

## AMYGDALAR NEURONAL ACTIVITY MEDIATES THE CARDIOVASCULAR RESPONSES EVOKED FROM THE DORSOLATERAL PERIAQUEDUCTAL GRAY IN CONSCIOUS RATS

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**Abstract**—There is ample evidence that both lateral/dorsolateral periaqueductal gray (l/dIPAG) and basolateral amygdala (BLA) are essential for the regulation of the autonomic responses evoked during innate reactions to threatening stimuli. However, it is not well established to what extent the BLA regulates the upstream functional connection from the l/dIPAG. Here we evaluated the role of the BLA and its glutamatergic receptors in the cardiovascular responses induced by l/dIPAG stimulation in rats. We examined the influence of acute inhibition of the BLA, unilaterally, by injecting muscimol on the cardiovascular responses evoked by the injection of N-methyl D-aspartate (NMDA) into the l/dIPAG. We also evaluated the role of BLA ionotropic glutamate receptors in these responses by injecting antagonists of NMDA and AMPA/kainate receptor subtypes into the BLA. Our results show that the microinjection of NMDA in the BLA increased the mean arterial pressure (MAP) and heart rate (HR). Injection of NMDA into the l/dIPAG caused similar increases in these variables, which was prevented by the prior injection of muscimol, a GABA<sub>A</sub> agonist, into the BLA. Moreover, injection of glutamatergic antagonists (2-amino-5-phosphopentanoate (AP5) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX)) into the BLA reduced the increase in MAP and HR induced by

l/dIPAG activation. Finally, the inhibition of the central amygdala neurons failed to reduce the cardiovascular changes induced by l/dIPAG activation. These results indicate that physiological responses elicited by l/dIPAG activation require the neuronal activity in the BLA. This ascending excitatory pathway from the l/dIPAG to the BLA might ensure the expression of the autonomic component of the defense reaction. © 2014 IBRO. Published by Elsevier Ltd. All rights reserved.

**Key words:** periaqueductal gray, basolateral amygdala, glutamatergic receptors, defense reaction.

### INTRODUCTION

It is generally agreed that specific neural circuits have developed in several organisms, that allow for a quick and strategic response to environmental threats. Specific pathways in the central nervous system (CNS) of mammals are activated during emotional or environmental stress, and induce various autonomic and endocrine responses (DiMicco et al., 2006; Szczepanska-Sadowska, 2008). These changes are accompanied by defensive behaviors including flight, freezing, defensive attack, and risk assessment (Blanchard and Blanchard, 1989a,b), which favor survival.

One of the brain regions involved in the integration of specific physiological changes associated with emotional behavior is the periaqueductal gray matter (PAG) (Bandler et al., 2000). Studies have shown that chemical stimulation of glutamatergic neurons in the caudal portion of the lateral/dorsolateral periaqueductal gray (l/dIPAG) induces tachycardia and increases locomotor activity, blood pressure, and body temperature; responses which are similar to those produced during emotional stress (Carrive, 1993; Bandler and Shipley, 1994; de Menezes et al., 2009). Such tachycardic and pressor responses are mediated by efferent neuronal projections from the PAG to autonomic brain centers (Lovick, 1993; McNaughton and Corr, 2004).

Furthermore, the PAG has been defined as a lower center of defensive reactions influenced by downstream afferent projections arriving from the hypothalamus (Bandler and Keay, 1996; Bernard and Bandler, 1998). However, studies show that the PAG could also be a source of excitatory ascending projections to the hypothalamus during defensive reactions. In this regard,

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**Abbreviations:** AP5, 2-amino-5-phosphopentanoate, NMDA receptor antagonist; BLA, basolateral amygdala; CEA, central amygdala; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione, AMPA/kainate receptor antagonist; DMH, dorsomedial hypothalamus; GABA<sub>A</sub>,  $\gamma$ -aminobutyric acid; HR, heart rate; l/dIPAG, lateral/dorsolateral periaqueductal gray; MAP, mean arterial pressure; NMDA, N-methyl D-aspartate, glutamatergic receptor agonist; PAG, periaqueductal gray; PBS, Phosphate-buffered saline; SEM, standard error of the mean; VTA, ventral tegmental area.

responses induced by stimulated l/dIPAG neurons are dependent on neuronal activity in the dorsomedial hypothalamus (DMH), suggesting that the PAG can produce changes in physiological parameters through upstream centers of neuronal activation, such as the hypothalamus (de Menezes et al., 2009; Horiuchi et al., 2009; Fontes et al., 2011).

Anatomical studies have also identified that both the dorsolateral (dIPAG) and the DMH form connections with the amygdala. Even though these connections appear to be indirect they play an important role in cardiovascular control during defense reactions. In fact, these three structures are believed to form an aversive system in the brain associated with anxiety (Graeff, 2004). The basolateral nucleus of the amygdala (BLA) has been identified as a site where GABAergic mechanisms play an important role in the regulation of cardiovascular function and behavior (Shekhar, 1993; Sanders and Shekhar, 1995). Additionally, it has been suggested that the BLA acts as an integration center for the anxiety states, and that glutamatergic neurotransmission is critical to the regulation of this condition (Campeau and Davis, 1995; Ledoux, 2012). Finally, glutamatergic stimulation of the BLA produces effects similar to those observed for the activation of l/dIPAG (Soltis et al., 1998). Altogether, these data suggest that the excitatory pathway between the dIPAG and the DMH could depend on the activation of BLA neurons.

There is ample evidence that the amygdala is critically involved in the regulation of innate and conditioned reactions to threatening stimuli (Ledoux, 1994; Campeau and Davis, 1995). Furthermore, it is frequently proposed that the PAG is downstream of the amygdala, directing motor outputs toward the proper defensive behavior (Ledoux, 2012). On the other hand, it is not well established to what extent the amygdala regulates the upstream functional connection from the l/dIPAG. Indeed, activation of the dIPAG leads to increased expression of c-Fos protein, a marker for neuronal activation, in the BLA (Ferreira-Netto et al., 2005).

Here, we evaluated role of the functional connection between the l/dIPAG and the BLA on the cardiovascular response evoked by the dIPAG. For that we examined the influence of acute inhibition of the BLA, by injecting muscimol, on responses evoked by the microinjection of the excitatory amino acid NMDA (N-methyl D-Aspartate) into l/dIPAG. We also evaluated the role of BLA ionotropic glutamate receptors in these responses by injecting 2-amino-5-phosphonopentanoate (AP5) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), antagonists of NMDA and AMPA/kainate receptor subtypes, respectively, into the BLA. To establish the anatomical specificity of the effect of muscimol in the BLA, we also tested the consequence of identical injection into the central amygdala (CEA) in parallel experiments. Characterizing the interaction between PAG and amygdala in triggering the defense reaction will lead to a better understanding of these structures' roles within a neural circuit that modulates reactions to threatening stimuli.

## EXPERIMENTAL PROCEDURES

### Ethical approval

All procedures were performed according to the regulations set forth by the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and according to the journal policies and regulations on animal experimentation and were approved by the ethics committee for animal research of the Federal University of Ouro Preto (CEUA-UFOP; # 2010/26). All efforts were made to minimize the number of animals used in the present study, and to avoid any unnecessary distress to the animals.

### Animals

Experiments were conducted on male Wistar rats ( $n = 46$ ) at the Federal University of Ouro Preto (Brazil). Animals were acquired from the University Centre of Animal Science, weighing approximately 200 g. The animals were housed in groups (three per cage), maintained in a dark-light cycle of 12 h, and with temperature maintained at 24 °C. Free access to food and water was allowed. In all experimental procedures, animals were handled carefully, in order to avoid stressful conditions. We assessed animal's stress level by observing no changes in heart rate and arterial pressure, as well as changes in rat behavior, during and after animal manipulation (data not shown).

### Surgical procedures

Surgical procedures started when rats reached a weight of  $300 \pm 20$  g. Rats were anesthetized ( $80 \text{ mg kg}^{-1}$  ketamine and  $11.5 \text{ mg kg}^{-1}$  xylazine, i.p., supplemented if needed) and guide cannulas (stainless steel) were implanted for microinjection of drugs, unilaterally, into the left BLA, CEA, and l/dIPAG as described previously (Shekhar and Keim, 1997; de Menezes et al., 2009). Briefly, rats were placed in a stereotaxic apparatus with the incisor bar positioned at 3.3 mm below the level of the interaural line. The guide cannulas were positioned according to the coordinates of the Paxinos and Watson atlas (Paxinos and Watson, 2007) using the bregma as a reference point; coordinates for the left BLA: 2.4 mm posterior, 4.6 mm lateral, 7.8 mm ventral; left CEA: 2.6 mm posterior, 4.0 mm lateral, 6.7 mm ventral; left l/dIPAG: 7.8 mm posterior, 0.7 mm lateral, 4.3 mm ventral. Two screws and dental acrylic secured the guide cannula, which was occluded with a stainless steel obturator. Animals were placed in individual cages for recovery. Rats were allowed at least 6 days for recovery before the beginning of the next surgery. After this period, a polyethylene catheter was inserted into the femoral artery (for cardiovascular measurements) of all animals under isoflurane anesthesia (2.5% isoflurane in 3 L/min  $\text{O}_2$ ; Cristalia, Itapira, Brazil). The catheter was tunneled subcutaneously and exteriorized on the back of the neck. At this time, the obturator of the guide cannula was removed. After surgery, analgesics (ketoflex 4 mg/kg, 0.1 ml/300 g s.c., Mundo Animal, Sao Paulo, Brazil) and antibiotics (a combination of: Benzathine benzylpenicillin, procaine benzylpenicillin, potassium benzylpenicillin, dihydrostrep-

tomycin sulfate, streptomycin sulfate, 0.2 ml/100 g, s.c.; Fort Dodge Animal Health, Sao Paulo, Brazil) were administered. The animals were maintained in individual cages in order to recover from anesthesia. Experimental procedures began 48 h after the last surgery.

