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Role of nitric oxide in the periaqueductal gray in defensive behavior in mice: influence of prior local N-methyl-D-aspartate receptor activation and aversive condition

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Abstract

Glutamate *N*-methyl-D-aspartate (NMDA) receptor activation within the dorsal column of the periaqueductal gray (dPAG) leads to antinociceptive, autonomic, and behavioral responses characterized as the fear reaction. Activation of NMDA receptors in the brain increases nitric oxide (NO) synthesis, and NO has been proposed to be a mediator of the aversive action of glutamate. This paper reviews a series of studies investigating the effects of neuronal NO synthase (nNOS) inhibition in the dPAG of mice in different aversive conditions. nNOS inhibition by infusion of Nω-propyl-L-arginine (NPLA) prevents fear-like reactions (e.g., jumping, running, freezing) induced by NMDA receptor stimulation within the dPAG and produces anti-aversive effects when injected into the same midbrain site in mice confronted with a predator. Interestingly, nNOS inhibition within the dPAG does not change anxiety-like behavior in mice exposed to the elevated plus maze (EPM), but it reverses the effect of an anxiogenic dose of NMDA injected into the same site in animals subjected to the EPM. Altogether, the results support a role for glutamate NMDA receptors and NO in the dPAG in the regulation of defensive behaviors in mice. However, dPAG nitrergic modulation of anxiety-like behavior appears to depend on the magnitude of the aversive stimulus. **Keywords:** periaqueductal gray matter (PAG), NMDA receptors, neuronal nitric oxide synthase (nNOS), elevated plus maze (EPM), rat exposure test (RET), mouse.

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Introduction

The dorsal portion of the periaqueductal gray (dPAG) has been identified as the principal substrate of aversive states in the midbrain (Graeff, 2004). Electrical or chemical stimulation of the dPAG elicits autonomic (e.g., tachycardia, defecation) and behavioral (e.g., jumping, running, immobility) responses characterized as the fear reaction. Additionally, many lines of evidence have indicated that in addition to fundamentally controlling fear-like fight and flight responses (for review, see Graeff, 2004), the PAG modulates less vigorous defensive responses related to anxiety, such as threat avoidance and risk assessment (Bertoglio & Zangrossi, 2006; Carvalho-Netto, Litvin, Nunes-de-Souza, Blanchard, & Blanchard, 2007; McNaughton & Corr, 2004; Mendes-Gomes & Nunes-de-Souza, 2005, 2009; Teixeira & Carobrez, 1999;).

Chemical stimulation of the PAG can be performed by local glutamate NMDA (*N*-methyl-D-aspartate) receptor activation. When injected into the dPAG, NMDA

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receptor agonists elicit fight and flight reactions in rats (e.g., Bandler, 1988; Bittencourt, Carobrez, Zamprogno, Tufik, & Schenberg, 2004) and mice (e.g., Beckett, Lawrence, Marsden, & Marshall, 1992), whereas local microinjection of 2-amino-7-phosphonoheptanoic acid (AP-7), a competitive NMDA receptor antagonist, produces antiaversive-like effects in the elevated plus maze (EPM; Guimarães, Carobrez, De Aguiar, & Graeff, 1991), a widely used animal model of anxiety.

