves the BNST (Crestani *et al.*, 2009; Resstel *et al.*, 2008). Recent studies have indicated that nitrergic neurotransmission acting *via* activation of the **neuronal isoform of the NOS enzyme (nNOS)** is a prominent local neurochemical mechanism involved in the control of stress-evoked cardiovascular responses exerted by the BNST (Barretto-de-Souza *et al.*, 2018; Hott *et al.*, 2017). For instance, blockade of nNOS, as well as of signalling mechanisms related to NO effects such as **soluble guanylyl cyclase (sGC)** and **PKG**, within the BNST, enhanced tachycardia and decreased the drop in tail skin temperature evoked by acute restraint stress (Barretto-de-Souza *et al.*, 2018). Nevertheless, the local mechanisms related to the control of cardiovascular responses to stress by nitrergic neurotransmission in the BNST remain unknown.

Presynaptic actions that stimulate the release of several neurotransmitters are a prominent mechanism mediating the effects of NO in the CNS (Ohkuma and Katsura, 2001; Philippu, 2016; Prast and Philippu, 2001). Indeed, earlier studies have shown that NO stimulated the release of noradrenaline (Ohkuma and Katsura, 2001; Philippu, 2016; Prast and Philippu, 2001). In this sense, antagonism of α_1-adrenoceptors within the BNST, but not of local α_2- and β-adrenoceptors, enhanced the increase in HR evoked by restraint, without affecting the increase in blood pressure (Crestani *et al.*, 2009). As with local nitrergic signalling (Barretto-de-Souza *et al.*, 2018), these results provided evidence of an inhibitory influence of α_1-adrenoceptors in the BNST in the control of restraint-evoked cardiovascular responses. Therefore, control of restraint-evoked cardiovascular responses by nitrergic neurotransmission in the BNST may be mediated by a facilitation of noradrenaline release within the BNST. However, a possible interaction between noradrenergic and nitrergic neurotransmission in the BNST has yet to be evaluated.

Influx of Ca^{2+} following activation of the **N-methyl-D-aspartate (NMDA)** glutamatergic receptor is involved in NO synthesis by nNOS in the CNS (Garthwaite, 2008; Garthwaite, 2016; Prast and Philippu, 2001). We recently reported that microinjection of a selective NMDA receptor antagonist into the BNST decreased the tachycardic response to restraint stress (Adami *et al.*, 2017). Although opposing roles of glutamatergic (facilitatory) and nitrergic (inhibitory) transmission in restraint-evoked tachycardia are implied, NO released from nNOS is described as a prominent signalling mechanism involved in the control of cardiovascular function by NMDA receptors in the brain. For instance, blockade of nNOS completely inhibited cardiovascular changes evoked by NMDA receptor activation in several structures in the CNS (Busnardo *et al.*, 2010; Martins-Pinge *et al.*, 2007; Resstel and Correa, 2006; Santini *et al.*, 2013; Tavares *et al.*, 2007). Nevertheless, an involvement of local nNOS in the control of cardiovascular function by NMDA receptors within the BNST has yet to be evaluated.

Based on the evidence presented above, this study aimed to test the hypothesis that nitrergic neurotransmission within the BNST plays a dual role in the control of cardiovascular responses evoked by acute restraint stress in rats, through interactions with different local neurochemical mechanisms. Thus, we tested the hypotheses that (i) the inhibitory influence of NO generated by nNOS within the BNST (previously demonstrated as an enhanced HR response following blockade of nNOS within the BNST) (Barretto-de-Souza *et al.*, 2018) is mediated by a facilitation of local noradrenergic neurotransmission and (ii) activation of local nNOS is involved in the facilitatory control of restraint-evoked cardiovascular changes by NMDA receptors in the BNST.

Methods

Animals

All animal care and experimental procedures were carried out according to protocols approved by the Ethical Committee for Use of Animals of the School of Pharmaceutical Science/UNESP (protocol #36/2016), which complies with Brazilian and international guidelines for animal use and welfare. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). Male Wistar rats with body weights ranging from 240 to 260 g (60 days old) were used. The animals were supplied by the animal breeding facility of the UNESP (Botucatu, SP, Brazil) and were housed in standard plastic cages with sawdust as bedding in a temperature-controlled room at 24°C in the Animal Facility of the Laboratory of Pharmacology, School of Pharmaceutical Sciences, São Paulo State University-UNESP. The animals had *ad libitum* access to granulated food and water and were maintained under 12h light/dark cycles (lights on between 07:00 and 19:00 h).

Surgical preparation

Five days before the trial, animals were anesthetized with tribromoethanol (250 $mg \cdot kg^{-1}$, i.p.). The scalp was anesthetized with 2% lidocaine, and the skull was exposed. Then, using a stereotaxic apparatus (Stoelting, Wood Dale, Illinois, USA), stainless-steel cannulae (26 G, 12 mm long) were bilaterally implanted into the BNST. Stereotaxic coordinates were as follows: antero-posterior = +8.6 mm from interaural coordinate; 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 cannulae to the skull. After surgery, the rats were treated with a poly-antibiotic formulation containing streptomycins and penicillins to prevent infection (560 $mg \cdot mL^{-1} \cdot kg^{-1}$, i.m.), and the nonsteroidal anti-inflammatory drug flunixin meglumine to provide post-operative analgesia (0.5 $mg \cdot mL^{-1} \cdot kg^{-1}$, s.c.).