### Cardiovascular measurements

The cannula inserted into the femoral artery of rats was used to obtain registration of cardiovascular parameters. It was connected to a pressure transducer MLT0699 (ADI Instruments, NSW, Sydney, Australia), which was connected to a signal amplifier ETH-400 (CB Sciences Inc., Dover, NH, USA). The pressure oscillations were captured, amplified, and converted into signals sent to an analog-to-digital converter (PowerLab/400, ADI Instruments, NSW, Sydney, Australia) and the pulsatile arterial pressure was recorded at 1000 Hz by the software Chart 7.0 for Windows (ADI Instruments, NSW, Sydney, Australia). MAP and HR were derived on-line from the pulsatile arterial pressure using beat-to-beat analysis.

### Experimental design

All experiments were performed in a room in which the temperature was maintained at 24–25 °C. On the day of the experiment, animals were brought to the experimental room, in their home cages, one hour prior to the beginning of the protocol. The experiment commenced only after stabilization of physiological parameters (HR and MAP) for at least 30 min. Microinjections were performed with a microinjector (30 gauge, 1 mm longer than the guide cannula) connected to a 5- $\mu$ l Hamilton syringe with Teflon tubing (ID 0.12 mm; OD 0.65 mm; Bioanalytical Systems, West Lafayette, IN, USA). The syringe was used to deliver 100 nl of injectate (drugs or vehicle (Phosphate-buffered saline (PBS))) over 20 s, approximately. The injector was removed 10 s after the injection procedure. The microinjection was considered successful if flow appeared immediately after removal of the microinjector, indicating that the injector was not obstructed. Each rat was subjected to two different trials 1 day apart, and in random order in which either vehicle (100 nl) or a drug was injected into the amygdala or PAG.

The first experimental group was conducted to re-evaluate whether the activation of the BLA region by injection of NMDA was capable of producing cardiovascular responses similar to those produced by the activation of the l/dIPAG. Thus, we examined the effect of injecting NMDA (30 pmol/100 nl, Sigma-Aldrich Brasil Ltda. Sao Paulo, Brazil) in the BLA region under physiological parameters of MAP and HR ( $n = 7$ ). To this end, each rat was subjected to two different trials 2 days apart and in random order in which we injected either NMDA or vehicle into the BLA region, unilaterally. This dose of NMDA was chosen based on the study of (Soltis et al., 1998) which demonstrated that microinjection of this dose into the BLA produces reliable increases in HR and MAP in conscious rats (Soltis et al., 1998).

The second series of experiments evaluated the role of neurons located in the BLA on the physiological

responses produced by activation of l/dIPAG. For that, we examined the effect of injection of muscimol (100 pmol/100 nl, Sigma-Aldrich Brasil Ltda. Sao Paulo, Brazil) into the BLA, unilaterally, on the cardiovascular responses induced by injecting NMDA (10 pmol/100 nl) into the l/dIPAG ( $n = 7$ ), ipsilaterally. Each rat was subjected to two different trials 2 days apart and in random order in which either vehicle (100 nl) or muscimol was injected into the BLA followed by injection of NMDA into the l/dIPAG five minutes later. This dose of NMDA was chosen based on the study of de Menezes and colleagues (de Menezes et al., 2009), which demonstrated that injecting this dose in the l/dIPAG produces reliable increases in HR and MAP in conscious rats. This dose of muscimol was chosen based on earlier studies demonstrating that a similar dose (80 pmol) in the BLA effectively reduced the physiological responses evoked by air jet stress (Stotz-Potter et al., 1996a,b).

The third series of experiments examined the role of BLA glutamatergic receptors on the increases in MAP and HR induced by l/dIPAG activation. For that, initially we tested the effect of injecting a combination of ionotropic glutamate receptor antagonists (AP5, 100 pmol/100 nl, and CNQ<sub>x</sub>, 100 pmol/100 nl, both from Sigma-Aldrich Brasil Ltda. Sao Paulo, Brazil) into the BLA, unilaterally, on the cardiovascular response induced by injecting NMDA (10 pmol/100 nl) into the l/dIPAG ( $n = 7$ ), ipsilaterally, five minutes later. We have also tested the effect of injecting AP5 (200 pmol/100 nl;  $n = 7$ ) or CNQ<sub>x</sub> (200 pmol/100 nl;  $n = 7$ ), separately, on the responses evoked by l/dIPAG activation. These doses of AP5 and CNQ<sub>x</sub> were chosen based on our previous findings (de Menezes et al., 2009), which demonstrated that injecting this doses in the DMH effectively reduced the physiological responses evoked by PAG activation.

In order to show that the reduction in the increase in MAP and HR induced by l/dIPAG activation, was due to the inhibition of the BLA, and not due to a decrease of neuronal excitability in the PAG region, we performed an experiment where over the course of two days we injected vehicle into the BLA, followed by microinjection of NMDA in l/dIPAG ( $n = 5$ ) five minutes later.

Finally, to evaluate the specificity of the chemical inhibition of BLA neurons, we conducted an experimental protocol, which evaluated the effect of microinjection of muscimol (100 pmol/100 nl) in the (CEA) on the physiological responses produced by injecting NMDA (10 pmol/100 nl) into the l/dIPAG ( $n = 6$ ) five minutes later.

### Statistical analysis

The data for HR and MAP values were recorded continuously. For all, but one group, statistical analysis and representation in figures, average changes from baseline were calculated for the interval of 3 min before the first microinjection (i.e. saline or muscimol), 5 min after the first injection and 7 min after the microinjection of NMDA. In the first experimental group, average changes from baseline were calculated for the interval of 6 min before the microinjection of vehicle or NMDA into the BLA and 9 min after the microinjection of

NMDA. The baseline MAP and HR values were obtained by averaging the values of the 5 min period prior to the first injection. All data analyzed were obtained from animals that had the injection sites confirmed by histology. Prism 5.0 (GraphPad Software, La Jolla, CA, USA) was used to analyze the data. Data are expressed as mean  $\pm$  standard error of the mean (SEM). Student's Paired *t*-test was used to analyze the changes from baseline within groups. A two-way (treatment and time as factors) repeated measures analysis of variance (ANOVA) with post hoc comparison with Bonferroni post-test was used to analyze differences between groups. The analyses were divided in 2–3 parts: baseline, treatment 1 and treatment 2. The significance threshold level was set at 0.05.

## RESULTS

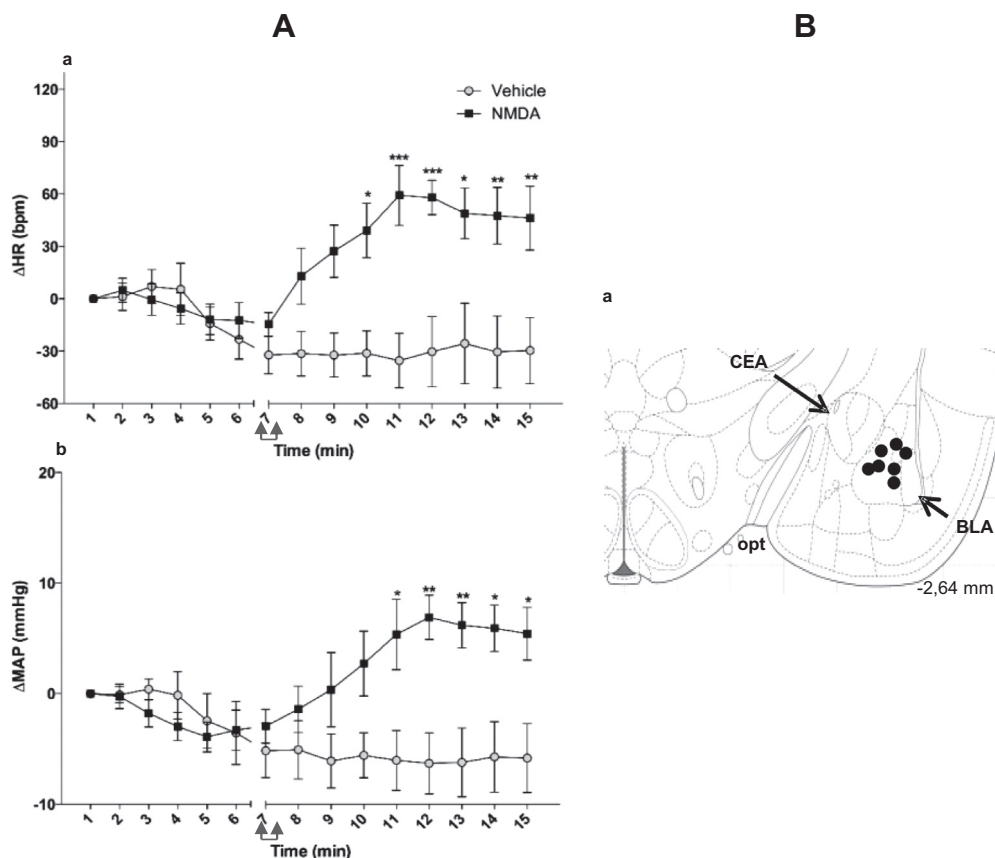
### Stimulation of the BLA produces cardiovascular responses similar to those obtained by I/dIPAG activation