Glutamate is a ubiquitous excitatory amino acid in the central nervous system. In addition to activating ionotropic NMDA receptors, glutamate is also able to activate two other ion channel-coupled receptors, the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor and kainate receptor (Heresco-Levy, 2003; Huntley, Vickers, & Morrison, 1994; Ozawa, Kamiya, & Tsuzuki, 1998; Seeburg, 1993), and a G-protein-coupled receptor (metabotropic receptor). These receptors are largely expressed in the PAG (Albin & Gilman, 1990; Onstott, Mayer, & Beitz, 1993; Schubert, Shaikh, & Siegel, 1996). Glutamate NMDA receptor activation leads to cellular calcium influx, which triggers a cascade of intracellular events, including activation of nitric oxide synthase (NOS), an enzyme that produces nitric oxide (NO) by converting L-arginine to L-citroline, with nicotinamide adenine dinucleotide phosphate (NADPH) and Ca^{2+} as co-factors (Garthwaite, Garthwaite, Palmer, & Moncada, 1989; Heresco-Levy, 2003; Mayer et al., 1991). NOS has at least three isoforms. Inducible NOS (iNOS) is involved in immunological reactions and activated by factors released during pathological events, such as cytokines that can induce a substantial release of NO. Two other NOS isoforms are constitutive forms that are present in vase endothelia (endothelial NOS [eNOS]) and neurons (neuronal NOS [nNOS]) (Guix, Uribesalgo, Coma, & Muñhoz, 2005; Lamas, Marsden, Li, Tempst, & Michel, 1992; Mungrue, Bredt, Stewart, & Husain, 2003; Prast & Philippu, 2001).

Similar to the proaversive effects produced by glutamate NMDA receptor agonists, injection of NO donors into the dorsolateral column of the PAG (dlPAG) produces fight and flight reactions in rats (De Oliveira, Del-Bel, & Guimarães, 2001). In contrast, administration of NOS inhibitors, guanylate cyclase inhibitors, and a NO scavenger in this same region induces anxiolytic-like effects in the EPM (De Oliveira & Guimarães, 1999; Guimarães, Beijamini, Moreira, Aguiar, & de Lucca, 2005; Guimarães, De Aguiar, Del Bel, & Ballejo, 1994).

This paper attempts to show that the roles played by glutamate NMDA receptors and the NO complex within the dPAG in defensive behavior in mice appear to depend on the magnitude or nature of the aversive stimuli. Whereas chemical stimulation of the dPAG elicits defensive reactions that are blocked by local infusion of a nNOS inhibitor, the behavioral defensive responses induced by a more naturalistic situation (e.g., exposure to the EPM) are sensitive to NOS inhibition only in animals pretreated with an NMDA receptor agonist in the same midbrain structure. However, when exposed to a more aversive situation (e.g., confrontation by a predator), inhibition of nNOS within the dPAG attenuates defensive-like behaviors in mice.

Defensive-like responses induced by chemical stimulation of the dPAG with NMDA infusion in mice: effects of nNOS inhibition

As widely demonstrated in many laboratories (e.g., Bandler & Carrive, 1988; Bittencourt et al., 2004; Blanchard & Blanchard, 1988; Carvalho-Netto, Markham, Blanchard, Nunes-de-Souza, & Blanchard, 2006), chemical stimulation of the dPAG (e.g., with NMDA infusion) induces a set of behavioral responses, such as jumping, running, and freezing, that last approximately 5 minutes. Immediately after (and sometimes *during*) intra-dPAG NMDA infusion, animals exhibit a sequence of apparently disoriented jumps intercalated with running. These explosive motor behaviors, especially jumping, last approximately 60-90 seconds and are followed by running intercalated with periods of freezing for 60-120 seconds. These defensive-like behaviors are followed by a sequence of non-aversively motivated behaviors (e.g., walking and rearing). Figure 1 illustrates the effects of intra-dPAG injection of NMDA (.04 nmol) on jumping and rearing frequency and on running and freezing time (in seconds) over 5 minutes in mice pretreated with saline or .2-.4 nmol N^{ω}-propyl-L-arginine (NPLA, a highly selective and potent inhibitor of nNOS; K_i = 57 nM) that displays 3158-fold and 149-fold selectivity for iNOS and eNOS, respectively (Zhang, Fast, Marletta, Martasek, & Silverman, 1997), in the same midbrain site. Intra-dPAG infusions of NPLA (.4 nmol) changed the defensive behaviors induced by intra-dPAG injections of NMDA. The excitatory amino acid induced a sudden sequence of jumping and running behaviors that were followed by a period of freezing. These results corroborate many previous studies (Bandler, 1988; Bandler & Carrive, 1988; Beckett et al., 1992; De Oliveira et al., 2001; Molchanov & Guimaraes, 1999), which demonstrated that intra-PAG injections of glutamate receptor agonists produce defensive reactions in rodents. NPLA antagonized these NMDA-induced behavioral effects, suggesting that NO release within the PAG plays a role in defensive behavior. Intra-dPAG infusion of NMDA did not alter rearing frequency, but higher-dose NPLA increased this vertical exploratory behavior, an effect that was independent of the treatment combination (i.e. saline or NMDA). The increase in rearing frequency suggests that this nNOS inhibitor (.2 and .4 nmol) does not provoke motor disruption, an effect previously reported with

