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Research Report

Nitric oxide modulates blood pressure through NMDA receptors in the rostral ventrolateral medulla of conscious rats

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ABSTRACT

The rostral ventrolateral medulla (RVLM) is an important site of cardiovascular control related to the tonic excitation and regulating the sympathetic vasomotor tone through local presympathetic neurons. Nitric oxide (NO) has been implicated in the modulation of neurotransmission by several areas of the central nervous system including the RVLM. However the pathways driving NO affects and the correlation between NO and glutamate-induced mechanisms are not well established. Here, we investigate the influence of NO on the cardiovascular response evoked by the activation of NMDA and non-NMDA glutamatergic receptors in the RVLM in conscious rats. For that, we examined the influence of acute inhibition of the NO production within the RVLM, by injecting the nonselective constitutive NOS inhibitor, L-NAME, on responses evoked by the microinjection of excitatory amino acids L-glutamate, NMDA or AMPA agonists into RVLM. Our results show that the injection of L-glutamate, NMDA or AMPA agonists into RVLM, unilaterally, induced a marked increase in the mean arterial pressure (MAP). Pretreatment with L-NAME reduced the hypertensive response evoked by the glutamate injection, and also abolished the pressor response induced by the injection of NMDA into the RVLM. However, blocking the NO synthesis did not alter the response produced by the injection of AMPA agonist. These data provide evidence that the glutamatergic neurotransmission within the RVLM depends on excitatory effects exerted by NO on NMDA receptors, and that this mechanism might be essential to regulate systemic blood pressure.

1. Introduction

Nitric oxidse (NO) has been implicated in the regulation of autonomic control and cardiovascular function. NO modulatory effect has been shown in several studies through many areas of the brain related to autonomic regulation, such as the paraventricular nucleus (PVN) (Busnardo et al., 2013; Martins-Pinge et al., 2012; Wang et al., 2005), the nucleus tractus solitarius (NTS) (da Silva et al., 2008; Dias et al., 2003, 2005; Patel et al., 2001), caudal ventrolateral medulla (CVLM) (de Castro et al., 2012) and rostral ventrolateral medulla (RVLM) (Chen et al., 2001; Guo et al., 2009; Martins-Pinge et al., 2007; Mayorov, 2007; Morimoto et al., 2000). These studies have shown that NO modulates neurotransmission within these critical areas for homeostatic control and essential to regulate the cardiovascular system.

In this regard, the RVLM provides a major tonic excitatory drive to the cardiovascular system, as an essential region to maintaining the baseline level of blood pressure (BP). Remarkably, the inhibition of the neuroactivity of neurons within this area produces a marked hypotension confirming that this area is essential to the regulation of sympathetic vasomotor tone (Dampney and Moon, 1980; Dampney, 1994; Dampney et al., 2003; Guyenet et al., 1990;

Abbreviations: AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid; AUC, area under curve; BP, blood pressure; COBEA, Brazilian Council for Animal Experimentation; CVLM, caudal ventrolateral medulla; sGC, soluble guanylase cyclase; cGMP, cyclic guanosine monophosphate; GLU, L-glutamate; HR, heart rate; L-NAME, N(G)-nitro-L-arginine methyl ester; MAP, mean arterial pressure; NIH, National Institutes of Health; NMDA, N-Methyl-D-Aspartate; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; PBS, Phosphate buffer saline; PVN, paraventricular nucleus; RVLM, rostral ventrolateral medulla

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McAllen, 1986; Menezes and Fontes, 2007).

Presympathetic neurons located in the RVLM are described as a site of NO action (Martins-Pinge et al., 2007; Mayorov, 2005), where specific neurons are reported to have nitric oxide synthases (NOS) and NO appear to play a functional role (Hirooka et al., 1996; Iadecola et al., 1993). However, NO effects on the RVLM remains controversial once NO has been shown to induce both inhibitory (Kishi et al., 2001; Nurminen et al., 1997) and excitatory effects on RVLM neurons (Hirooka et al., 1996; Martins-Pinge et al., 1997; Mayorov, 2005; Morimoto et al., 2000). These authors have proposed mechanisms for NO actions in the RVLM by a GABAergic (NO inhibitory affects) or glutamatergic (NO excitatory effect) pathway. However, experiments using conscious rats showed a simpaexcitatory modulatory role of NO within the RVLM by glutamate-induced mechanism, in which NO modulates the action of this neurotransmitter leading to cardiovascular changes (Chen et al., 1997, 2001; Martins-Pinge et al., 2007; Mayorov and Head, 2002). In this regard, one possible mechanism proposed was that NO interacts with both subtypes of glutamatergic ionotropic receptors N-Methyl-D-aspartate (NMDA)-receptors, non-NMDA-receptors (i.e. AMPA and Kainate) within RVLM promoting the cardiovascular regulation. However these studies were made in anesthetized animals (Chan et al., 2003; Chen et al., 2001; Morimoto et al., 2000).

Although there is abundant evidence that NO can modulate the glutamatergic neurotransmission within RVLM, few studies have shown a correlation between NO and glutamatergic pathway on the autonomic and cardiovascular control in conscious animals. Moreover, there is no study showing the specific nature of the NO interaction with glutamatergic receptors by the RVLM in conscious models.

Here, we investigate the influence of NO on cardiovascular response evoked by the activation of NMDA and non-NMDA glutamatergic receptors in conscious rats. For that we examined the influence of acute inhibition of NO production within the RVLM by injecting the nonselective constitutive NOS inhibitor, L-NAME, on responses evoked by the microinjection of excitatory amino acids L-glutamate, NMDA or AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) into RVLM. Characterizing the interaction between NO and the glutamatergic receptors within the RVLM will lead to a better understanding of the mechanism of the excitatory neurotransmission within this important nucleus.

2. Results

2.1. Injection of L-NAME into the RVLM does not change MAP and HR

In order to evaluate the cardiovascular effects of the NOS blockade within the RVLM, we performed an injection of L-NAME into the RVLM of conscious rats. We did not observe any changes on cardiovascular basal values after a unilateral L-NAME injection (Table 1). Baseline values of MAP and HR were also not different when evaluated all groups (Table 2).

Table 1
Effect of L-NAME on values of MAP and HR-Values are means ± S.E.M. MAP (mean arterial pressure) and HR (heart rate); n = 6. Values of MAP and HR were measured during at least 2 min: before L-NAME, after L-NAME and 10 min after L-NAME. Statistical analyses by one-way ANOVA, Bonferroni post-hoc.