One day before the experiment, animals were again anesthetized with tribromoethanol (250 $mg \cdot kg^{-1}$, 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, New Jersey, USA) was implanted into the abdominal aorta *via* the femoral artery for cardiovascular recording. The catheter was tunnelled under the skin and exteriorized on the animal's dorsum. After surgery, the nonsteroidal anti-inflammatory drug flunixin meglumine was administered for post-operation analgesia (0.5 mg·mL^{-1}·kg^{-1}, s.c.). The animals were kept in individual cages during the post-operative period and the trial.

Arterial pressure and heart rate recording

The catheter implanted into the femoral artery was connected to a pressure transducer (DPT100, Utah Medical Products Inc., Midvale, UT, USA). Pulsatile arterial pressure was recorded using an amplifier (Bridge Amp, ML221, ADInstruments, Australia) connected to a computerized data acquisition system (PowerLab 4/30, ML866, ADInstruments, Australia) using an appropriate program (Lab Chart PRO, ADInstruments, Australia). The values of mean arterial pressure (MAP) and heart rate (HR) were obtained from the pulsatile arterial pressure recordings.

Tail cutaneous temperature measurement

Tail skin temperature recordings were performed using a thermal camera Multi-Purpose imager (IRI4010, InfraRed Integrates Systems Ltd, Northampton, UK). The analysis was performed using a software for thermographic analysis, and temperature was represented by colour intensity variations (Busnardo *et al*., 2013; Vianna and Carrive, 2005). For image analysis, the temperature values were obtained at five points along the animal's tail, and the mean was calculated for each recording.

Restraint stress

Restraint stress consisted of introducing the animals into plastic cylindrical tubes (diameter = 6.5 cm, length = 15 cm), which were ventilated by 0.5 in. holes that comprised approximately 20% of the tube. In the present study, restraint stress session lasted 30 min (Adami *et al*., 2017; Barretto-de-Souza *et al*., 2018; Choi *et al*., 2007), after which animals were returned to their home cages. Each animal underwent only one session of stress in order to avoid habituation. This model of stress in rats has been widely used for several years (Bali and Jaggi, 2015; Buynitsky and Mostofsky, 2009).

Drug microinjections into the brain

Injection needles (33G, Small Parts, USA) used for the microinjection of drugs into the BNST were 1 mm longer than the guide cannulae fixed to the skull. The needle was connected to a 2 μL syringe (7002KH, Hamilton, USA) using PE-10 tubing.

Experimental design

Rats in all experimental protocols were brought to the experimental room in their own cages. Animals were allowed at least 60 min to adapt to the experimental room conditions, such as sound and illumination, before starting the experiments. The experimental room was temperature controlled (24°C) and acoustically isolated from the other rooms. Cardiovascular recordings began at least 20 min before the onset of the restraint and continued throughout the period of stress. The tail skin temperature was measured 10, 5 and 0 min before the restraint for baseline values and every 5 min during restraint.

In each protocol, animals were randomly distributed among the experimental groups. Only animals in which the microinjection sites reached the BNST were included in the study. Researchers were not blinded to treatment groups during experiments and data analysis once all data were obtained *via* direct recording of physiological parameters, so that analysis did not include any subjective evaluation.

Involvement of local noradrenergic neurotransmission in the control of cardiovascular responses to restraint stress by NO in the BNST. This protocol aimed to investigate whether the control of restraint-evoked cardiovascular responses by nitrergic neurotransmission in the BNST is mediated by a facilitation of local noradrenergic neurotransmission. The control of cardiovascular responses to restraint stress by noradrenergic neurotransmission in the BNST is selectively mediated by activation of local α_1-adrenoceptors (Crestani *et al*., 2009). Therefore, independent sets of rats received intra-BNST microinjections of either the selective α_1-adrenoceptor antagonist **WB4101** (1.5 nmol/200 nL) or vehicle (saline, 200 nL), followed 5 min later by microinjection of the NO donor NOC-9 (75 nmol/200 nL) or vehicle (Tris–HCl, 200 nL) into the BNST (Crestani *et al*., 2009; Faria *et al*., 2016). Five minutes after the second pharmacological treatment of the BNST, animals in all experimental groups were exposed to the standard 30 min session of restraint stress.

Forty-six animals were used in this experiment. Histological analysis confirmed that microinjection sites reached the BNST in 34 animals, which were included in the present study. The individual samples of each experimental group are presented in Table 1.

Involvement of local nNOS in the control of cardiovascular responses to restraint stress by NMDA receptors in the BNST. This protocol aimed to investigate the involvement of local NO release from nNOS in the control of cardiovascular responses to acute restraint stress by the NMDA receptors within the BNST. For this, independent sets of rats received bilateral intra-BNST microinjections of either the selective nNOS inhibitor, **N$^{\omega}$propyl-L-arginine (NPLA**; 0.1 nmol/100 nL) or vehicle (saline, 100 nL), followed 5 min later by the microinjection of NMDA (0.1 nmol/100 nL) or vehicle (saline, 100 nL) into the BNST (Alves *et al*., 2009; Bali and Jaggi, 2015; Buynitsky and Mostofsky, 2009; Martins-Pinge *et al*., 2012). Five minutes after the second pharmacological treatment of the BNST, animals in all experimental groups were subjected to the standard restraint stress.

Thirty-six animals were used in this protocol. Histological analysis confirmed that microinjection sites reached the BNST in 26 animals, which were included in the present study. The individual samples of each experimental group are presented in Table 1.

