



Anxiety-like responses induced by nitric oxide within the BNST in mice: Role of CRF1 and NMDA receptors



M.P. Faria^{a,b}, T.T. Miguel^c, K.S. Gomes^b, R.L. Nunes-de-Souza^{a,b,*}

^a Programa Interinstitucional de Pós-Graduação em Ciências Fisiológicas, Universidade Federal de São Carlos and Universidade Estadual Paulista, Araraquara, SP, Brazil

^b Laboratório de Farmacologia, Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista, 14800-903 Araraquara, SP, Brazil

^c Farmacologia, Universidade Federal de Uberlândia, Uberlândia, MG, Brazil

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ABSTRACT

It has been shown that the bed nucleus of the stria terminalis (BNST) of rats contains nitrergic neurons, which are activated during animal exposure to aversive stimuli. The BNST is also populated by glutamatergic and corticotrophin releasing factor (CRFergic) neurons, which in turn are activated under stressful situations. Here we investigated the anxiogenic-like effects of intra-BNST injections of a nitric oxide (NO) donor, NOC-9 in mice. The role of CRFergic and glutamatergic systems on defensive behavior induced by NOC-9 was investigated with previous intra-BNST infusion of different doses of CP376395, a CRF type 1 receptor antagonist (CRF1), or AP-7, an NMDA (N-methyl-D-aspartate) receptor antagonist. Anxiety-like behavior was assessed immediately and 5 min after intra-BNST drug injection, exposing mice to a novel arena and to the elevated plus-maze (EPM; an anxiogenic situation). Results showed that NOC-9 provoked a short period (≈ 150 s) of freezing behavior in the novel arena and increased anxiety in the EPM. Both CP and AP-7 attenuated the anxiogenic-like effects of NOC-9 in the EPM without changing freezing behavior in the novel arena. When given alone (i.e. without prior intra-BNST injection of NOC-9), AP-7 (0.20 nmol), but not CP (0.75, 1.50, or 3.00 nmol), attenuated anxiety in mice exposed to the EPM. These results suggest that CRF1 and NMDA receptors located within the BNST differentially modulate aversive effects induced by NO production in this limbic forebrain structure.

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Introduction

Animal exposure to aversive stimuli induces fear- and anxiety-like behaviors (e.g., fight, flight, freezing and vocalization) as well as neuro-endocrine (e.g., ACTH and corticosterone release) and autonomic (e.g., tachycardia, tachypnea, increased blood pressure and defecation) activations (Blanchard and Blanchard, 2003; Blanchard et al., 1993; Graeff, 1990). These defensive reactions are coordinated by various areas of the brain aversive system (Graeff, 1990). For instance, the midbrain periaqueductal gray, the hypothalamus, and the amygdaloid complex have been implicated in the modulation of behavioral, hormonal, and autonomic responses induced by dangerous and threatening situations (Bandler and Depaulis, 1991; Behbehani, 1995; Brandao et al., 2003; Carrive et al., 1997; Fanselow, 1991; Graeff et al., 1993; Lang et al., 1998; Lovick, 2000; Walker and Carrive, 2003).

The amygdaloid complex has been widely investigated as an important modulator site of defensive responses, such as freezing, the startle reflex, antinociception, and heart rate alteration (Rosen and

Schulkin, 1998), and its extensive connections to the bed nucleus of the stria terminalis (BNST) have raised substantial interest in the identification of the role of the BNST in the modulation of anxiety-related responses (Alheid, 2003; Davis, 1998; Heimer, 2003; Sahuque et al., 2006; Schulkin et al., 2005; Ventura-Silva et al., 2012). For instance, electrical stimulation of the BNST elicits behavioral and endocrine changes that are similar to those induced by environmental stressors (Casada and Dafny, 1991; Dunn, 1987). In contrast, pharmacological inactivation of the BNST decreases the expression of conditioned or unconditioned responses induced by aversive situations (Sahuque et al., 2006).

Although many neurotransmitters (e.g., catecholamines, serotonin, and excitatory/inhibitory amino acids) have been implicated in the modulation of anxiety-like responses (Carobrez et al., 2001; Molchanov and Guimaraes, 2002; Vianna et al., 2001), the so-called atypical neurotransmitters, endocannabinoids and nitric oxide (NO), as well as the peptide corticotrophin-releasing factor (CRF) have more recently gained attention as important candidates in the modulation of emotional states (Esplagues, 2002; Fogaca et al., 2012; Guimaraes et al., 2005; Howlett et al., 2002; Piomelli, 2003). NO is a diffusible gas produced by the nitric oxide synthase (NOS) enzyme, through conversion of L-arginine to L-citrulline, using nicotinamide adenine dinucleotide phosphate (NADPH) and Ca^{2+} as co-factors (Lohse et al., 1998;

* Corresponding author at: Laboratório de Farmacologia, Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista, 14800-903 Araraquara, SP, Brazil.

E-mail address: souzam@fcar.unesp.br (R.L. Nunes-de-Souza).

Mayer et al., 1991). Among the three main NOS isoforms, neuronal NOS (nNOS) is the constitutive form expressed in neurons (de Oliveira et al., 2000). Proaversive effects (e.g., fight and flight reactions) have been demonstrated after injection of NO donors [e.g., SIN-1 (3-morpholino-sylnomine hydrochloride) and NOC-9 (6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-hexanamine)] into the periaqueductal gray (PAG) in rats and mice (Guimaraes et al., 2005; Miguel et al., 2012). However, while SIN-1 produces peroxynitrite, a substance that can provoke cytotoxic effects and cause other NO-independent cellular effects (Del Carlo and Loeser, 2002; Morot Gaudry-Talarmain et al., 1997), NOC-9, which is relatively stable at alkaline pH (>10.0), releases NO at physiological pH (7.4) without producing peroxynitrite (Ambalavanan et al., 1999; Del Carlo and Loeser, 2002; Seccia et al., 1996). Conversely, intra-PAG injection of nNOS inhibitors, guanylate cyclase inhibitors, and an NO scavenger provokes anxiolytic-like effects in rats exposed to the elevated plus-maze (EPM) (Guimaraes et al., 1994). In mice, intra-PAG injection of a highly selective and potent nNOS inhibitor, N ω -propyl-L-arginine (NPLA), attenuates defensive behavior in the rat exposure test, a prey–predator interaction test (Carvalho-Netto et al., 2009) and blocks NMDA (N-methyl-D-aspartate)-induced anxiogenic-like effects in the EPM (Nunes-de-Souza et al., 2010).

The anxiogenic effects of NO may be related to its interference with the release of neurotransmitters, such as acetylcholine, GABA, dopamine, serotonin, glutamate, and CRF in distinct brain areas related to the defensive response (de Oliveira et al., 2000; Moreira and Guimaraes, 2004; Moreira et al., 2004). For instance, Guimaraes et al. (2005) have demonstrated that glutamate receptor antagonists are able to attenuate the proaversive effect of NO donors in the PAG. We have also observed that blocking the CRF type 1 receptor (CRF1) within the mouse PAG attenuates the proaversive effects produced by local injection of NOC-9, a NO donor (Miguel et al., 2012).