To confirm that the activation of BLA neurons is capable of producing increases in HR and MAP comparable to

those induced by PAG activation, we injected an NMDA receptor agonist into the BLA. Injection of NMDA into the BLA produced increases in HR and MAP, relative to vehicle treatment (for treatment, HR:  $F_{(1,96)} = 16.89$ ,  $p < 0.05$ ; and MAP:  $F_{(1,96)} = 10.59$ ,  $p < 0.05$ ) (Fig. 1), as expected (Soltis et al., 1998). These changes were accompanied by increases in locomotor activity as previously reported (Soltis et al., 1998), which were not quantified. Post-mortem histology confirmed that injection sites were located in the BLA (Figs. 1 and 8).

### GABAergic inhibition of BLA reduces the cardiovascular response evoked by I/dIPAG activation

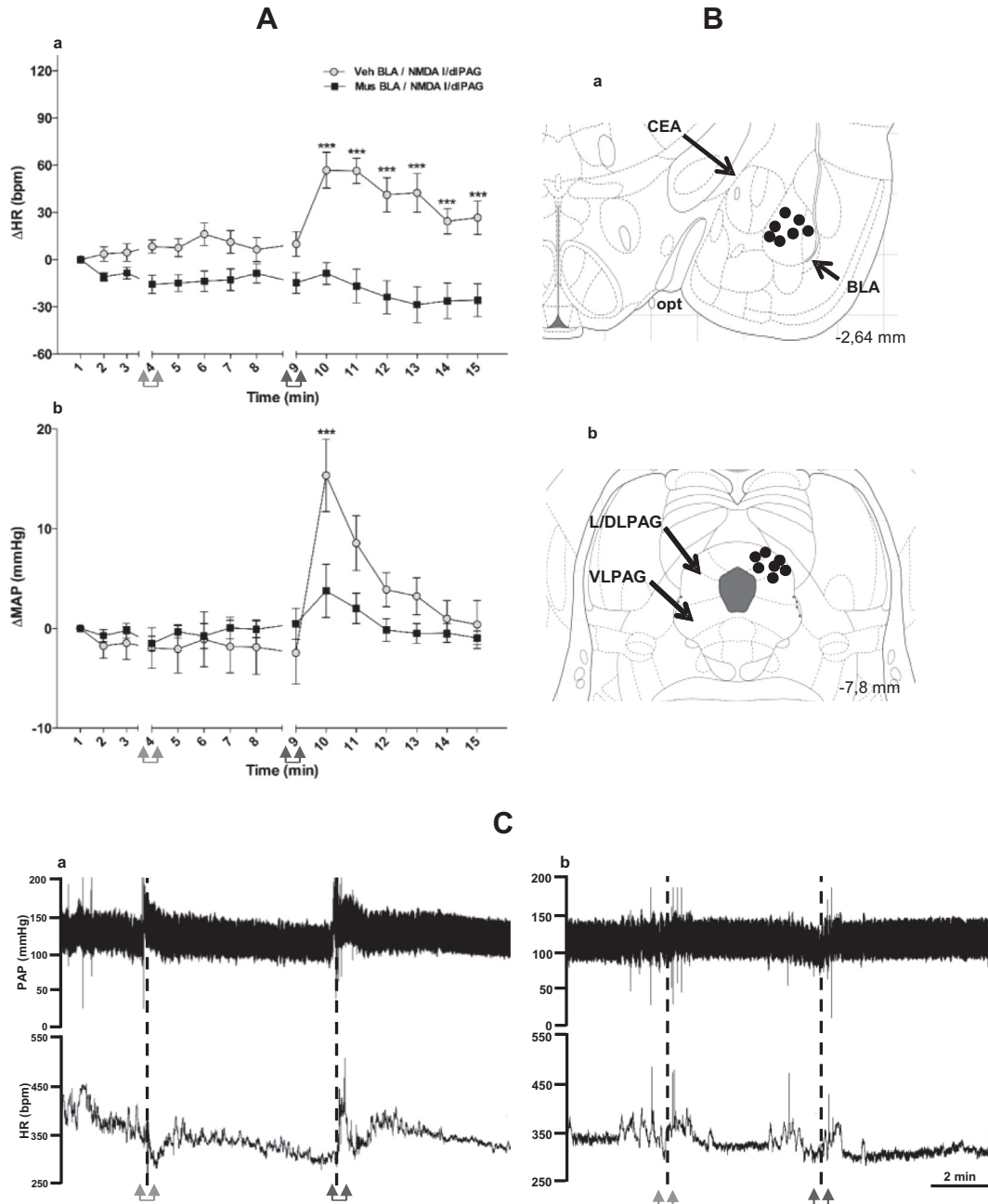
To evaluate the role of BLA neurons on the physiological responses produced by I/dIPAG activation, we injected muscimol (a GABA<sub>A</sub> receptor agonist) (100 pmol/100 nl) or vehicle into the BLA, and subsequently injected NMDA (10 pmol/100 nl) into the I/dIPAG. Injection of either muscimol or vehicle did not alter HR ( $7 \pm 9$  vs.  $-2 \pm 10$  bpm,  $p > 0.05$ ) or MAP values ( $1 \pm 1$  vs.  $0 \pm 1$  mmHg,  $p > 0.05$ ). However, injecting muscimol



**Fig. 1.** Cardiovascular response evoked by the injection of NMDA into the BLA. (A) Mean changes in HR (a) and MAP (b) as a result of vehicle (100 nl, open circles) versus NMDA (30 pmol/100 nl, filled squares) injection into the BLA ( $n = 7$ ). Baseline HR: vehicle,  $334 \pm 12$  bpm; NMDA,  $398 \pm 16$  bpm. Baseline MAP: vehicle,  $108 \pm 2$  mmHg; NMDA,  $104 \pm 4$  mmHg. \* $p < 0.05$  and \*\* $p < 0.01$ , respectively, show significant differences between the responses from the injection of vehicle or NMDA into the BLA by a two-factor repeated measures ANOVA followed by the Bonferroni post hoc test. Arrows indicate the injection of vehicle or NMDA into the BLA; (B) Sites of injection in the BLA in all experiments. Schematic coronal sections of the rat brain adapted from the atlas of Paxinos and Watson (Paxinos and Watson, 2007) illustrating approximate sites of injections into the BLA for all experiments for which data are reported. Numbers indicate distance from the bregma in millimeters. Filled circles represent injections of NMDA (30 pmol/100 nl) or vehicle into the BLA. Abbreviations: BLA (basolateral amygdala); HR (heart rate); MAP (mean arterial pressure).

into the BLA resulted in a significantly smaller increase in HR and MAP evoked by NMDA injection into the l/dIPAG, relative to vehicle treatment (for treatment, HR:

$F_{(1,72)} = 9.21$ ,  $p < 0.05$  and MAP:  $F_{(1,71)} = 6.94$ ,  $p = 0.0218$ ) (Fig. 2). The increase in HR and MAP evoked by l/dIPAG activation were accompanied by the