Effect of bilateral microinjections of NPLA into the BNST on cardiovascular responses to acute restraint stress in rats pretreated

Table 1

Basal parameters of MAP, HR and tail skin temperature (T) before and after pharmacological treatment of the BNST

		MAP		HR		Skin temperature	
Groups	***n***	**Before**	**After**	**Before**	**After**	**Before**	**After**
VEH + VEH	9	106 ± 3 *t* = 0.6	106 ± 2	401 ± 15 *t* = 1	378 ± 14	29.1 ± 0.8 *t* = 0.3	29.4 ± 0.6
WB + VEH	8	102 ± 2 *t* = 0.5	104 ± 2	365 ± 6 *t* = 1.3	350 ± 8	30.4 ± 0.8 *t* = 0.5	29.9 ± 0.7
VEH + NOC9	9	114 ± 2 *t* = 0.6	116 ± 3	386 ± 15 *t* = 0.8	370 ± 9	26.2 ± 0.7 *t* = 1.9	28.0 ± 0.7
WB + NOC9	8	110 ± 2 *t* = 0.7	109 ± 1	368 ± 12 *t* = 0.3	363 ± 8	31.4 ± 0.7 *t* = 0.8	30.7 ± 0.5
VEH + VEH	8	105 ± 3 *t* = 0.03	105 ± 2	390 ± 11 *t* = 0.4	399 ± 15	27.5 ± 0.3 *t* = 1.1	26.9 ± 0.5
NPLA+VEH	6	104 ± 2 *t* = 0.4	106 ± 2	389 ± 10 *t* = 0.3	396 ± 15	27.4 ± 0.5 *t* = 0.2	27.2 ± 0.9
VEH + NMDA	6	111 ± 1 *t* = 0.2	109 ± 3	387 ± 10 *t* = 0.2	392 ± 12	27.6 ± 0.7 *t* = 1.7	26.3 ± 0.4
NPLA+NMDA	6	107 ± 1 *t* = 0.3	106 ± 2	390 ± 10 *t* = 0.5	398 ± 12	27.5 ± 0.6 *t* = 1.7	27.3 ± 0.5
WB + SAL	8	103 ± 2 *t* = 0.8	105 ± 2	345 ± 6 *t* = 1.6	360 ± 6	29.5 ± 0.6 *t* = 0.4	29.2 ± 0.5
WB + NPLA	11	98 ± 2 *t* = 1.8	102 ± 1	352 ± 6 *t* = 0.5	358 ± 8	30.8 ± 0.5 *t* = 1.5	30.2 ± 0.3

Data are expressed as means ± SEM. Values of *t* are shown as calculated for Student's *t*-test. No significant differences between values before and after treatments were found

with WB4101 into the BNST. To confirm the involvement of nNOS in mechanisms within the BNST related to a facilitatory control of restraint-evoked cardiovascular responses, we investigated the effect of nNOS blockade in the absence of local neurochemical mechanisms related to the inhibitory influence of nNOS (i.e. noradrenergic neurotransmission). For this, the selective nNOS inhibitor NPLA (0.4 nmol/100 nL) or vehicle (saline, 100 nL) was microinjected bilaterally into the BNST of animals pretreated locally with the selective α_1-adrenoceptor antagonist WB4101 (10 nmol/100 nL) (Barretto-de-Souza *et al.*, 2018; Crestani *et al.*, 2009). Five minutes after the second pharmacological treatment of the BNST, animals in all experimental groups underwent the 30 min of restraint stress.

Twenty-one animals were used in this experiment. Histological analysis confirmed that microinjection sites reached the BNST in 19 animals, which were included in the present study. The individual samples of each experimental group are presented in Table 1.

Histological determination of the microinjection sites

At the end of each experiment, rats in all experimental groups were anesthetized with urethane (250 mg·mL^{-1}·200 g^{-1} body weight, i.p.) and 1% Evans blue dye at an equal volume to drug injections was microinjected into the brain as a marker of the injection sites. The brains were then removed from the cranial cavity and post-fixed in 10% formalin solution for at least 48 h at 4°C. Then, serial 40-μm-thick sections of the BNST region were cut using a cryostat (CM1900, Leica, Wetzlar, Germany). The sites of injection sites were analysed using the Atlas of Paxinos and Watson (1997) as a reference.

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2018). All analysis were performed using the GraphPad Prim 7 software. Data are presented as mean ± SEM. Initially, all data were submitted to D'Agostino-Pearson omnibus test to assess the sample variance, which indicated a homogeneity of the data in all experimental groups. Then, the basal values of MAP, HR and tail skin temperature before and after the pharmacological treatments were compared using Student's *t*-test. The time-course curves of cardiovascular changes during restraint stress were analysed using two-way ANOVA, with treatment as main factor and time as repeated measurement, followed by Bonferroni's *post hoc* test. Statistical significance was set at $P < 0.05$.

Materials

N^{ω}-propyl-L-arginine (NPLA) a selective nNOS inhibitor, supplied by Tocris (Westwoods Business, Park Ellisville, MO,

USA); WB4101, a selective α_1-adrenoceptor antagonist; NMDA; tribromoethanol and urethane, supplied by Sigma-Aldrich (St Louis, MO), were dissolved in saline (0.9% NaCl). NOC-9 (Sigma-Aldrich) was dissolved in 1 M Tris–HCl solution. NOC-9 solution was prepared at pH 10 to prevent NO release before it reached the brain tissue. NOC-9 is relatively stable at an alkaline pH (>10.0) and produces NO at physiological pH (7.4) (Keefer et al., 1996). The pH of other drugs was adjusted to 7.4. Flunixin meglumine (Banamine®; Schering-Plough, Cotia, SP, Brazil) and the poly-antibiotic preparation (Pentabiotico®; Fort Dodge, Campinas, SP, Brazil) were used as provided.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b,c).