Very few studies have investigated the role of nitrergic transmission within the BNST in the context of aversive reactions. Previous studies have demonstrated that the nitrergic neurons located within the BNST are activated by aversive stimuli in rats (Beijamini and Guimaraes, 2006; Guimaraes et al., 2005). Moreover, glutamatergic (McElligott and Winder, 2008) and CRFergic neurons have been identified in the BNST (Cummings et al., 1983; Ju et al., 1989; Morin et al., 1999; Sakanaka et al., 1987; Swanson et al., 1983). However, while the anxiogenic-like effects of NO seem to be modulated by NMDA-glutamate and CRF1 receptors within other limbic brain structures (e.g., PAG, inferior colliculus) (de Araujo Moreira et al., 2003; Guimaraes et al., 2005; Miguel et al., 2012; Miguel and Nunes-de-Souza, 2008), it remains unclear whether these interactions play a role within the BNST on the modulation of anxiety-like responses. Taken together, these lines of evidence suggest that the facilitation of nitrergic neurotransmission in the BNST may induce anxiogenic-like behavior, which may in turn be modulated by CRF and NMDA-glutamate receptors. In this paper, we investigate the effects of intra-BNST injection of NOC-9, a NO donor, on the behavior of mice exposed to a novel arena and to the EPM. The effects of intra-BNST injection of CRF1 or NMDA receptor antagonists, n-(1-ethylpropyl)-3,6-dimethyl-2-(2,4,6-trimethylphenoxy)-4-pyridinamine hydrochloride (CP376395) and 2-amino-7-phosphonoheptanoic acid (AP-7), on the behavioral effects induced by local injection of NOC-9 are also investigated.

Materials and methods

Ethics

Experimental protocols were conducted according to the ethical principles of the Brazilian College of Animal Experimentation (COBEA) and approved by the local Research Ethics Committee (CEP/FCF/CAR-UNESP; protocol number 09/2011).

Subjects

Subjects were 360 male Swiss mice (Univ. Estadual Paulista – UNESP, SP, Brazil) weighing 25–35 g at testing. Mice were housed in groups of 10 per cage (size: 41 × 34 × 16 cm) and maintained on a 12-h light–dark cycle (lights on at 07:00 a.m.) in a temperature-controlled environment (23 ± 2 °C). Food and water were available *ad libitum*, except during brief testing periods. All mice were naïve at the beginning of the experiments and were used only once.

Drugs

Drugs used were NOC-9 [6-(hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-hexanamine], a diazeniumdiolate (NONOate), quick-releasing NO donor (Tocris Cookson Inc., Ballwin, USA). NOC-9 spontaneously dissociates in a pH-dependent, first-order process with half-lives of 1 min and 3 min at 37 °C and 22–25 °C, respectively (pH 7.4), to liberate 2 mol of NO per mole of parent compound (Hrabie et al., 1993; Keefer et al., 1996). The main physiological pathway for the degradation of nitric oxide acts via the heme group (Seregelyes et al., 2004). The NO diffuses very quickly throughout the membranes to the lumen of the vessels, where it reacts with hemoglobin (Hb) forming nitrates and met-hemoglobin (metHb) (Guix et al., 2005). Other drugs were CP 376395 [n-(1-ethylpropyl)-3,6-dimethyl-2-(2,4,6-trimethylphenoxy)-4-pyridinamine hydrochloride], a CRF1 receptor antagonist (Tocris Cookson Inc., Ballwin, USA) and AP-7 (2-amino-7-phosphonoheptanoic acid), a NMDA glutamatergic receptor antagonist. Doses were based on pilot and previous studies (Nunes-de-Souza et al., 2010; Sahuque et al., 2006). The NOC-9 was dissolved in a TRIS solution, pH 10.00. The rationale for dissolving NOC-9 in a pH 10.00 solution was to prevent NO formation until the drug reached the cerebral issue, where it would be produced at physiological pH (7.4) (Seccia et al., 1996). The other drugs were dissolved in 0.9% physiological saline, which was used as a control.

Surgery and microinjection

Each mouse was bilaterally implanted with 7-mm stainless steel guide cannulae (26-gauge; Insight Equipamentos Científicos Ltd., Brazil) under anesthesia with intraperitoneal injection of ketamine (100 mg/kg) plus xylazine (10 mg/kg). Guide cannulae were fixed to the skull with dental acrylic and jeweler's screws. The stereotaxic coordinates for the BNST were 0.5 mm anterior to bregma, ± 3.0 mm lateral to the midline, and 3.2 mm ventral to the skull surface (Paxinos and Franklin, 2001), with the guide cannulae angled 32°. Guide cannulae were positioned +1.0 mm dorsally to the structure. A dummy cannula (33 gauge, stainless steel wire; Fishtex Industry and Commerce) was inserted into each guide cannula to reduce the incidence of occlusion. Immediately after surgery, animals received an intramuscular injection of penicillin-G benzathine (Pentabiotic, 56.7 mg/kg in a 0.1 mL volume; Fort Dodge, Campinas, SP, Brazil) and a subcutaneous injection of the anti-inflammatory analgesic Banamine (0.5 mg/kg flunixin meglumine in a volume of 0.3 mL).

Five to seven days after surgery, different solutions were bilaterally injected into the BNST through microinjection units (33-gauge stainless steel cannula; Insight Equipamentos Científicos Ltd., Brazil) that extended 1.0 mm beyond the tip of the guide cannula. Each microinjection unit was attached to a 2- μ L Hamilton microsyringe via polyethylene tubing (PE-10). The microinjection procedure consisted of gently restraining the animal, removing the dummy cannula, inserting the injection unit in situ and proceeding with the microinjection, leaving the needle for a further 30 s after the microinjection was completed. The drug/vehicle volume delivered was 0.2 μ L/side. Confirmation of a successful procedure was obtained by monitoring the movement of a small air bubble in the PE-10 tubing.

Behavioral assessment

Assessment of the freezing behavior

Given that freezing has been suggested as a defensive behavior observed after NO donor injection into areas of the brain defense system (Guimaraes et al., 2005; Joca and Guimaraes, 2006; Miguel et al., 2012; Moreira et al., 2004), we recorded the time spent freezing as a measure of anxiety-like response induced by NOC-9. Thus, immediately after intra-BNST injection of NOC-9, mice were individually placed in a novel arena (a glass holding cage; 30 × 20 × 25 cm) and the time (in seconds) spent freezing was recorded during a 5-min period. Freezing was defined as the complete absence of movement except for breathing while the animal exhibited a characteristic tense posture. All sessions were recorded by a laterally mounted camera (JVC “Everio” GZ-MS110) positioned approximately 1 m from the apparatus, linked to a monitor and DVD recorder. The sessions were scored blind to treatment by a highly trained observer (intrarater reliability ≥ 0.90).

