



Excitatory Amino Acid Receptors Mediate Asymmetry and Lateralization in the Descending Cardiovascular Pathways from the Dorsomedial Hypothalamus

Carlos Henrique Xavier^{1,2*}, Danielle Ianzer², Augusto Martins Lima², Fernanda Ribeiro Marins², Gustavo Rodrigues Pedrino¹, Gisele Vaz², Gustavo Batista Menezes⁴, Eugene Nalivaiko³, Marco Antônio Peliky Fontes²

1 Laboratório de Fisiologia e Terapêutica Cardiovascular, Departamento Ciências Fisiológicas, Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, GO, Brazil, **2** Departamento de Fisiologia e Biofísica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil, **3** School of Biomedical Sciences and Pharmacy, University of Newcastle, Newcastle, NSW, Australia, **4** Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

Abstract

The dorsomedial hypothalamus (DMH) and lateral/dorsolateral periaqueductal gray (PAG) are anatomically and functionally connected. Both the DMH and PAG depend on glutamatergic inputs for activation. We recently reported that removal of GABA-ergic tone in the unilateral DMH produces: *asymmetry*, that is, a right- (R-) sided predominance in cardiac chronotropism, and *lateralization*, that is, a greater increase in ipsilateral renal sympathetic activity (RSNA). In the current study, we investigated whether excitatory amino acid (EAA) receptors in the DMH–PAG pathway contribute to the functional interhemispheric difference. In urethane (1.2 to 1.4 g/kg, i.p.) anesthetized rats, we observed that: (i) nanoinjections of N-methyl D-aspartate (NMDA 100 pmol/100 nl) into the unilateral DMH produced the same right-sided predominance in the control of cardiac chronotropy, (ii) nanoinjections of NMDA into the ipsilateral DMH or PAG evoked lateralized RSNA responses, and (iii) blockade of EAA receptors in the unilateral DMH attenuated the cardiovascular responses evoked by injection of NMDA into either the R- or left- (L-) PAG. In awake rats, nanoinjection of kynurenic acid (1 nmol/100 nL) into the L-DMH or R- or L-PAG attenuated the tachycardia evoked by air stress. However, the magnitude of stress-evoked tachycardia was smallest when the EAA receptors of the R-DMH were blocked. We conclude that EAA receptors contribute to the right-sided predominance in cardiac chronotropism. This interhemispheric difference that involves EAA receptors was observed in the DMH but not in the PAG.

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* Email: carlosxavier@ufg.br

Introduction

Asymmetries are the left-right differences in the properties of central nervous system and are found at different levels of the neuraxis [1,2]. There is evidence for interhemispheric specialization of autonomic control in humans [3,4]. During stress and emotional arousal, most brain regions are influenced asymmetrically, which unbalances sympathetic outflow to the heart and improves the chances of cardiac ectopies [5,6].

Studies have highlighted the involvement of diencephalic and mesencephalic regions in the organization of physiological responses to stress [for review, see [7,8]]. Anatomic and functional data have clearly revealed that the hypothalamus and the lateral/dorsolateral regions of the periaqueductal gray (PAG) are connected [9–17].

The dorsomedial hypothalamus (DMH) region plays a key role in organizing physiological responses to emotional stress [18]. DMH neurons are excited by nanoinjections of either GABA_A antagonists or excitatory amino acid (EAA) agonists [8]. The

inhibition of DMH neurons attenuates the responses evoked by exposure to emotional stress [19]. Inversely, DMH disinhibition via the blockade of local GABA_A receptors produces a series of effects that mimic stress-related physiological responses, such as behavioral changes, thermogenesis, tachycardia and the pressor response (for review, see [8]). Previous studies have demonstrated that this selective GABA-ergic blockade results in DMH activation that allows for a predominant local excitatory input [20,21]. These findings suggest that both phenomena should happen simultaneously to produce the aforementioned responses.

The PAG is thought to be an exit synaptic relay for defensive reactions [22,23]. Similar to the DMH, the PAG also mediates physiological responses to stress [7,24]. The PAG is responsive to efferent excitatory inputs [25] from higher regions [26], such as the DMH [12]. Activation of the PAG with the excitatory amino acid (EAA) agonist N-methyl D-aspartate (NMDA), especially in the lateral and dorsolateral columns, evokes behavioral changes and sympathetically mediated increases in body temperature, heart rate (HR) and arterial pressure [12,13]. Recent studies have

revealed a functional projection between the DMH and the PAG [12,13,27]. The tachycardia evoked by the unilateral DMH depends on EAA receptors in the PAG, particularly NMDA receptors [12]. In addition to the fact that DMH-evoked responses relays in PAG [8], the activity of DMH neurons is similarly required for PAG-evoked responses [13,27]. In this regard, DMH-PAG connections seem to be a bilateral excitatory pathway that allows setting specific responses.

We have previously reported that the cardiovascular responses evoked by nanoinjections of a GABA_A antagonist into unilateral (left or right) DMH have the following characteristics: i) asymmetry – the right DMH drives full cardiac performance (chronotropy and inotropy) and tail vasoconstriction; and ii) lateralization – either side of the DMH seems to prominently exert autonomic control over the ipsilateral hemibody [18,28,29]. Thus, the question of whether excitatory input contributes to the asymmetry and lateralization at the level of the DMH and PAG arises. In the present study, we compared the cardiovascular responses evoked by activation and blockade of EAA receptors in the right and left sides of the DMH and PAG. A portion of our results have been presented in abstract form [30].

Results

Photomicrographs of coronal sections of the DMH and PAG exemplifying injection sites targeted in our experiments are, such as an schematic drawing on the injections sites obtained in all experiments are shown in Figure S1. The baseline values that were obtained before the initiation of the experiments are shown in table 1. There were no differences in the baseline values that were sampled prior to the administration of the central nanoinjections of each experimental series.

