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Pharmacology, Biochemistry and Behavior 83 (2006) 296–301
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
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Flight reactions induced by injection of glutamate *N*-methyl-D-aspartate receptor agonist into the rat dorsolateral periaqueductal gray are not dependent on endogenous nitric oxide

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Received 9 November 2005; received in revised form 1 February 2006; accepted 11 February 2006
Available online 6 March 2006

Abstract

Glutamate *N*-methyl-D-aspartate (NMDA) receptors and the enzyme neuronal nitric oxide synthase (nNOS) are significantly expressed in the midbrain dorsolateral periaqueductal gray (dlPAG). Local injections of either NMDA-receptor agonists or nitric oxide (NO) donors induce flight reactions in rats. Since the activation of NMDA receptors in the brain increases the synthesis of NO, the present work was conducted to test the hypothesis that the flight reaction induced by intra-dlPAG administration of NMDA would be mediated by endogenous NO. Male Wistar rats with cannulas aimed at the dlPAG received intracerebral injections of L-NAME (NOS inhibitor, 100–200 nmol), carboxy-PTIO (NO scavenger, 1–3 nmol) or ODQ (guanylate cyclase inhibitor, 1–3 nmol). Saline or NMDA (0.1 nmol) was injected 10 min later and the behavioral changes were recorded for 2 min in the injection box. Intra-dlPAG injection of NMDA produced flight reactions characterized by crossings and jumps. Contrary to the initial hypothesis, these effects were not prevented by pretreatment with L-NAME, carboxy-PTIO or ODQ. Although the NO pathway may mediate some effects induced by NMDA receptor activation in the brain, the present results suggest that the administration of NMDA into the dlPAG induces flight reactions by mechanisms that are independent of endogenous NO.

Keywords: Aversion; Flight; Anxiety; Glutamate; Nitric oxide; Periaqueductal gray

1. Introduction

Glutamate is the primary excitatory neurotransmitter in the mammalian nervous system. Approximately 60% of neurons utilize this amino acid as neurotransmitter (for review, see Javitt, 2004). Glutamate receptors are divided into two main families, namely metabotropic and ionotropic receptors. Based on preferential agonist compounds three subtypes of glutamate ionotropic receptors have been proposed. They include *N*-methyl-D-aspartate (NMDA), kainate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) subtypes (Ozawa et al., 1998).

In several brain regions NMDA receptors are connected with the Ca^{+2}/calmodulin-dependent enzyme neuronal nitric oxide synthase (nNOS). This enzyme is activated by the calcium influx produced by the activation of glutamate receptors and synthesizes the neurotransmitter nitric oxide (NO) from L-arginine (Garthwaite et al., 1988, 1989). NO may diffuse to pre- and postsynaptic neurons (Edelman and Gally, 1992; Snyder and Bredt, 1991), activating the enzyme guanylate cyclase (GC) and mediating the glutamate-linked enhancement of cGMP (Bredt and Snyder, 1989; Garthwaite et al., 1988; Knowles et al., 1989).

Glutamate receptors are widely expressed in the midbrain periaqueductal gray (PAG, Albin et al., 1990). This structure has been divided into dorsomedial, dorsolateral (dlPAG), lateral and ventrolateral columns (Carrive, 1993). The dorsal columns of the PAG are proposed to be part of a neural substrate responsible for the elaboration of active defensive behaviors (Graeff, 1981, 1994). Electrical stimulation of these columns or local injection of glutamate induces flight reactions that are similar to unconditioned fear responses to proximal danger (Bandler and Carrive, 1988; Krieger and Graeff, 1985). Based on criteria of face validity and pharmacological predictability, this behavioral response has been proposed as an animal model of panic attacks (Deakin

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doi:10.1016/j.pbb.2006.02.010

and Graeff, 1991; Jenck et al., 1995; Schenberg et al., 2001). Further supporting a role for glutamate in aversive behaviors mediated by the PAG, local injection of an NMDA-receptor antagonist (AP7) induces antiaversive effects in animal models of anxiety, such as the elevated plus maze (Guimarães et al., 1991) or the Vogel punished licking test (Molchanov and Guimarães, 2002). Moreover, administration of the AMPA/kainate receptor antagonist (CNQX) into this structure also induces anxiolytic-like effect in the elevated plus maze (Matheus and Guimarães, 1997).