Results

Involvement of local noradrenergic neurotransmission in the control of cardiovascular responses to restraint stress by NO in the BNST

Photomicrograph of a coronal brain section depicting bilateral microinjection sites in BNST of a representative animal is presented in Figure S1. Bilateral treatment of the BNST with the selective α_1-adrenoceptor antagonist WB4101 and/or the NO donor NOC-9 did not affect baseline values of either MAP, HR or tail skin temperature (Table 1). Nevertheless, acute restraint stress evoked a sustained increase in both MAP and HR and decreased the tail skin temperature (Figure 1). Analysis also indicated a significant effect of pharmacological treatments of the BNST in MAP and HR responses to restraint stress, but without affecting the drop on tail skin temperature (Figure 1). Significant treatment × time interaction was identified for MAP and HR, but not for the skin temperature. *Post hoc* analysis did not reveal an effect of WB4101, when it was given alone (WB4101 + veh group) in any parameters analysed (Figure 1). However, intra-BNST injection of NOC-9 alone (veh + NOC-9 group) decreased the increase of MAP and HR evoked by restraint stress (Figure 1). This effect of NOC-9 on HR, but not on MAP, was inhibited in animals pretreated with WB4101 injected into the BNST (WB + NOC-9 group) (Figure 1). Diagrammatic representations showing the microinjection sites into the BNST of all animals used in this protocol are presented in Figure 1.

Involvement of local NO signalling in the control of cardiovascular responses to restraint stress by NMDA in the BNST

Bilateral microinjections into the BNST of the selective nNOS inhibitor NPLA and/or NMDA did not affect baseline values of either MAP, HR or tail skin temperature (Table 1). However, acute restraint stress evoked a sustained increase in both MAP and HR and decreased the tail skin temperature (Figure 2). Further analysis indicated a significant effect

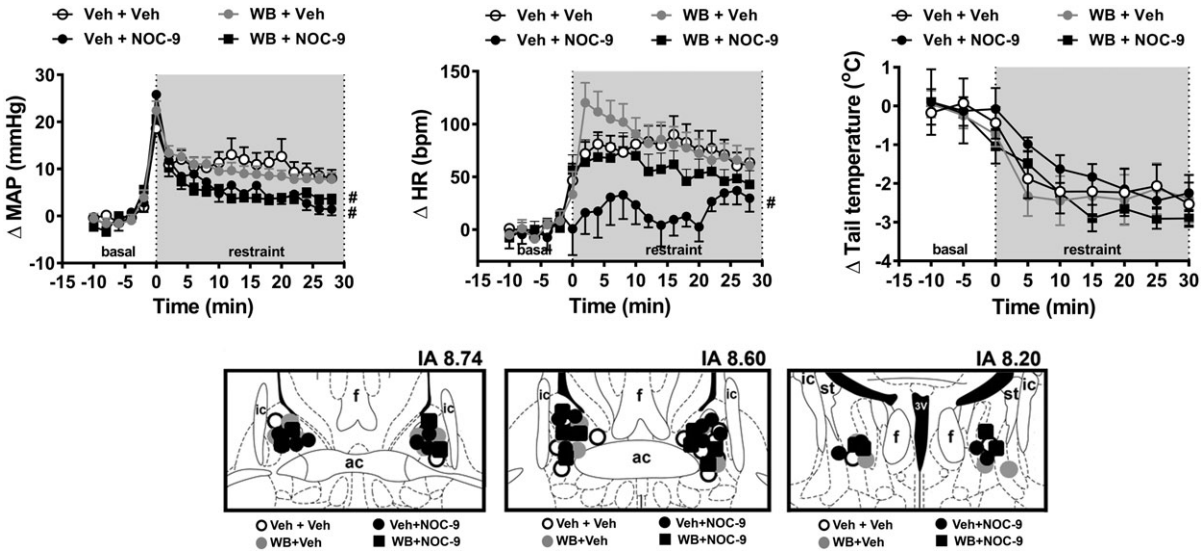

Figure 1

(upper Figure) Effect of BNST treatment with the selective α_1-adrenoceptor antagonist WB4101 (WB) and/or the NO donor NOC-9 on cardiovascular responses to acute restraint stress. Time course curves of change on MAP (ΔMAP), HR (ΔHR) and tail skin temperature (Δ tail temperature) evoked by acute restraint stress in animals treated bilaterally into the BNST with either Veh + Veh ($n = 9$), WB + Veh ($n = 8$), Veh + NOC-9 ($n = 9$) or WB + NOC-9 ($n = 8$). Shaded area indicates the period of restraint. Data shown are means ± SEM. $^{\#}P < 0.05$, significantly different from the Veh + Veh group, over the whole restraint period; two-way ANOVA followed by Bonferroni *post hoc* test. (lower Figure) Diagrammatic representation based on the rat brain atlas of Paxinos and Watson (1997) indicating the microinjection sites into the BNST of the groups Veh + Veh, WB + Veh, Veh + NOC-9 and WB + NOC-9. 3V, third ventricle; ac, anterior commissure; f, fornix; IA, interaural coordinate; ic, internal capsule; st, stria terminalis.

