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Contrasting effects of nitric oxide and corticotropin-releasing factor within the dorsal periaqueductal gray on defensive behavior and nociception in mice

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Abstract

The anxiogenic and antinociceptive effects produced by glutamate N-methyl-D-aspartate receptor activation within the dorsal periaqueductal gray (dPAG) matter have been related to nitric oxide (NO) production, since injection of NO synthase (NOS) inhibitors reverses these effects. dPAG corticotropin-releasing factor receptor (CRFr) activation also induces anxiety-like behavior and antinociception, which, in turn, are selectively blocked by local infusion of the CRF type 1 receptor (CRFr1) antagonist, NBI 27914 [5-chloro-4-(N-(cyclopropyl)methyl-N-propylamino)-2-methyl-6-(2,4,6-trichlorophenyl)aminopyridine]. Here, we determined whether i) the blockade of the dPAG by CRFr1 attenuates the anxiogenic/antinociceptive effects induced by local infusion of the NO donor, NOC-9 [6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-hexanamine], and ii) the anxiogenic/antinociceptive effects induced by intra-dPAG CRF are prevented by local infusion of N^{ω}-propyl-L-arginine (NPLA), a neuronal NOS inhibitor, in mice. Male Swiss mice (12 weeks old, 25-35 g, N = 8-14/group) were stereotaxically implanted with a 7-mm cannula aimed at the dPAG. Intra-dPAG NOC-9 (75 nmol) produced defensive-like behavior (jumping and running) and antinociception (assessed by the formalin test). Both effects were reversed by prior local infusion of NBI 27914 (2 nmol). Conversely, intra-dPAG NPLA (0.4 nmol) did not modify the anxiogenic/antinociceptive effects of CRF (150 pmol). These results suggest that CRFr1 plays an important role in the defensive behavior and antinociception produced by NO within the dPAG. In contrast, the anxiogenic and antinociceptive effects produced by intra-dPAG CRF are not related to NO synthesis in this limbic midbrain structure.

Key words: Nitric oxide; Corticotropin-releasing factor; Periaqueductal gray; Defensive behavior; Antinociception; Mice

Introduction

Electrical or chemical stimulation of the dorsal portion of the midbrain periaqueductal gray (dPAG) matter in rats and mice induces vigorous flight and vertical jumping alternating with freezing behavior (1,2). These aversive responses are quite similar to escape reactions elicited by natural predators (3) and, in general, such a defensive behavioral profile is accompanied by antinociception (4). This type of environmentally induced pain inhibition has been suggested to be an adaptive response of animals to cope with situations of imminent danger (4).

A broad range of neurotransmitters released into the PAG have been shown to play important roles in the mediation of defensive behaviors. For instance, it has been shown that serotonin, gamma-aminobutyric acid (GABA), glutamate and neuropeptides such as opioid and corticotropin-releasing factor (CRF) receptor agonists and antagonists change defensive behavior when injected into this midbrain structure (5,6).

As a ubiquitous excitatory amino acid in the CNS, glutamate activates the ionotropic N-methyl-D-aspartate receptor (NMDAr), as well as two other ion channel-coupled receptors, the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor and kainate receptor, and some metabotropic receptor subtypes, the G-protein-coupled receptors (7). The NMDAr is largely expressed in the PAG and its activation with exogenous NMDA elicits fight/flight reactions and antinociception in rats (8) and mice (1). NMDAr activation leads to calcium influx into the cell, which triggers a cascade of intracellular events, including activation of nitric oxide synthase (NOS), an enzyme that produces nitric oxide (NO) by conversion of L-

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arginine to L-citrulline, using nicotinamide adenine dinucleotide phosphate (NADPH) and Ca^{2+} as co-factors (7,9). Among the three main NOS isoforms, the neuronal NOS (nNOS) is a constitutive form expressed in neurons (10).

Similarly to the proaversive effects produced by glutamate-NMDAr agonists, injection of NO donors into the dPAG produces fight and flight reactions in rats (11). Conversely, intra-dPAG injection of NOS inhibitors, guanylate cyclase inhibitors and an NO scavenger provokes anxiolytic-like effects in rats exposed to the elevated plus-maze (12). 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 (13), and reverses both the defensive-like behavior and the antinociception induced by local infusion of NMDA (1). These results are consistent with previous findings indicating that there are a large number of NOS immunoreactive neurons in the dPAG of rats and mice (14,15).

Furthermore, it has been shown that NO interferes with the release of several neurotransmitters (e.g., acetylcholine, GABA, glutamate, dopamine, serotonin, and CRF) in distinct brain areas related to the defensive response (10,16). The neuropeptide CRF is strongly linked to stress-related responses, since stress episodes are able to induce CRF release into the brain (17) and to activate the hypothalamic-pituitary-adrenal (HPA) axis (18). CRF-containing neurons and CRF 1 and 2 receptor subtypes (CRFr1 and CRFr2) have usually been identified in the PAG (19). In addition, previous studies have related this neuropeptide to anxiety- and stress-mediated responses, since intracerebroventricular (*icv*) and intra-dPAG CRF microinjections produce anxiogenic effects (20,21). Moreover, injection of non-selective CRFr antagonists into this midbrain structure prevents anxiety-like behavior elicited by restraint and cold stress and ethanol withdrawal in rats exposed to the elevated plus maze (EPM) (22-24).