Experiment 1. Unilateral activation of the DMH and PAG increased heart rate, blood pressure and renal sympathetic activity

The results obtained and typical examples are grouped in figures 1 and 2. Nanoinjection of NMDA into the unilateral DMH (Figure 1) and PAG (Figure 2) positively affected autonomic and cardiovascular parameters. These effects were evident one minute after the nanoinjections and continued for approximately 5 minutes. We observed mean arterial pressure (MAP) increases after unilateral injections of NMDA into the DMH or PAG (Figures 1B and 2B; $P < 0.05$). However no differences were found in the ranges of these responses when nanoinjections into the left or right side within the same experimental group were compared (Δ MAP DMH: Right = 13 ± 2 vs. Left side = 14 ± 3 ; PAG: Right = 16 ± 3 vs. Left side = 18 ± 4 mmHg; Figures 2B and 3B).

Although unilateral nanoinjections of NMDA into either side of the DMH or PAG increased RSNA as sampled in the left renal nerve, greater responses were found after injections into the left DMH and PAG; i.e., greater responses were observed after injections that were ipsilateral to the side in which RSNA was sampled (Δ RSNA DMH: Right = 30 ± 7 vs. Left side = 66 ± 13 ; PAG: Right = 26 ± 3 vs. Left side = $92 \pm 14\%$; $P < 0.05$; Figures 1A and 2A). Nanoinjections into the DMH and PAG also increased HR ($P < 0.05$ vs. baseline; Figures 1C and 2C), but injections into the Right-DMH provoked greater chronotropy effect (Δ HR DMH: Right = 59 ± 5 vs. Left side = 28 ± 4 bpm; $P < 0.05$; Figure 1C). The tachycardia evoked by nanoinjections of NMDA into the ipsilateral PAG was similar to that observed after injections into the contralateral side (Δ HR PAG: Right = 39 ± 6 vs. Left side = 45 ± 6 bpm; Figure 2C).

Table 1. Baseline values of mean arterial pressure (MAP) and heart rate (HR) collected before nanoinjections into unilateral DMH or PAG.

| | Anesthetized | | | | Non-Anesthetized | | | |
|------------|--------------|-------------|-------------|--------------|------------------|-------------|--------------|-------------|
| | NMDA DMH | | NMDA PAG | | Kyn DMH | | Kyn PAG | |
| | Left | Right | Left | Right | Left | Right | Left | Right |
| MAP (mmHg) | 93 ± 3 | 94 ± 2 | 96 ± 6 | 98 ± 7 | 102 ± 3 | 105 ± 5 | 106 ± 4 | 108 ± 8 |
| HR (bpm) | 319 ± 13 | 325 ± 4 | 353 ± 9 | 357 ± 10 | 321 ± 8 | 334 ± 9 | 346 ± 13 | 333 ± 9 |

There were no differences between basal values found before nanoinjections between L- and R- within each experimental series.
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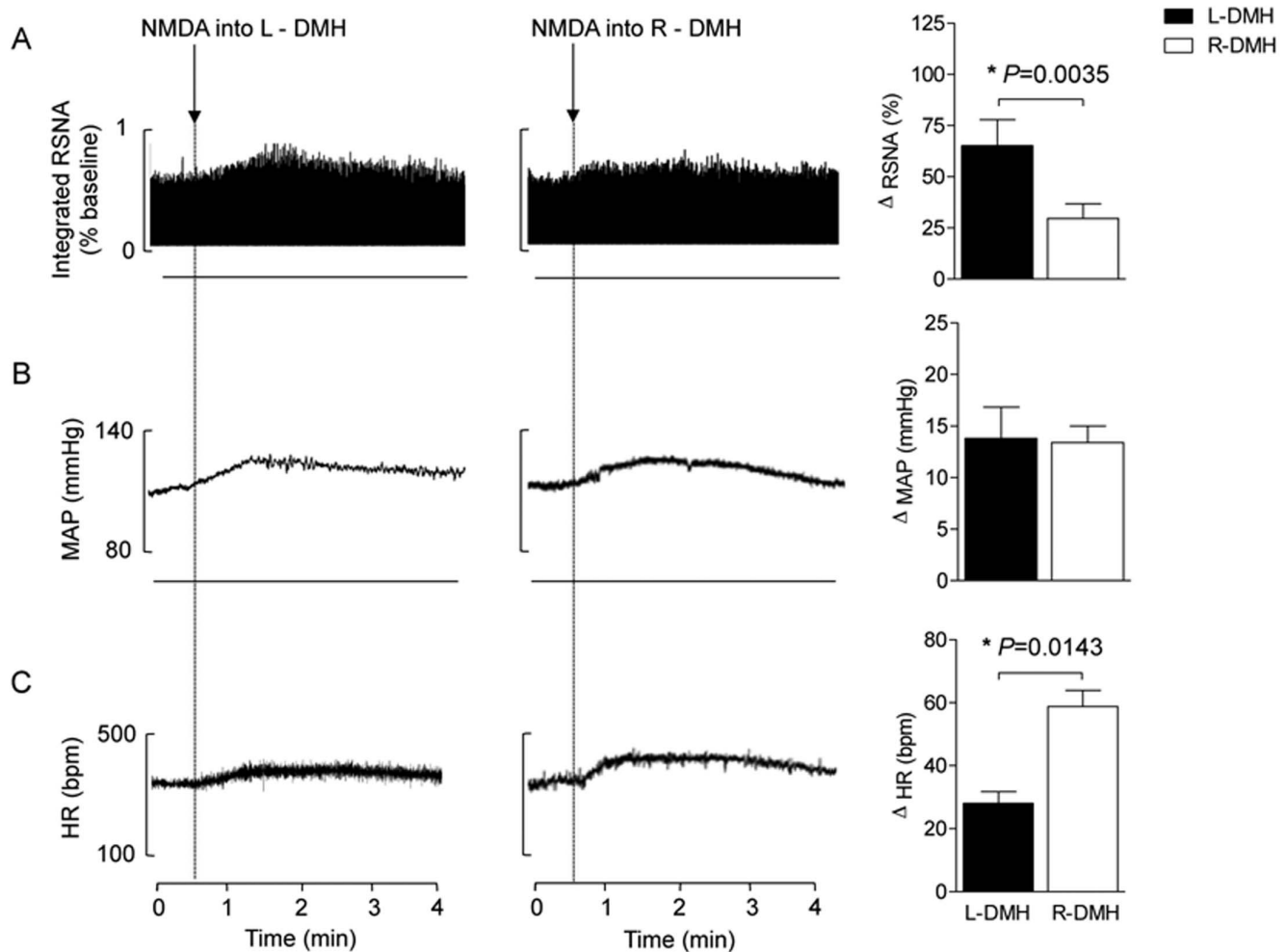