While glutamate receptors are distributed along all columns of the rat PAG, the enzyme nNOS is selectively expressed in the dlPAG (Onstott et al., 1993). Similar to glutamate agonists, local injections of NO-donors produce flight reactions (De Oliveira et al., 2000a). On the contrary, administration into this region of NOS inhibitors (Guimarães et al., 1994), guanylate cyclase inhibitors (De Oliveira and Guimarães, 1999; Aguiar et al., 2004; Guimarães et al., 2005) or a nitric oxide scavenger (Aguiar et al., 2004) induces anxiolytic-like effects in the elevated plus-maze model. Altogether, these results suggest that NO might also mediate aversive behaviors in the dlPAG.

Although NO has been proposed as a mediator of the aversive action of glutamate in this region (for review, see De Oliveira et al., 2001), this hypothesis has not been directly addressed. Podhorna and Brown (2000) observed that in the ultrasonic vocalization model the NO-pathway mediates the anxiogenic effect of systemic injections of NMDA. On the other hand, ionotropic glutamate receptors mediate the flight reaction induced by a NO-donor into the dlPAG (Moreira et al., 2004). While this later result suggests that the aversive effect of NO in the dlPAG depends on NMDA-receptor activation, the opposite has not been tested in this brain region. Therefore, the objective of this work was to test the hypothesis that the aversive reactions induced by glutamate NMDA receptor in the dlPAG involve the activation of the nitric oxide pathway.

2. Material and methods

2.1. Subjects

Male Wistar rats weighing 220–240 g at the beginning of each experiment were housed in pairs in a temperature-controlled room (24 ± 1 °C) under standard laboratory conditions with free access to food and water and a 12 h light/12 h dark cycle (lights on at 06:30 h a.m.). All the experiments were conducted between 8 a.m. and 11 a.m. Procedures were conducted in conformity with the Brazilian Society of Neuroscience and Behavior guidelines for the care and use of laboratory

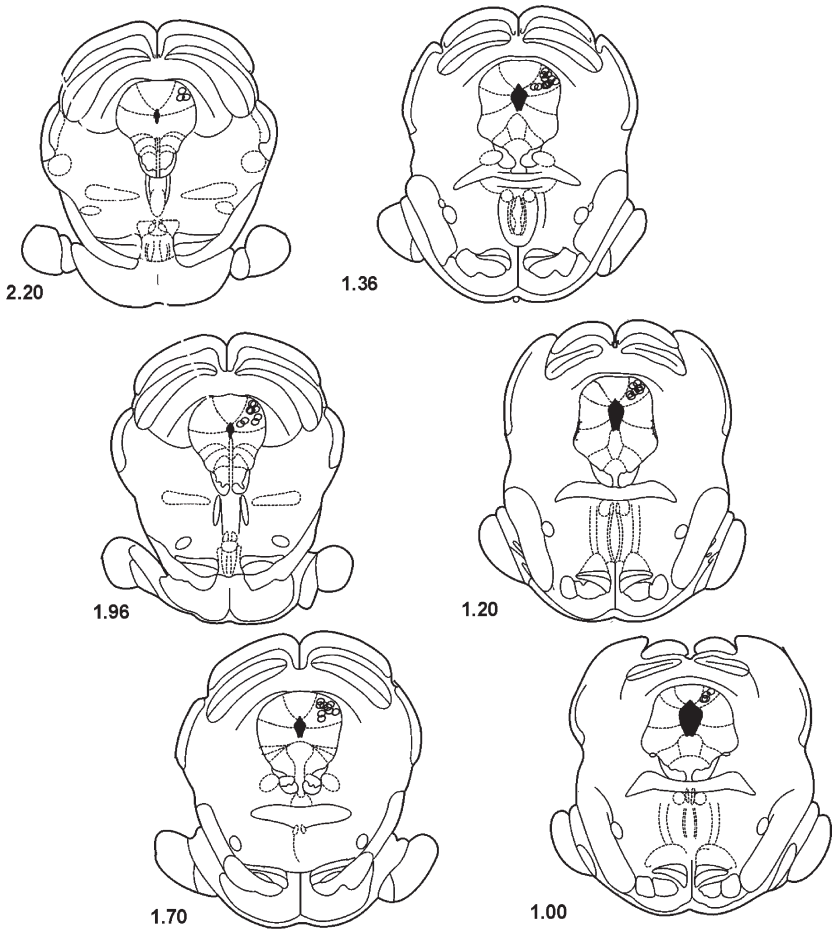

Fig. 1. Histological localization of injection sites (0.2 μL, open circles) in diagrams based on the atlas of Paxinos and Watson (1997).