We have recently observed that the blockade of CRFr1 with NBI 27914 [5-chloro-4-(N-(cyclopropyl)methyl-N-propylamino)-2-methyl-6-(2,4,6-trichlorophenyl)aminopyridine], a potent and selective CRFr1 antagonist (25), within the mouse dPAG abolishes the anxiogenic and antinociceptive effects produced by local injection of CRF (25). Thus, considering that intra-dPAG NO donors produce anxiogenic-like effects (11) and that NO diffusion elicits CRF release (16,26), the present study investigated whether the blockade of CRFr1 would attenuate the anxiogenic and antinociceptive effects of NO donors within this midbrain structure (Experiment 1). In addition, we attempted to show whether the anxiogenic and antinociceptive effects produced by intra-dPAG CRF are attenuated by prior local injection of the nNOS inhibitor NPLA (Experiment 2). Given that intra-dPAG injection of NOC-9 [6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-hexanamine] provokes explosive defensive behavior, the behavioral test was carried out in a glass cage (Experiment 1). Although intra-dPAG infusion of CRF also increases defensive behavior, this reaction is not so extreme; thus, the behavioral test used in Experiment 2 was reliably assessed with the EPM.

Material and Methods

Subjects

Male adult Swiss mice (Universidade Estadual Paulista, UNESP, Brazil) weighing 25-35 g at testing were used in the study. Mice were housed in groups of 10 per cage (41 x 34 x 16 cm) and maintained under a normal 12-h light-dark cycle (lights on at 7:00 am) in a temperature-controlled environment (23 ± 2°C). Food and water were freely available except during the brief test periods. All mice were naive at the beginning of the experiments.

Drugs

Drugs and doses used were as follows: NBI 27914 (Tocris Cookson Inc., USA), a potent and selective (Ki = 1.7 nM) CRFr1 antagonist (2 nmol) (25), NOC-9 (Sigma-Aldrich, Brazil), a peroxynitrite production-free NO donor (75 nmol) (11), CRF (150 pmol; Sigma-Aldrich) (25), and NPLA (Tocris Cookson Inc.), a highly selective and potent inhibitor of nNOS (Ki = 57 nM), which displays 3158- and 149-fold selectivity over iNOS and eNOS, respectively (0.4 nmol) (1). Doses were based on previous studies (1,11,21,25,27). NBI was dissolved in dimethylsulfoxide (70% DMSO in physiological saline), NOC-9 in 1 M Tris solution, pH 10, to prevent NO release before it reaches brain tissue (11) and CRF and NPLA in physiological saline (0.9% NaCl).

Surgery and microinjection

Mice were implanted with a 7-mm stainless steel guide cannula (26 gauge; Insight Equipamentos Científicos Ltda., Brazil) under anesthesia with 80 mg/kg ketamine plus 8 mg/kg xylazine (a muscle relaxant). A guide cannula was fixed to the skull using dental acrylic and jeweler's screws. Stereotaxic coordinates, based on the Paxinos and Franklin Atlas (28) for the dPAG, were 4.1 mm posterior to the bregma, 1.3 mm lateral to the midline, and 2.2 mm ventral to the skull surface, with the guide cannula angled 26° to the vertical and its tip positioned 1 mm above the target site. A dummy cannula (33 gauge, stainless steel wire; Fishtex Industry and Commerce of Plastics Ltda., Brazil), inserted into each guide cannula immediately after surgery, served to reduce the incidence of occlusion. Postoperative analgesia was provided for 3 days by adding 200 mg/mL acetaminophen to the drinking water (final concentration = 0.16 mg/mL).

Five to 7 days after surgery, the various solutions were injected into the dPAG using microinjection units (33-gauge stainless steel cannulae; Insight Equipamentos Científicos Ltd.), which extended 1.0 mm beyond the tip of the guide cannula. Each microinjection unit was attached to a 5-µL Hamilton microsyringe via polyethylene tubing (PE-10), and drug administration was controlled by an infusion pump (BI 2000, Insight Equipamentos Científicos Ltd.) programmed to deliver 0.2 µL over a period of 30 s. The microinjection procedure

consisted of gently restraining the animal, removing the dummy cannula and inserting the injection unit, which remained *in situ* for a further 30 s after the injection. Successful infusion was confirmed by monitoring the movement of a small air bubble in the PE-10 tubing.