	MAP, mmHg	HR, bpm
Before L-NAME	120 ± 3	348 ± 24
L-NAME	120 ± 3	351 ± 20
After L-NAME	117 ± 3	363 ± 18

Table 2
Baseline values of MAP and HR- Values are means ± S.E.M. MAP (mean arterial pressure) and HR (heart rate); n = 6 each group. Values of MAP and HR were measured during at least 2 min before GLU, NMDA and AMPA. Statistical analyses by one-way ANOVA, Bonferroni post-hoc.

	MAP, mmHg	HR, bpm
Control	118 ± 3	406 ± 17
GLU	113 ± 4	409 ± 15
NMDA	112 ± 2	404 ± 11
AMPA	109 ± 3	394 ± 19

2.2. Inhibition of NO synthesis reduce the pressor response induced by the injection of L-glutamate into RVLM

To evaluate the role of NO in the cardiovascular response induced by the injection of L-glutamate into the RVLM, we injected vehicle or L-NAME into the RVLM, unilaterally, and subsequently injected L-glutamate into the RVLM. The injection of L-glutamate into the RVLM induced an increase in MAP followed by a bradycardia with maximum peak at ~30 s and 60 s, respectively. L-NAME injection into the RVLM attenuated the increase in the MAP induced by the injection of L-glutamate when we evaluated the time course response (for treatment GLU vs. GLU L-NAME, ΔMAP: $F_{(1,\ 50)}=23.8$, $p<0.001$) and did not change the bradycardia induced by L-glutamate (for treatment, ΔHR: $F_{(1,\ 50)}=0.32$, $p>0.05$) (Fig. 1). L-NAME pretreatment also decreased the delta maximum of the hypertensive response evoked by GLU (ΔMAP max: GLU 25.47 ± 3.77 mmHg vs. 6.978 ± 3.03 mmHg GLU L-NAME, p = 0.0087) but did not change HR (ΔHR max: GLU 51.52 ± 17.77 bpm vs. −39.34 ± 14.96 bpm GLU L-NAME, p = 0.9372). We also performed analysis of the area under curve/time (AUCt) followed by *t*-test. We observed that L-NAME pretreatment also decreased AUCt for hypertension evoked by GLU (GLU 46.9 ± 9.543 vs. 9.101 ± 6.488 GLU L-NAME, p = 0.0084) but did not change AUCt in the HR responses (GLU 138.1 ± 52.86 vs. 104.3 ± 33.06 GLU L-NAME, p = 0.5996).

To show that the reduction in the cardiovascular response was induced by the reduction in NO concentration, and not by a decline of the excitability of the RVLM neurons, we injected vehicle twice, instead of injecting L-NAME, and subsequently injected L-glutamate. Injection of L-glutamate produced similar increase in MAP and decrease in HR, on both occasions in the time course response (for treatment GLU vs. GLU vehicle, ΔMAP: $F_{(1,\ 50)}=0.19$, $p>0.05$and ΔHR: $F_{(1,\ 50)}=0.99$ $p>0.05$) (Fig. 2) and for delta maximum of BP and HR induced by GLU (ΔMAP max: GLU 19.98 ± 2.493 mmHg vs. 17.07 ± 2.64 mmHg GLU vehicle, p = 0.5887) and (ΔHR max: GLU −32.34 ± 32.21 bpm vs. −45.13 ± 19.78 bpm GLU vehicle, p = 0.5887). We also did not find changes in AUCt for the hypertensive and HR response evoked by GLU before and after vehicle (GLU 39.82 ± 10.15 vs. 32.63 ± 10.37 GLU vehicle, p = 0.6310) AUCt for HR (GLU vs. 133.9 ± 57.29 vs. 78.91 ± 37.87 GLU vehicle, p = 0.4419).

2.3. Inhibition of NO synthesis affects the pressor response induced by the activation of NMDA receptors, but does not change the response induced by the activation of AMPA receptors, in the RVLM

For this analysis, we sought to evaluate whether the decreased cardiovascular response produced by glutamate after NO synthesis blockade were dependent on both, AMPA and NMDA receptors activity, or due to a specific receptor activity. For that, we evaluated the effect of the NOS blocker, on the response evoked by the activation of specific glutamatergic receptors NMDA or AMPA.

