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Research Report

Anxiogenic-like effects induced by NMDA receptor activation are prevented by inhibition of neuronal nitric oxide synthase in the periaqueductal gray in mice

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ABSTRACT

Glutamate-NMDA (*N*-methyl-D-aspartate) receptor activation within the periaqueductal gray (PAG) leads to antinociceptive, autonomic and behavioral responses characterized as the fear reaction. We have recently demonstrated that the vigorous defensive-like behaviors (e.g. jumping and running) and antinociception induced by intra-PAG injection of *N*-methyl-D-aspartate (NMDA) were completely blocked by prior infusion of N^{ω}-propyl-L-arginine (NPLA), a specific neuronal nitric oxide synthesis (nNOS) enzyme inhibitor, into the same midbrain structure. It remains unclear however, whether the inhibition of nNOS within the mouse PAG changes the anxiety-like behavior *per se* or the effects of the inhibition of nNOS depend on the suppression of downstream of glutamate-NMDA receptor activation. This study investigated whether intra-PAG infusion of NPLA (i) attenuates anxiety in the elevated plus-maze (EPM) and (ii) antagonizes the anxiogenic-like effects induced by intra-PAG injection of NMDA. Test sessions were videotaped and subsequently scored for conventional indices of anxiety (percentage of open arm entries and percentage of open arm time) and locomotor activity (closed arm entries). Results showed that intra-PAG infusions of NPLA (0.2, 0.4 or 0.8 nmol/0.1 μl) did not alter significantly any behavioral response in the EPM when compared to control group (Experiment 1). Intra-PAG infusion of NMDA (0 and 0.02 nmol/0.1 μl; a dose that does not provoke vigorous defensive behaviors *per se* in mice) significantly reduced open arm exploration, confirming an anxiogenic-like effect (Experiment 2). When injected into the PAG 10 min prior local NMDA injection (0.02 nmol/0.1 μl), NPLA (0.4 nmol/0.1 μl) was able to revert the anxiogenic-like effect of glutamate-NMDA receptor activation. Neither intra-PAG infusion of NMDA nor NPLA altered closed arm entries, a widely used measure of locomotor activity in the EPM. These results suggest that intra-PAG nitric oxide synthesis

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Abbreviations: NADPHnicotinamide adenine dinucleotide phosphate; NMDAN-methyl-D-aspartate; NPLAN^{ω}-propyl-L-arginine; NOnitric oxide; NOSnitric oxide synthase; eNOSendothelial nitric oxide synthase; iNOSinducible nitric oxide synthase; nNOSneuronal nitric oxide synthase; PAGPeriaqueductal gray; dPAGdorsal periaqueductal gray; dlPAGdorsolateral periaqueductal gray; EPMelevated plus-maze; AP-72-amino-7-phosphonoheptanoic acid; CNScentral nervous system; AMPAα-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; RETrat exposure test

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does not play a role on anxiety-like behavior elicited during EPM exposure; however its synthesis is important for the proaversive effects produced by activation of glutamate-NMDA receptors located within this limbic midbrain structure.

1. Introduction

Electrical or chemical stimulation of the midbrain periaqueductal gray (PAG) elicits autonomic (e.g., tachycardia, defecation) and behavioral (e.g., jumping, running, immobility) responses characterized as fear reaction. In addition, many lines of evidence have indicated that besides fundamentally controlling fear-like responses—fight and flight (for review, see Graeff, 2004), the PAG modulates less vigorous defensive responses related to anxiety such as threat avoidance and risk assessment (Teixeira and Carobrez, 1999; McNaughton and Corr, 2004; Mendes-Gomes and Nunes-de-Souza, 2005; Bertoglio and Zangrossi, 2006; Carvalho-Netto et al., 2007).

Chemical stimulation of the PAG can be carried out by local glutamate-NMDA (*N*-methyl-D-aspartate) receptor activation, for example with the exogenous excitatory aminoacid *N*-methyl-D-aspartate (NMDA). When injected into the dorsal columns of the PAG (dPAG), NMDA elicits fight and flight reactions in rats (e.g., Bandler, 1988; Bittencourt et al., 2004) and mice (e.g., Miguel and Nunes-de-Souza, 2006), 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 test (EPM; Guimarães et al., 1991), a widely used animal model of anxiety.