Defensive response analysis

Immediately after the microinjection procedure (see also General procedure, Experiment 1), each mouse was placed in a glass cage (30 x 21 x 25 cm) to record defensive and exploratory behavior for a 5-min period. The recorded defensive behavior consisted of time spent (in seconds) running [i.e., trotting (running but keeping the same pattern as walking) and galloping (fast running, alternating anterior and posterior limb pairs)] and freezing (complete absence of movement except breathing while the animal exhibits a characteristic tense posture), and frequency of jumps (i.e., upward leaps directed to the wall of the glass cage). The exploratory behavior variables recorded were time of locomotion (i.e., slow walking with elevation of trunk and tail and out of phase stance and swing movements of the contralateral limbs) and frequency of rearing (standing on hind limbs, with both forelimbs off the floor; this measure included both unsupported rearing, and rearing against the wall).

This test was recorded with a camera-TV-DVD system and behavior was subsequently scored by a trained observer.

Elevated plus-maze

The basic EPM design was closely similar to that originally described by Lister (29) and consisted of two open arms (30 x 5 x 0.25 cm) and two closed arms (30 x 5 x 15 cm) connected by a common central platform (5 x 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.

After drug administration (see General procedure; Experiment 2) into the dPAG (Figure 1), each mouse was placed in an individual holding cage and then 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. The videotaped test sessions lasted 5 min and, between subjects, the maze was thoroughly cleaned with 20% alcohol and dry cloths. All experiments were performed under normal laboratory illumination (1 x 60 W yellow incandescent lamp positioned approximately 1.80 m above the EPM floor), during the light phase of the light-dark cycle. Videotapes were scored by a trained observer using an ethological analysis package developed by the group of Dr. S. Morato, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, USP (Brazil). Behavioral parameters consisted of both conventional spatiotemporal and ethological measures (30). Conventional measures were the frequencies of open- and closed-arm entries (entry = all four paws into an arm) and the time spent in the open arms of the maze. These data were used to calculate the percentage of open-arm entries [(open / total) x 100] and percentage of time spent in each zone of the maze [(time in compartment / 300) x 100]. Ethological measures are reported as frequency scores for open-arm end exploration (OAEE = entering the 10-cm distal section of the open arm from the central square), head dipping (HD = exploratory movement of head/shoulders over the side of the maze) and stretched-attend postures (SAP: exploratory posture in which the body is stretched forward then retracted to the original position without any forward locomotion). In view of the importance of the thigmotactic cues for patterns of plus-maze exploration, HD and SAP were further differentiated by recording where on the maze they were displayed. Consistent with earlier reports (30), the closed arms and the central platform were designated “protected” areas together (i.e., offering relative security), while the open arms were designated “unprotected” areas. Data for the HD and SAP measures are thus reported as separate protected and unprotected scores.

Nociception test

Nociception was assessed by the formalin test (1). The formalin test causes a two-phase nociceptive response (31). The first phase begins immediately after formalin injection and lasts approximately 5 min. It results from the direct stimulation of nociceptors (31). The second phase begins 20 min after the injection and lasts approximately 40 min. This phase is caused by C-fiber activation (32) and also involves a period of sensitization, during which inflammatory phenomena occur (32). In the present study, 50 µL formalin (2.5% formaldehyde) was injected into the dorsal surface of the right hind paw of the mouse, which was placed in a glass holding cage (30 x 20 x 25 cm). Given that the second phase of the nociceptive response lasts a long time (about 40 min), in the present study we chose this phase of the formalin test to assess the effects of drugs injected into the dPAG.

Therefore, the nociceptive response was recorded by measuring the time (in seconds) spent licking the paw injected with formalin during the second phase, between 25 and 35 min after injection (a period of 10 min).

General procedure

Experiment 1: effects of combined intra-dPAG injections of NBI 27914 and NOC-9 on defensive and exploratory behavior and nociception in mice

Defensive and exploratory behavior. Mice received an intra-dPAG injection of NBI 27914 (pretreatment: 0 or 2 nmol/0.2 µL) and, 10 min later, an injection of NOC-9 (treatment: 0 or 75 nmol/0.2 µL) at the same midbrain site. Immediately after the intra-dPAG NOC-9 injection, each mouse was placed in the glass cage to record defensive and exploratory behavior (see details in section “Defensive response analysis”).

Nociception test. Formalin (50 µL) was injected into the hind paw of mice 48 h after the evaluation of defensive and exploratory behavior in the glass cage. Fifteen minutes after this injection, each mouse received an intra-dPAG injection

of NBI 27914 (pretreatment: 0 or 2 nmol/0.2 µL) and, 10 min later, an injection of NOC-9 (treatment: 0 or 75 nmol/0.2 µL) at the same midbrain site. Immediately after intra-dPAG NOC-9 injection, mice were placed individually in the glass holding cage for 10 min (i.e., 25-35 min after the formalin injection) to record the time spent licking the affected paw.