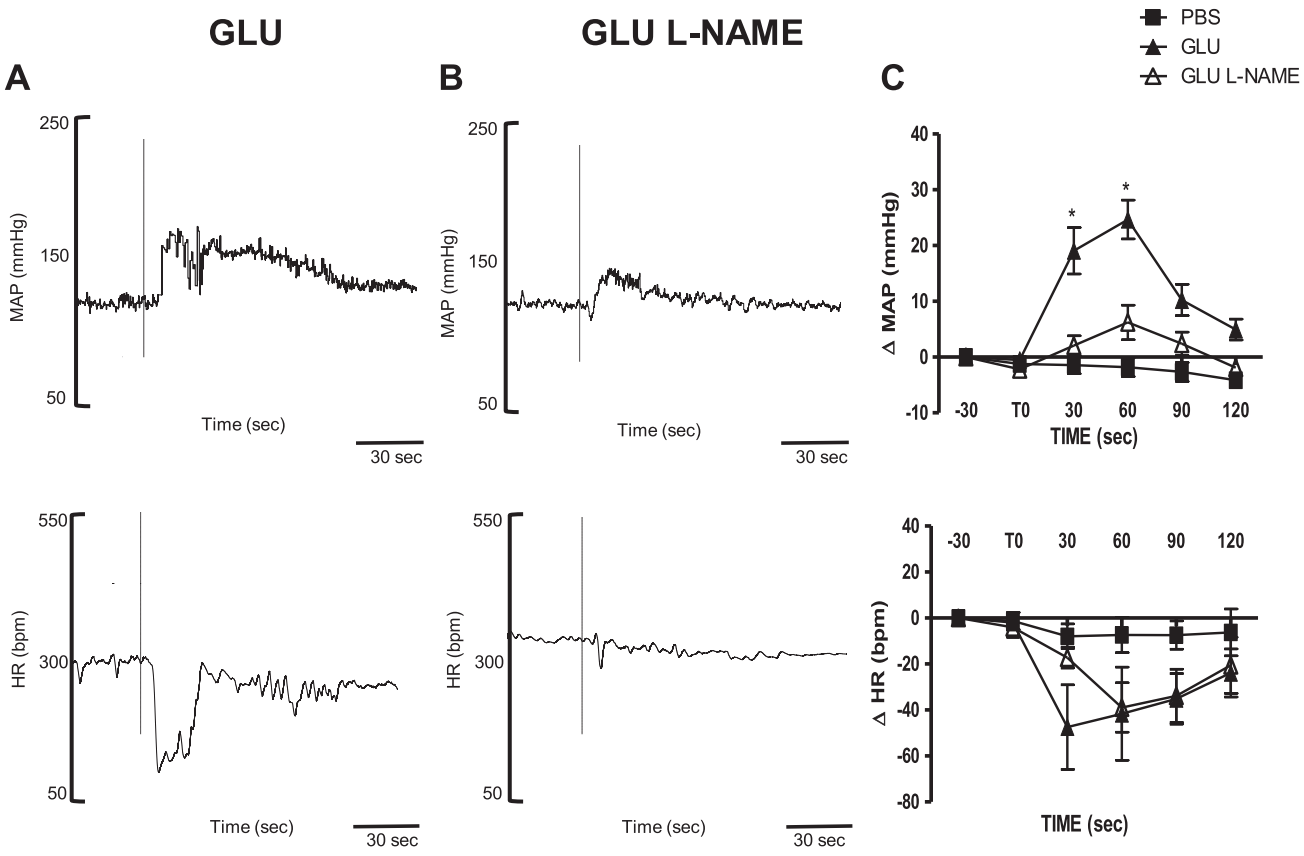

Fig. 1. Mean Arterial Pressure (mmHg) and Heart Rate records in response to L-glutamate microinjection before (A) and after L-NAME microinjection (B). The line illustrates the time of microinjections. (C) Mean Arterial Pressure (MAP, mmHg) and (B) Heart rate (HR, bpm) levels in response to PBS microinjection (PBS) and L-glutamate (GLU) before and after L-NAME microinjection (GLU L-NAME). Symbols represent mean ± S.E.M. *Statistical difference between GLU and GLU L-NAME ($p < 0.05$, two-way ANOVA, Bonferroni post-hoc) (n = 6).

The injection of NMDA agonist within RVLM promoted an increase in MAP with a maximum peak between ~60 s and 90 s after the injection, but did not alter HR. The injection of L-NAME into the RVLM attenuated the increase in MAP induced by the activation of NMDA receptors (for treatment NMDA vs. NMDA L-NAME, ΔMAP: $F_{(1, 50)} = 10.72$, $p < 0.01$). On the other hand, the injection of L-NAME did not significantly alter the HR response induced by the injection of NMDA (for treatment NMDA vs. NMDA L-NAME, ΔHR: $F_{(1, 50)} = 0.78$ $p > 0.05$) (Fig. 3). L-NAME decreased also the delta maximum of the hypertensive response evoked by NMDA (ΔMAPmax: NMDA 21.39 ± 3.84 mmHg vs. 8.527 ± 2.28 mmHg NMDA L-NAME, p = 0.026) but no changes in HR were observed (ΔHRmax: NMDA 83.71 ± 28.74 bpm vs. −29.61 ± 21.92 bpm NMDA L-NAME, p = 0.1320). Similar results were observed for the AUCt responses evoked by NMDA, L-NAME pretreatment prevented BP increases (NMDA 59.23 ± 11.17 NMDA L-NAME, p = 0.0063) but did not change HR (NMDA vs. 191.1 ± 91.63 vs. 101.5 ± 83.24 NMDA L-NAME, p = 0.4858).

The increase in MAP elicited by the injection of the AMPA agonist (maximum peak between ~60 s and 90 s) was not attenuated by a previous injection of L-NAME (for treatment AMPA vs. AMPA L-NAME, ΔPAM: $F_{(1, 50)} = 1.75$ $p > 0.05$). The treatment with L-NAME did not change the HR response induced by the injection of AMPA into the RVLM (for treatment AMPA vs. AMPA L-NAME, ΔHR: $F_{(1, 50)} = 0.84$ $p > 0.05$) (Fig. 4). L-NAME did not change delta maximum of the hypertensive response evoked by AMPA (ΔMAP max: AMPA 31.92 ± 4.619 mmHg vs. 20.34 ± 2.92 mmHg AMPA L-NAME, p = 0.1320) similarly we did not find any change on HR response before or after L-NAME (ΔHR max: AMPA 88.86 ± 24.12 bpm vs. −50.72 ± 32.72 bpm AMPA L-NAME, p = 0.5887). The AUCt for the hypertensive response evoked by AMPA was not reduced by L-NAME pretreatment (AMPA 73.53 ± 15.58 vs. 50.13 ± 9.187 AMPA L-NAME, p = 0.2248) as did not change HR (AMPA vs. 166.3 ± 63.01 vs. 88.76 ± 69.33 AMPA L-NAME, p = 0.4272).

To determine the difference on the effect of the reduction in NO synthesis in the RVLM in the cardiovascular response induced by the activation of both glutamatergic receptors, we evaluated the difference in the hypertensive response to NMDA agonist and AMPA agonist after the injection of L-NAME using the percentage of the hypertensive responses before the treatment with the NOS blocker. We found that only 28.7% ± 5.8 of the maximum pressor response evoked by NMDA was maintained after NOS blocked versus 56.34% ± 7.73 of the AMPA pressor response that was maintained after the L-NAME microinjection. Similar response were observed for the AUCt analysis, L-NAME reduced 71.214% ± 4.115 of NMDA AUCt hypertensive response but reduced just 31.83% ± 7.97 of AMPA AUCt response.

The histological sections demonstrate the site of microinjections in RVLM, they were examined microscopically and compared to the rat brain atlas (Paxinos and Watson, 1986) and Paxinos and Watson (2007) as is shown in Fig. 5.

3. Discussion

In this study we investigated the mechanism of interaction between the NO and the glutamatergic pathway on the cardiovascular