Elevated plus maze

The basic EPM design was very similar to that originally described by Lister (1987) and comprised of two open arms (30 × 5 × 0.25 cm) and two closed arms (30 × 5 × 15 cm) connected via a common central platform (5 × 5 cm). The apparatus was constructed from wood (floor) and transparent glass (clear walls) and was raised to a height of 38.5 cm above floor level.

Five or ten minutes after intra-BNST injection, each mouse was placed on an individual holding cage and subsequently transported to the maze. Testing commenced by placing the subject on the central platform of the maze (facing an open arm), after which the experimenter immediately withdrew to an adjacent laboratory. Test sessions were 5 min in duration and the maze was thoroughly cleaned with 20% alcohol between subjects. All experiments were performed under normal laboratory illumination (1 × 60 W yellow incandescent lamp positioned approximately 1.80 m above the EPM floor), during the light phase of the light–dark cycle. All sessions were recorded by a vertically mounted camera (JVC “Everio” GZ-MS110) positioned approximately 1 m from the apparatus, linked to a monitor and DVD recorder. Animals were tested in an order counterbalanced for treatment condition.

The sessions were scored blind to treatment by a highly trained observer (intrarater reliability ≥ 0.90) using the software “X-plo-rat 2005”, developed by Dr. Morato's group at Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, USP (the software can be freely downloaded at <http://scotty.fclrp.usp.br/X-Plo-Rat.html>). Behavioral parameters comprised the conventional spatiotemporal measures: frequencies of open and closed arm entries (entry = all four paws into an arm) and the time spent in the open arm of the maze. These data were used to calculate percentage of open arm entries $[(\text{open} / \text{total}) \times 100]$ and the percentage of time spent in each zone of the maze [e.g. (time in compartment / 300) × 100].

General procedure

Experiments 1–3: effects of intra-BNST NOC-9, CP376395 or AP-7 microinjections on the behavior of mice exposed to the novel arena and EPM. Five to seven days after surgery, mice were transported to the experimental room and left undisturbed for at least 30 min prior to testing. Each mouse was then bilaterally microinjected with NOC-9 (Exp. 1: 0, 18.75, 37.50 or 75.0 nmol/side; $n = 81$), CP376395 (Exp. 2: 0, 0.75, 1.50 or 3.00 nmol/side; $n = 69$) or AP-7 (Exp. 3: 0, 0.05, 0.10 or 0.20 nmol/side; $n = 58$) into the BNST. All three experiments were independently conducted. Immediately after NOC-9 injection, each mouse was placed into the glass holding cage (a novel arena) to record the time spent (in seconds) freezing for a period of 5 min. Given that a pilot study indicated that intra-BNST injection of CP 376395 or AP-7

alone failed to induce freezing behavior, the animals treated with these drugs (Exp. 2 and Exp. 3) were not exposed to the novel arena. Immediately after being tested for their freezing response induced by NOC-9 (Exp. 1) or 10 min after CP 376395 (Exp. 2) or AP-7 (Exp. 3) injections, animals were individually placed in the EPM and the anxiety indices [percentage of open arm entries (%OE) and percentage of open arm time (%OT)] and locomotion (frequency of closed arm entries) were recorded for a 5-min period.

Experiment 4: effects of intra-BNST CP 376395 on the behavioral effects produced by local injection of NOC-9. Five to seven days after surgery, 72 mice were transported to the experimental room and left undisturbed for at least 30 min prior to testing. The highest dose of CP 376395 devoided of per se effect (observed in Experiments 2) was used.

Animals were then bilaterally injected with CP 376395 (pretreatment: 0 or 3.00 nmol/side) into the BNST and 10 min later they received NOC-9 (treatment: 0 or 75.00 nmol/side) microinjection into the same site. Immediately after the intra-BNST NOC-9 injection, each mouse was placed into the novel arena to measure the freezing time (in seconds) for a period of 5 min. Immediately after being tested for the freezing response, animals were individually exposed to the EPM and the behavioral measures of anxiety (% open arm entries and % open arm time) and locomotion (closed arm entries) were recorded for 5 min.

Experiment 5: effects of intra-BNST AP-7 on the behavioral effects produced by local injection of NOC-9. The current experiment was identical to experiment 4, except that 80 mice were bilaterally injected with AP-7 (pretreatment: 0 or 0.05 nmol/side) into the BNST and 10 min later received NOC-9 (treatment: 0 or 75.00 nmol/side) microinjection into the same site. The dose of AP-7 was based on the lack of effect on anxiety observed in Experiment 3.

Histological analysis

At the end of testing, all animals received a 0.2 μL intra-BNST infusion (bilaterally) of 1% Evans blue through the same microinjection procedure as for the drugs. Animals were then sacrificed in a CO₂ chamber and their brains were removed and sliced on a cryostat. Slices were inspected under a stereoscope to verify cannula placement and dye spreading using the Paxinos and Franklin (2001) Atlas as a reference. Microinjections were considered positive when injection units reached the BNST bilaterally. Data from injection sites outside the BNST were excluded from the study.

Statistical analysis

All results were initially subjected to Levene's test for homogeneity of variance. Where Levene's test yielded significant heterogeneity, results were log-transformed, then confirmed for homogeneity of variance before being subjected to one- or two-way analysis of variance (ANOVA). Finally, data were subjected to the Duncan post hoc test. While one-way ANOVA was carried out to analyze data from Experiments 1, 2 and 3, two-way ANOVA was used in Experiments 4 and 5 (factor 1: pretreatment; factor 2: treatment). Eta-squared (η^2) effect size was calculated by dividing the sum of squares of the effect of interest by the total sums of squares for all effects, interactions, and error in the ANOVA (Cohen, 1973). In Experiments in which all results from the group treated with vehicle (Veh; Exp. 1) or saline + vehicle (Sal + Veh; Exps. 4 and 5) and exposed to the novel arena were zero, the data did not present a normal distribution. In these cases, a non-parametric (Kruskal–Wallis analysis followed by the Mann–Whitney comparison test) approach was used. Eta-squared (η^2) effect size for Kruskal–Wallis was calculated according to the method described by Cohen (2008). In all cases, a p value of ≤ 0.05 was required for significance.