Experiment 2: effects of combined intra-dPAG injections of NPLA and CRF on anxiety-like behavior and nociception in mice

Anxiety test. Mice received intra-dPAG injection of NPLA (pretreatment: 0 or 0.4 nmol) and, 10 min later, CRF was injected (treatment: 0 or 150 pmol) into the same midbrain site. Ten minutes later, each mouse was placed on the EPM to record conventional and ethological parameters (for details see section "Defensive response analysis") for a period of 5 min.

Nociception test. Forty-eight hours after the EPM test, formalin was injected into the hind paw of each mouse and, 5 min later, they received an intra-dPAG microinjection of NPLA (pretreatment: 0 or 0.4 nmol). After a further 10 min, the mice received an injection of CRF (0 or 150 pmol) and, 10 min later, they were placed individually in a glass holding cage for 10 min (i.e., 25-35 min after the formalin injection) to record the time (in seconds) spent licking the affected paw.

Importantly, all animals that had received an intra-dPAG injection of NOC-9 (75 nmol in Experiment 1) or CRF (150 pmol in Experiment 2) on day 1 (defensive and exploratory behavior and EPM test, respectively) received vehicle on day 3 (nociception test) and vice versa.

Histological analysis

At the end of testing, all animals received a 0.2-µL intra-dPAG infusion of 1% Evans blue by the same microinjection procedure as used for the drugs. Animals were then sacrificed in the CO_2 chamber, their brains removed and injection sites checked histologically by reference to the Paxinos and Franklin Atlas (28). Data from animals with injection sites outside the dPAG were excluded from data analysis. Final sample sizes ranged from 8 to 11 (defensive and exploratory behaviors) and 8 (nociception test) animals per group in Experiment 1 and from 9 to 14 (anxiety test) and 8 to 11 (nociception test) animals per group in Experiment 2.

Statistical analysis

All results were initially subjected to Levene's test for homogeneity of variance. Where the Levene test yielded significant inhomogeneity, results were transformed to their log, square root or cube root and confirmed for homogeneity of variance before being subjected to two-way analysis of variance (ANOVA; factor 1 = pretreatment and factor 2 = treatment), followed by the *post hoc* Duncan test. In those cases (Experiment 1 = running, jumping, and freezing) where the Levene test remained significant even after all transformations, data were analyzed by Kruskal-Wallis non-parametric ANOVA followed by the Dunn test. In all cases, a P value ≤ 0.05 was required for significance.

Ethics

The 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, Universidade Estadual Paulista, resolution 10/2006).

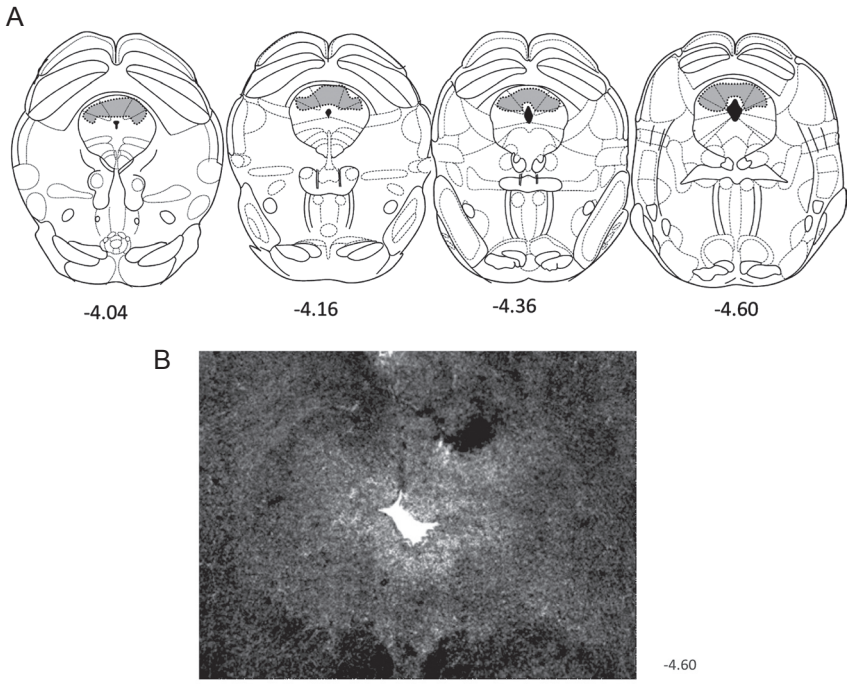

Figure 1. *A*, Diagram of mouse brain sections, based on Paxinos and Franklin (28), showing microinfusion sites within the midbrain dorsal periaqueductal gray (dPAG). The gray area corresponds to the whole area in which microinjections were placed in the various slices (distance from bregma in mm) described in the Paxinos and Franklin Atlas (reproduced with permission). *B*, Photomicrograph of a midbrain coronal section from a representative subject showing an injection site into the dPAG (dark stain: Evan blue). The section was -4.60 mm from bregma.