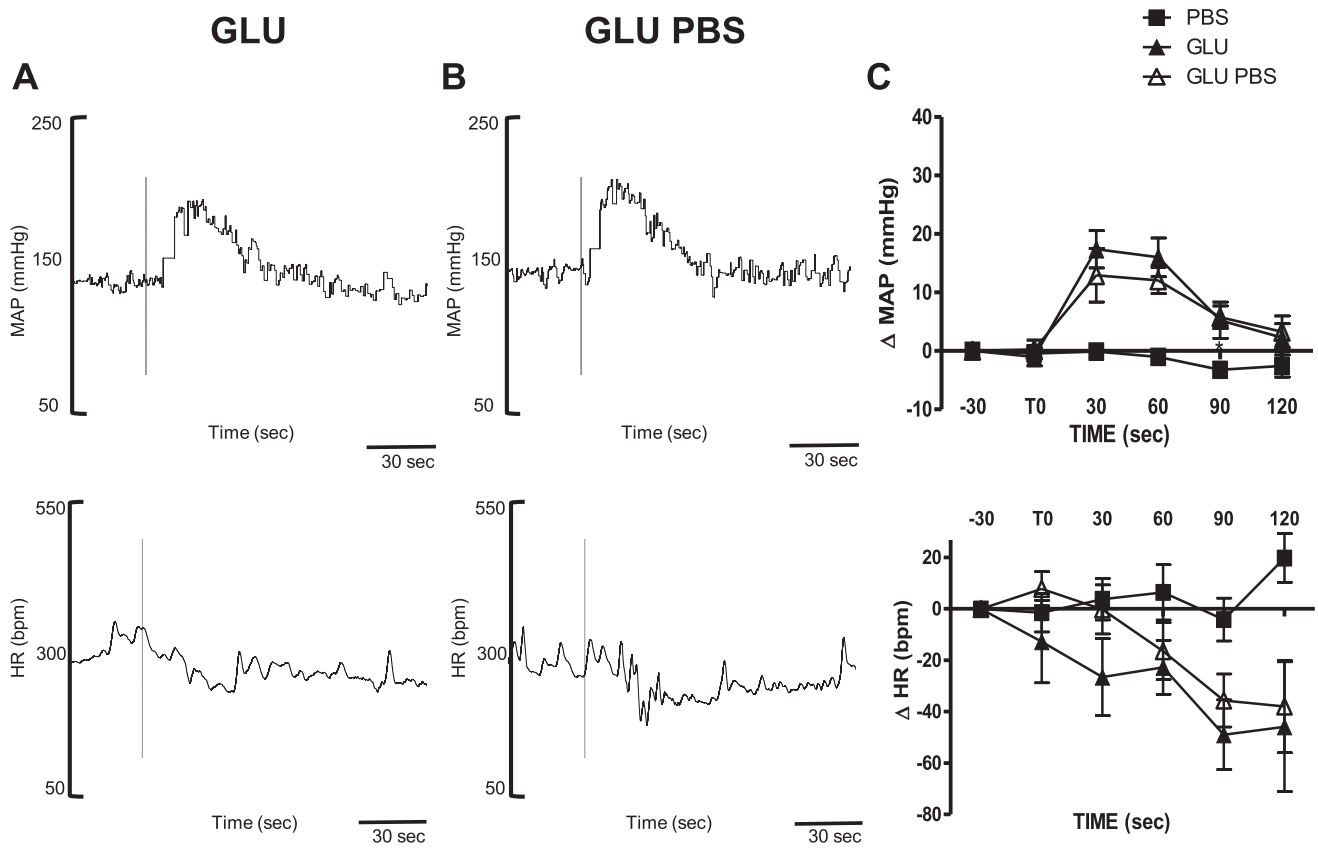

Fig. 2. Mean Arterial Pressure (mmHg) and Heart Rate records in response to Glutamate microinjection before (A) and after PBS microinjection (B). The line illustrates the time of microinjections. (C) Mean Arterial Pressure (MAP, mmHg) and (B) Heart rate (HR, bpm) levels in response to PBS microinjection (PBS) and L-glutamate (GLU) before and after PBS microinjection (GLU PBS). Symbols represent mean ± S.E.M. *Statistical difference between GLU and GLU PBS ($p < 0.05$, two-way ANOVA, Bonferroni post-hoc) (n=6).

control by examining the NO modulatory effect on ionotropic receptors within the RVLM in conscious rats. Our results showed that inhibition of NO production attenuated the pressor response induced by the injection of L-glutamate and that NMDA receptors are the main via of NO action within RVLM. Our data provide evidence that the glutamatergic neurotransmission within the RVLM depends on excitatory effect exerted by NO on NMDA receptors, and that this mechanism might be essential to regulate systemic blood pressure.

First, we confirmed that in conscious rats the injection of L-glutamate into RVLM increases BP and promotes bradycardia, and that the BP increases evoked by L-glutamate is dependent of NO production, since we observed a decrease of glutamate-induce hypertension after blocking NO synthesis. Studies have shown contradictory results related to cardiovascular control for both L-glutamate and NO within the RVLM. These results are especially antagonistic when comparing experiments performed in conscious animals versus experiments using animals under anesthesia. Martins-Pinge et al. (1997) and Sakima et al. (2000) showed that the L-glutamate microinjection into the RVLM induce an increase in BP accompanied by a significant decrease in HR of conscious rats (Martins-Pinge et al., 1997; Sakima et al., 2000), while the L-glutamate microinjection in anesthetized rats evoked an increase in blood pressure accompanied by a discrete tachycardia (Sakima et al., 2000). The NO effects within the RVLM appear with similar conflict. Studies have shown excitatory effects of NO through the RVLM of conscious animals (Martins-Pinge et al., 2007; Mayorov, 2005) and an inhibitory modulation by the RVLM of anesthetized animals (Ishide et al., 2003; Zanzinger et al., 1995).

We have also shown that NO blocker, L-NAME, does not change the bradycardia induced by the L-glutamate injection, as described previously by Mayorov and colleagues, 2005 in conscious rabbits. It is possible that NO has no modulatory effect on the cardiac response by L-glutamate within RVLM. It is also likely that injections of glutamate and its agonists can spread to the nucleus ambigus producing activation of parasympathetic preganglionic neurons explaining the decrease in heart rate. However, we observed a discrete reduction in the bradycardia induced by L-glutamate after L-NAME and no change of the L-glutamate response after vehicle. In this regard, we believe that the discrete decrease on glutamate-induced bradycardia after L-NAME could be due to the decrease of the hypertensive response and consequent reduction of the reflex bradycardia.

Importantly, the unilateral microinjection of the NOS blocker, L-NAME, did not change the basal BP and HR in the present study. The lack of changes in the cardiovascular parameters after the unilateral blocking of NO endogenous production may be caused by an offset from the contralateral site of RVLM as demonstrated previously by Morimoto et al., 2000 where injections of L-NAME into the RVLM, bilaterally, decreased blood pressure, HR and sympathetic responses but unilateral injections did not change these parameters (Morimoto et al., 2000).