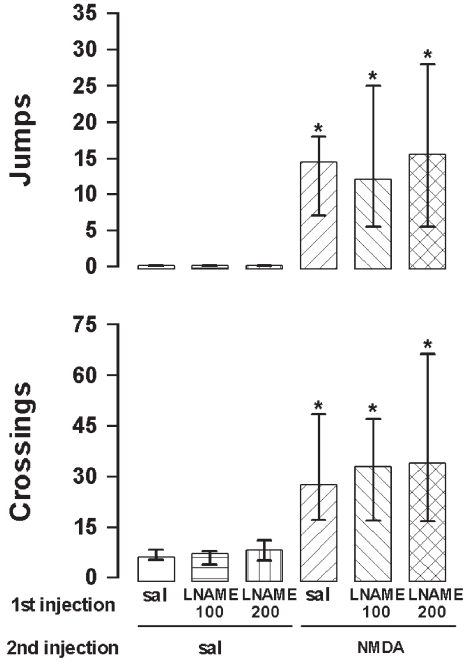

Fig. 2. Effects of saline+saline ($n=6$), L-NAME 100 nmol+saline ($n=6$), L-NAME 200 nmol+saline ($n=7$), saline+NMDA 0.1 nmol ($n=16$), L-NAME 100 nmol+NMDA ($n=13$) or L-NAME 200 nmol+NMDA ($n=10$) injected into the dlPAG. Each bar represents the median±interquartil range for the number of jumps and crossings. Asterisks signal significant difference from saline+saline group (Kruskall–Wallis followed by Mann–Whitney, $p<0.05$).

animals, which are in compliance with international laws and policies. All efforts were made to minimize animal suffering.

2.2. Drugs

NMDA (*N*-methyl-D-aspartic acid; Sigma) 0.1 nmol/0.2 μL, the NOS inhibitor L-NAME (Nitro-L-arginine-*N*-methyl ester hydrochloride; RBI) 100 and 200 nmol/0.2 μL and the NO-scavenger Carboxy-PTIO ((*S*)-3-Carboxy-4-hydroxyphenylglicine; RBI) 1 and 3 nmol/0.2 μL, were dissolved in saline (0.9% NaCl); the specific GC-inhibitor ODQ (1*H*-[1,2,4]Oxadiazolol [4,3-a]quinoxalin-1-one; RBI) 1 and 3 nmol/0.2 μL, was dissolved in DMSO. The solutions were prepared immediately before use and were kept on ice and protected from the light during the experimental session. The NMDA dose was based on a pilot study and was the smallest one, in our experimental conditions, that induced consistent flight reactions. The doses of L-NAME, Carboxy-PTIO and ODQ were chosen based on previous works showing anxiolytic effects of these drugs after injection into the dorsal PAG (Guimarães et al., 1994, 2005; Teixeira, 2002; Aguiar et al., 2004).

2.3. Apparatus

The experiments were carried out in a Plexiglas box (29×19×34 cm) and in a circular open arena (60 cm in diameter with a 60 cm high Plexiglas wall) located in a sound-attenuated, temperature-controlled (25±1 °C) room, illuminated with three 40 W fluorescent bulbs placed 4 m above the apparatus. The rats were videotaped inside the Plexiglas box and their behavior was analyzed by a trained observer. In the arena, the distance moved was analyzed with the help of the Ethovision software (version 1.9; Noldus, the Netherlands). This software detects the position of the animal in the open arena and calculates the distance moved.

2.4. Surgery

Rats were anesthetized with 2.5% 2,2,2-tribromoethanol (10 mg/kg, i.p.) and fixed in a stereotaxic frame. A stainless steel guide cannula (0.6 mm OD) was implanted unilaterally on the right side aimed at the dlPAG (coordinates: AP=0 from lambda, L=1.9 mm at an angle of 16°, D=4.0 mm). The cannula was attached to the bones with stainless steel screws and acrylic cement. An obturator inside the guide cannulae prevented obstruction.