systemic injections of other NOS inhibitors (Del Bel, da Silva, & Guimaraes, 1998; Del Bel, Souza, Guimaraes, da Silva, & Nucci-da-Silva, 2002; Del Bel, da Silva, Guimaraes, & Bermudez-Echeverry, 2004). Intra-dPAG NPLA injections appear to selectively reduce NMDA-induced behavioral responses (e.g., jumping, running, freezing) in mice. Altogether, these results suggest that NO, synthesized after glutamate NMDA receptor activation within the dPAG, may modulate defensive behaviors in this midbrain structure.

However, intra-dPAG NPLA fails to alter defensive behavior when mice are exposed to a more naturalistic situation (e.g., the EPM). The results shown below suggest that the role played by NO in emotional responsiveness appears to be dependent on glutamate NMDA receptor activation, at least within the mouse dPAG, and the type of aversive stimulus exposure.

Inhibition of nNOS within the dPAG fails to alter anxiety-like behavior in the mouse EPM

Figure 2 shows that inhibition of NO synthesis within the mouse dPAG neither increased nor decreased anxiety-like behavior in the EPM. Intra-dPAG infusion of .4 nmol NPLA completely blocked vigorous defensive-like behaviors (e.g., jumping and running) induced by NMDA infusion (.04 nmol) into the same site (Figure 1), and intra-dPAG NPLA infusions of this nNOS inhibitor at doses of .2, .4, and .8 nmol failed to attenuate anxiety-like behavior in the EPM (Figure 2). The failure of intra-dPAG NPLA to affect anxiety-like behavior suggests that the aversive experience in the EPM is not sufficient to induce an anxiogenic amount of NO synthesis within the mouse dPAG, suggesting that intra-dPAG NO likely does not play a role in anxiety-like behavior elicited during EPM exposure in mice.

Inhibition of nNOS within the dPAG attenuates anxiety-like behavior induced by intra-dPAG NMDA infusion in the mouse EPM

Although intra-dPAG NPLA failed to alter indices of anxiety in the mouse EPM, NO synthesis appears to be important for the anxiogenic-like effects induced by local infusion of NMDA. We found that intra-dPAG injection of NMDA at a dose that did not produce any vigorous defensive-like behavior (.02 nmol/.1 µl) led mice to explore the open arms of the EPM less