Figure 1. Representative chart records and changes in RSNA (A), MAP (B) and HR (C) evoked by the nanoinjection of NMDA (100 pmol/100 nl) into the right (white bars) and left (black bars) DMH in the first experimental series (n=5). * $P<0.05$ Right- vs. Left-DMH (Mann Whitney non-parametric test). doi:10.1371/journal.pone.0112412.g001

Experiment 2. EAA receptors in the unilateral DMH contributed to the HR, MAP and RSNA responses evoked from the PAG

In contrast to the observations of experiment 1, blockade of the EAA receptors in the left DMH completely abolished the increases in RSNA, MAP and HR that were evoked by injections of NMDA into either the ipsilateral or contralateral PAG (Δ RSNA: Right = -1 ± 2 vs. Left side = $3 \pm 5\%$; Δ MAP: Right = 1 ± 2 vs. Left side = 2 ± 1 mmHg; and Δ HR: Right = 3 ± 5 vs. Left side = 4 ± 1 bpm, Figure 3). We found no differences in asymmetry or lateralization in the control of HR or RSNA.

Experiment 3. EAA receptors in the unilateral DMH and PAG are involved in stress-induced tachycardic and pressor responses in non-anaesthetized rats

Figure 4 (panels A and C) shows the time courses of the responses over the entire durations of the experiments. Panels B and D show the peak changes in HR and MAP that were observed during stress exposure. Compared to the amplitudes of the control responses (bilateral vehicle bars – Figure 4B), nanoinjections of kynurenic acid into either the right or left-sides of the DMH or PAG attenuated the tachycardia evoked by stress. However, stress-

evoked tachycardia was dramatically reduced when the EAA receptors in the Right-DMH were blocked (Δ HR DMH: Right = 12 ± 18 vs. Left side = 87 ± 17 ; PAG: Right = 73 ± 15 vs. Left side = 74 ± 12 ; bilateral vehicle: 132 ± 9 bpm; $P<0.05$ – Figure 4B). Blockade of the EAA receptors in either the Right or Left-sides of the DMH and PAG did not alter the amplitudes of the pressor responses induced by stress (Δ MAP DMH: Right = 12 ± 4 vs. Left side = 17 ± 3 ; PAG: Right = 18 ± 4 vs. Left side = 18 ± 5 ; bilateral vehicle: 19 ± 3 mmHg – Figure 4D).

Experiment 4. Anatomic pathways between the DMH and PAG

Photomicrographs depicting the injection sites of retrobeads into the right DMH and left PAG and labeled neurons are shown in Figure 5A and B. In the left DMH, neurons labeled with red beads that were originally injected into the left PAG were observed. Similarly, in the right PAG, neurons labeled green with beads injected into the right DMH were observed.

Discussion

The major findings of this study are as follows: i) NMDA nanoinjected into the Right-DMH evoked greater cardiac