2.5. Procedure

Seven days after surgery the animals were randomly assigned to one of the treatment groups. Intracerebral injections were performed with a thin dental needle (0.3 mm OD) introduced through the guide cannula until its tip was 1.0 mm below the cannula end. A volume of 0.2 μL was injected in 20 s using a microsyringe (Hamilton, USA) connected to an infusion pump (Kd Scientific, USA). A polyethylene catheter (PE 10) was interposed between the upper end of the dental needle and the microsyringe. The rats received injections into the dlPAG of

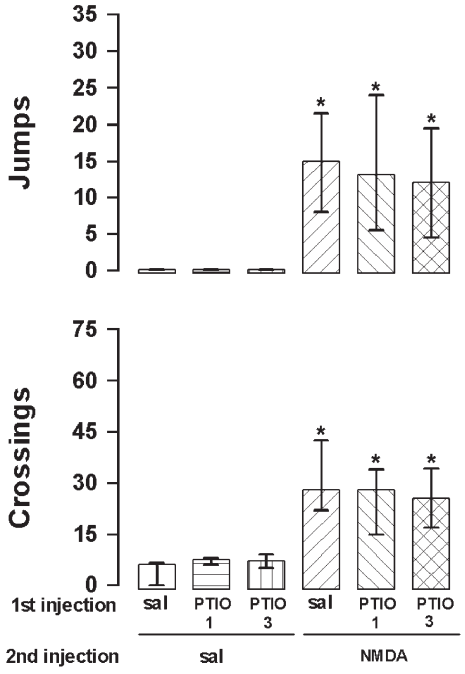

Fig. 3. Effects of saline+saline ($n=5$), Carboxy-PTIO 1 nmol+saline ($n=7$), Carboxy-PTIO 3 nmol+saline ($n=8$), saline+NMDA 0.1 nmol ($n=13$), Carboxy-PTIO 1 nmol+NMDA ($n=8$) or Carboxy-PTIO 3 nmol+NMDA ($n=5$) injected into the dlPAG. Further specifications as in Fig. 2.

vehicle, L-NAME, Carboxy-PTIO or ODQ followed, 10 min later, by a second injection of saline or NMDA. Animal behavior was videotaped for 2 min from the beginning of the second injection and the number of jumps towards the top edges of the box and the number of crossings inside the box were counted by a trained observer. Thereafter they were immediately placed inside the open arena and the distance moved during 5 min was measured.

2.6. Histology

After the behavioral tests the rats were sacrificed under deep urethane anesthesia and perfused through the left ventricle of the heart with isotonic saline followed by 10% formalin solution. After that, a dental needle was inserted through the guide cannula and 0.2 μL of fast-green was injected. The brains were removed and, after a minimum period of 3 days immersed in a 10% formalin solution, 50 μm sections were obtained in a Cryostat (Cryocut 1800). The injection sites were identified in diagrams from the Paxinos and Watson's atlas (Paxinos and Watson, 1997). The injection sites can be seen in Fig. 1. Rats that received injections outside the aimed area were excluded from analysis.

2.7. Statistical analysis

The total number of jumps and crossings was analyzed by Kruskal–Wallis followed by the Mann–Whitney test. These data are presented as median±interquartil range (IR). The total distance moved was analyzed by one-way analysis of variance (ANOVA) followed by the Duncan test. Differences were considered significant at $p<0.05$ level.