In order to confirm that L-NAME induced an attenuation of BP increases evoked by L-glutamate injection, we performed a control experiment using vehicle instead of injecting L-NAME before L-glutamate injections. The responses induced by L-glutamate before or after vehicle yielded similar increase in BP and decrease in HR showing that there was no lesion in the region or excitotoxic

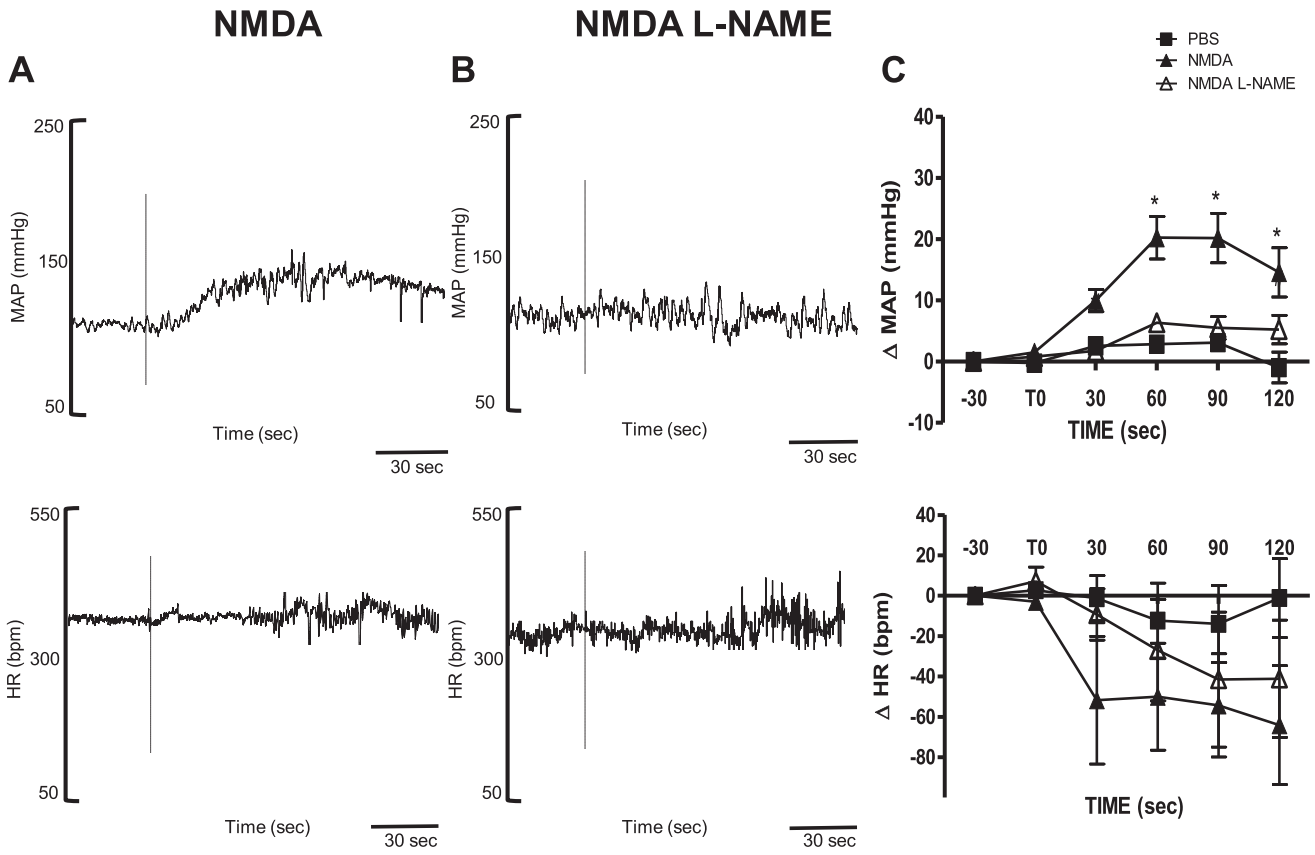

Fig. 3. Mean Arterial Pressure (mmHg) and Heart Rate records in response to NMDA microinjection before (A) and after L-NAME microinjection (B). The line illustrates the time of microinjections. (C) Mean Arterial Pressure (MAP, mmHg) and (B) Heart rate (HR, bpm) levels in response to PBS microinjection (PBS) and NMDA microinjection (NMDA) before and after L-NAME microinjection (NMDA L-NAME). Symbols represent mean ± S.E.M. * Statistical difference between NMDA and NMDA L-NAME ($p < 0.05$, two-way ANOVA, Bonferroni post-hoc) (n=6).

neuronal death as consequence of the injections. Our results, in conjunction with the previous results, indicate that the pressor response induced by L-glutamate into RVLM is dependent of the NO-glutamate pathway interaction. However, it was not known which glutamatergic receptor, NMDA and non-NMDA, within the RVLM, could be distinctly influenced by the presence of NO.

Thus, we next tested the role of NO on the cardiovascular response caused by the activation of two types of glutamatergic receptors, NMDA and AMPA receptors. Our experiments showed that the blockade of NOS does not alter the response evoked by the injection of AMPA, previous experiments, with anesthetized cats, showed that L-NAME pretreatment promoted attenuation of the pressor response and reduction of the vertebral nerve activity induced by AMPA activation (Chen et al., 2001), showing that NO could modulate the AMPA receptors activation. The contradiction between Chen et al., 2001 and the present study can be explained by the different species used for each experiment, and also by the effects of anesthetics that can strongly influence the cardiovascular responses related with NO pathways (Martins-Pinge et al., 2007). In this regard, several experiments involving the RVLM observed differences in the central cardiovascular control between anesthetized rats versus conscious animals (Menezes and Fontes, 2007; Sakima et al., 2000). As discussed above, in anesthetized animals NO plays a inhibitory effect within the RVLM (Zanzinger et al., 1995), however in conscious animals aqueous NO injection into the RVLM promoted increase in MAP (Mayorov, 2005). Moreover, a study demonstrated that the blockade of NO synthesis reduced the sympathetic response evoked by the injection of glutamate (Martins-Pinge et al., 2007), indicating an excitatory modulation of NO in RVLM neurons. These controversial responses may have source in the fact that anesthesia influence the neurotransmission, particularly by modulating GABA neurotransmission (Bachelard et al., 1990).

In this study we showed that NO within the RVLM has a facilitatory effect on NMDA receptors activation, once blood pressure increase was modulated by NO presence. The interaction between NO and NMDA receptors in brain areas such as the NTS and in the cerebellum have been demonstrated in previous studies. These studies describe the interaction between NO and NMDA and also the specificity of NMDA receptors to promote activation of soluble guanylate cyclase as well as synthesis of cyclic guanosine monophosphate (cGMP) in a mechanism independent of AMPA or metabotropic glutamatergic receptors (Bredt and Snyder, 1989; Chianca et al., 2004; Garthwaite et al., 1989). Interesting Wu et al. (2001) showed that stimulation of NMDA receptors increase NO synthesis showing the possible connections in a modulatory pathway linking both NO and NMDA receptors. This study also demonstrated the involvement of NO synthetized by neuronal nitric oxide synthase (nNOS) and soluble guanylate cyclase on the regulation of the cardiovascular responses induced by NMDA activation (Wu et al., 2001).