Results

Brain injection sites

Figure 1 shows schematic brain sections based on the Paxinos and Franklin Atlas (28), indicating microinfusion sites within the midbrain dPAG, and a photomicrograph of a midbrain coronal section of a

representative subject, showing an injection site within the dPAG of the mouse.

Experiment 1: effects of prior intra-dPAG injection of NBI 27914 on the behavior and antinociception produced by local infusion of NOC-9 at the same site

Figure 2A-E show the effects of intra-dPAG injection of NBI 27914 on the defensive and exploratory behavior produced by local infusion of NOC-9. Kruskal-Wallis ANOVA revealed significant differences in running ($H = 23.09$; $P < 0.05$), jumping ($H = 20.10$; $P < 0.05$) and freezing ($H = 22.06$; $P < 0.05$) measures. The *post hoc* Dunn test revealed that intra-dPAG NOC-9 (vehicle + NOC-9) increased the time spent running (2A) and freezing (2C) and the frequency of jumps (2B), relative to the control group (vehicle + vehicle; $P < 0.05$). Prior intra-dPAG injection of NBI 27914 (NBI + NOC-9) completely blocked the effects of NOC-9 (vehicle + NOC-9) on running and jumping and tended to prevent the freezing induced by the NO donor. Importantly, intra-dPAG NBI alone (i.e., NBI + vehicle) did not change any behavior. Regarding the exploratory behavior locomotion (Figure 2D), two-way ANOVA revealed significant effects for the pretreatment factor ($F_{1,32} = 8.29$; $P < 0.05$) and treatment factor ($F_{1,32} = 11.76$; $P < 0.05$), but no pretreatment x treatment interaction ($F_{1,32} = 0.95$; $P = 0.34$). *Post hoc* comparisons revealed that NOC-9 (vehicle + NOC-9) reduced locomotion compared to the control group (vehicle + vehicle). Pretreatment with NBI 27914 reduced the effect of NOC-9 (NBI + NOC-9 *vs* vehicle + NOC-9; $P < 0.05$), but did not alter this exploratory behavior when given alone (NBI + vehicle). Two-way ANOVA revealed significant effects regarding rearing frequency (Figure 2E) only for the treatment factor ($F_{1,32} = 6.35$; $P < 0.05$), without showing differences for the pretreatment factor ($F_{1,32} = 0.26$; $P = 0.61$) or pretreatment x treatment interactions ($F_{1,32} = 0.006$; $P = 0.94$). The *post hoc* Duncan test revealed that intra-dPAG NOC-9 reduced the rearing frequency of the control group (vehicle + vehicle).

Figure 3 shows the effects of intra-dPAG injection of NBI 27914 on the antinociceptive effect produced by local infusion of NOC-9. Two-way ANOVA revealed significant differences for the pretreatment factor ($F_{1,28} = 4.05$; $P < 0.05$), treatment factor ($F_{1,28} = 10.60$; $P < 0.05$) and pretreatment x treatment interaction ($F_{1,28} = 5.12$; $P < 0.05$). The *post hoc* Duncan test showed that NOC-9 produced antinociception (vehicle + NOC-9 *vs* vehicle + vehicle) while