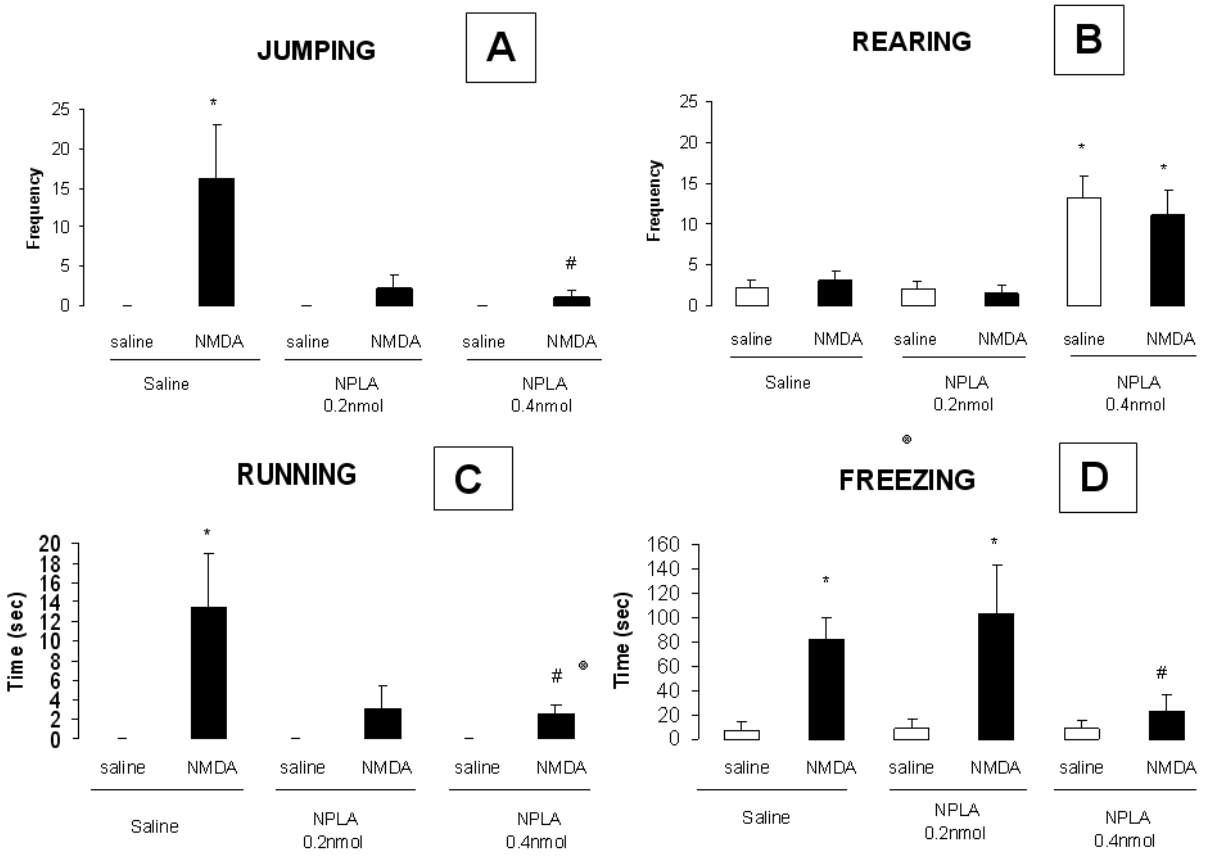

Figure 1. Effects of intra-dPAG injection of NMDA (0.04 nmol) on (A) jumping frequency, (B) rearing frequency, (C) running time (s), and (D) freezing time (s) over 5 min in mice pretreated with saline or NPLA (0.2-0.4 nmol) into the same midbrain site (n = 9-14; see text for details). *$p < .05$, compared with Saline+Saline; $^{\#}p < .05$, compared with Saline+NMDA (adapted from Miguel & Nunes-de-Souza, 2006).

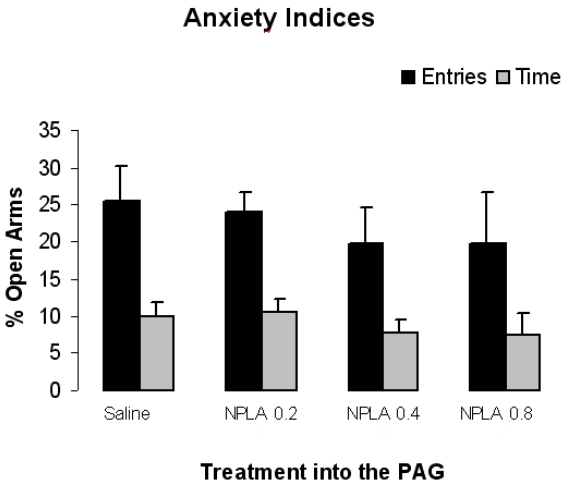

Figure 2. Effects of NPLA microinjection (0, .2, .4, and .8 nmol/0.1 µl; n = 9-15) into the dPAG on the percentage of open arm entries and percentage of open arm time in the EPM. Data are expressed as mean ± SEM (reproduced from Miguel & Nunes-de-Souza, 2008).