Additionally, hypertensive effects of AMPA into RVLM were not statistically reduced after L-NAME injection. In order to confirm that NO effect was mainly mediated by NMDA receptors we compared the cardiovascular response induced by NMDA before and after L-NAME. We found that the inhibition of the NO production reduced the maximum hypertensive response induced by NMDA injection in approximately 71%, a remarkable reduction of hypertensive response. This result strongly suggests that NO functional action in the RVLM is mainly mediated by NMDA

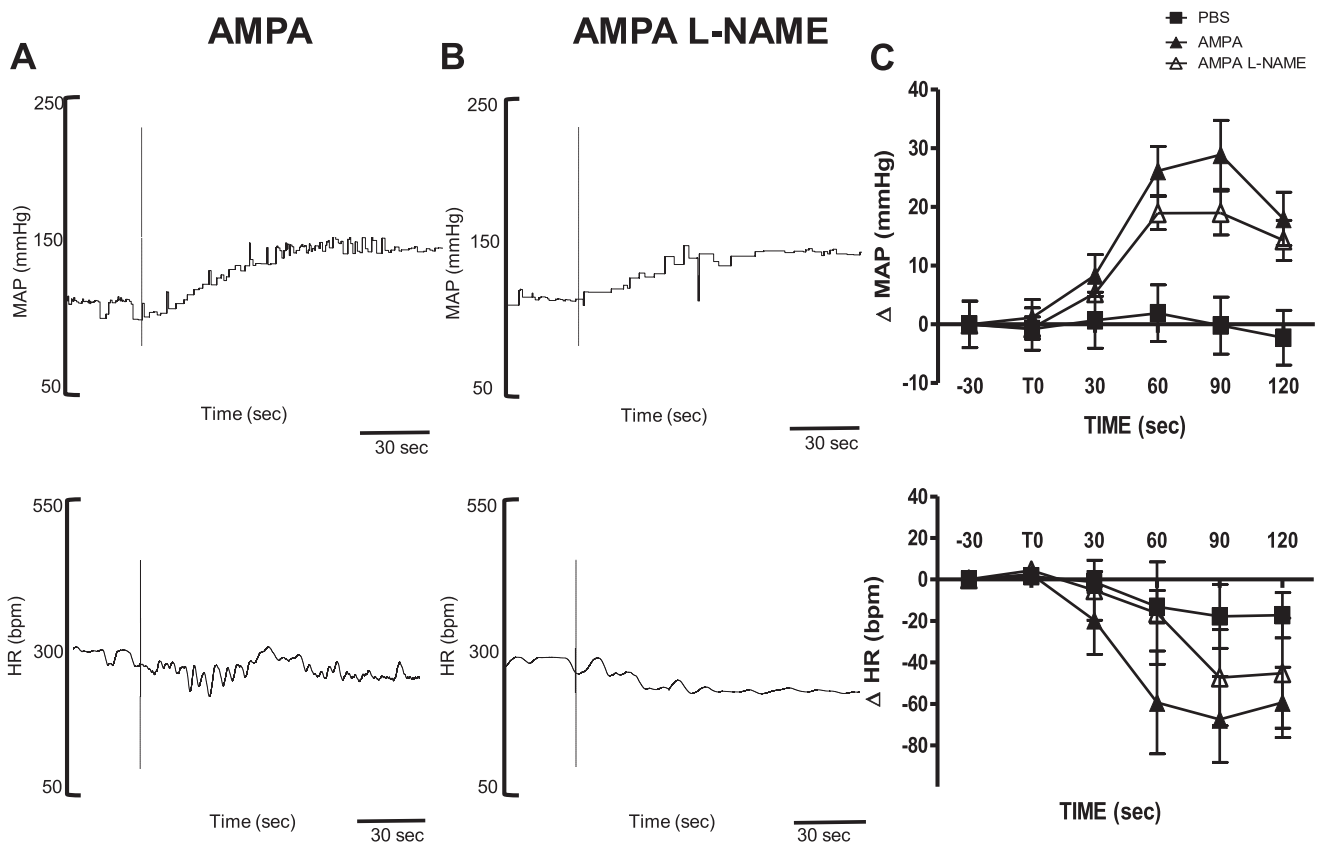

Fig. 4. Mean Arterial Pressure (mmHg) and Heart Rate records in response to AMPA microinjection before (A) and after L-NAME microinjection (B). The line illustrates the time of microinjections. (C) Mean Arterial Pressure (MAP, mmHg) and (B) Heart rate (HR, bpm) levels in response to PBS microinjection (PBS) and AMPA microinjection (AMPA) before and after L-NAME microinjection (AMPA L-NAME). Symbols represent mean ± S.E.M. Statistical difference between AMPA and AMPA L-NAME ($p < 0.05$, two-way ANOVA, Bonferroni post-hoc) (n = 6).

receptors. Noticeably, we observed a small reduction in the magnitude of AMPA hypertensive response after L-NAME injection, thus when an AMPA or NMDA agonists are injected they can lead a downstream increase of local glutamate release, which in turn may activate these receptors (Chen et al., 2001). The inactivation of NMDA receptors after treatment with L-NAME may contribute to produce reduction of AMPA magnitude response.

The present study cannot determine the specific pathway between the NO and the NMDA postsynaptic activation into RVLM, but experiments in vitro, using whole-cell path clamp, recording RVLM neurons showed an increase of amplitude of excitatory postsynaptic currents in a bath preparation of L-arginine. The same study showed a selective effect of NO through Ca^{2+} (Huang et al., 2003). Based on our findings and previous studies, we suggested a possible pathway of NO interaction with NMDA receptors in the RVLM. Studies have shown that NO can mediate the cyclic GMP production by the activation of soluble guanylate cyclase (Arnold et al., 1977; Estevez et al., 1998; Huang et al., 2003; Martins-Pinge et al., 1999; Wu et al., 2001) NO may regulate the synthesis of cGMP, which modulates directly the postsynaptic activation of NMDA receptors allowing the influx of calcium, thus inducing nNOS activation leading the NO neuronal production following by cGMP synthesis and NMDA activation in a positive feedback (Fig. 6). However, it is also possible that NO may have a direct effect on NMDA receptors via direct nitrosylation. A previous study, recording cortical neuron in vitro preparation, showed that NO may have a physiological function through NMDA receptors that is non-depend of cGMP (Lei et al., 1992).