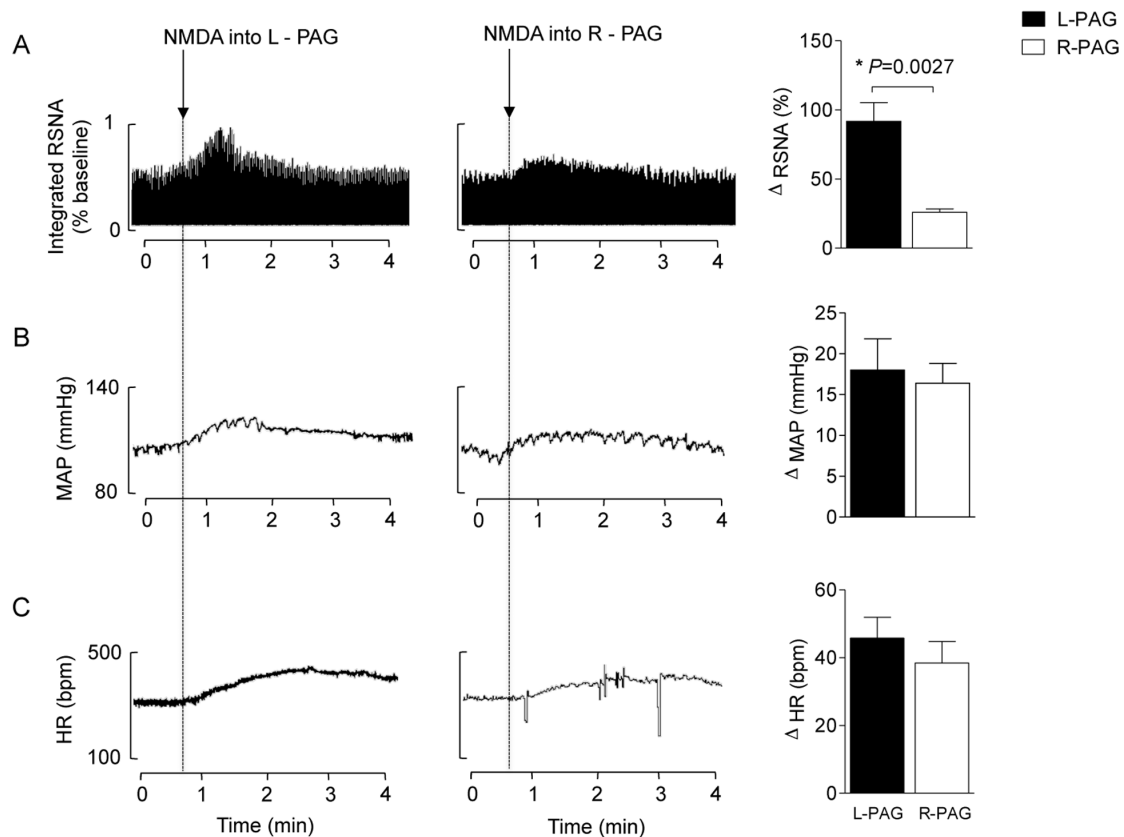


Figure 2. Representative chart records and changes in RSNA (A) (recorded in the left renal nerve), MAP (B) and HR (C) evoked by nanoinjections of NMDA (100 pmol/100 nl) into the right (white bars) and left (black bars) PAG in the first experimental series (n = 5). $*P < 0.05$ Right- vs. Left-PAG (Mann Whitney non-parametric test).
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chronotropic changes than did nanoinjections into the Left-DMH; ii) nanoinjection of NMDA into the unilateral DMH and PAG evoked lateralized increases in RSNA; and iii) blockade of EAA receptors in the right DMH nearly abolished stress-evoked tachycardia, whereas identical blockade in the Left-DMH, Left- and Right-PAG only attenuated the tachycardic response.

The present data, obtained in rats, extend previous findings showing that the right hypothalamus governs full cardiac performance [28,29,31]. We recently demonstrated that disinhibition of the Right-DMH produces greater tachycardia compared to disinhibition of the Left-DMH. Additionally, inhibition of the Right-DMH nearly abolished stress-evoked tachycardia [28]. The removal of GABA-ergic tone (disinhibition) implicates in predominant local excitatory input to the DMH, mainly by recruiting inotropic glutamate receptors [20,21], which changes the sympathetic output [8] and regional blood flow to different target organs [28,32]. The present study demonstrates that this asymmetric cardiac control by GABA_A receptors also involves EAA receptors in the DMH. Together with the greater positive chronotropy that is evoked by NMDA injections into Right-DMH, the lack of cardiac reactivity during the stress trial under the blockade of EAA receptors in the right side highlights the involvement of excitatory input in DMH asymmetry. Whether these differential cardiac responses are governed by the amygdala [33] or other sources of excitatory input to the DMH [34] remains to be revealed.

Newly described findings suggest that the greater chronotropic response that is evoked by removal of GABA-ergic tone in the Right-DMH [28] may involve differential glutamatergic inputs.

Several mechanisms could account for this difference. One possibility is that this functional asymmetry results from an unknown characteristic of the DMH neurons in the right side, which may be determined by differences in electrophysiological properties or the densities of glutamatergic receptors. It is also important to consider other possible functional and neuroanatomical differences between the right and left pathways that descend from the DMH, including relays other than the PAG, and the organization of sympathetic projections to the heart. Because the DMH projects and provides glutamatergic outputs to other nuclei that are involved in the control of cardiovascular responses, such as rostral ventrolateral medulla (RVLM) [35] and raphe pallidus (RP) [36], it is plausible that these lower relays are involved. Additionally, the right and left cardiac sympathetic neurons and nerves differ in terms of function [37–40], and the stimulation of the components on the right side is more likely to evoke positive chronotropic responses [39,41,42].

We found that nanoinjection of NMDA into the unilateral DMH or PAG evoked greater responses in the ipsilateral renal nerve. This finding indicates that the control of RSNA by the DMH and PAG is lateralized. For technical reasons, RSNA was recorded in the left nerve. We recognize that simultaneous recordings of both nerves would be more appropriate; however, in a previous study, we also observed that blockade of GABA_A receptors in the DMH also evokes greater increases in the ipsilateral nerve [28]. Therefore, the current data together with data from our previous study support our hypothesis of a certain degree of hypothalamic lateralization in the control of RSNA [28].