**Fig. 2.** Effect of muscimol injection in the BLA on the responses evoked from the l/dIPAG. (A) Mean changes in HR (a) and MAP (b) as a result of vehicle (100 nl, open circles) versus muscimol (100 pmol/100 nl, filled squares) injection into the BLA, followed by NMDA (6 pmol/100 nl) into l/dIPAG ( $n = 7$ ). Baseline HR: vehicle,  $339 \pm 12$  bpm; muscimol,  $352 \pm 6$  bpm. Baseline MAP: vehicle,  $113 \pm 2$  mmHg; muscimol,  $114 \pm 2$  mmHg.  $*p < 0.05$  and  $**p < 0.01$ , respectively, show significant differences between the responses from the injection of muscimol and/or vehicle into the BLA by a two-factor repeated measures ANOVA followed by the Bonferroni post hoc test. Light gray arrows indicate the injection of vehicle or muscimol into the BLA; Dark gray arrows indicate the injection of NMDA into the l/dIPAG. (B) Sites of injection in the BLA and the l/dIPAG in all experiments. Schematic coronal sections of the rat brain adapted from the atlas of Paxinos and Watson (Paxinos and Watson, 2007) illustrating approximate sites of injections into the BLA (a) and l/dIPAG (b) for all experiments for which data are reported. Numbers indicate distance from the bregma in millimeters. Filled circles represent injections of muscimol (100 pmol/100 nl) or vehicle into the BLA and the injection of NMDA into the l/dIPAG. (C) Original recordings showing the effects of vehicle (left panel) or muscimol (right panel) injections into the BLA on the cardiovascular changes evoked by the injection of NMDA into l/dIPAG (a, b). Light gray arrows indicate the injection of vehicle or muscimol into the BLA; Dark gray arrows indicate the injection of NMDA into the l/dIPAG. Recordings of pulsatile arterial pressure (PAP; top trace) and HR (bottom trace) are shown. Abbreviations: BLA (basolateral amygdala); HR (heart rate); l/dIPAG (lateral/dorsolateral periaqueductal gray); MAP (mean arterial pressure); Mus (muscimol); Veh (vehicle).

increase in animal's locomotor activity as previously reported (de Menezes et al., 2009). Although we have not quantified this increase, injection of muscimol into BLA clearly attenuated this increase in locomotor activity. Post-mortem histology confirmed that injection sites were located in the BLA and l/dIPAG (Fig. 2 and Fig. 8).

### Glutamatergic receptor inhibition within BLA reduces the cardiovascular response evoked by l/dIPAG activation

For this analysis, we wanted to evaluate the role of the glutamatergic receptor on the physiological responses produced by l/dIPAG activation. First, we analyzed the effect of the inhibition of two types of receptors together, NMDA and AMPA/kainate, on these responses. Next, we evaluated the role of each of these receptors separately.

*Inhibition of NMDA and AMPA/kainate receptors.* Injection of either a cocktail of AP5 (a NMDA receptor antagonist) and CNQ<sub>x</sub> (a AMPA/kainate receptor antagonist) or vehicle did not alter HR ( $3 \pm 13$  vs.  $0 \pm 12$  bpm,  $p > 0.05$ ) and MAP ( $0 \pm 1$  vs.  $1 \pm 1$  mmHg,  $p > 0.05$ ). On the other hand, injecting cocktail resulted in a significantly smaller increase in HR and MAP evoked by NMDA injection in the l/dIPAG, as relative to vehicle treatment (for treatment, HR:  $F_{(1,60)} = 15.09$ ,  $p < 0.05$ ; and for interaction between treatment and time MAP:  $F_{(6,60)} = 3.48$ ,  $p < 0.05$ ) (Fig. 3). Post-mortem histology confirmed that injection sites were located in the BLA and l/dIPAG (Fig. 3 and Fig. 8).

*Inhibition of NMDA receptors.* Injection of either AP5 or vehicle did not alter HR ( $9 \pm 4$  vs.  $0 \pm 11$  bpm,  $p > 0.05$ ) and MAP ( $1 \pm 1$  vs.  $-5 \pm 3$  mmHg,  $p = 0.1146$ ,  $p > 0.05$ ). Nevertheless, injecting AP5 resulted in a significantly smaller increase in HR and MAP evoked by NMDA injection in the l/dIPAG, as relative to vehicle treatment (for treatment, HR:  $F_{(1,72)} = 46.72$ ,  $p < 0.05$  and MAP:  $F_{(1,72)} = 6.29$ ,  $p < 0.05$ ) (Fig. 4). Post-mortem histology confirmed that injection sites were located in the BLA and l/dIPAG (Fig. 4 and Fig. 8).

*Inhibition of AMPA/kainate receptors.* Injection of either CNQ<sub>x</sub> or vehicle did not alter HR ( $-22 \pm 8$  vs.  $3 \pm 14$  bpm,  $p > 0.05$ ) and MAP ( $-4 \pm 1$  vs.  $-2 \pm 3$  mmHg,  $p > 0.05$ ). However, injecting CNQ<sub>x</sub> resulted in a significantly smaller increase in HR and MAP evoked by NMDA injection in the l/dIPAG, as relative to vehicle treatment (and for interaction between treatment and time, HR:  $F_{(6,84)} = 3.02$ ,  $p < 0.05$ ; and for treatment MAP:  $F_{(1,84)} = 6.44$ ,  $p < 0.05$ ) (Fig. 5). Post-mortem histology confirmed that injection sites were located in the BLA and l/dIPAG (Fig. 5 and Fig. 8).

To show that the reduction in the cardiovascular response induced by the l/dIPAG was due to the inhibition of the neuroactivity within the BLA, and not by a decline of the excitability of the PAG, we injected vehicle into the BLA on two consecutive days, and

subsequently injected NMDA into the l/dIPAG. Injection of NMDA produced similar increases in HR and MAP on both days (for treatment, HR:  $F_{(1,48)} = 0.00002$ ,  $p > 0.05$  and MAP:  $F_{(1,48)} = 1.43$ ,  $p > 0.05$ ) (Fig. 6). Post-mortem histology confirmed that injection sites were located in the l/dIPAG (Fig. 6 and Fig. 8).

### GABAergic inhibition of CEA did not affect the cardiovascular response evoked by l/dIPAG activation

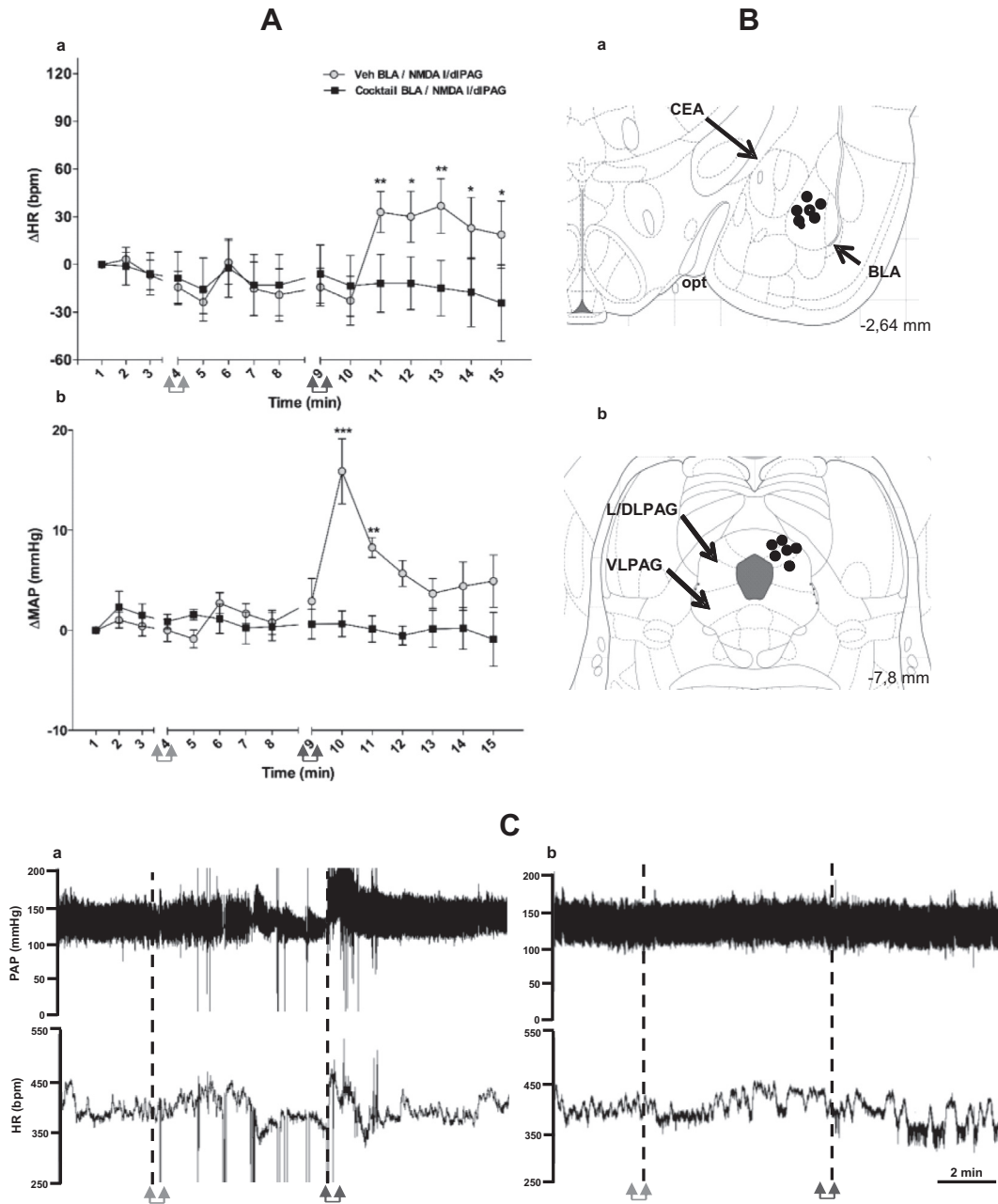
To identify the specificity produced by GABAergic inhibition of BLA neurons on the physiological responses produced by l/dIPAG activation, we injected muscimol or vehicle into the CEA, and subsequently injected NMDA into the l/dIPAG. Injection of either muscimol or vehicle did not alter HR ( $-4 \pm 9$  vs.  $-5 \pm 7$  bpm,  $p > 0.05$ ) and MAP ( $0 \pm 1$  vs.  $-1 \pm 1$  mmHg,  $p > 0.05$ ). Moreover, injecting muscimol did not reduce the HR and MAP changes caused by NMDA injection, as relative to vehicle treatment (for treatment, HR:  $F_{(1,70)} = 0.19$ ,  $p > 0.05$  and MAP:  $F_{(1,70)} = 0.26$ ,  $p > 0.05$ ) (Fig. 7). Post-mortem histology confirmed that injection sites were located in the CEA and l/dIPAG (Fig. 7 and Fig. 8).