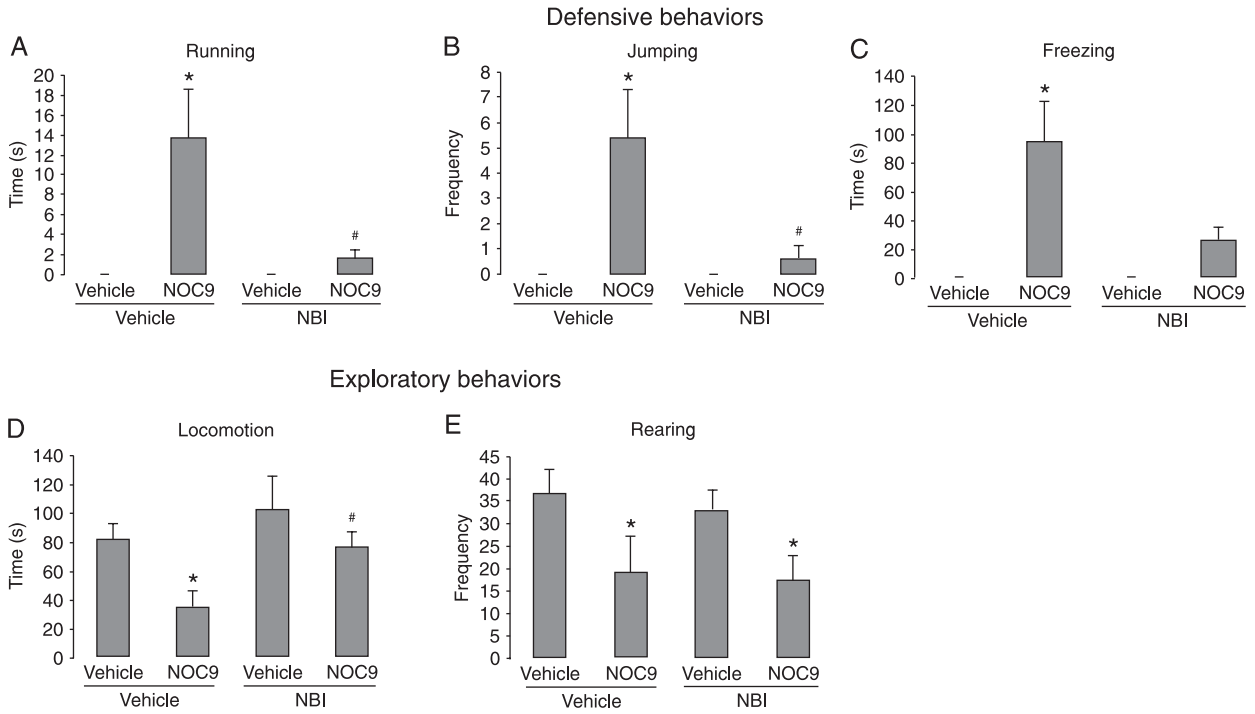

Figure 2. Effect of intra-dPAG microinjections of NBI 27914 (0 or 2 nmol) and NOC-9 (0 or 75 nmol) upon defensive [running (*A*), jumping (*B*), and freezing (*C*)] and exploratory [locomotion (*D*) and rearing (*E*)] behavior of mice. Mice received an intra-dPAG injection of NBI 27914 (pretreatment: 0 or 2 nmol/0.2 µL) and, 10 min later, an injection of NOC-9 (treatment: 0 or 75 nmol/0.2 µL) at the same midbrain site and were then tested immediately. Data are reported as means ± SEM, N = 8-11 per group. dPAG = dorsal periaqueductal gray; NBI 27914 = [5-chloro-4-(N-(cyclopropyl)methyl-N-propylamino)-2-methyl-6-(2,4,6-trichlorophenyl)aminopyridine]; NOC-9 = [6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-hexanamine]. *$P < 0.05$ compared to control group (vehicle + vehicle), and #$P < 0.05$ compared to vehicle + NOC-9 group (Kruskal-Wallis followed by the Dunn *post hoc* test or two-way ANOVA followed by the Duncan *post hoc* test).

NBI 27914 pretreatment completely blocked this effect of NOC-9, without changing the nociceptive response when given alone (NBI + vehicle).

Experiment 2: effects of combined intra-dPAG injections of NPLA and CRF on anxiety-like behavior and nociception in mice

Figure 4A shows the effects of intra-dPAG injections of first NPLA (0 or 0.4 nmol) and then CRF (0 or 150 pmol) on the behavior of mice exposed to the EPM. Two-way ANOVA revealed significant effects of the treatment (CRF) factor for both indices of anxiety (%OE, open-arm entry: $F_{1,38} = 14.53$; $P < 0.05$; %OT, open-arm time: $F_{1,38} = 9.92$; $P < 0.05$) but did not show any effect for the pretreatment (NPLA) factor (%OE: $F_{1,38} = 0.06$; $P = 0.81$; %OT: $F_{1,38} = 0.03$; $P = 0.85$) or pretreatment x treatment interaction (%OE: $F_{1,38} = 0.48$; $P = 0.49$; %OT: $F_{1,38} = 1.42$; $P = 0.24$). *Post hoc* analysis revealed that CRF (vehicle + CRF) decreased both indices of anxiety (%OE and %OT, $P < 0.05$) compared to the control group (vehicle + vehicle), and these effects were not blocked by prior intra-dPAG injection of NPLA. In addition, one-way ANOVA did not reveal any effect of the pretreatment factor ($F_{1,38} = 0.016$; $P = 0.90$), treatment factor ($F_{1,38} = 0.005$; $P = 0.94$) or pretreatment x treatment interaction ($F_{1,38} = 0.67$; $P = 0.42$) on closed-arm entries.

As shown in Figure 4B, ANOVA revealed some significant effects of CRF treatment on ethological measures (protected SAP: $F_{1,38} = 4.61$, $P < 0.05$; unprotected SAP: $F_{1,38} = 14.17$, $P < 0.05$; unprotected HD: $F_{1,38} = 12.77$, $P < 0.05$; OAEE: $F_{1,38} = 9.22$, $P < 0.05$). *Post hoc* comparisons