Glutamate is a ubiquitous excitatory amino acid in the CNS. Besides activates the ionotropic NMDA receptor, glutamate also activates other two ion channel coupled receptors, the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor and kainate receptor (Heresco-Levy, 2003; Ozawa et al., 1998; Huntley et al., 1994; Seeburg, 1993) and a G-protein coupled receptor (metabotropic receptor). These receptors are largely expressed in the PAG (Schubert et al., 1996; Onstott et al., 1993; Albin and Gilman, 1990). Glutamate-NMDA receptor activation leads to cellular calcium influx which triggers a cascade of intracellular events including activation of nitric oxide synthase (NOS), enzyme that produces nitric oxide (NO) by conversion of L-arginine to L-citroline, having NADPH (nicotinamide adenine dinucleotide phosphate) and Ca^{2+} as co-factors (Heresco-Levy, 2003; Mayer et al., 1991; Garthwaite et al., 1989). There are at least three NOS isoforms: an inducible (iNOS) involved in immunological reactions, activated by factors released in pathological events, such as cytokines that can induce large releasing of NO. Two other NOS are constitutive forms, which are present in vase endothelium (endothelial NOS—eNOS) and in neurons (neuronal NOS—nNOS) (Guix et al., 2005; Mungrue et al., 2003; Prast and Philippu, 2001; Lamas et al., 1992).

Similarly 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 et al., 2000). On the other hand, administration in this same region of NOS inhibitors, guanylate cyclase inhibitors and a NO scavenger (Guimarães et al., 2005; De Oliveira and Guimarães, 1999; Guimarães et al., 1994) provokes anxiolytic-like effects in the EPM.

In a recent study Miguel and Nunes-de-Souza (2006) showed that intra-PAG injection of N^{ω}-propyl-L-arginine (NPLA), a specific nNOS inhibitor, completely blocked the vigorous defensive behavioral reactions (e.g., jumping, running, immobility) and antinociception induced by NMDA infusion into the same site in mice. However, it remains unclear whether the inhibition of nNOS within the mouse PAG changes the anxiety-like behavior *per se* or the effects of the inhibition of nNOS depend on the suppression of downstream of glutamate-NMDA receptor activation. This study investigated whether intra-PAG infusion of NPLA (i) attenuates anxiety in the elevated plus-maze and (ii) antagonizes the anxiogenic-like effects induced by intra-PAG injection of NMDA (at a dose that does not provoke vigorous defensive behaviors *per se* in mice).

2. Results

Histology confirmed that a total of 108 mice had cannula placements in the PAG (Fig. 1). Forty-six mice were used in the Experiment 1 [Saline (n=15), NPLA 0.2 nmol (n=12), 0.4 nmol (n=10), 0.8 nmol (n=9)], 31 mice were used in the Experiment 2 [Saline (n=15) and NMDA 0.02 nmol (n=16)] while 31 mice were used in the Experiment 3 [saline+saline (n=8), saline+NMDA (n=8), NPLA+saline (n=7), NPLA+NMDA (n=8)].

2.1. *Experiment 1: lack of effects of intra-PAG injection of NPLA on the behavior of mice in the EPM*

Fig. 2 shows that intra-PAG injection of NPLA (0, 0.2, 0.4 or 0.8 nmol/0.1 μl) neither changed the anxiety indices [one-way ANOVA for percent open arm entries ($F_{3,42}$=0.65, p>0.05), percent open arm time ($F_{3,42}$=0.53, p>0.05)] nor affected locomotor activity [one-way ANOVA for closed arm entries ($F_{3,42}$=1.54, p>0.05)] in the EPM.

2.2. *Experiment 2: anxiogenic-like effects of intra-PAG injection of NMDA in mice exposed to the EPM*

The effects of intra-PAG injection of NMDA (0.02 nmol/0.1 μl) on the behavior of mice exposed to the EPM are shown in Fig. 3. Student *t*-test revealed that injection of NMDA into the PAG decreased percent of open arm entries [*t*-test (29)=2.16, p<0.05)] and percent open arm time [(*t*-test (29)=3.00, p<0.05)] while did not significantly change closed arm entries [(*t*-test (29)=−0.59, p>0.05)].

2.3. *Experiment 3: blockade of the intra-PAG NMDA anxiogenic-like effects produced by prior local infusion of NPLA*

Fig. 4 shows the effects of intra-PAG NPLA pretreatment on anxiogenic-like responses to local infusion of NMDA. Two-