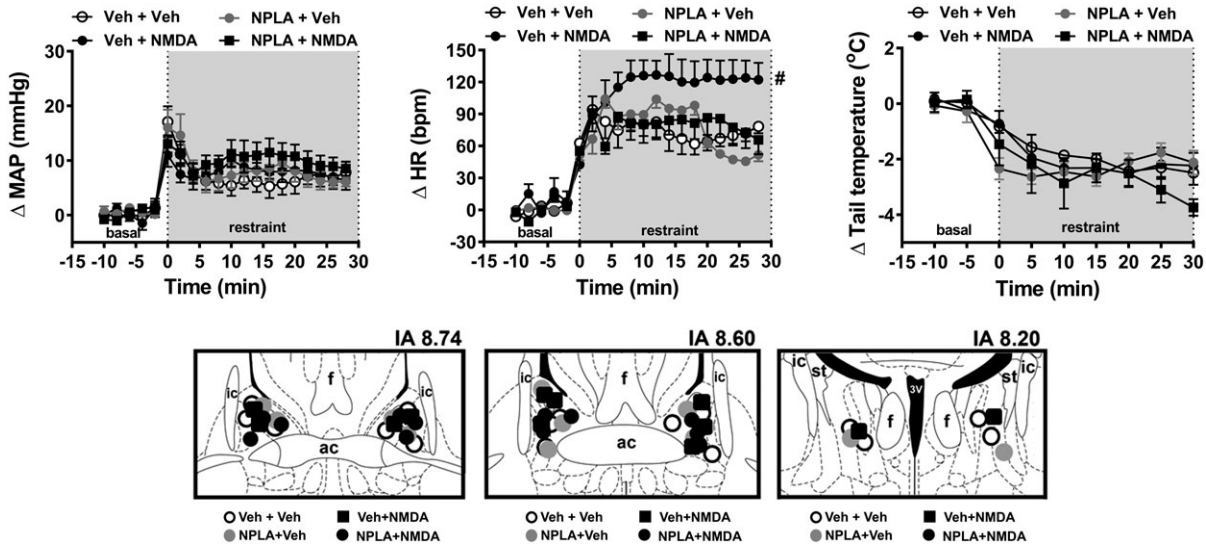

Figure 2

(upper Figure) Effect of BNST treatment with the selective nNOS inhibitor NPLA and/or the glutamatergic agonist NMDA on cardiovascular responses to acute restraint stress. Time course curves of change on MAP (ΔMAP), HR (ΔHR) and tail skin temperature (Δ tail temperature) evoked by acute restraint stress in animals treated bilaterally into the BNST with either Veh + Veh ($n = 8$), NPLA+Veh ($n = 6$), Veh + NMDA ($n = 6$) or NPLA+NMDA ($n = 6$). Shaded area indicates the period of restraint. Data shown are means ± SEM. $^{\#}P < 0.05$, significantly different from the Veh + Veh group over the whole restraint period; two-way ANOVA followed by Bonferroni *post hoc* test. (lower Figure) Diagrammatic representation based on the rat brain atlas of Paxinos and Watson (1997) indicating the microinjection sites into the BNST of the groups Veh + Veh, NPLA+Veh, Veh + NMDA and NPLA+NMDA. 3V, third ventricle; ac, anterior commissure; f, fornix; IA, interaural coordinate; ic, internal capsule; st, stria terminalis.

of pharmacological treatments of the BNST on restraint-evoked tachycardia, but not in MAP and tail skin temperature responses (Figure 2). Analysis also indicated a treatment × time interaction for HR and MAP, but not for and skin temperature. *Post hoc* analysis did not reveal an effect of NPLA given alone (NPLA+veh group) on any of the parameters analysed (Figure 2). However, bilateral microinjection of NMDA into the BNST (veh + NMDA group) enhanced the restraint-evoked tachycardia (Figure 2) and this effect of NMDA on HR was not observed in animals pretreated with NPLA into the BNST (NPLA+NMDA group) (Figure 2). Figure 2 presents diagrammatic representations showing microinjection sites into the BNST of all animals used in this protocol.

Effect of bilateral microinjection of NPLA in the BNST in cardiovascular responses to acute restraint stress in rats pretreated with WB4101 in the BNST

Bilateral treatment of the BNST with the selective nNOS inhibitor NPLA in animals locally pretreated with the selective α_1-adrenoceptor antagonist WB4101 did not affect baseline values of either MAP, HR or tail skin temperature (Table 1). Nevertheless, acute restraint stress evoked a sustained increase in both MAP and HR and decreased the tail skin temperature (Figure 3). Two-way ANOVA analysis indicated that bilateral microinjection of NPLA into the BNST of animals locally pretreated with WB4101 decreased the restraint-evoked increase in MAP and HR, but without affecting the tail skin temperature (Figure 3). The analysis also indicated a treatment × time interaction for MAP and HR, but not for tail skin temperature. Diagrammatic representations showing microinjection sites into the BNST of all animals used in this protocol are presented in Figure 3.

Discussion

This study shows for the first time an interaction of nitrergic neurotransmission within the BNST with local glutamatergic and noradrenergic neurotransmission. Furthermore, the present results are the first to indicate a dual role of nitrergic neurotransmission in the BNST in the control of cardiovascular responses evoked by aversive threats. Indeed, we observed that bilateral microinjection of the NO donor NOC-9 into the BNST decreased the restraint-evoked increase in HR and arterial pressure. The inhibitory influence of NOC-9 in HR, but not in arterial pressure, was inhibited by local BNST pretreatment with the selective α_1-adrenoceptor antagonist WB4101. Moreover, microinjection of NMDA into the BNST enhanced the tachycardic response to restraint, and this effect was abolished by local pretreatment with the selective nNOS inhibitor NPLA. An involvement of nNOS in the BNST in local facilitatory control of cardiovascular responses to restraint stress was further reinforced by demonstration that microinjection of the nNOS inhibitor in animals pretreated with the α_1-adrenoceptor antagonist (the local neurochemical mechanism related to the inhibitory influence of nNOS) decreased the restraint-evoked increase in HR and arterial pressure.