Results

Injections into the BNST

Fig. 1 shows (A) a sketched series of coronal brain sections, indicating microinfusion sites within the BNST, and (B) a schematic diagram (left) and a representative photomicrograph (right) of the microinfusion sites within the BNST of mice used in the present study (Paxinos and Franklin, 2001). Histology confirmed that a total of 168 (46.95%) mice had accurate cannula placements in the BNST. A total of 37 (45.67%) animals were used to investigate the effects of intra-BNST NOC-9 microinjection [Experiment 1: vehicle ($n = 10$); 18.75 nmol ($n = 10$); 37.5 nmol ($n = 8$); 75.00 nmol ($n = 9$)]. Thirty-five (50.72%) animals were used to assess the effects of CP 376395 microinjections into the BNST [Experiment 2: saline ($n = 9$); 0.75 nmol ($n = 8$); 1.50 nmol ($n = 9$); 3.00 nmol ($n = 9$)]. Thirty (51.72%) animals were used to assess the effects of AP-7 microinjections into the BNST [Experiment 3: saline ($n = 8$); 0.05 nmol ($n = 7$); 0.10 nmol ($n = 8$); 0.20 nmol ($n = 7$)]. Thirty (41.66%) animals were used in Experiment 4 [saline-vehicle ($n = 9$); saline-75.00 nmol NOC-9 ($n = 6$); 3.00 nmol CP-vehicle ($n = 7$); 3.00 nmol CP-75.00 nmol NOC-9 ($n = 8$)]. Finally, 36 (45.00%) animals were necessary to reveal the effects of AP-7 in the modulation of anxiety-like behavior [Experiment 5: [saline-vehicle ($n = 8$); saline-75.00 nmol NOC-9 ($n = 9$); 0.05 nmol AP-7-vehicle ($n = 9$); 0.05 nmol AP-7-75.00 nmol NOC-9 ($n = 10$)].

Experiment 1: freezing and anxiogenic-like effects produced by intra-BNST NOC-9 injection in mice

Fig. 2A shows the effects of intra-BNST injections of NOC-9 (0, 18.75, 37.50 or 75.00 nmol) on the time spent freezing. The Kruskal–Wallis test followed by the Mann–Whitney comparison test revealed that all doses of NOC-9 produced freezing in mice exposed to the novel arena [$H(3,37) = 20.61$; $\eta^2 = 0.79$; $p < 0.0001$]. Fig. 2B shows the anxiety-like indices (%OE and %OT) recorded during a 5-min period in the EPM in mice bilaterally microinjected with NOC-9 (0, 18.75, 37.50 or 75.00 nmol) into the BNST. One-way ANOVA followed by Duncan's test revealed that NOC-9 (37.50 or 75.00 nmol) decreased the percentage of open-arm time [$F(3,33) = 3.27$; $\eta^2 = 0.23$; $p = 0.03$] without changing significantly the percentage of open-arm entries [$F(3,33) = 1.71$; $\eta^2 = 0.13$; $p = 0.18$] and the frequency of closed-arm entries [$F(3,33) = 0.21$; $\eta^2 = 0.02$; $p = 0.88$].

Experiment 2: intra-BNST CP 376395 injection fails to alter the behavior of mice exposed to the EPM

Fig. 3 shows the anxiety-like indices recorded during a 5-min period in the EPM in mice bilaterally microinjected with CP 376395 (0, 0.75, 1.50 or 3.00 nmol) into the BNST. One-way ANOVA did not reveal a significant treatment effect for the percentage of open-arm entries [$F(3,31) = 0.04$; $\eta^2 = 0.004$; $p = 0.98$] or percentage of open-arm time [$F(3,31) = 0.75$; $\eta^2 = 0.07$; $p = 0.52$]. One-way ANOVA also revealed that intra-BNST injection of CP 376395 failed to alter closed-arm entries [$F(3,31) = 0.60$; $\eta^2 = 0.05$; $p = 0.62$].

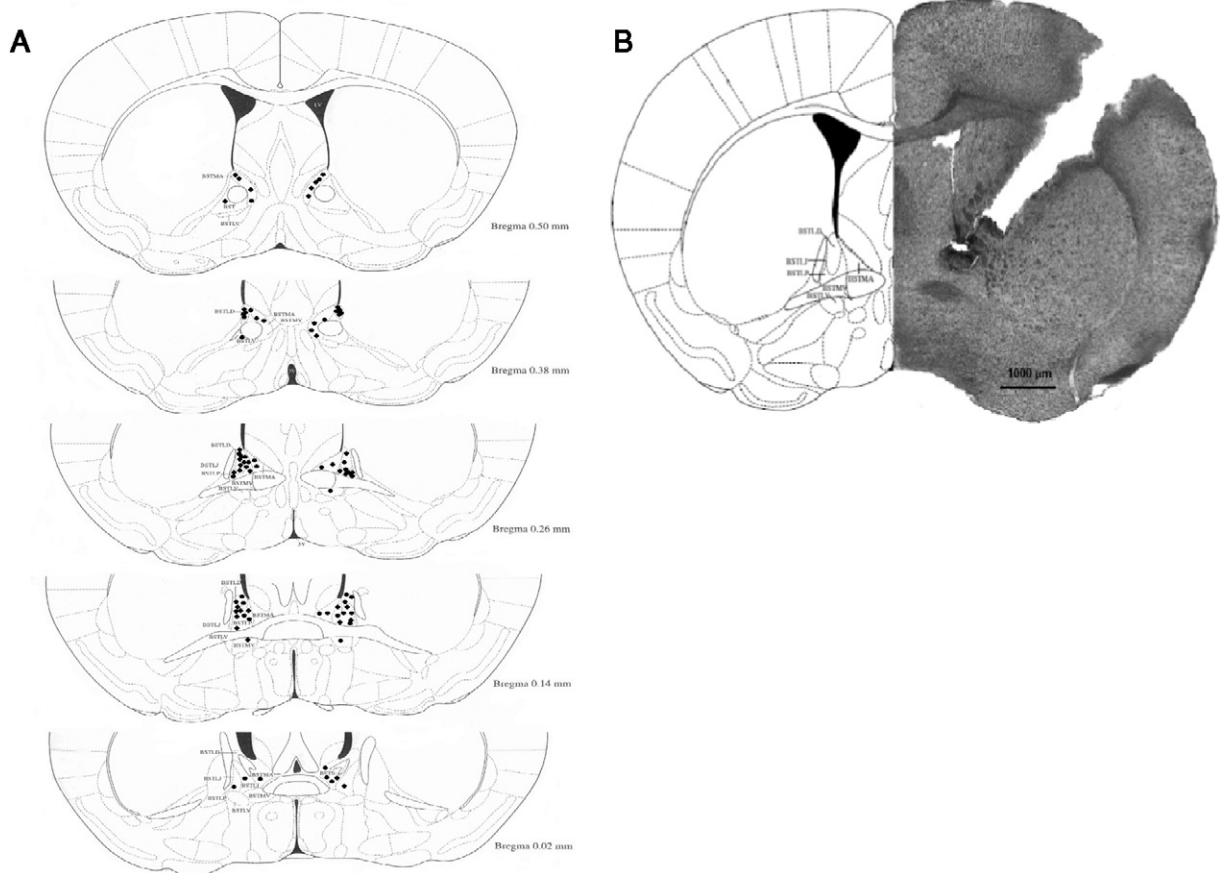


Fig. 1. (A) Schematic representation of microinjections sites within the bed nucleus of stria terminalis (BNST) of the mouse. The number of the dots in the figure is less than the total number of mice because of the overlaps. (B) Schematic diagram (left) and a representative photomicrograph (right) of a coronal section from a representative subject showing the injection site into the BNST (Paxinos and Franklin, 2001). Abbrev.: BST: bed nucleus of stria terminalis; LD: lateral-dorsal; LJ: lateral-juxtacapsular; LP: lateral-posterior; LV: lateral-ventral; MA: medial-anterior; MV: medial-ventral.