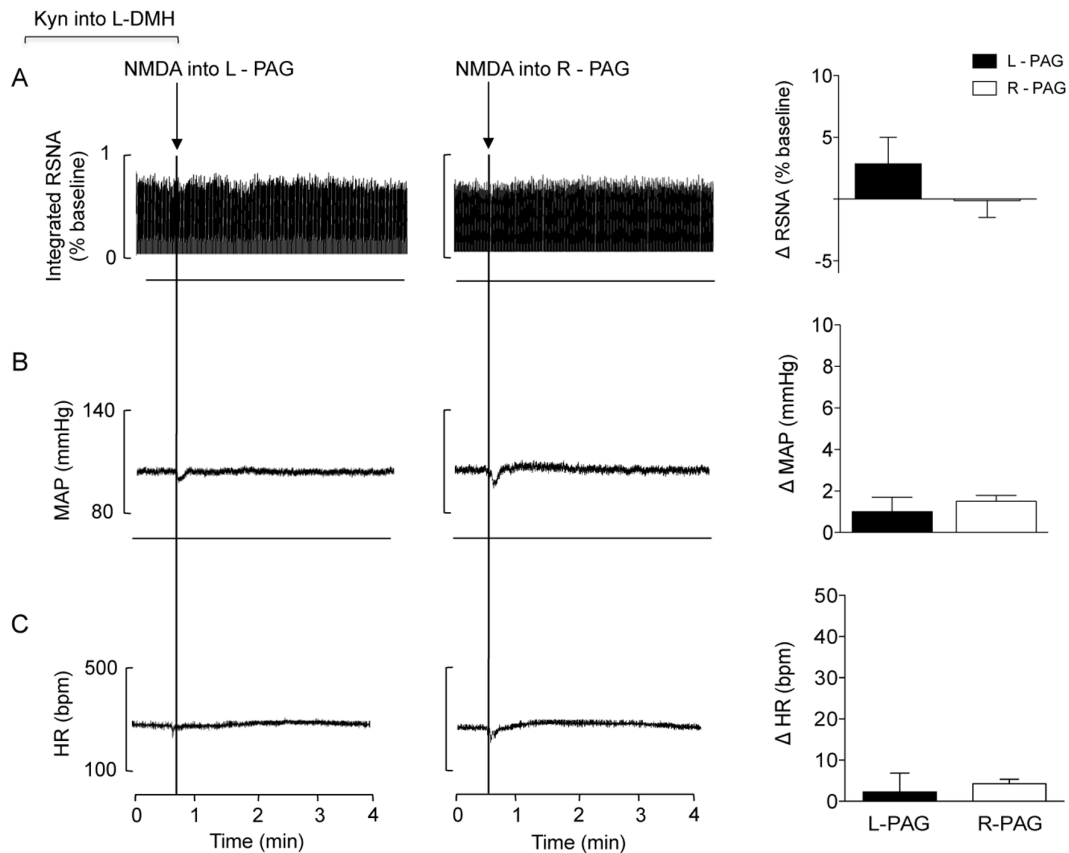


Figure 3. Representative chart records and changes in RSNA (A) (recorded in the left renal nerve), MAP (B) and HR (C) evoked by nanoinjections of NMDA (100 pmol/100 nl) into the right (white bars) and left (black bars) PAG after blockade of EAA receptors in the left DMH in the second experimental series (n = 5; Mann Whitney non-parametric test).

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In fact, substantial evidences in the literature support the existence of lateralization in the control of RSNA and other autonomic nerves [43–46]. Previous anatomical studies further support this idea of lateralization. In this regard, the descending pathways from the DMH are organized as ipsilateral mirrors [9,47]. Accordingly, Zaretskaia and colleagues clearly demonstrated the recruitment of predominantly ipsilateral relays after DMH disinhibition [48]. However, the pattern of lateralization in the autonomic control of renal nerves by PAG differed from that observed after exteroceptive stimulation in conscious rats [23]. Also, it has been reported that unilateral nanoinjection of a nitric oxide donor into the PAG recruits ipsilateral DMH neurons, which suggests that the PAG-DMH pathway underlies organized responses that may not be the consequence of generalized arousal [49]. Because the PAG is one of the descending pathways from the DMH [8,18] and the activity of DMH neurons is required for PAG-evoked responses [13,27], we suggest that the functional lateralization between the DMH and PAG composes a collateral background facilitation system for controlling RSNA. Our anatomic findings extend this idea of DMH–PAG bidirectional connection.

Inhibition of the PAG attenuated stress-induced tachycardia, as previously described [24]. In our experiments, we observed that there was no asymmetry in the cardiac control by PAG, which may indicate that EAA receptors in both sides of PAG participate equally in cardiac reactivity to stress. Also, the blockade of EAA receptors in the Left-DMH attenuated the cardiovascular responses evoked by either ipsilateral or contralateral PAG stimulation. Because the right DMH remains functioning after

the blockade of the contralateral DMH, we suggest that the PAG-DMH pathway may be involved in the organization of lateralized but not asymmetric responses. Additionally, unilateral PAG stimulation failed to reveal any cardiac dominance, which indicates that the excitatory input from the PAG is not involved in asymmetric cardiac control – as observed for the DMH. We also confirmed that the PAG indeed acts via the DMH [13,27]. However, an important question requires further investigation; i.e., whether the descending pathways from the DMH are the pathways that govern the PAG-evoked responses.

A growing body of research has reported on the interactions between the DMH and PAG in the organization of cardiovascular responses to emotional stress. Our recent results add the contribution of EAA receptors in the DMH–PAG pathway to the evidence suggesting that interhemispheric differences are capable of modifying cardiovascular control. Undoubtedly, further studies are necessary to better understand the pathways and mechanisms underlying this functional interhemispheric difference. Studying the differential autonomic responses that are organized by each side of the brain may improve the knowledge about the interindividual variability of stress-evoked responses.

Methods

4. 1 General procedures

All experiments were performed on male Wistar rats (250–320 g; 10–12 weeks) that were bred at the animal facilities of the Biological Sciences Institute (CEBIO, UFMG, Belo Horizonte,