Recent results from our group have shown an involvement of nitrergic signalling in the BNST in the control of

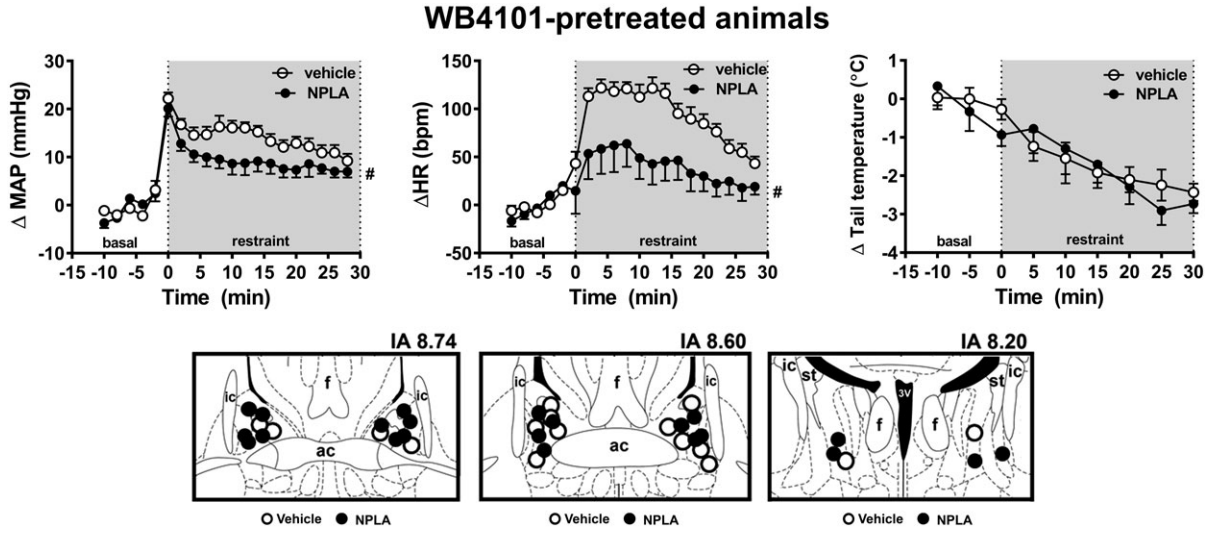

Figure 3

(upper Figure) Effect of BNST treatment with the selective nNOS inhibitor NPLA on cardiovascular responses to acute restraint stress in rats pretreated with the selective α_1-adrenoceptor antagonist WB4101 into the BNST. Time course curves of change on MAP (ΔMAP), HR (ΔHR) and tail skin temperature (Δ tail temperature) evoked by acute restraint stress in animals locally pretreated with WB4101 and subjected to bilateral microinjection into the BNST of either NPLA ($n = 11$) or vehicle ($n = 8$). Shaded area indicates the period of restraint. Data shown are means ± SEM. $^{\#}P < 0.05$, significantly different from the Veh + Veh group, over the whole restraint period; two-way ANOVA followed by Bonferroni *post hoc* test. (lower Figure) Diagrammatic representation based on the rat brain atlas of Paxinos and Watson (1997) indicating the microinjection sites into the BNST of vehicle and NPLA in animals pretreated locally with WB4101. 3V, third ventricle; ac, anterior commissure; f, fornix; IA, interaural coordinate; ic, internal capsule; st, stria terminalis.

cardiovascular responses to restraint stress (Barretto-de-Souza *et al*., 2018), as microinjection of either a nonselective NOS inhibitor or a selective nNOS inhibitor into the BNST enhanced the restraint-evoked HR increase. Local BNST treatment with inhibitors of signalling mechanisms related to NO effects such as sGC and PKG also enhanced HR response to restraint. Taken together, these results provided evidence of an inhibitory role of nitrergic signalling within the BNST in the cardiovascular responses to restraint stress. The decreased HR and arterial pressure responses reported in the present study in animals that received NOC-9 administration into the BNST provides further evidence of a predominantly inhibitory role of nitrergic neurotransmission in the BNST in the cardiovascular responses evoked by restraint. However, more importantly, the present findings indicate that the inhibitory control of HR response by nitrergic signalling in the BNST is mediated by a facilitation of local noradrenergic neurotransmission acting *via* local α_1-adrenoceptors. These data are also compatible with previous evidence that noradrenergic neurotransmission in the BNST, acting *via* α_1-adrenoceptors plays an inhibitory role in restraint-evoked tachycardia (Crestani *et al*., 2009). Moreover, our results are supported by reports that NO stimulated the release of noradrenaline in limbic structures such as the hippocampus and cerebral cortex (Ohkuma and Katsura, 2001; Philippu, 2016; Prast and Philippu, 2001). However, to the best of our knowledge, the present findings constitute the first evidence of an interaction between nitrergic and noradrenergic neurotransmissions within the BNST.

The decrease in arterial pressure response evoked by NOC-9 was not affected by local α_1-adrenoceptor antagonism. This finding is in line with a previous report that BNST noradrenergic neurotransmission plays an inhibitory role in tachycardia, without affecting the arterial pressure in response to restraint (Crestani *et al*., 2009). Therefore, the modulation of restraint-evoked pressor response by nitrergic neurotransmission in the BNST seems to be mediated by mechanisms other than local noradrenergic neurotransmission. One possible option is cholinergic neurotransmission, as activation of muscarinic receptors in the BNST inhibited the cardiovascular responses to restraint stress (Gouveia *et al*., 2016). Although there are reports of an NO-induced release of acetylcholine in limbic structures such as the hippocampus and prefrontal cortex (Ohkuma and Katsura, 2001; Prast and Philippu, 2001), a possible interaction between nitrergic and cholinergic neurotransmissions within the BNST has never been evaluated. Therefore, further studies are needed to clarify the mechanisms related to the inhibitory influence of nitrergic neurotransmission in the BNST in the pressor response to restraint stress.