## DISCUSSION

In this study, we evaluated the influence of BLA on the cardiovascular response produced by the activation of l/dIPAG. Our results showed that neuronal excitation in the l/dIPAG produces cardiovascular changes through the activation of glutamate receptors present in the BLA. Our data provide evidence that the BLA is downstream of the l/dIPAG, and directs the autonomic components of the defense reaction resulting from the activation of l/dIPAG.

First, we confirmed that the activation of glutamate receptors in the BLA with NMDA increased both HR and MAP. This was also found by Soltis et al. (1998) and Sanders and Shekhar (1995) for BLA neuron activation, as well as by others after l/dIPAG activation and DMH disinhibition (Carrive, 1993; da Silva et al., 2003, 2006; de Menezes et al., 2006, 2008, 2009). Interestingly, studies have shown that the cardiovascular responses triggered by l/dIPAG activation depend on the neuronal activity in the DMH (de Menezes et al., 2009; Horiuchi et al., 2009). Noteworthy, the activation of the dIPAG leads to increased expression of c-Fos protein, a marker for neuronal activation, in the BLA (Ferreira-Netto et al., 2005). Therefore, if the amygdala shares connections with the PAG it is possible that there is an indirect ascending pathway between the l/dIPAG and the DMH, which uses the BLA as an integrator in the control of the cardiovascular response induced by PAG activation.

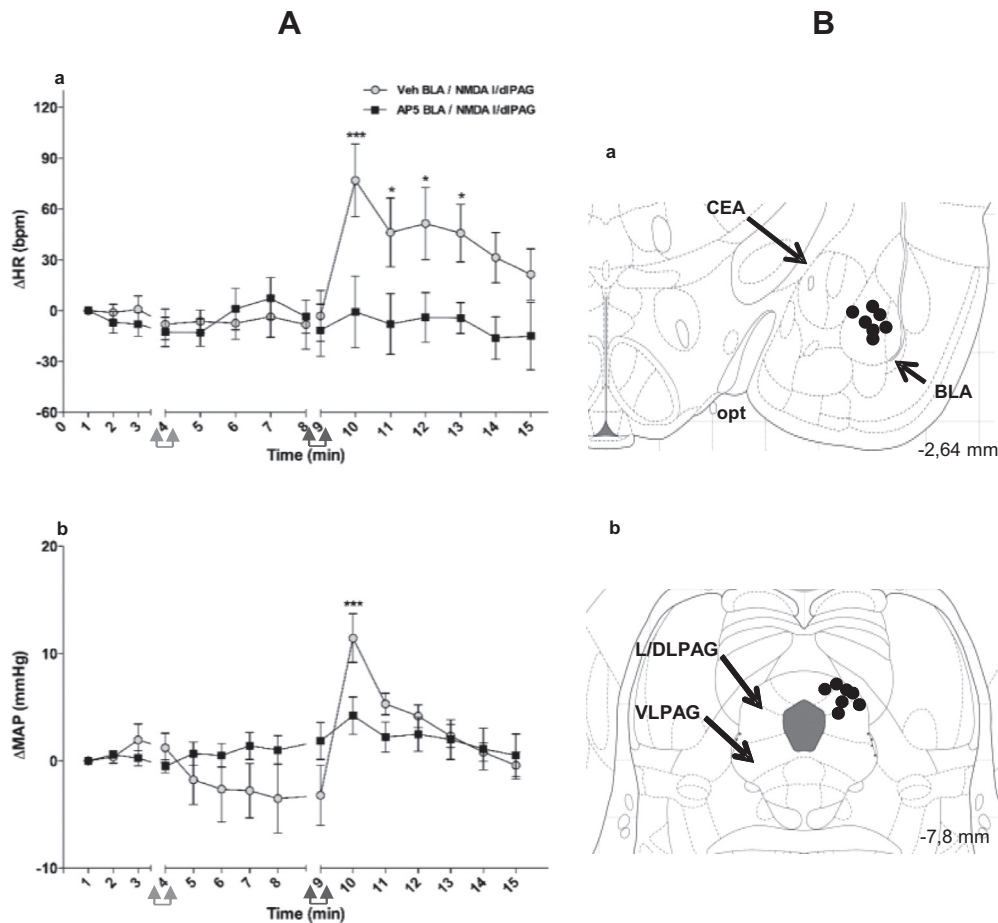
To further study this possibility, we evaluated the role of BLA neurons in the physiological responses produced by l/dIPAG activation. When we injected muscimol into the BLA, the increase in HR and MAP was significantly smaller than that regularly evoked by the NMDA injection into the l/dIPAG. Our results indicate that neuroactivity within the BLA is necessary for the



**Fig. 3.** Effect of glutamatergic inhibition of BLA on the increase in MAP and HR evoked by activation of the l/dIPAG. (A) Mean changes in HR (a) and MAP (b) as a result of vehicle (100 nl, open circles) versus cocktail (100 pmol/100 nl, filled squares) injection into the BLA, followed by NMDA (6 pmol/100 nl) into l/dIPAG ( $n = 7$ ). Baseline HR: vehicle,  $355 \pm 20$  bpm; cocktail,  $374 \pm 19$  bpm. Baseline MAP: vehicle,  $120 \pm 3$  mmHg; cocktail,  $119 \pm 2$  mmHg. \* $p < 0.05$  and \*\* $p < 0.01$ , respectively, show significant differences between the responses from the injection of cocktail and/or vehicle into the BLA by a two-factor repeated measures ANOVA followed by the Bonferroni post hoc test. Light gray arrows indicate the injection of vehicle or cocktail into the BLA; Dark gray arrows indicate the injection of NMDA into the l/dIPAG. (B) Sites of injection in the BLA and the l/dIPAG in all experiments. Schematic coronal sections of the rat brain adapted from the atlas of Paxinos and Watson (Paxinos and Watson, 2007) illustrating approximate sites of injections into the BLA (a) and l/dIPAG (b) for all experiments for which data are reported. Numbers indicate distance from the bregma in millimeters. Filled circles represent injections of cocktail (100 pmol/100 nl) or vehicle into the BLA and the injection of NMDA into the l/dIPAG. (C) Original recordings showing the effects of vehicle (left panel) or cocktail (right panel) injections into the BLA on the cardiovascular changes evoked by the injection of NMDA into l/dIPAG (a, b). Light gray arrows indicate the injection of vehicle or cocktail into the BLA; Dark gray arrows indicate the injection of NMDA into the l/dIPAG. Recordings of pulsatile arterial pressure (PAP; top trace) and HR (bottom trace) are shown. *Abbreviations:* BLA (basolateral amygdala); HR (heart rate); l/dIPAG (lateral/dorsolateral periaqueductal gray); MAP (mean arterial pressure); Mus (muscimol); Veh (vehicle).

manifestation of the cardiovascular responses induced by l/dIPAG activation. Synthesis of the present results with the previous findings indicates that l/dIPAG neurons represent upstream mediators for sympathoexcitatory

responses evoked by the disinhibition of the BLA (Sajdyk and Shekhar, 1997; Soltis et al., 1998), and that this pathway plays a role in the increases in MAP and HR during fight or flight responses.