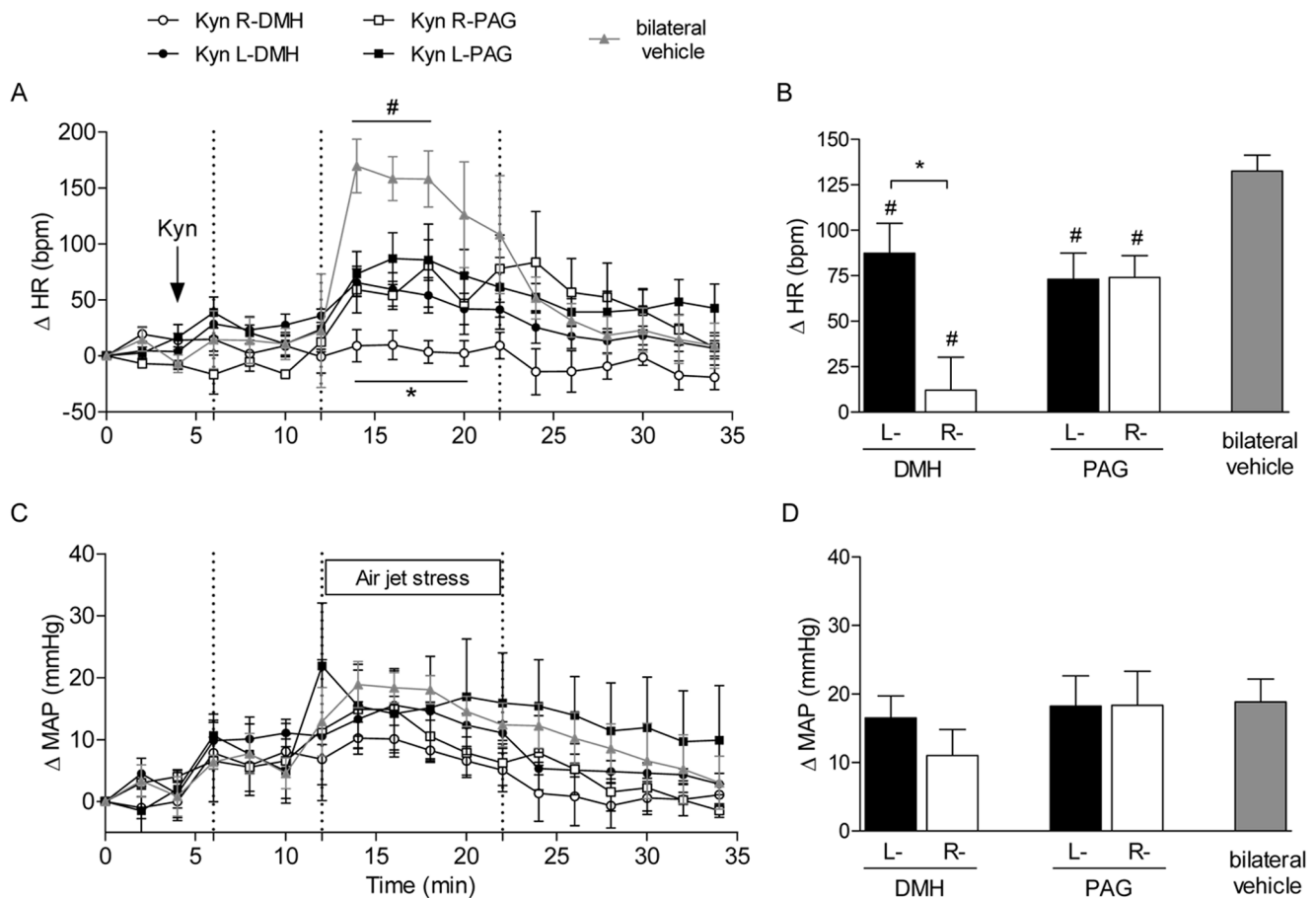


Figure 4. Results of the third experimental series. Panels A and C: time courses of HR and MAP before and after nanoinjection of kynurenic acid (1 nmol/100 nl) into the right (open circles) and left (black circles) DMH (n = 5) and right (open squares) and left (black squares) l/dl PAG (n = 5) and after bilateral vehicle nanoinjection (gray triangles; n = 10). * $P < 0.05$ Right (R-) vs. Left (L-) DMH; # $P < 0.05$ vehicle compared to R- or L-DMH and R- or L-PAG (two-way ANOVA followed by Newman Keuls post hoc tests). **Panels B and D:** peak changes in HR and MAP during the stress trials in animals that had previously been nanoinjected with kynurenic acid (1 nmol/100 nl) into right (open bars) and left (black bars) DMH or l/dl PAG and after the bilateral nanoinjection of vehicle (gray bars). * $P < 0.05$ R vs. L and # $P < 0.05$ vs. bilateral vehicle (one-way ANOVA followed by Newman-Keuls post hoc tests).
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MG, Brazil) and were conducted in accordance with the guidelines established by our local committee and in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. This study was approved by the institutional animal care and use committee – (Comitê de Ética em Experimentação Animal - CETEA/UFMG protocol number 137/06). All efforts were made to minimize the number of animals used. Animals were housed in individual home cages (47 cm×31 cm×16 cm) and had free access to food and water.

We recorded the following cardiovascular parameters in two different experimental conditions: i) in the *anesthetized* condition, we recorded HR, MAP and renal sympathetic nerve activity (RSNA); and ii) in the *non-anesthetized* condition, we recorded HR and MAP.

4.2 Experiments in anesthetized animals

General procedures. Rats were anesthetized with urethane (1.2 to 1.4 g/kg i.p.). The trachea was cannulated to maintain an open airway. The adequacy of the anesthesia was verified by the absence of a withdrawal response to nociceptive stimulation of the hindpaw. Supplemental doses of urethane (0.1 g/kg i.v.) were given when necessary. Body temperature was maintained near

37.0°C with a heating lamp. The head was positioned in a stereotaxic frame (Stoelting, Wood Dale, IL, USA) with the tooth bar fixed at −3.3 mm below the interaural line. Using a low rotation drill, small craniotomies were made for the insertion of thin-tip graduated glass pipettes into the DMH (3.2 mm posterior, 0.6 mm lateral and 8.5 mm ventral) and the PAG (7.0 mm posterior, 0.6 mm lateral and 4.8 mm ventral).