In conclusion, the present study provides further evidence that the NO plays a sympathoexcitatory role on blood pressure control within the RVLM, furthermore our data suggest that the NO exerts an excitatory effect within the RVLM through changes in NMDA receptor activity. However, further studies are awaited to determine the molecular pathway of NO modulatory effect on NMDA receptors in the RVLM neurons to control blood pressure.

4. Experimental procedures

4.1. Animals

The experiments were performed in conscious, freely moving, adult male Fischer rats (n = 24), weighing 280–320 g, and bred and housed at the Center for Animal Science, Federal University of Ouro Preto. They were kept in individual cages on a 12 h light/dark cycle, at a controlled room temperature (23 °C), and diet and filtered water ad libitum. All experiments protocols were in accordance to the Brazilian Council for Animal Experimentation (COBEA). All procedures were approved by the institutional ethics committee for animal research of the Federal University of Ouro Preto (CEUA-UFOP; no – 19/2011), and were performed according to the regulations set forth by the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (NIH publication 80–23, revised in 1996).

4.2. Surgeries

Nine days before the experiments rats were implanted under Ketamine and Xilazine solution (80 mg/kg; 7 mg/kg; i.m.) anesthesia with metal guide cannulas into the rostral ventrolateral

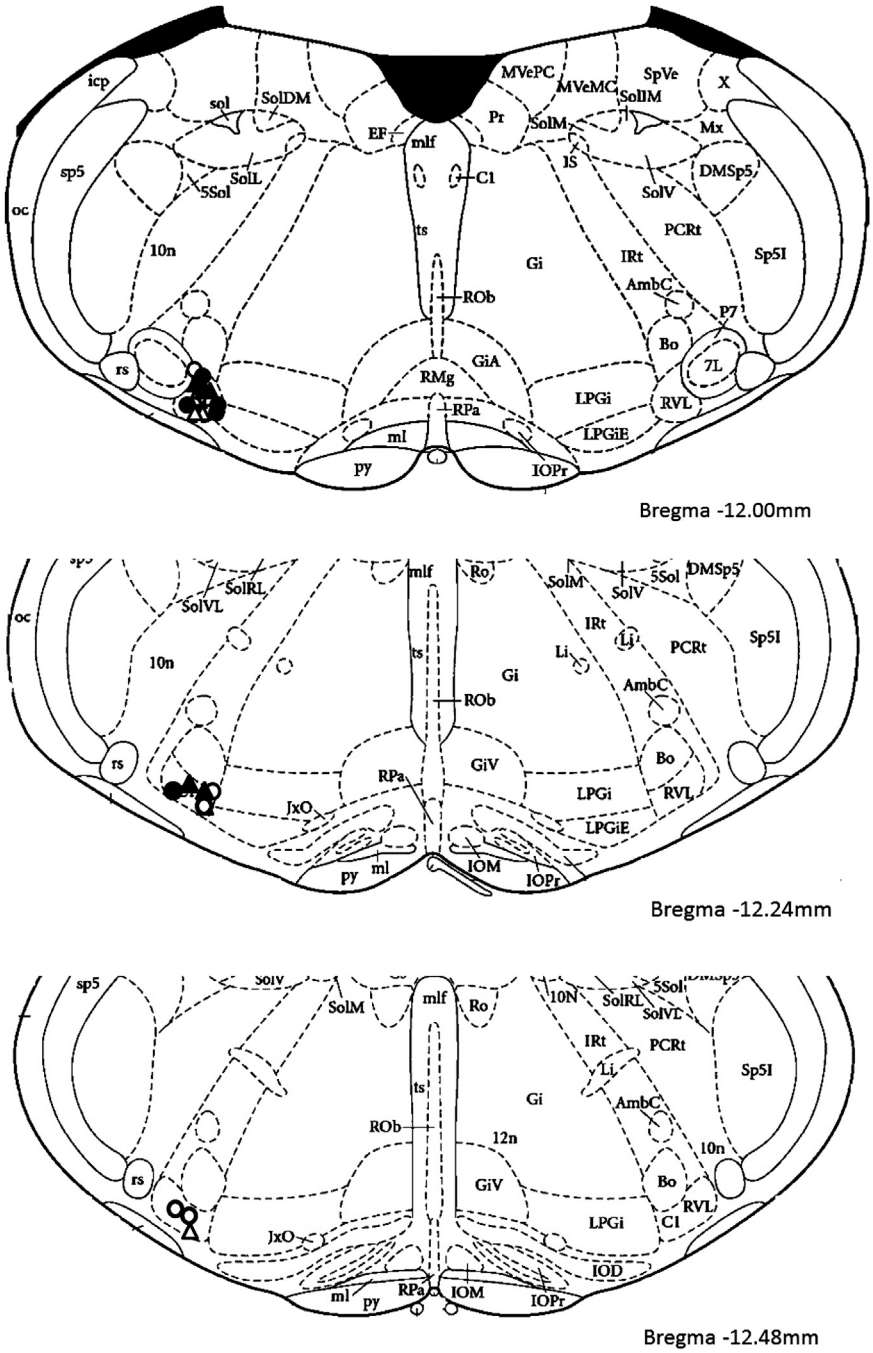

Fig. 5. Photomicrograph exemplifying a brain coronal sections from control group (open circles), GLU group (black circles), NMDA group (open triangles) and AMPA group (black triangles) with their respective microinjection sites into RVLM. Schematic coronal sections of the rat brain were adapted from the atlas of Paxinos and Watson (2007).

medulla (stereotaxic coordinates: AP −3.1; LL ±1.8; DV −6.8 (Paxinos and Watson, 1986). Forty-eight hours before the experiments, under anesthesia (isoflurane 2–3.5% − 2 L/min of O_2), a polyethylene catheter was implanted into the femoral artery for blood pressure and heart rate measurements. The catheter was tunneled through the subcutaneous and exteriorized on the back of the neck.

The animals were kept in individual cages with free access to water and food pellets. Rats received prophylactic treatment with antibiotics (Veterinary Pentabiotic – penicillin: 48.000UI; streptomycin: 20 mg and dihydrostreptomycin: 20 mg/kg; i.m.) and anti-inflammatory (Ketoprofen: 4 mg/kg; i.m.) drugs were performed in order to prevent post-surgical infections and inflammation, respectively.