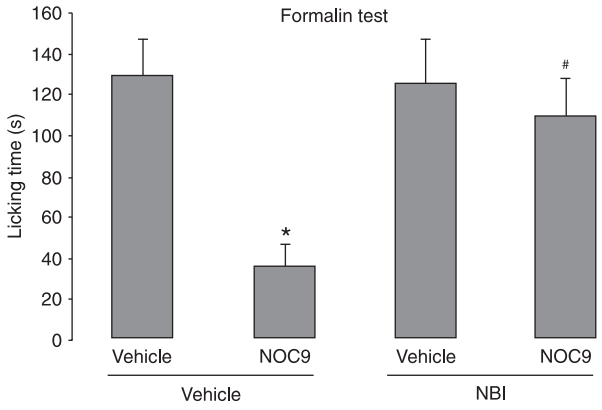

Figure 3. Effect of intra-dPAG microinjections of NBI 27914 (0 or 2 nmol) and NOC-9 (0 or 75 nmol) on the time (in seconds) spent licking the formalin affected paw in mice (nociceptive response). Mice received an intra-dPAG injection of NBI 27914 (pretreatment: 0 or 2 nmol/0.2 µL) and, 10 min later, an injection of NOC-9 (treatment: 0 or 75 nmol/0.2 µL) at the same midbrain site and were then tested immediately. Data are reported as means ± SEM, N = 8 per group. dPAG = dorsal periaqueductal gray; NBI 27914 = [5-chloro-4-(N-(cyclopropyl)methyl-N-propylamino)-2-methyl-6-(2,4,6-trichlorophenyl)aminopyridine]; NOC-9 = [6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-hexanamine]. *P < 0.05 compared to control group (vehicle + vehicle) and #P < 0.05 compared to vehicle + NOC-9 group (two-way ANOVA followed by the Duncan *post hoc* test).

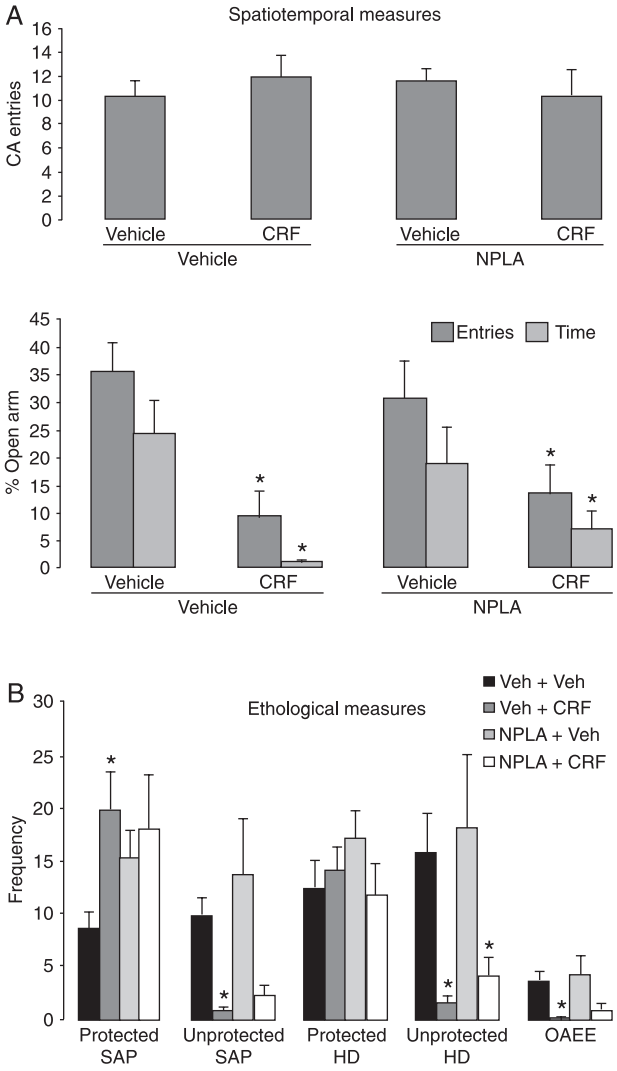

Figure 4. Effects of intra-dPAG microinjections of NPLA (0 or 0.4 nmol) and CRF (0 or 150 pmol) on (*A*) spatiotemporal measures: frequency of closed-arm (CA) entries (upper panel), percentage of open-arm entries and time (lower panel) and (*B*) ethological measures: frequency of protected and unprotected stretched-attend posture (SAP), protected and unprotected head dipping (HD) and open-arm end exploration (OAEE) in mice exposed to the elevated plus-maze. Mice received intra-dPAG injection of NPLA (pretreatment: 0 or 0.4 nmol) and, 10 min later, CRF was injected (treatment: 0 or 150 pmol) into the same midbrain site. Testing started 10 min after the second injection. Data are reported as means ± SEM, N = 9-14 per group. dPAG = dorsal periaqueductal gray; NPLA = N^{ω}-propyl-L-arginine; CRF = corticotropin-releasing factor. *P < 0.05 compared to control group (vehicle (Veh) + vehicle; two-way ANOVA followed by the Duncan *post hoc* test).

indicated that previous treatment with NPLA was not able to reverse the CRF effect on these measures (protected SAP: P = 0.70; unprotected SAP: P = 0.71; unprotected HD: P = 0.64; OAEE: P = 0.56). In addition, neither CRF nor NPLA significantly changed the frequency of protected HD ($F_{1,38}$ = 0.47, P = 0.49; Figure 4B).