Catheters were placed into a femoral artery to record MAP and HR and into a femoral vein for supplementary anesthesia. The left renal nerve and left renal artery were isolated and covered with mineral oil. Subsequently, the renal nerve was placed on a silver bipolar electrode. The RSNA signal was amplified (10K), filtered (100–1000 Hz), displayed on an oscilloscope and monitored by via an audio amplifier. The filtered nerve activity signal was rectified, integrated (resetting every second), displayed online and acquired using Powerlab 4/20 LabChart 7.1 (ADInstruments, Sydney, Australia). All data were digitized at 1 kHz. The noise of the recording system was determined *post mortem* and subtracted from the RSNA values obtained during the experiment. After a 20-minutes stabilization period, the experiments were performed as described below.

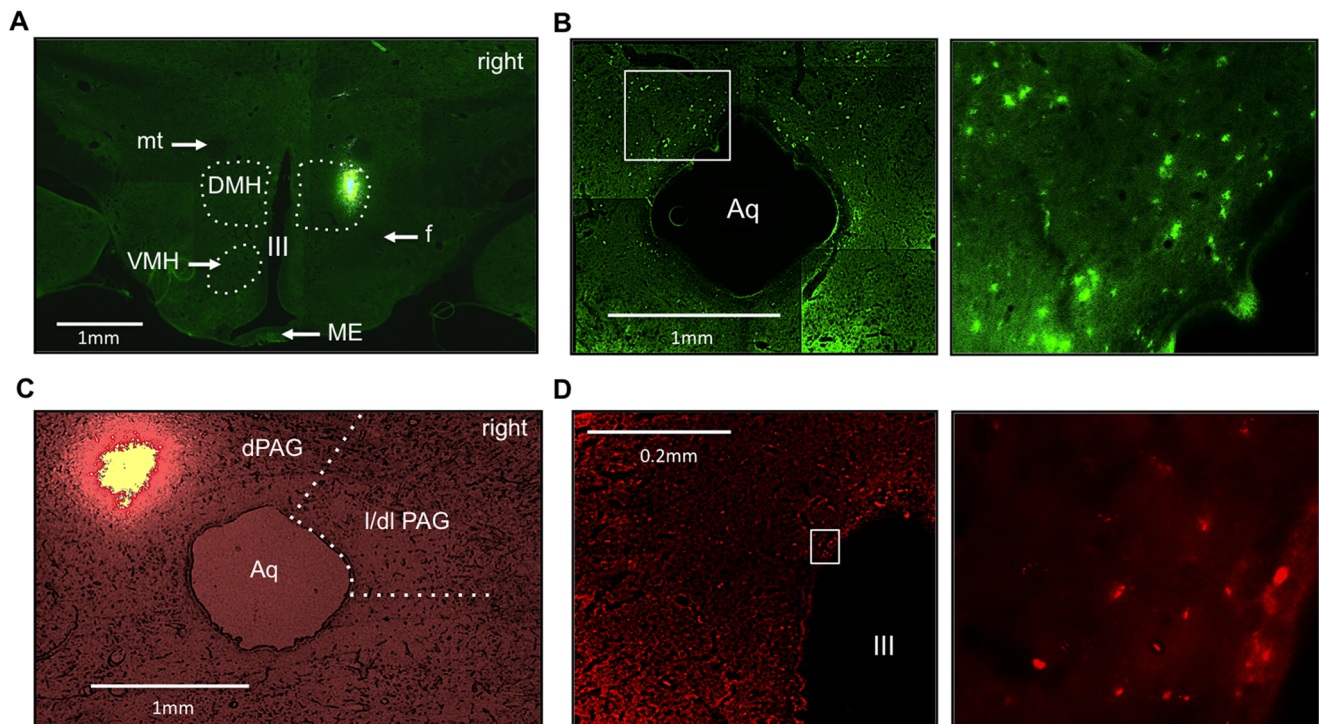


Figure 5. Photomicrographs of rat brain slices depicting spots from animals injected with green and red retrobeads at the level of (A) the DMH and (C) the PAG, respectively. Zoom view of the (B) PAG and (D) DMH neurons that were retrogradely labeled with green and red retrobeads, respectively. DMH: dorsomedial hypothalamus; ME: median eminence; mt: mammillothalamic tract; f: fornix; VMH: ventromedial hypothalamus; III: third ventricle; l/dlPAG: lateral/dorsolateral periaqueductal gray; Aq: aqueduct.

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Experiment 1. Effects of unilateral stimulation of the DMH and PAG on HR, MAP and RSNA. We evaluated the changes in HR, MAP and RSNA that were evoked by nanoinjection of NMDA (100 pmol/100 nl) into the left or right DMH and PAG. These experiments were performed in two separate groups of animals ($n=5$ each) that received nanoinjections into the DMH or PAG. The side of the first unilateral DMH or PAG injection was chosen randomly. If NMDA was injected into the R-DMH, the subsequent injection was into the L-DMH. The same strategy was adopted for nanoinjections into the PAG. Between nanoinjections, we waited a minimum of 15 minutes or until the variables returned to baseline.

Experiment 2. Contribution of the EAA receptors of the unilateral DMH to the PAG-evoked responses. Based on previous reports that examined the influence of DMH neurons on the responses evoked from the PAG [13,27], we aimed to reveal whether blockade of EAA receptors in the unilateral DMH could modify the range of the responses evoked by stimulation of either the unilateral or contralateral PAG ($n=5$). Following a 20-minutes stabilization period, we nanoinjected kynurenic Acid (Kyn, an antagonist of ionotropic AMPA, NMDA and kainate glutamate receptors; 1 nmol/100 nl) into the left DMH. After 15 minutes, we performed the first injection of NMDA (100 pmol/100 nl) into the unilateral PAG (the side was randomly chosen). Fifteen minutes later or when the variables returned to baseline, the second nanoinjection was made into the contralateral PAG.