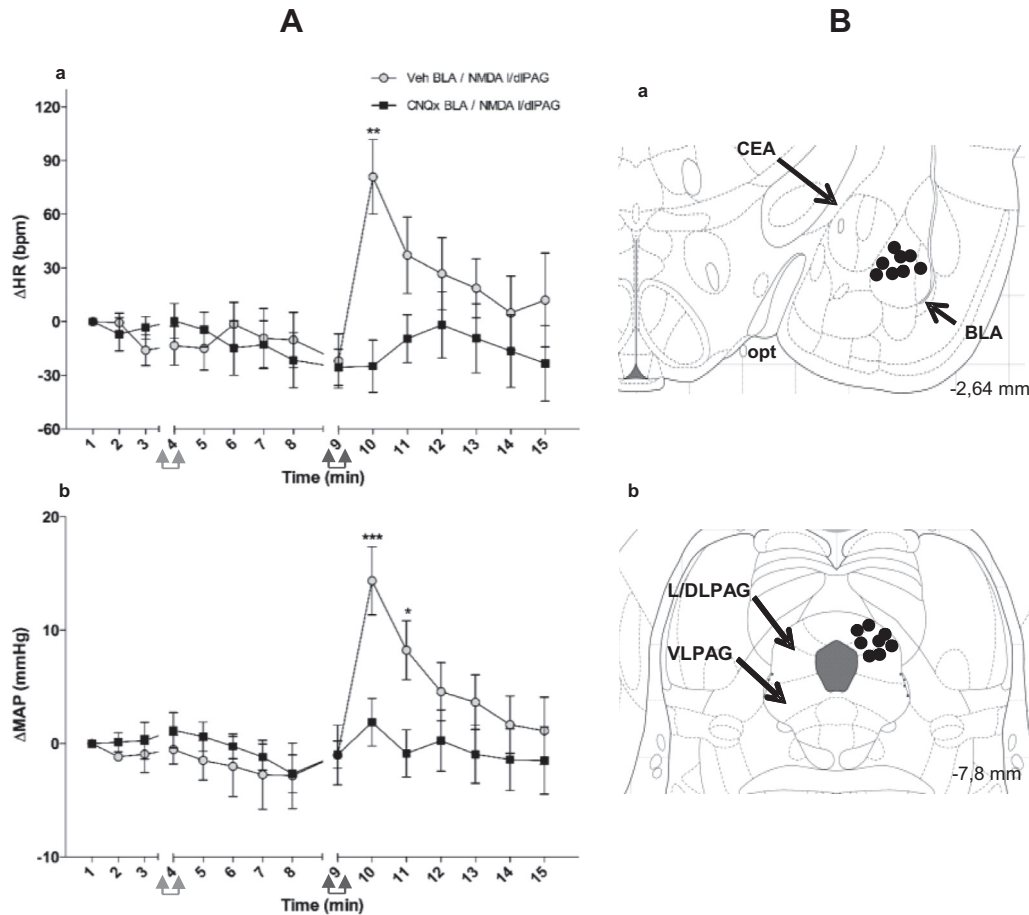
**Fig. 4.** Effect of the NMDA receptor antagonist in the BLA on the increase in MAP and HR evoked by activation of the l/dIPAG. (A) Mean changes in HR (a) and MAP (b) as a result of vehicle (100 nl, open circles) versus AP5 (100 pmol/100 nl, filled squares) injection into the BLA, followed by NMDA (6 pmol/100 nl) into l/dIPAG ( $n = 7$ ). Baseline HR: vehicle,  $340 \pm 14$  bpm; AP5,  $351 \pm 16$  bpm. Baseline MAP: vehicle,  $116 \pm 6$  mmHg; AP5,  $112 \pm 5$  mmHg. \* $p < 0.05$  and \*\* $p < 0.01$ , respectively, show significant differences between the responses from the injection of AP5 and/or vehicle into the BLA by a two-factor repeated measures ANOVA followed by the Bonferroni post hoc test. Light gray arrows indicate the injection of vehicle or AP5 into the BLA; Dark gray arrows indicate the injection of NMDA into the l/dIPAG. (B) Sites of injection in the BLA and the l/dIPAG in all experiments. Schematic coronal sections of the rat brain adapted from the atlas of Paxinos and Watson (Paxinos and Watson, 2007) illustrating approximate sites of injections into the BLA (a) and l/dIPAG (b) for all experiments for which data are reported. Numbers indicate distance from bregma in millimeters. Filled circles represent injections of AP5 (100 pmol/100 nl) or vehicle into the BLA and the injection of NMDA into the l/dIPAG. Abbreviations: BLA (basolateral amygdala); HR (heart rate); l/dIPAG (lateral/dorsolateral periaqueductal gray); MAP (mean arterial pressure); Mus (muscimol); Veh (vehicle).

The ascending connection between the dIPAG and the BLA is probably indirect since this PAG column does not project, directly, to the BLA (Cameron et al., 1995a). This pathway possibly uses the ventral tegmental area (VTA) as a relay nucleus. The dIPAG sends direct projections to the VTA, which in turn sends projections to the BLA (Cameron et al., 1995b; de la Mora et al., 2010; Swason, 1982). It is worth noticing that the VTA to BLA pathway modulates conditioned fear through the activation of D2 receptors in the BLA (de Oliveira et al., 2011). Notably, the dIPAG have different connections and possibly distinctive functions from the lateral and dorsomedial columns (Vianna and Brandão, 2003; Kamon ligaya et al., 2010) where injections made in our study were also located. The injection volume (100 nl) used in our study is relatively large, thus is possible that the increases in MAP and HR induced by the NMDA

injection into the PAG were caused, mainly, by the activation of the dIPAG neurons, even though the activation of the lateral PAG column neurons produced similar increases in the HR when compared to the response produced by the dIPAG activation (using a much smaller volume – 15 nl) in anesthetized rats (Kamon ligaya et al., 2010).

Because we observed increases in locomotor activity after activation of either BLA or l/dIPAG, it could be suggested that increases in HR and MAP were secondary to sympathetic support of behavioral activity. Although we agree that locomotor activity can increase HR and blood pressure by itself, it is well known that such behavioral changes are an integrated component of mechanisms underlying aversive reactions under threatening stimuli, and the changes in cardiovascular parameters are primary elements that enable the active





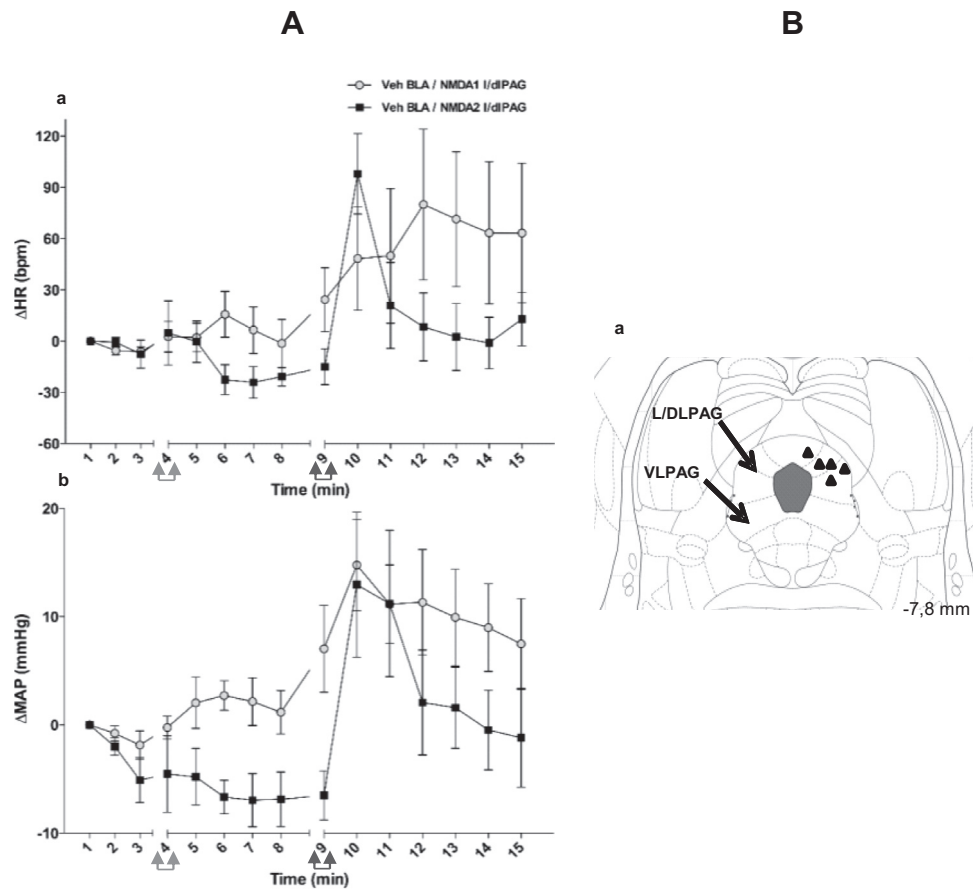
**Fig. 5.** Effect of AMPA/kainate receptor antagonist in the BLA on the increase in MAP and HR evoked by activation of the l/dIPAG. (A) Mean changes in HR (a) and MAP (b) as a result of vehicle (100 nl, open circles) versus CNQ<sub>x</sub> (200 pmol/100 nl, filled squares) injection into the BLA, followed by NMDA (6 pmol/100 nl) into l/dIPAG ( $n = 7$ ). Baseline HR: vehicle,  $364 \pm 10$  bpm; CNQ<sub>x</sub>,  $386 \pm 7$  bpm. Baseline MAP: vehicle,  $109 \pm 4$  mmHg; CNQ<sub>x</sub>,  $109 \pm 2$  mmHg. \* $p < 0.05$  and \*\* $p < 0.01$ , respectively, show significant differences between the responses from the injection of CNQ<sub>x</sub> and/or vehicle into the BLA by a two-factor repeated measures ANOVA followed by the Bonferroni post hoc test. Light gray arrows indicate the injection of vehicle or CNQ<sub>x</sub> into the BLA; Dark gray arrows indicate the injection of NMDA into the l/dIPAG. (B) Sites of injection in the BLA (a) and the l/dIPAG (b) for all experiments for which data are reported. Numbers indicate distance from bregma in millimeters. Filled circles represent injections of CNQ<sub>x</sub> (100 pmol/100 nl) or vehicle into the BLA and the injection of NMDA into the l/dIPAG. *Abbreviations:* BLA (basolateral amygdala); HR (heart rate); l/dIPAG (lateral/dorsolateral periaqueductal gray); MAP (mean arterial pressure); Mus (muscimol); Veh (vehicle).