(Figure 3). This anxiogenic-like effect of NMDA was characterized by a selective reduction in the percentage of open arm entries and percentage of open arm time, the two main measures used as indices of anxiety in the EPM (e.g., File, 1992; Rodgers & Johnson, 1995). These results corroborate previous findings that demonstrated an anxiogenic-like effect induced by glutamate NMDA receptor activation within the midbrain PAG in rodents (e.g., Bandler, 1988; Bittencourt et al., 2004).

Interestingly, when injected into the dPAG, NPLA completely blocked the enhancement of anxiety-like behavior induced by intra-PAG NMDA. As shown in Figure 4, intra-dPAG NPLA reversed the anxiogenic-like effects produced by local infusion of NMDA. Importantly, animals that received intra-dPAG injection of NPLA (NPLA + saline) did not exhibit any significantly different behavior in the mouse EPM compared with the control group (saline + saline). NMDA-injected animals (NMDA + saline), in turn, confirmed the results shown in Figure 3, showing an anxiogenic-like profile of this NMDA receptor agonist. These results indicate that glutamate NMDA receptor activation within the mouse PAG induces NO synthesis, which in turn leads to enhanced anxiety-like behavior in the EPM.

The mechanisms involved in the apparently contrasting effects of intra-dPAG NPLA on anxiety-like behavior (i.e., blockade of the anxiogenic-like effects of NMDA but inability to attenuate anxiety-like behavior when injected alone) are not clear. In fact, the existing data suggest that glutamatergic activation appears to be necessary to observe the effects of NO. However, this does not exclude a possible intrinsic anxiogenic-like effect of glutamate release during the exposure of mice to the EPM. Measuring glutamate release or NMDA receptor activation within the dPAG during the exposure of mice to the EPM would be interesting. In this context, we recently found that glutamate NMDA receptor blockade by intra-dPAG infusion of the NMDA receptor antagonist AP-7 attenuated anxiety-like behavior in mice exposed to the EPM (Figure 5). Intra-dPAG injection of AP-7 (.2 nmol/.1 µl) increased open arm exploration without affecting closed arm entries (results not shown), suggesting a selective effect of this NMDA receptor antagonist on anxiety-like behavior. This anxiolytic-like profile produced by NMDA receptor blockade within the PAG was previously demonstrated in rats exposed to the EPM (Guimarães et al, 1991; Molchanov & Guimarães, 2002).

The mechanisms underlying these apparently contrasting effects of intra-dPAG AP-7 and NPLA on anxiety-like behavior remain unclear. The results showing that intra-dPAG injection of AP-7 and NPLA

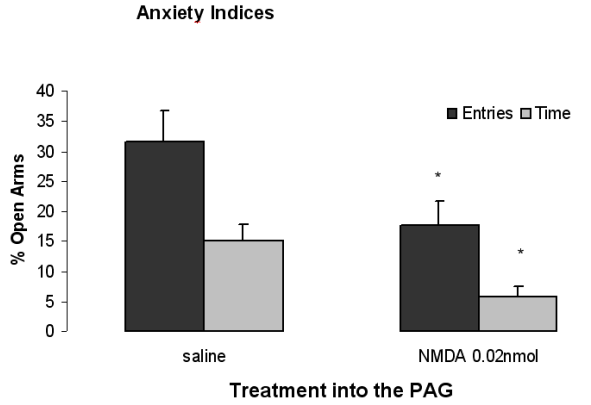

Figure 3. Effects of NMDA microinjection (0 and .02 nmol/.1 µl; n = 15-16) into the dPAG on the percentage of open arm entries and percentage of open arm time in the EPM. Data are expressed as mean ± SEM. *p < .05, compared with control group (saline) (reproduced from Miguel & Nunes-de-Souza, 2008).