4.3 Experiments in non-anesthetized animals

General procedures. Under tribromoethanol anesthesia (250 mg/kg i.p.), the rats were placed in a stereotaxic frame (Stoelting, Wood Dale, IL, USA) and bilateral stainless steel guide

cannulas (22 gauge, 16 mm length) targeted toward the DMH or PAG were inserted as previously described [28]. After the surgical procedures, the animals were allowed to recover in individual home cages for at least three days. Subsequently, the animals were anesthetized again with tribromoethanol (250 mg/kg i.p.), and a polyethylene catheter (0.011 ID, Clay Adams, Parsippany, NJ, USA) was inserted into a femoral artery to record MAP and HR. The catheter was routed subcutaneously to the nape of the neck where it was exteriorized and secured. Rats were then allowed to recover for 24-h before the experiments began. All animals for which data were reported remained in good health conditions throughout the course of surgical procedures and experimental protocol as assessed by appearance, behavior, and maintenance of body weight.

Experiment 3. Contributions of EAA receptors of the unilateral DMH and PAG to the stress-induced tachycardic and pressor responses. We assessed the contributions of EAA receptors in the unilateral DMH or PAG to the cardiovascular responses evoked by acute stress exposure. In one group of animals ($n=5$), bilateral guide cannulas were targeted only to the DMH. In another group of animals ($n=5$), guide cannulas were targeted only to the bilateral PAG (the coordinates are provided below). Both the DMH and PAG groups were subjected to the following experimental procedures. After 20 minutes of baseline HR and MAP monitoring, the EAA antagonist Kyn (1 nmol/100 nl) was injected unilaterally into the DMH or PAG. The rats were then placed into a plastic restrainer (60-mm inner diameter) and subjected to an air jet (10 l/min) directed toward their heads for 10 min. Subsequently, the animals remained in the restrainer for an additional 10 min. The air jet stress was repeated on 3 consecutive days. On the first day, the side of the first

nanoinjection was chosen randomly. On the second day, the nanoinjection was contralateral to the site targeted on the first day. As a control, on the third day, the stress trial was repeated after bilateral nanoinjection of vehicle (NaCl 0.9%, 100 nl) in all animals. After the surgeries, a single doses of antibiotics [Pentabiotic (Benzathine Benzilpeniciline, Sodium Benzilpeniciline, Potassium Benzilpeniciline, Procaine Benzilpeniciline and Estreptomycin) 5 mg/kg; 0.2 ml] and analgesics [Banamine Pet (Flunixin Meglumine) 1.1 mg/kg] were injected (i.m.).

4.4 Anatomic tracings

Experiment 4. Anatomic pathways between the DMH and PAG. Anesthetic procedures were as described for Experiment 2. The craniotomy procedures used to access the DMH and PAG were identical to those used in Experiment 1. The procedures for the neuronal tracings were as previously described [50]. Undiluted green and red Retrobeads (Luma Fluor) were nanoinjected (100 nl) into the right DMH and left PAG, respectively. Seven days later, the brain were removed, maintained in 4% PFA for 24 h and then transferred to a 30% sucrose solution for 48 h. Brain slices (40 μ m) containing the DMH and PAG regions were taken and mounted on silanized glass slides. Microscopic analyses were performed using excitation maximums of 460 and 530 nm and emission maximums of 505 and 590 nm for the green and red beads, respectively. Procedures for analgesia and antibiotic injections were the same described above (Experiment 3).

4.5 Injections and drugs

The following drugs were utilized in the physiological experiments: i) NMDA (100 pmol/100 nl); ii) the EAA receptor antagonist kynurenic acid (1 nmol/100 nl); and iii) vehicle (NaCl 0.9%, 100 nl). The drugs were purchased from Sigma. As control, some injections of vehicle (NaCl 0.9%) – 100 nL were performed. The coordinates for the nanoinjections into the DMH were – 3.2 mm posterior and \pm 0.6 mm lateral to bregma with a depth of –8.5 mm. The coordinates for the PAG nanoinjections were – 7.0 mm posterior and \pm 0.6 mm lateral to bregma at a depth of – 4.8 mm [51].

4.6 Histology

At the end of the experiments (1–3), alcian blue dye (2%, 100 nl) was nanoinjected into the DMH and PAG for subsequent

histological confirmation [52,53]. Subsequently, the rats were euthanized by overdoses of anesthetic. The atlas of Paxinos & Watson was used as reference to confirm the injection sites in the DMH and PAG [51].

4.7 Analyses

Comparisons between maximal changes (sampled before and after) evoked by nanoinjections into DMH or PAG were determined by Mann Whitney non-parametric test. In air jet experiments, comparisons of the stress-evoked changes between groups (sampled every 2 min) were performed with two-way analysis of variance (factors drug and time) followed by Newman Keuls post hoc test. Peak changes in HR and MAP induced by air jet were compared by one-way analysis of variance followed by Newman Keuls post hoc test. We used Mann Whitney test and ANOVAs as appropriate (see figure legends for details). The level of significance was taken as $P < 0.05$. The data are expressed as the mean \pm SEM.

Supporting Information

Figure S1 Photomicrographs of rat brain slices and schematic drawing depicting the injection sites at the level of the DMH (column A) and PAG (column B). mt: mammillothalamic tract; f: fornix; III: third ventricle; l/dlPAG: lateral/dorsolateral periaqueductal gray; Aq: aqueduct. White squares: experiment 1. Black squares: experiment 2. White circles: experiment 3. (TIF)

Author Contributions

Conceived and designed the experiments: CHX EN MAPF. Performed the experiments: CHX DI FRM GRP AML GBM GV. Analyzed the data: CHX EN MAPF. Contributed reagents/materials/analysis tools: CHX GBM EN MAPF. Wrote the paper: CHX EN MAPF.

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