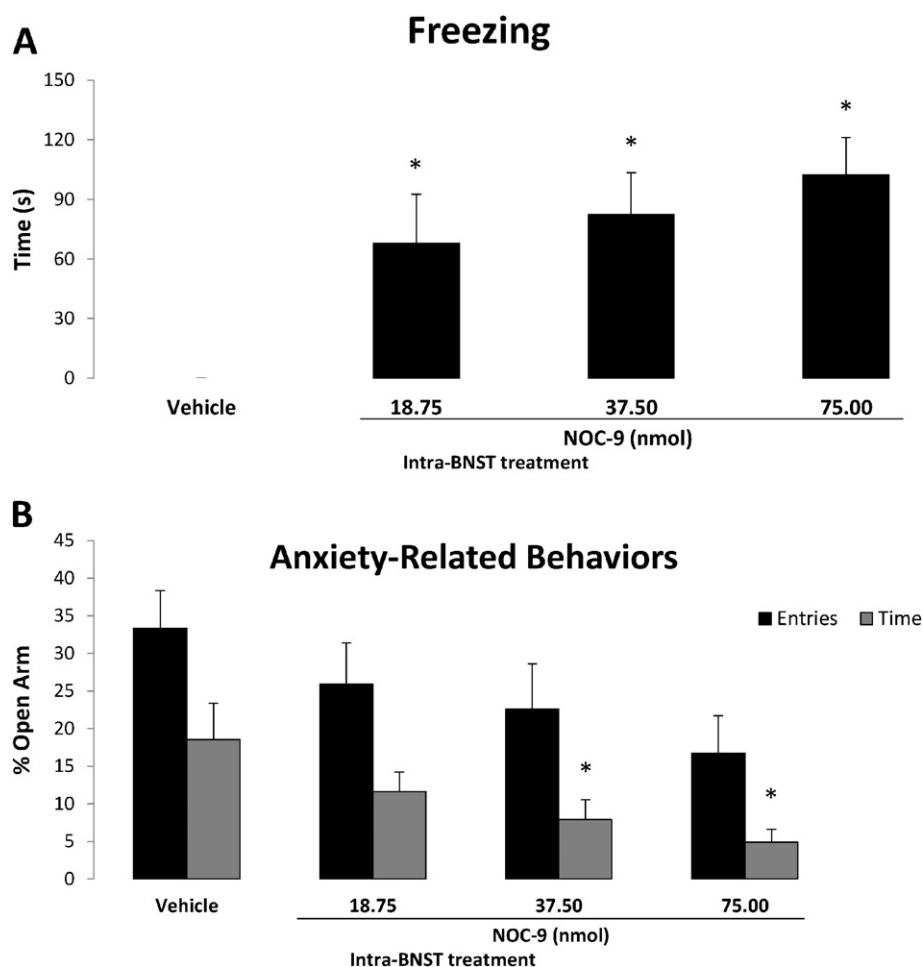


Fig. 2. Intra-BNST injections of NOC-9 (0, 18.75, 37.50, or 75.00 nmol/0.2 μ L) provoke (A) freezing assessed in the novel arena and (B) anxiety-related responses in mice exposed to the EPM. Bars represent means (\pm SEM); $n = 8$ –10 per group. * $p < 0.05$ compared to the vehicle group.

Experiment 3: anxiolytic-like effects of AP-7 microinjected into the BNST of mice exposed to the EPM

Fig. 4 shows the anxiety-like indices recorded during a 5-min period in the EPM in mice bilaterally microinjected with AP-7 (0, 0.05, 0.10 or

0.20 nmol) into the BNST. One-way ANOVA followed by Duncan's test revealed that AP-7 (0.20 nmol) increased both the percentage of open-arm entries [$F(3,26) = 3.85$; $\eta^2 = 0.31$; $p = 0.02$] and percentage of open-arm time [$F(3,26) = 6.54$; $\eta^2 = 0.43$; $p = 0.002$]. Importantly,

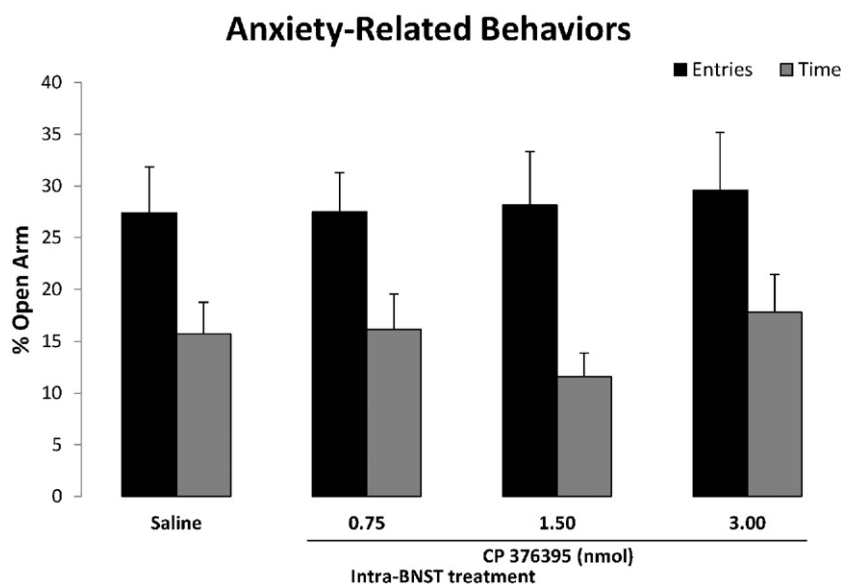


Fig. 3. Lack of effects of CP 376395 microinjection (0, 0.75, 1.50, or 3.00 nmol/0.2 μ L) into the BNST on anxiety-related responses in mice exposed to the EPM. Bars represent means (\pm SEM); $n = 8$ –9 per group.

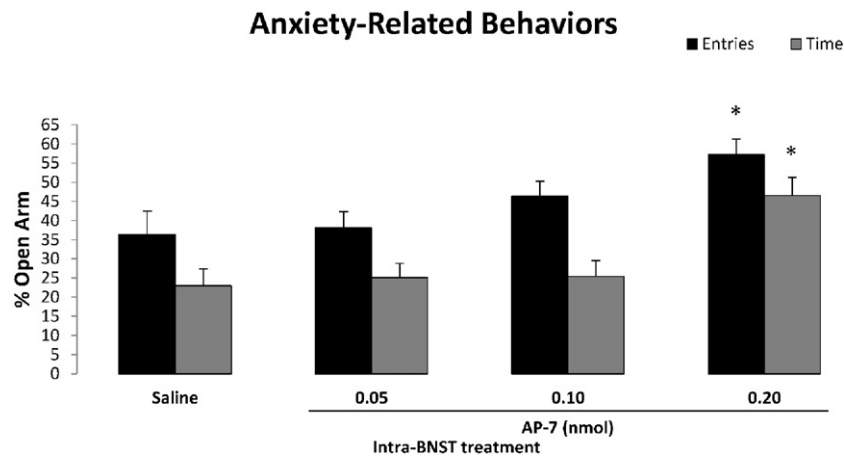


Fig. 4. Anxiolytic-like effects of AP-7 microinjection (0, 0.05, 0.10, or 0.20 nmol/0.2 μ L) into the BNST in mice exposed to the EPM. Bars represent means (\pm SEM); $n = 7$ –8 per group. * $p < 0.05$ compared to the saline group.

intra-BNST injections of AP-7 did not change the frequency of closed-arm entries [$F(3,26) = 1.40$; $\eta^2 = 0.14$; $p = 0.26$].