Activation of nNOS in response to influx of Ca^{2+} following activation of the NMDA receptor is the best characterized mechanism underlying NO synthesis in the CNS (Garthwaite, 2008; Garthwaite, 2016; Prast and Philippu, 2001). Nevertheless, contrary to the inhibitory influence of nitrergic neurotransmission in restraint-evoked cardiovascular responses, we recently reported a facilitation of the HR response to restraint by NMDA receptors in the BNST (Adami *et al*., 2017). The enhanced tachycardia reported in the present study following microinjection of NMDA into the BNST further reinforces the idea of a facilitatory role of local glutamatergic neurotransmission in cardiac responses to restraint stress. However, the present results provide the first evidence of a role of local nitrergic signalling in the

control of stress-evoked cardiovascular responses by NMDA receptors within the BNST. This finding is in line with previous evidence of a prominent role of nNOS activation in the control of cardiovascular function by the NMDA receptor in other CNS structures (Busnardo *et al.*, 2010; Martins-Pinge *et al.*, 2007; Resstel and Correa, 2006; Santini *et al.*, 2013; Tavares *et al.*, 2007).

Although the present findings indicate nNOS as part of local signalling pathways related to a facilitatory influence in cardiovascular responses to stress, this is not the predominant role of nitrergic neurotransmission. Indeed, as discussed above, the microinjection of either nNOS inhibitors (Barretto-de-Souza *et al.*, 2018) or NO donors (see Figure 1) provides evidence of a predominantly inhibitory role of this neurochemical mechanism within the BNST. However, we observed in the present study that the microinjection of the nNOS inhibitor, in the absence of the local neurochemical mechanism related to the inhibitory influence of nNOS (i.e. noradrenergic neurotransmission acting *via* α_1-adrenoceptors) unmasked the facilitatory role of nNOS, as shown by the decreased arterial pressure and HR responses to restraint (see Figure 3). This finding confirms that nNOS may act within the BNST to counteract the predominantly inhibitory influence.