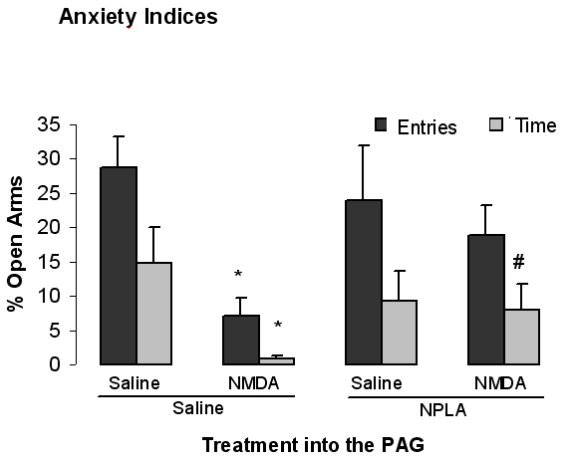

Figure 4. Effects of combined microinfusions of NPLA (0 and .4 nmol/.1 µl) and NMDA (0 and .02 nmol/.1 µl) into the dPAG on the percentage of open arm entries and percentage of open arm time in the EPM. Data are expressed as mean ± SEM (n = 7-8). *p < .05, compared with control group (saline + saline); #p < .05, compared with saline + NMDA group (adapted from Miguel & Nunes-de-Souza, 2008).

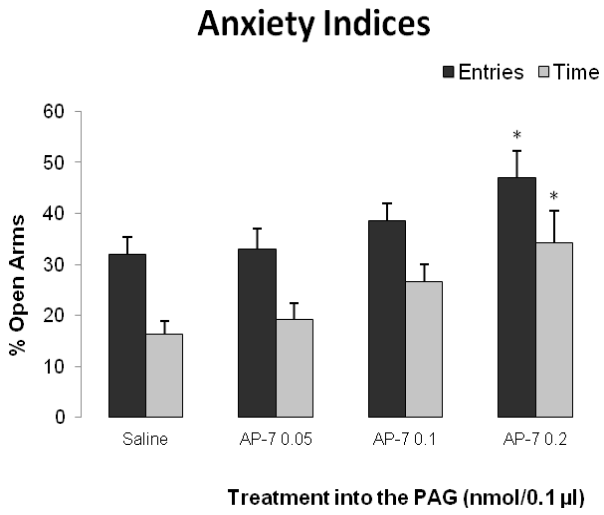

Figure 5. Effects of AP-7 microinjection (0, .05, .1, and .2 nmol/.1 µl; n = 8-15) into the dPAG on the percentage of open arm entries and percentage of open arm time in the EPM. Data are expressed as mean ± SEM. *p < .05, compared with control group (saline).

attenuated and did not alter, respectively, anxiety-like behavior when injected alone suggest that the anxiogenic-like effect of glutamate NMDA receptor activation may not be completely dependent on NO synthesis. The anxiogenic-like effect of NO appears to depend on NMDA receptor "hyperactivation" within the mouse dPAG, whereas glutamate NMDA receptor activation appears to play a tonic role in anxiety-like behavior. If so, then NMDA receptor activation would lead to neuronal excitation without affecting NO release. Ca^{2+} influx via NMDA receptor activation has been demonstrated to trigger subsequent and persistent changes in the expression of AMPA receptors, and these receptors are responsible for a substantial portion of basal excitatory postsynaptic potential (e.g., MacDonald, Jackson, & Beazely, 2006).

Considering that intra-dPAG nNOS inhibition attenuated anxiety-like behavior only in animals pretreated with an NMDA receptor agonist, dPAG nitrergic modulation of defensive behavior may depend on the magnitude of the aversive stimulus to which mice are subjected. To test this hypothesis, we investigated the effects of intra-dPAG infusion of NPLA on defensive behavior in mice confronted by a predator (rat).