coping strategy (Bandler et al., 2000). In addition, previous studies have shown that activation of dIPAG and BLA in anesthetized rats evoke increase in HR, blood pressure and sympathetic activity (al Maskati and Zbrozyna, 1989; Horiuchi et al., 2009).

Data from our study and others (Soltis et al., 1997, 1998) show that injection of glutamatergic agonists into the BLA leads to increase in HR and MAP. These findings confirm that the BLA is innervated by glutamatergic projections. Therefore, the cardiovascular responses produced by PAG activation could depend mainly on glutamatergic projections between the l/dIPAG and the BLA.

To further test the proposed glutamatergic excitatory projection pathway from the PAG to the BLA, we next tested the role of two types of glutamatergic receptors in the BLA (i.e., NMDA and AMPA/kainate), together and separately, in this pathway. Inhibiting BLA neurons by injecting a glutamatergic receptor antagonist cocktail

abolished the cardiovascular changes produced by l/dIPAG activation. Next, when we inhibited BLA neurons by injecting AP5 (a NMDA receptor antagonist), the response produced by l/dIPAG activation were almost completely eliminated. Similarly, injecting CNQ<sub>x</sub> (an AMPA/kainate receptor antagonist) also resulted in a significant reduction in the response. In line with these findings, in a study by Soltis and colleagues (Sajdyk and Shekhar, 1997), separately injecting AP5 or CNQ<sub>x</sub> into the BLA reduced the increases in HR and blood pressure induced by the blockade of GABAA receptors in the BLA. Thus, the joint activation of both glutamatergic receptors in the BLA is needed in order to generate the cardiovascular responses induced by l/dIPAG activation. Our data suggest that the circuit between the l/dIPAG and BLA, whose activation results in an array of physiological and behavioral changes, is excitatory relying on the activation of glutamatergic receptors.

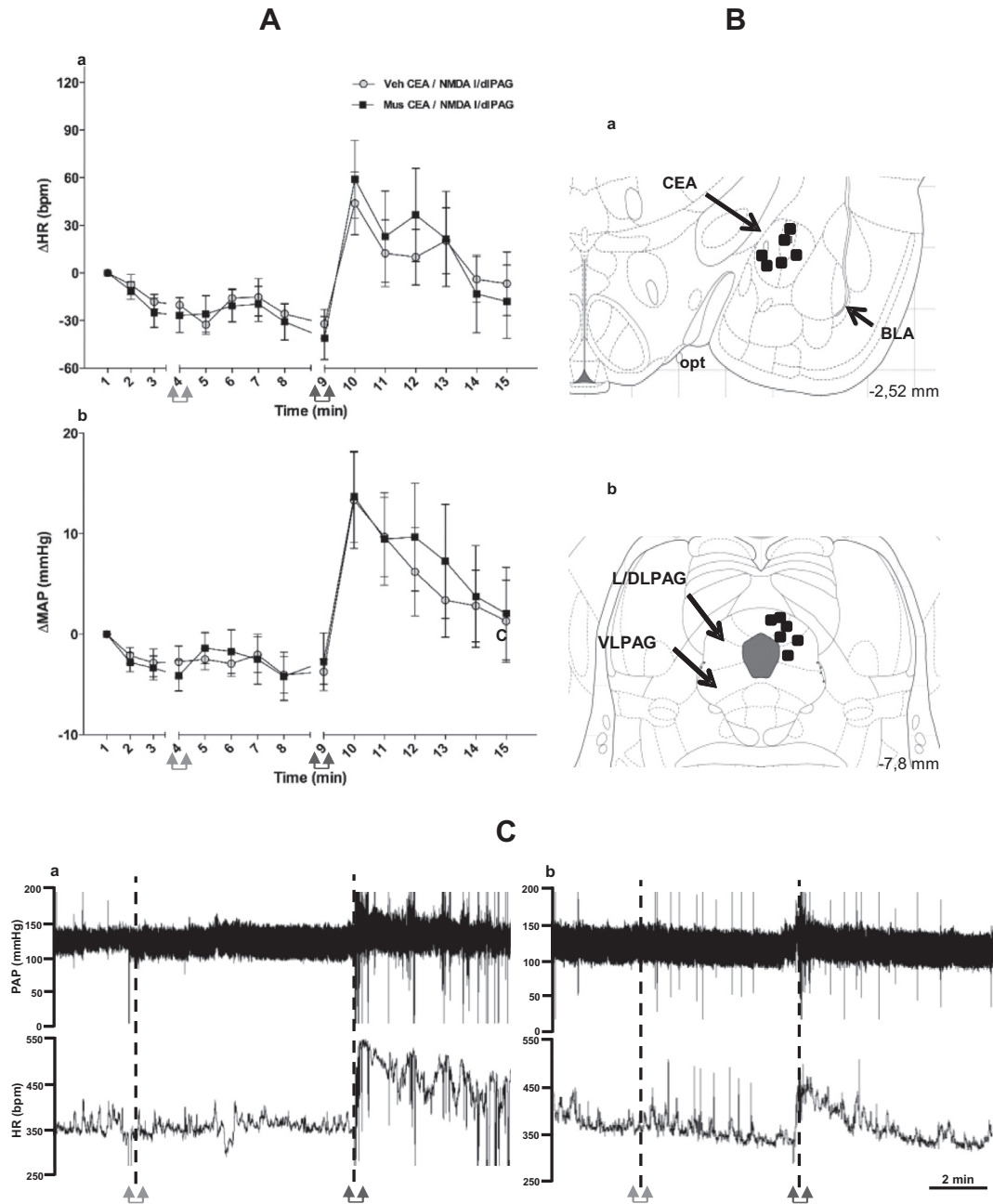


**Fig. 6.** Effect of repeated injections of NMDA into the l/dIPAG on the increase in MAP and HR evoked by the activation of this region. (A) Mean in HR (A) and MAP (B) as a result of vehicle injection into the BLA, followed by NMDA into l/dIPAG ( $n = 5$ ) in two consecutive days. Baseline HR:  $316 \pm 12$  bpm;  $345 \pm 13$  bpm. Baseline MAP:  $107 \pm 2$  mmHg;  $103 \pm 4$  mmHg. Light gray arrows indicate the injection of vehicle into the BLA; Dark gray arrows indicate the injection of NMDA into the l/dIPAG. (B) Sites of injection in the BLA and the l/dIPAG in all experiments. Schematic coronal sections of the rat brain adapted from the atlas of Paxinos and Watson (Paxinos and Watson, 2007) illustrating approximate sites of injections into the BLA (a) and l/dIPAG (b) for all experiments for which data are reported. Numbers indicate distance from bregma in millimetres. Filled triangles represent injections of vehicle (100 pmol/100 nl) into the BLA and the injection of NMDA into l/dIPAG. Abbreviations: BLA (basolateral amygdala); HR (heart rate); l/dIPAG (lateral/dorsolateral periaqueductal gray); MAP (mean arterial pressure); Mus (muscimol); Veh (vehicle).

The present data cannot discard alternative explanations for the relationship between the dIPAG and the BLA in this context. One possible explanation would be that key neurons from both regions converge to common DMH neurons related to the cardiovascular responses evoked from either region. Importantly, inhibition of the DMH can reduce the cardiovascular response produced by the activation of both regions (Soltis et al., 1998; de Menezes et al., 2009). Thus, loss of background facilitation from either region would effectively lessen the responses produced by the other.