Experiment 4: intra-BNST CP 376395 blocks the anxiogenic-like (but not the freezing) effects provoked by local injection of NOC-9

Fig. 5A shows the lack of effect of intra-BNST injection of CP 376395 (pretreatment: 0 or 3.00 nmol) on the time spent freezing induced by local injection of NOC-9 (treatment: 0 or 75.00 nmol). The Kruskal–Wallis test followed by the multiple comparisons test confirmed that NOC-9 produced freezing [$H(3,30) = 25.68$; $\eta^2 = 0.79$; $p < 0.0001$], an effect that was not prevented by prior intra-BNST injection of CP 376395 ($p > 0.90$).

Fig. 5B shows the effects of intra-BNST CP 376395 (pretreatment: 0 or 3.00 nmol) injected prior to NOC-9 (treatment: 0 or 75.00 nmol) administration into the same site on the anxiety-related measures in mice exposed to the EPM. Regarding the percentage of open-arm entries, two-way ANOVA revealed significant differences for pretreatment [$F(1,26) = 4.29$; $\eta^2 = 0.11$; $p = 0.05$] and treatment [$F(1,26) = 7.57$; $\eta^2 = 0.19$; $p = 0.01$] factors, without showing any significant effects for pretreatment versus treatment interaction [$F(1,26) = 2.26$; $\eta^2 = 0.05$; $p = 0.14$]. Regarding the percentage of open-arm time, two-way ANOVA revealed significant differences for pretreatment [$F(1,26) = 4.60$; $\eta^2 = 0.11$; $p = 0.04$] and treatment [$F(1,26) = 5.40$; $\eta^2 = 0.13$; $p = 0.03$] factors and a tendency for pretreatment \times treatment

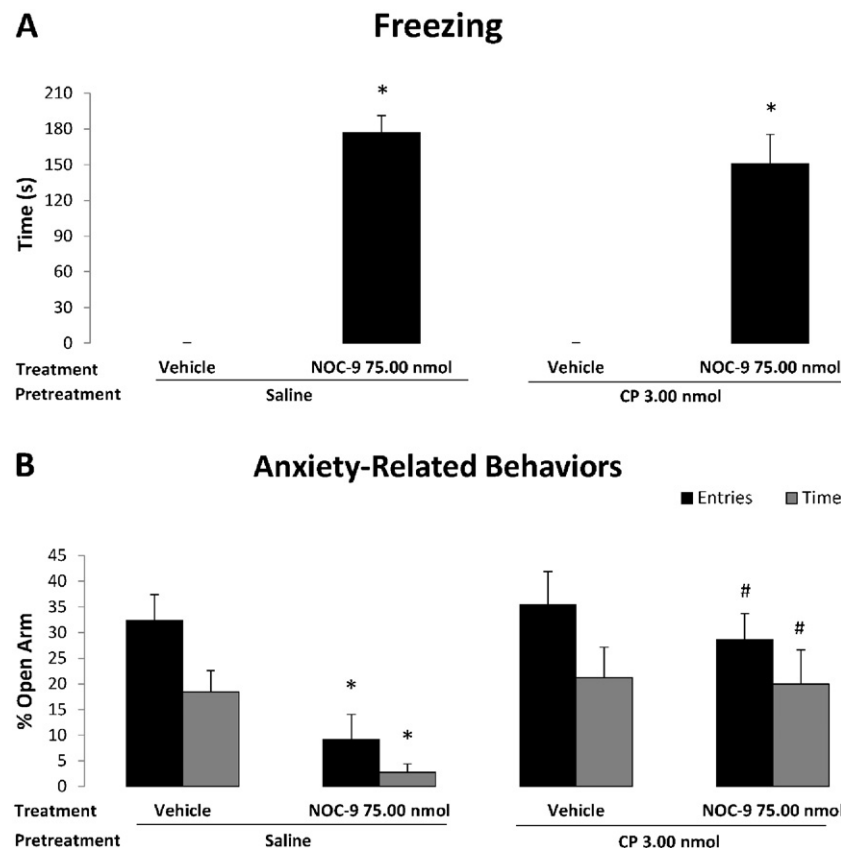


Fig. 5. Intra-BNST microinjection of CP 376395 (0 or 3.00 nmol) (A) does not change freezing but (B) attenuates the anxiogenic-like effects induced by local injection of NOC-9 (0 or 75.00 nmol) in mice. Bars represent means (\pm SEM); $n = 6$ –9 per group. * $p < 0.05$ compared to the control group (saline + vehicle) and to the saline + NOC-9 group, respectively.

interaction [$F(1,26) = 3.76$; $\eta^2 = 0.09$; $p = 0.06$]. Duncan's post hoc test showed that NOC-9 decreased both the percentage of open-arm entries and the percentage of open-arm time (saline + NOC-9 vs. saline + vehicle; $p < 0.05$). Although two-way ANOVA did not reveal a clear pretreatment \times treatment interaction effect, Duncan's post hoc test revealed that the anxiogenic-like effects of NOC-9 were not observed in mice pretreated with the CRF1 antagonist at the same site (Fig. 5B). In addition, two-way ANOVA revealed that the closed-arm entries measure was not changed by any drug injected into the BNST [$F(1,26) \leq 1.44$; $\eta^2 \leq 0.05$; $p = 0.24$].

Experiment 5: intra-BNST AP-7 blocks the anxiogenic-like (but not the freezing) effect provoked by local injection of NOC-9

Fig. 6A shows the effects of intra-BNST AP-7 (pretreatment: 0 or 0.05 nmol) microinjected 10 min before local injection of NOC-9 (treatment: 0 or 75.00 nmol) on time spent freezing displayed by mice exposed to the novel arena. The Kruskal–Wallis test followed by the multiple comparisons test confirmed that NOC-9 produced freezing [$H(3,36) = 29.27$; $\eta^2 = 0.77$; $p < 0.0001$], an effect that was not prevented by prior intra-BNST injection of AP-7 ($p > 0.99$).