Role of glutamate NMDA receptors and nitric oxide within the periaqueductal gray on defensive behaviors in mice confronted by a predator

The rat exposure test (RET, Figure 6) is an animal model of anxiety based on the predator-prey (rat-mouse) interaction. The RET was developed and validated to facilitate the measurement of avoidance and risk assessment behaviors in mice (Yang et al., 2004). Testing procedures are conducted in a clear polycarbonate cage (exposure chamber) covered with a black polycarbonate lid. The exposure chamber is divided into two equally sized compartments by a wire mesh screen (surface and predator compartment). The home cage is a box made of black Plexiglas on three sides and clear Plexiglas on the fourth side to facilitate videotaping. The home chamber is connected to the exposure cage by a clear Plexiglas tube tunnel. Rats have been shown to be predators of mice both in nature and in the laboratory (Calvo-

Figure 6. Photograph of rat exposure test apparatus. The predator (rat) is placed in the right half of the exposure cage, which is divided into two equally sized compartments by a wire mesh screen (surface and predator compartments) (reproduced from Amaral et al., 2010).

Torrent, Brain, & Martinez, 1999; O'Boyle 1974, 1975). When confronted by rats, both wild and laboratory mice show clear innate defensive behaviors (Blanchard et al., 1998). Recent studies have attempted to identify possible neurotransmitter systems and hormonal changes (e.g., plasma corticosterone) involved in the modulation of defensive responses in mice exposed to the RET (Amaral, Gomes, & Nunes-de-Souza, 2010; Carvalho-Netto et al., 2007; Litvin, Pentowski, Blanchard, & Blanchard, 2007; Martinez, Carvalho-Netto, Amaral, Nunes-de-Souza, & Canteras, 2008).

In this context, we recently found that NPLA infusion into the dPAG attenuates the avoidance of the predator in the RET (Carvalho-Netto, Gomes, Amaral, & Nunes-de-Souza, 2009). Figure 7 shows the dose-response curve for NPLA treatment. The highest dose (.4 nmol) increased the time in the surface compartment and in contact with the wire screen barrier between the mouse and predator. Additionally, intra-dPAG NPLA (.4 nmol) markedly reduced the duration of freezing behavior and risk assessment behavior, indicating that NLPA significantly reduced the spatiotemporal (avoidance) and ethological (freezing and risk assessment) measures of the RET, supporting the hypothesis of a potential role for the nitric oxide system in the dPAG in the regulation of anxiety-like behavior. Moreover, intra-dPAG NPLA also reversed the proaversive-like effect of NMDA injected into the same structure, suggesting a modulatory role for NO in defensive behavior induced by glutamate NMDA receptor activation within the dPAG (Carvalho-Netto et al., 2009).

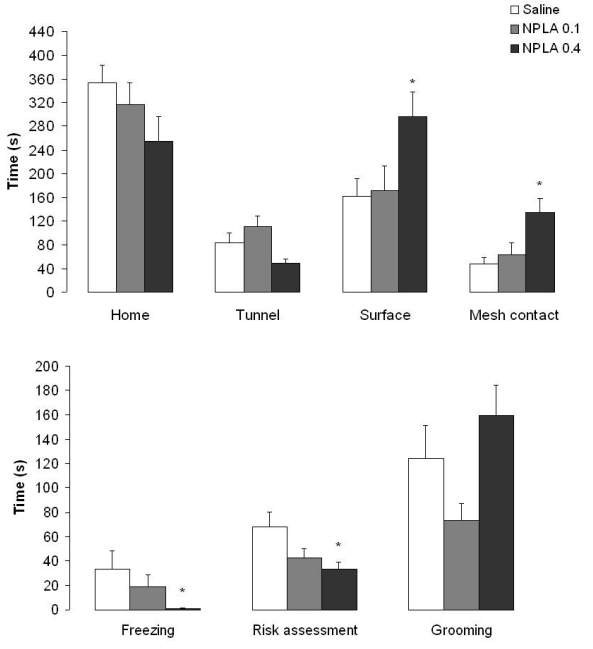

Figure 7. Effects of NPLA microinjection (.1 and .4 nmol/.1 µl; n = 9-15) into the dPAG on the behaviors of mice in the RET. Each bar represents the mean ± SEM. *p < .05, compared with control group (reproduced from Carvalho-Netto et al., 2009).