The procedures for cerebral cannula and femoral catheter placement have been described in detail elsewhere (Menezes and Fontes, 2007; Rodrigues et al., 2012; Silva et al., 2013).

4.3. Experimental design

During experiments, eight days after brain surgery mean arterial pressure and heart hate were recorded in conscious rats; a stable baseline for mean arterial pressure (MAP) and heart rate (HR) was achieved for at least 30 min prior to microinjection. Injections into the RVLM were performed, unilaterally, with a 5 μL Hamilton syringe connected by polyethylene tubing (PE-10 Intramedic, Clay Adams) to injector needles (dental needle, G30, 19.1 mm of length).

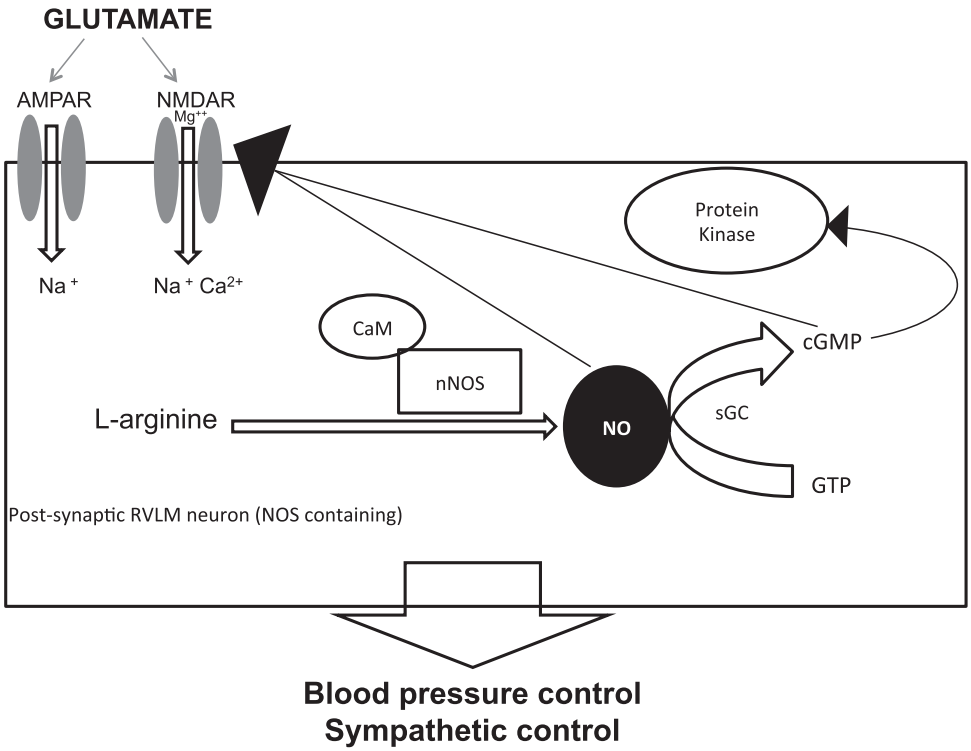

Fig. 6. Schematic representation of hypothetical mechanisms of NO modulation on postsynaptic NMDA receptors in a RVLM neuron (NOS containing). Glutamate activates both NMDA and AMPA receptors to promote cardiovascular responses however, NO selectively modulate NMDA receptors in the RVLM mediating blood pressure control. Two pathways are suggested to be the mechanism of NO effects on NMDA receptors: First, NO synthetized by L-arginine through nNOS regulates the synthase of cGMP which modulates directly the NMDA receptors activation than calcium (Ca^{2+}) influx by a feedforward mechanism. This pathway suggests that NO modulates NMDA activation through cGMP, which can also affect molecular signalization to various cellular events by protein kinases in the RVLM neurons. Second mechanism suggested is that NO acts directly to NMDA receptors inducing nitrosylation of NMDA receptors and modulating these receptors by a mechanism independent of cGMP.

The first experiment was designed to re-evaluate whether NO modulates the changes in MAP and HR induced by the injection of L-glutamate into the RVLM. For that, after recording baseline values of MAP and HR, we injected phosphate buffer saline (PBS; 100nL) into the RVLM. Ten min later we injected L-glutamate, 1nmol/100nL, into the RVLM to evaluate its cardiovascular response. Then, 10 min later, we injected vehicle (PBS and propyleneglycol in a ratio of 6:4) or the nonselective constitutive NOS inhibitor, N(G)-nitro-L-arginine methyl ester (L-NAME) (15 nmol/100 nL; Martins-Pinge et al., 2007) to evaluate the effects of NO on the cardiovascular responses induced by L-glutamate. Fifteen minutes after vehicle or L-NAME, we re-injected glutamate (1 nmol/100 nl) into RVLM (n=6).

The second series of experiments evaluated the involvement of NO on the modulation of the cardiovascular response induced by the activation of the subtypes of glutamate receptors NMDA and non-NMDA in the RVLM. To that end, we executed the same protocol used in the previous series, but instead of injecting L-glutamate, we injected NMDA (10 pmol/100 nL), a NMDA glutamatergic receptor agonist (n=6), or AMPA, 3 pmol/100 nl, a non-NMDA glutamatergic receptor agonist (n=6) before and after L-NAME (15 nmol/100 nL) to elucidate a possible glutamatergic receptors selectivity of the NO modulatory effect on RVLM neurons in the cardiovascular control.

After recordings, animals were sacrificed, and Evans blue (100 nL) dye was injected to confirm the injection sites. Brains were removed, post fixed seven days in formalin followed of 24 h in 20% sucrose for cryoprotection. Brains were then sectioned at 50 μm in a cryostat. The slices were mounted on glass slides. After drying, the slices were stained with Neutral Red and visualized in an optical microscope for confirmation sites of the injection. Rats without confirmed histology were discarded from the study.

4.4. Statistical analyses

Statistical analysis was done using Prism software (version 5; GraphPad Software Inc., La Jolla, CA, USA). Values are given as means ± SEM calculated by the values mean obtained during each 30 s of recording, the prior 30 s before each one injection was used as basal period. Significant differences were determined using Student's *t*-test followed by Mann Whitney post-test, one-way and two-way ANOVA with multiple comparisons followed by the Bonferroni post-test. Differences were considered significant at a *P*-value below 0.05.

Contributions

NLSM, and RCDM designed the study. NLSM performed the experiments. NLSM, FCSS, DACJR and RCDM analyzed and interpreted the data. NLSM, FCSS, DACJR and RCDM wrote the manuscript.

Financial disclosure

All authors have no conflicts of interest to report.

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