Fig. 6B shows the effects of intra-BNST AP-7 (0 or 0.05 nmol) injected prior to NOC-9 (0 or 75.00 nmol) injection into the same site on the percentage of open-arm entries and the percentage of open-arm time of mice exposed to the EPM. Regarding the percentage of open-arm entries, two-way ANOVA revealed significant differences for pretreatment factor [$F(1,32) = 4.83$; $\eta^2 = 0.11$; $p = 0.03$] and for pretreatment versus treatment interaction [$F(1,32) = 4.76$; $\eta^2 = 0.11$; $p = 0.03$], but not for treatment factor [$F(1,32) = 1.78$; $\eta^2 = 0.04$; $p = 0.19$]. Duncan's post hoc test revealed that NOC-9 (saline + NOC-9) decreased the percentage of open-arm entries compared to control (saline + vehicle; $p < 0.05$). Moreover, AP-7 microinjection blocked the anxiogenic-like effect of NOC-9 (AP-7 + NOC-9 vs. saline + NOC-9; $p < 0.05$; Fig. 6B). Regarding the percentage of open-arm time, two-way ANOVA revealed significant effects for treatment

factor [$F(1,32) = 4.90$; $\eta^2 = 0.11$; $p = 0.03$] and pretreatment versus treatment interaction [$F(1,32) = 6.71$; $\eta^2 = 0.15$; $p = 0.01$], but not significant changes for pretreatment factor [$F(1,32) = 1.13$; $\eta^2 = 0.02$; $p = 0.29$]. Duncan's post hoc test confirmed the anxiogenic-like effect produced by intra-BNST NOC-9 (saline + NOC-9 vs. saline + vehicle; $p < 0.05$). More importantly, pretreatment with AP-7 prevented this anxiogenic-like effect, since the percentage of open-arm time was significantly higher in the AP-7 + NOC-9 group than in the saline + NOC-9 group ($p < 0.05$; Fig. 6B). Regarding closed-arm entries, there were significant changes for treatment factor [$F(1,32) = 6.24$; $\eta^2 = 0.16$; $p < 0.05$], but no effects were shown for pretreatment factor [$F(1,32) = 0.01$; $\eta^2 = 0.0003$; $p > 0.05$] and pretreatment versus treatment factor interaction [$F(1,32) = 0.05$; $\eta^2 = 0.001$; $p > 0.05$]. Post hoc analysis did not confirm any significant differences among groups compared to control (saline + vehicle; $p < 0.05$).

Discussion

The main results of this study showed that increasing NO production within the BNST suddenly provokes freezing behavior and anxiogenic-like responses in mice exposed to the novel arena and EPM, respectively. While the anxiogenic-like effect of NOC-9 was attenuated by CP 376395, freezing remained unchanged in mice previously treated with this CRF1 receptor antagonist into the BNST. However, when given alone (i.e., without intra-BNST injection of NOC-9), CP 376395 was unable to change the behavioral profile of mice exposed to the EPM. A similar pharmacological profile was observed when AP-7, a NMDA receptor antagonist, was injected before the NO donor. At a dose devoid of intrinsic effects on anxiety, AP-7 did not change NOC-9-induced freezing, but completely blocked NOC-9 effects on behavior recorded in the EPM. However, in contrast to the lack of effects on anxiety observed with CP 376395, the highest dose of AP-7 was able to produce anxiolysis when injected alone into the BNST.

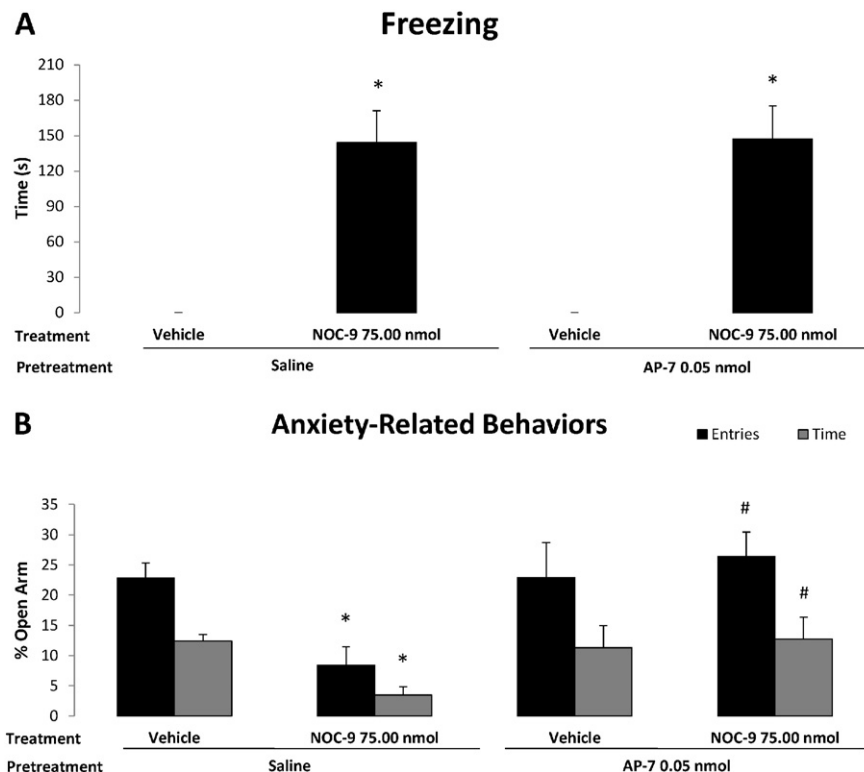


Fig. 6. Intra-BNST microinjection of AP-7 (0 or 0.05 nmol) (A) does not change freezing but (B) attenuates the anxiogenic-like effects induced by local injection of NOC-9 (0 or 75.00 nmol) in mice. Bars represent means (\pm SEM); $n = 8$ –10 per group. *,# $p < 0.05$ compared to the control group (saline + vehicle) and to the saline + NOC-9 group, respectively.

Intra-BNST injections of NOC-9 (at all doses) were able to produce freezing and anxiogenic-like effects. Freezing was observed immediately after the NO donor injection and lasted about 2–3 min (data not shown). When exposed to the EPM 5 min after intra-BNST NOC-9 injection, mice displayed anxiogenic-like behavior. Given that the BNST has a dense population of NMDA glutamate receptors (Guimaraes et al., 2005; McElligott and Winder, 2008) as well as CRF neurons (Cummings et al., 1983; Ju et al., 1989; Morin et al., 1999; Sakanaka et al., 1987; Swanson et al., 1983), we investigated whether the anxiogenic-like effect induced by the increase in NO release (induced by local injection of NOC-9) would depend on glutamate and/or CRF actions at their respective receptors.