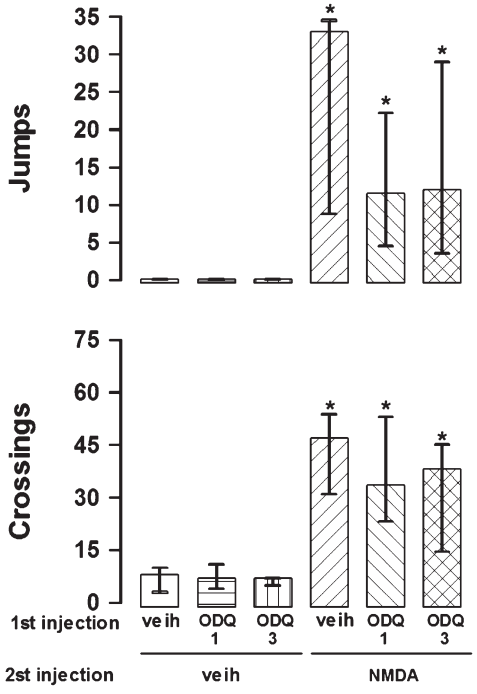

Fig. 4. Effects of DMSO+saline ($n=4$), ODQ 1 nmol+saline ($n=5$), ODQ 3 nmol+saline ($n=7$), saline+NMDA 0.1 nmol ($n=8$), ODQ 1 nmol+NMDA ($n=8$) or ODQ 3 nmol+NMDA ($n=5$) injected into the dlPAG. Further specifications as in Fig. 2.

Table 1
Mean±S.E.M. distance moved (cm) measured over a period of 5 min in a circular arena 2 min after the last drug injection

Experiment 1			
Saline+saline	961.8±91.9	Saline+NMDA	973.3±121.7
L-NAME (100)+saline	759.4±111.7	L-NAME (100)+NMDA	965.9±115.3
L-NAME (200)+saline	894.2±96.0	L-NAME (200)+NMDA	1094.9±153.5
Experiment 2			
Saline+saline	1152.3±99.9	Saline+NMDA	855.4±123.1
Carboxy-PTIO (1)+saline	1097.5±84.2	Carboxy-PTIO (1)+NMDA	946.6±191.6
Carboxy-PTIO (3)+saline	808.1±120.2	Carboxy-PTIO (3)+NMDA	734.2±156.2
Experiment 3			
DMSO+saline	869.5±98.7	DMSO+NMDA	964.6±131.3
ODQ (1)+saline	1343.8±145.6	ODQ (1)+NMDA	1091.4±128.4
ODQ (3)+saline	852.6±90.9	ODQ (3)+NMDA	1128.9±430.5

The number inside the parenthesis indicated the drug dose (in nmol) used. NMDA was injected at the dose of 0.1 nmol. There are no differences among groups.

3. Results

Injection of NMDA 0.1 nmol into the dlPAG produced flight reactions characterized by jumps towards the top edges of the box and by an increased number of crossings during the 2 min observational period. The effect of pretreatment with L-NAME can be seen in Fig. 2. L-NAME 100 or 200 nmol failed to prevent the flight reaction induced by NMDA ($p=0.0585$ and $p=0.0066$ for crossing and jumps, respectively; Kruskal–Wallis). Similarly, as can be seen in Fig. 3, the effects of NMDA were not prevented by pretreatment with Carboxy-PTIO ($p=0.0441$ and $p=0.002$ for crossing and jumps, respectively; Kruskal–Wallis). Finally, these parameters were not modified by ODQ pretreatment ($p=0.0002$ and $p=0.0001$ for crossing and jumps, respectively; Kruskal–Wallis; Fig. 4). None of the aforementioned treatments changed the total distance moved in the open arena after the initial flight reaction (Table 1).

4. Discussion

As expected, microinjection of NMDA into the dlPAG of rats induced a vigorous flight reaction characterized by crossings and jumps that lasted for 2 min after the beginning of the injection. This is in agreement with previous work reporting defensive reactions induced by this compound in the PAG (Bittencourt et al., 2004). These effects are also observed after local injection of glutamate or trans-(±)-1-amino-1,3-cyclopentanedicarboxylic acid (t-ACPD), a metabotropic glutamate receptor agonist (Krieger and Graeff, 1985; Molchanov and Guimarães, 1999), supporting a role for glutamate in defensive reactions mediated by the dorsal PAG.

Different columns of the PAG are involved in reactions to distinct kinds of stressors (Graeff, 1981, 1994). While responses to physical stress are triggered either from the ventrolateral (visceral stressors) or from the lateral (somatic stressors) PAG, the dlPAG is proposed to be a component of a circuit that triggers active emotional coping to innate psychological stress (Bandler et al., 2000). Accordingly, rats exposed to a live predator show an increase in Fos-like immunoreactivity (Canteras and Goto, 1999) and nitric oxide activity (Chiavegatto et al., 1998) in the dlPAG. Moreover, restraint stress increases nNOS mRNA expression (De Oliveira et al., 2000b) and NADPHd activity (Krukoff and Khalili, 1997) in this structure.