Final considerations

Based on previous studies (for review, see De Oliveira et al., 2001; Guimarães et al. 2005) and the present discussion, the effects of NPLA may represent an inhibition of NO production mediated by endogenous glutamatergic activation via NMDA receptors. Indeed, NMDA receptor activation has been established as the main stimulus for NO production in the central nervous system (for review, see Esplugues, 2002), and reciprocal regulatory mechanisms between these two neuronal pathways (glutamatergic and nitrergic) are likely to occur in the dPAG (Lin, Kang, Wan, Huang, & Tseng, 2000). An elegant study reported by Beijamini and Guimarães (2006) showed that exposure to a cat activated NOS-expressing neurons in the rat dPAG, an effect that was attenuated by prior intracerebroventricular microinjection of AP-7, a competitive NMDA receptor antagonist.

However, the failure of intra-dPAG NPLA to affect anxiety-like behavior in the EPM suggests that nitrergic neurotransmission located within this midbrain structure appears to not be recruited during the exposure of mice to this widely used animal model of anxiety. The scope of the present review was not to extensively compare the roles of glutamate NMDA receptors and NO in the dPAG on defensive behaviors in rats and mice. The existing literature suggests that the defensive response evaluated in the mouse EPM does not depend on NO synthesis within this limbic midbrain structure.

Previous studies have shown that mice exhibit a different behavioral defensive profile compared with rats (Blanchard et al., 1997; Blanchard, Griebel, & Blanchard, 2001; Carvalho-Netto & Nunes-de-Souza, 2004; Gomes et al., 2009; Jardim, Nogueira, Graeff, & Nunes-de-Souza, 1999), suggesting species-specific neurobiological mechanisms. The inability of intra-dPAG NPLA to affect anxiety-like behavior in the mouse EPM contrasts with previous findings reported with the rat EPM. For example, Guimarães et al. (1994) showed that administration of N^G-nitro-L-arginine methyl ester (L-NAME) and L-N^G-nitro arginine (L-NOARG) into the dPAG produced anxiolytic-like effects in the rat EPM. Altogether, these results suggest that the different effects of NO formation in the dPAG in rats and mice reflect a higher aversive state generated by the EPM in rats compared with mice. Importantly, however, L-NAME and L-NOARG are not selective nNOS inhibitors because they also inhibit eNOS (Pfeiffer, Leopold, Schmidt, Brunner, & Mayer, 1996). Considering the evidence indicating the presence of eNOS within the PAG (Iwase et al., 2001; Paakkari & Lindsberg, 1995), mechanisms other than, or in addition to, those involving nNOS in the rodent PAG may play a role in the modulation of anxiety-like behavior. Supporting this hypothesis are findings showing that eNOS also contributes to long-term potentiation (LTP)

in the hippocampus (Hopper & Garthwaite, 2006). These authors emphasized the importance of an integrated action of both isoenzymes (nNOS and eNOS) in LTP. Synaptic plasticity and neurochemical and behavioral evidence demonstrate the relevance of eNOS in other biological processes previously believed to be exclusive of nNOS action (Demas et al., 1999; Doreulee et al., 2003; Frisch et al., 2000; Haul, Godecke, Schrader, Haas, & Luhmann, 1999; Kano, Shimizu-Sasamata, Huang, Moskowitz, & Lo, 1998). Altogether, these findings suggest that the role of NO in anxiety-like behavior in the EPM appears to depend on the combined action of both enzyme isoforms within the mouse PAG. Further studies are required to confirm the anxiolytic-like profile of eNOS in this midbrain structure.

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