NOC-9 produces an intracellular reaction at physiological pH, triggering a NO release without peroxynitrite formation, that could damage neuronal membranes (Seccia et al., 1996). The aversive effect of NOC-9 has already been highlighted in previous studies involving its microinjection in other brain sites, such as the inferior colliculus, amygdala and PAG (Braga et al., 2009; Miguel et al., 2012). For instance, intra-PAG injections of NOC-9 elicited explosive motor behaviors (e.g., jumping and running) followed by freezing and pain inhibition (Miguel et al., 2012). However, as observed in the present study, when injected into the BNST, NOC-9 seems to elicit more subtle defensive behaviors. Although this NO donor produces freezing, it seems to be unable to provoke jumping and running when injected into the BNST. One could argue that the enhancement of NO production might induce freezing and anxiety wherever a NO donor is injected into any brain limbic structures. Recent results with NOC-9 injections into the medial prefrontal cortex (mPFC) have suggested that this assumption is not entirely true. Intra-mPFC injections of NOC-9 induce anxiety in mice exposed to the EPM, however this NO donor did not provoke any other anxiogenic-like behavior (results not published). Together, these findings corroborate previous evidence showing that defensive behavior would be coordinated by a hierarchical brain defensive system (McNaughton and Corr, 2004). Those authors have argued that while the anxiety state is mediated mainly by forebrain structures (e.g. prefrontal dorsal stream, posterior cingulate, septo-hipocampal system, and amygdala), the fear state involves more caudal structures (e.g. medial hypothalamus and periaqueductal gray). Although it would be expected that both rostral and caudal limbic structures play a role in the modulation of anxiety and fear, the BNST seems to be a forebrain area involved in the modulation of more subtle defensive behaviors. Curiously, as far as we know, the effects of NOC-9 injection into the BNST on defensive behavior had never been tested prior to our study.

We have recently demonstrated that fear-like behaviors (e.g., jumping, running, freezing) induced by intra-PAG injection of NOC-9 were completely blocked by prior local injection of NBI 27914, a CRF1 antagonist, suggesting that NO production may lead to CRF release within this midbrain area. The hypothesis that the aversive effects induced by NO production could be mediated by CRF1 receptors within the BNST was tested in the present study through combined local injections of CP 376395 and NOC-9 in mice. We decided to focus on CRF receptor type 1 given the results obtained by our group in the PAG (Miguel and Nunes-de-Souza, 2011) and amygdala (not published) showing a role of CRF1 (but not CRF2) on the modulation of anxiety-like behavior. Interestingly, while intra-BNST CP 376395 was incapable of changing the freezing induced by local injection of NOC-9 (Fig. 5A), this CRF1 antagonist attenuated the anxiogenic-like behavior produced by the NO donor in mice exposed to the EPM. Although statistics had revealed only a tendency for pretreatment \times treatment interaction ($p = 0.06$), post hoc tests confirmed that CP 376395 impaired the effects of NOC-9 on anxiety indices (Fig. 5B), without changing closed-arm entries, a widely used measure of general activity (Cruz et al., 1994; Rodgers and Johnson, 1995). These results indicate that the anxiogenic-like effects produced by NOC-9 in the BNST are attenuated by the blockade of CRF1 receptors, suggesting that the neuropeptide CRF plays an important role in the modulation of anxiety-related

behaviors induced by NO release. Evidence showing that NO may release CRF was shown by Raber et al. (1995) who described an increase of CRF release into the amygdala and hypothalamus after treatment with nitroprusside, a NO donor. However, as shown in the present study, CRF1 receptors located within the BNST do not seem to be involved in the modulation of the NO-induced freezing. This lack of effects of CP 376395 is in contrast to those observed when the CRF1 receptors are blocked in the midbrain PAG. Briefly, intra-PAG NOC-9-induced freezing was absent in mice that received prior local injection of NBI 27914, a CRF1 receptor antagonist (Miguel et al., 2012).

These contrasting results observed with intra-BNST combined injections of NOC-9 and CP 376395 on freezing (in the novel arena) versus anxiety-related behavior (in the EPM) suggest that CRF neurotransmission within this forebrain limbic site could be mainly involved in the modulation of more subtle anxiety-like responses. This assumption is supported by previous studies showing that CRF injection into various brain areas induces an anxiogenic effect devoid of motor explosive reaction, such as jumping and running (Miguel et al., 2014; Miguel and Nunes-de-Souza, 2011; Sahuque et al., 2006). In other words, this peptide might be modulating less intense and aversive behavioral responses (e.g., avoidance to the open arms of the EPM) rather than freezing response.

In contrast to what we observed with CP 376395, intra-BNST injection of AP-7 alone attenuated anxiety-like responses in mice exposed to the EPM (Fig. 4). At the highest dose used (0.20 nmol), this NMDA receptor antagonist increased %OE and %OT, suggesting an anxiolytic-like effect. These results are suggestive that glutamate plays a tonic role in the modulation of anxiety (assessed in the mouse EPM) by acting at NMDA receptors located within the BNST. Interestingly, when injected before NOC-9, 0.05 nmol of AP-7, a dose devoid of intrinsic effects on anxiety (see Fig. 4), produced a similar effect to that observed with the blockade of CRF1 receptors (Fig. 6A/B). In other words, intra-BNST AP-7 (0.05 nmol) did not block NOC-9 induced freezing (Fig. 6A), however, it attenuated the main anxiogenic-like effects provoked by this NO donor in mice exposed to the EPM (Fig. 6B). In line with the above assumption that NO would lead to CRF release into the BNST, these results also suggest that the increase in NO production would stimulate glutamate release in this forebrain structure. Finally, we have hypothesized that glutamate, which is usually related to explosive motor reactions modulated by NMDA receptors (Aguiar et al., 2006; Miguel and Nunes-de-Souza, 2006; Molchanov and Guimaraes, 1999), could play a role in the freezing response induced by NOC-9 injected into the BNST. Surprisingly, despite attenuating the anxiogenic-like effect of NOC-9, AP-7 failed to change the freezing behavior induced by the NO donor, suggesting a dissociative mediation of glutamate in these NO-induced defensive responses. It is noteworthy, however, that we have used only a single dose of AP-7 to investigate the role of NMDA receptors in the modulation of the freezing induced by NOC-9. Further studies employing higher doses of AP-7 need to be conducted to clarify the role of the NMDA receptors located within the BNST in the modulation of this defense behavior.

In conclusion, present results are suggestive that both CRF (at CRF1 receptors) and glutamate (at NMDA receptors) play a role in the modulation of anxiety through the downstream mechanistic action of nitric oxide released within the BNST. Interestingly, while local blockade of NMDA receptors attenuated per se the anxiety-like behavior, intra-BNST injections of the CRF1 antagonist did not change anxiety indices in mice exposed to the EPM. Therefore, it seems reasonable to suggest that glutamate (but not CRF) plays a tonic role at NMDA receptors in the modulation of anxiety in mice exposed to the EPM. The role of CRF1 receptors in this emotional state seems to depend on the hyperactivation of nitrergic neurons of this limbic area, which was mimicked in the present study, with local injection of a NO donor. Finally, while both CRF1 and NMDA receptors are involved in the anxiogenic-like effects produced by NO in mice exposed to the EPM, neither CRF (at CRF1 receptor) nor glutamate (at NMDA receptor) seem to play a role in

NO-induced freezing. Further studies are needed to clarify the underlying mechanisms of these apparently discrepant behavioral effects of NO within the BNST.

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