The PAG, along with the medial hypothalamus and the amygdala, constitutes a system that has been traditionally grouped as a brain aversion system (Graeff, 1994). The current views hold that fear and anxiety are represented in all levels of this system, although they can be discriminated either by the distance from the source of danger or by the defensive direction (Sewards and Sewards, 2002; McNaughton and Corr, 2004). Accordingly with this proposal, anxiety might occur when an animal approaches a dangerous situation or environment, while fear and panic might be related to escape reactions, such as in the present experiments. Considering the distance from the aversive stimulus, the amygdala and the medial hypothalamus would mediate responses to distal threat while the dorsal columns of the PAG would elaborate escape reactions to proximal danger (McNaughton and Corr, 2004). Accordingly, electrical or chemical stimulation of these columns elicits a core of behaviors such as running, jumps and galloping that resembles responses to proximal innate fear stimuli (Blanchard and Blanchard, 1988; Schenberg et al., 2001). Therefore, the ethological parameters quantified in the present experiments have been proposed as candidates for panic-like behaviors in rats (Vargas and Schenberg, 2001).

These aforementioned brain areas, among others, may contribute to the vigorous behavioral reaction described in our results. Employing Fos-protein expression as a neural marker for the activation of brain structures (Morgan and Curran, 1991), it was observed that induction of flight reaction by NMDA-injection into the dlPAG activates hypothalamic areas related to defensive reactions, such as the dorsomedial part of the ventromedial nucleus, the premammillary dorsal nucleus and the lateral and anterior nuclei. Moreover, there was also a significant activation in the central and medial amygdala nuclei, in midbrain structures such as the dorsal PAG, the inferior and superior colliculi, the median raphe nucleus and the locus coeruleus (Ferreira-Netto et al., 2005). All these structures are activated in response to various aversive stimuli and are implicated in different components of panic, fear or anxiety reactions (Silveira et al., 1993; Dielenberg et al., 2001).

Concerning the role of NO, drugs that release this compound induce flight reactions similar to glutamate agonists when injected into the dlPAG (De Oliveira et al., 2000a). In addition, NO increases glutamate release in different brain structures (Lin et al., 1999, 2000) and NO-induced flight reactions in the dlPAG are prevented by ionotropic glutamate receptor antagonists (Moreira et al., 2004). This suggests that NO may act by increasing glutamate release, the latter being the final mediator of NO-induced effects. Moreover, the present results suggest that some effects of glutamate do not involve NO formation. In accordance with this possibility, the induction of long-term potentiation by NMDA was not modified by manipulations enhancing or inhibiting NO formation in a study with hippocampal slices (Hopper et al., 2004). In addition, the inhibition of neurotransmitter release mediated by NMDA receptor was not prevented by Carboxy-PTIO (Sequeira et al., 2001).

These results do not discard that NO may be important in the dlPAG in other circumstances, such as in anxiety-related behaviors. Although the systemic administration of NO-modulators has produced contradictory results (Volke et al., 1995; Li et al., 2003), more consistent data have been found in experiments employing intracerebral injections. In the dlPAG, the NO-inhibitor L-NAME induced an anxiolytic effect in the elevated plus-maze (Guimarães et al., 1994). Similar results were obtained with the NO-scavenger Carboxy-PTIO and the GC inhibitor ODQ (Aguiar et al., 2004; Guimarães et al., 2005). Moreover, injection of L-NAME or the nNOS selective inhibitor 7-Nitroindazole in the medial amygdala induced anxiolytic effects both in the elevated plus-maze and in the light–dark test (Forestiero et al., 2006).

In summary, selective NMDA-receptor activation in the dlPAG caused flight reactions that were not prevented by the nNOS inhibitor L-NAME, the NO scavenger, Carboxy-PTIO or the GC inhibitor ODQ. Although NO may have a facilitatory role in defensive reactions, the effect of exogenously administered NMDA into the dlPAG is independent of endogenous NO.

Acknowledgments

This research was supported by grants from CAPES, CNPq and FAPESP (02/13197-2). We thank J.C. de Aguiar and E.T. Gomes for the excellent technical support.

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