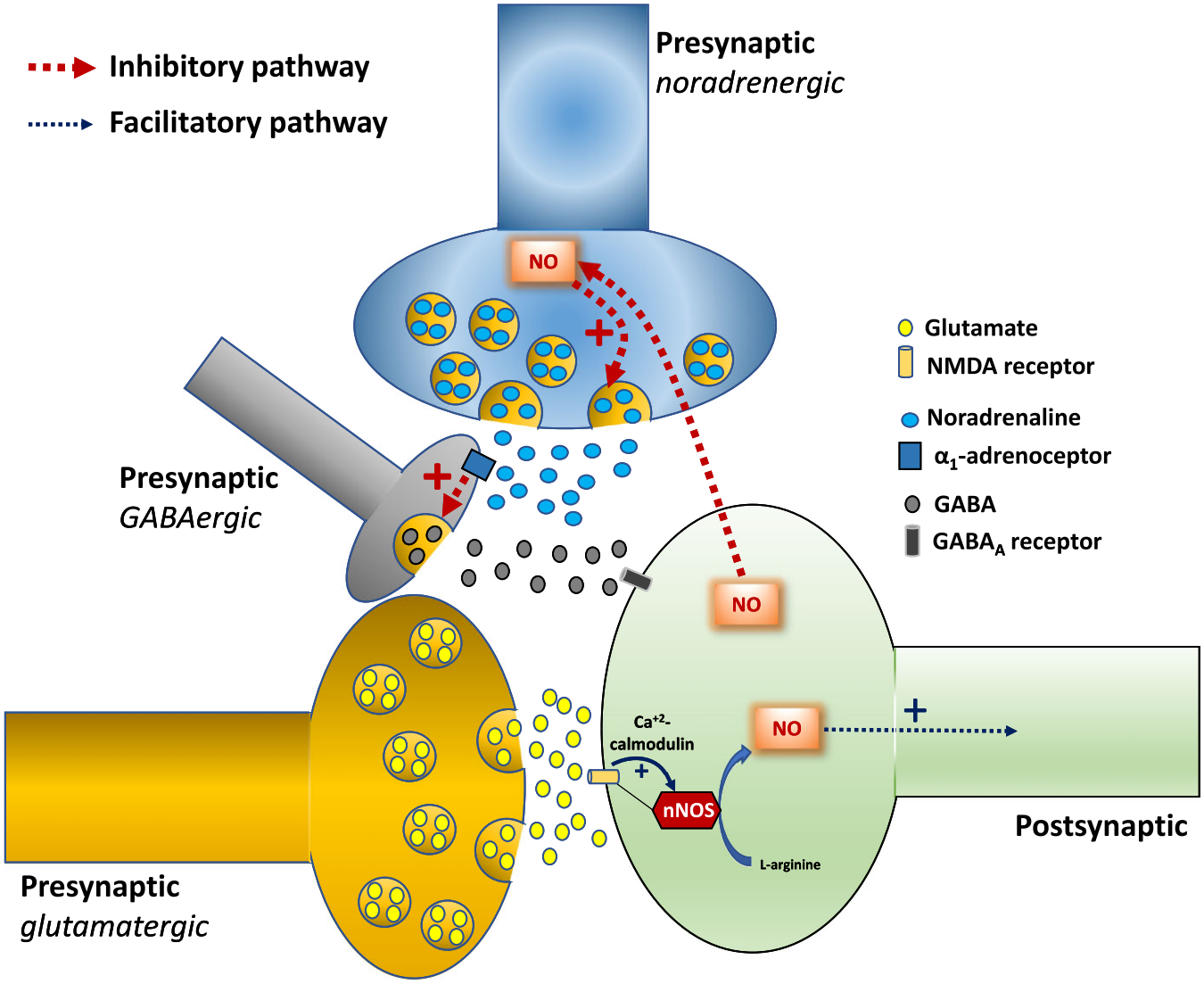

Figure 4

Schematic representation illustrating the local mechanisms by which NO released from nNOS into the BNST modulates the cardiovascular responses to restraint stress. (Inhibitory mechanism) The red dotted arrows indicate the pathway related to the predominant inhibitory influence of BNST nitrergic neurotransmission in the control of cardiovascular responses to restraint. The NO synthesized by nNOS stimulates the release of noradrenaline which in turn facilitates local GABA release *via* activation of α_1-adrenoceptors in GABAergic terminals. The GABA evokes inhibitory postsynaptic currents (IPSCs) *via* activation of the $GABA_A$ receptor. (Facilitatory mechanism) The blue arrows indicate the facilitatory pathway of nitrergic neurotransmission in the BNST in controlling the cardiovascular responses to restraint stress. The NO synthesized by nNOS is a prominent signalling mechanism involved in the effects of NMDA receptor activation (yellow channel). The thickness of the arrows indicates the predominance of the pathways (i.e. inhibitory and facilitatory mechanism). See text for further details.

The predominantly inhibitory influence of nitrergic neurotransmission in the BNST corroborates reports of protective cardiovascular effects of nitrergic neurotransmission in the CNS (Martins-Pinge *et al.*, 2012; Sharma and Patel, 2017; Stern *et al.*, 2003). Central networks, providing inhinbitory control of cardiovascular responses during aversive threats, are important as they allow precise response control, fine tuning and functional state stabilization of the target organ, thus reducing the amplitude of the response (Berntson *et al.*, 1991; Paton *et al.*, 2005). Therefore, nNOS activation within the BNST may be an important mechanism counteracting excessive cardiac activation during stress. The nNOS involvement in the facilitatory control of restraint-evoked cardiovascular responses, mediated by NMDA receptors, is in line with previous findings of the pro-aversive effects of NO in the brain (Calixto *et al.*, 2008; Guimaraes *et al.*, 2005; Silva *et al.*, 2012).

Corticolimbic structures such as the hippocampus, amygdala and medial prefrontal cortex have little direct anatomical connections with primary stress effector regions in hypothalamus and brainstem (Myers, 2017; Ulrich-Lai and Herman, 2009). However, outputs from these regions converge on the BNST (Dong *et al.*, 2001a; Myers *et al.*, 2014) and, in turn, BNST neurons project to hypothalamic and brainstem nuclei controlling autonomic activity (Dong *et al.*, 2001b; Dong and Swanson, 2006). Thus, the BNST has been proposed as a relay station between processing of emotional information by corticolimbic structures, and elaboration of physiological and behavioural responses to stress by hypothalamic and brainstem regions (Crestani *et al.*, 2013; Myers, 2017; Ulrich-Lai and Herman, 2009). Limbic inputs to the BNST are predominantly glutamatergic and GABAergic (Myers *et al.*, 2014). In this context, the present study provides the first evidence of an involvement of nNOS activation in the control of stress responses by glutamatergic inputs in the BNST acting *via* NMDA receptors. However, the present study provides evidence that the nitrergic neurotransmission in the BNST acts predominantly by modulating information from noradrenergic inputs within the BNST. An interaction between noradrenergic and glutamatergic neurotransmissions within the BNST has been reported (Silberman and Winder, 2013), thus supporting a prominent role of nitrergic neurotransmission acting *via* facilitation of noradrenergic neurotransmission in the processing of limbic information in the BNST for elaboration of cardiovascular responses.

The opposing roles of nitrergic neurotransmission in the BNST in the control of stress-evoked cardiovascular changes acting *via* modulation of local noradrenergic (inhibitory) and glutamatergic (stimulatory) are supported by earlier findings that noradrenaline evoked predominantly an inhibitory influence in activity of neurons within the BNST (Casada and Dafny, 1993). Accordingly, activation of local α_1-adrenoceptors inhibited the activity of neurons within the BNST by presynaptically facilitating local GABAergic neutransmission (Dumont and Williams, 2004). Therefore, the predominant inhibitory role of NO in cardiovascular changes to stress is possibly mediated by a facilitation of local noradrenergic neurotransmission, which in turn facilitates local GABAergic terminals *via* activation of α_1-adrenoceptors. A schematic representation outlining the mechanism by which NO within the BNST inhibits (*via* noradrenergic interaction) and facilitates (*via* glutamatergic interaction) the restraint-evoked cardiovascular responses is presented in Figure 4.

In summary, the results of the present study provide evidence that nitrergic neurotransmission in the BNST plays a dual role in the cardiovascular responses to stress. A predominantly inhibitory influence in HR responses is mediated by a facilitation of local noradrenergic neurotransmission acting *via* α_1-adrenoceptors. However, inhibition of arterial pressure response seems to be mediated by mechanisms others than local noradrenergic neurotransmission. Furthermore, our data suggest that local NO release from nNOS is part of the signalling pathway related to the facilitatory control of HR response to restraint stress by NMDA receptors within the BNST.

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Author contributions

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

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Figure S1 Photomicrograph of a coronal brain section depicting bilateral microinjection sites in the bed nucleus of the stria terminalis (BNST) of a representative animal. Arrows indicate the microinjection sites. ac – anterior commissure; cc – corpus callosum; f – fornix; LV – lateral ventricle.