In order to confirm that the reduction in the cardiovascular response induced by the l/dIPAG was in fact due to the inhibition of BLA activity and not by a decline in l/dIPAG excitability, we injected NMDA into the l/dIPAG on both experimental days, which yielded similar increases in HR and MAP. These data indicate that the neurons within the l/dIPAG respond similarly to repeated NMDA injections, confirming that the dose of NMDA used in our study does not cause excitotoxic neuronal death.

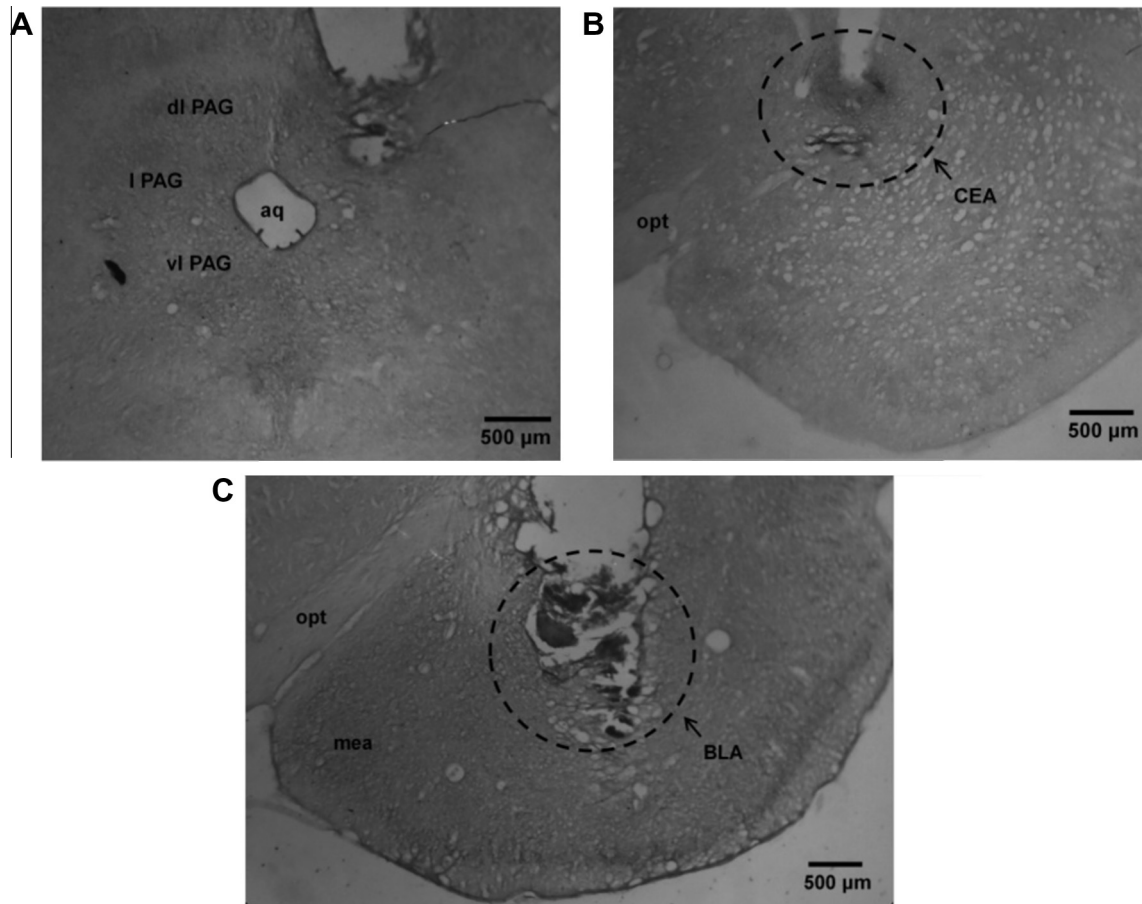
In order to test whether the l/dIPAG responses depend specifically on the BLA, we performed the same procedure described above with the CEA region, as previous experiments with retrograde tracers have shown that the CEA and the anterolateral part of the basal amygdala project to the PAG (Gray and Magnuson, 1992; Gabbott, 2003; Gabbott et al., 2003, 2005). In addition, other studies have shown that the CEA participates in the cardiovascular responses associated with the learning of stressful stimuli (Kapp et al., 1979; Gentile et al., 1986), and has been shown to modulate efferent projections to the PAG (Shaikh et al., 1991, 1994). In our study, we observed that the activation of GABAergic receptors in the CEA did not affect the HR and MAP responses induced by l/dIPAG activation. This result shows that CEA neurons do not contribute to the responses generated by l/dIPAG activation, which could be explained by the differential involvement of amygdaloid nuclei in regulating unconditioned and conditioned behaviors (Ruiz-Martinez et al., 2006). Similarly, this same response pattern was observed when we tested GABAergic inhibition of regions in the



**Fig. 7.** Effect of GABAergic inhibition of CEA on the increase in MAP and HR evoked by activation of the l/dIPAG. (A) Mean in HR (A) and MAP (B) as a result of vehicle versus muscimol injection into the CEA, followed by NMDA into l/dIPAG ( $n = 6$ ). Baseline HR: vehicle,  $381 \pm 10$  bpm; muscimol,  $374 \pm 10$  bpm. Baseline MAP: vehicle,  $117 \pm 5$  mmHg; muscimol,  $113 \pm 3$  mmHg. Light gray arrows indicate the injection of vehicle or muscimol into the CEA; Dark gray arrows indicate the injection of NMDA into the l/dIPAG. (B) Sites of injection in the CEA (a) and l/dIPAG (b) for all experiments. Schematic coronal sections of the rat brain adapted from the atlas of Paxinos and Watson (Paxinos and Watson, 2007) illustrating approximate sites of injections into the CEA (a) and l/dIPAG (b) for all experiments for which data are reported. Numbers indicate distance from the bregma in millimeters. Filled squares represent injections of muscimol (100 pmol/100 nl) or vehicle into BLA and the injection of NMDA into l/dIPAG. (C) Original recordings showing the effects of vehicle (left panel) or muscimol (right panel) injections into BLA on the cardiovascular changes evoked by the injection of NMDA into l/dIPAG (a, b). Light gray arrows indicate the injection of vehicle or muscimol into the CEA; Dark gray arrows indicate the injection of NMDA into the l/dIPAG. Recordings of pulsatile arterial pressure (PAP; top trace) and HR (bottom trace) are shown. *Abbreviations:* CEA (central amygdala); HR (heart rate); l/dIPAG (lateral/dorsolateral periaqueductal gray); MAP (mean arterial pressure); Mus (muscimol); Veh (vehicle).

vicinity but outside of the BLA. When we injected muscimol into these regions, the cardiovascular responses induced by l/dIPAG activation were not compromised (data not

shown), indicating that the responses are specific to the BLA and not the result of the diffusion of muscimol to nearby regions.



**Fig. 8.** Examples of typical sites of injections into the periaqueductal gray, basolateral and central amygdala. (A) The site of microinjection in the dorsolateral column of the PAG. (B) The site of microinjection in the CEA. (C) The site of microinjection in the BLA. *Abbreviations:* aq (aqueduct); opt (optic tract); mea (medial amygdala); CEA (central amygdala); BLA (basolateral amygdala); dlPAG (dorsal of the periaqueductal gray); lPAG (lateral of the periaqueductal gray); viPAG (ventrolateral of the periaqueductal gray).

## CONCLUSION

In this study, we showed that BLA activity plays a critical role in the modulation of physiological responses caused by l/dlPAG activation, and that these responses specifically depend on the glutamatergic activity of BLA neurons. Importantly, the same role was not observed for another region of the amygdala, the CEA. Thus, knowing that the autonomic responses evoked by l/dlPAG activation depends on neuronal activity in the DMH (de Menezes et al., 2009; Horiuchi et al., 2009), and that the cardiovascular responses induced by BLA activation also depends on DMH neurons (Soltis et al., 1998), we propose that the excitatory pathway between the dlPAG and the DMH, uses the BLA as a relay site. It is plausible that an anomalous activity of this circuit would lead to an enhanced excitation of the BLA, which in turn would over activate the DMH, increasing the vulnerability to emotional disorders, such as anxiety. Comprehension of the circuit responsible for controlling the cardiovascular changes during the defense reaction can be the key for understanding the pathophysiology of stress-related cardiovascular disorders. Our findings contribute to the understanding of this circuitry, which may help in the future development of more specific and

effective therapeutic strategies for the treatment of various anxiety disorders and the cardiovascular disorders associated with them.

*Acknowledgments*—This work was supported by FAPEMIG, PRONEN, the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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*(Accepted 22 October 2014)*  
*(Available online 4 November 2014)*