Figure 5 shows the effects of combined injections of first NPLA (0 or 0.4 nmol) and then CRF (0 or 150 pmol) into the dPAG on time spent licking the paw affected by the 2.5% formalin injection. Two-way ANOVA did not reveal significant differences for the pretreatment (NPLA) factor ($F_{1,30}$ = 0.91; P = 0.34) or for pretreatment x treatment interaction ($F_{1,30}$ = 0.13; P = 0.72), but did show a difference for the treatment (CRF) factor ($F_{1,30}$ = 20.71; P < 0.05). *Post hoc* comparisons revealed that CRF (vehicle + CRF) reduced the time spent licking the affected paw, compared to the control group (vehicle + vehicle), an effect that was not blocked by intra-dPAG pretreatment with NPLA.

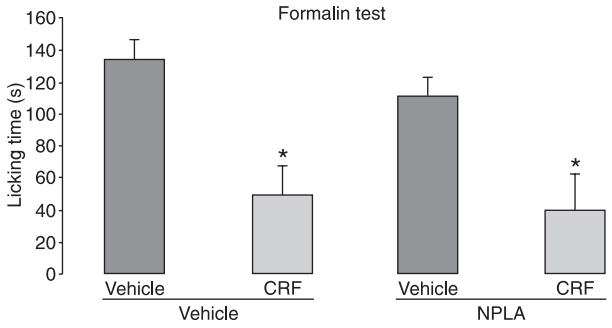

Figure 5. Effects of intra-dPAG microinjections of NPLA (0 or 0.4 nmol) and CRF (0 or 150 pmol) on the time (in seconds) spent licking the formalin-treated paw. Mice received intra-dPAG injection of NPLA (pretreatment: 0 or 0.4 nmol) and, 10 min later, CRF was injected (treatment: 0 or 150 pmol) into the same midbrain site. Testing started 10 min after the second injection. Data are reported as means ± SEM, N = 8-11 per group. dPAG = dorsal periaqueductal gray; NPLA = N^{ω}-propyl-L-arginine; CRF = corticotropin-releasing factor. *P < 0.05 compared to control group (vehicle + vehicle; two-way ANOVA followed by the Duncan *post hoc* test).

Discussion

The results of the present study show that intra-dPAG injection of NOC-9 produces defensive-like behavior (running, jumping, and freezing) and inhibits the nociceptive response elicited during the formalin test in mice. Both defensive behavior and antinociception induced by this NO donor were attenuated by prior intra-dPAG injection of the CRFr1 antagonist NBI 27914. In contrast, the anxiogenic and antinociceptive effects produced by intra-dPAG injection of CRF were not changed by prior injection of the nNOS inhibitor NPLA into this midbrain site.

The defensive-like behavior induced by intra-dPAG NOC-9 in mice has been reported following intra-dPAG injection of this NO donor in rats (11). The cited investigators reported that intra-dPAG NOC-9 also reduced distance traveled. In agreement with those findings, the present study showed that intra-dPAG NOC-9 also reduced exploratory behavior, as indicated by a decrease in rearing frequency and locomotion time. Here, the decrease in locomotion and rearing following NOC-9 injection might have been a consequence of the increased freezing time. In other words, the exhibition of running, jumping and freezing induced by NOC-9 seemed to concur with the expression of exploratory behavior. Regarding the underlying mechanisms related to the aversive effects induced by the increase in NO synthesis, previous findings have emphasized the involvement of cGMP. Briefly, it has been shown that intra-PAG injection of ^{1}H-[1,2,4]oxadiazolol[4,3-a]quinoxalin-1-one] (ODQ), a selective soluble guanylyl cyclase (a cGMP synthesis enzyme) inhibitor, attenuates the aversive effects produced by local infusion of NOC-9 (11).

We have recently observed that intra-dPAG injection of CRF produces anxiety and antinociception in mice, both effects being completely blocked by prior local infusion of the CRFr1 antagonist, NBI 27914 (25). Importantly, the anxiogenic and antinociceptive effects of CRF were not changed by intra-dPAG injection of antisauvagine-30 (25), a CRFr2 antagonist, suggesting that CRFr1 (but not CRFr2) located within the dPAG plays a role in the mediation of anxiety and pain inhibition in mice. Here, we investigated whether the blockade of CRFr1 would also attenuate the defensive behavior and antinociception observed with intra-dPAG injection of NOC-9. As shown in Figures 2 and 3, intra-dPAG injection of NBI 27914 prevented the behavioral and antinociceptive effects induced by the NO donor, suggesting that these aversive effects caused by an enhancement in NO synthesis are attenuated by CRFr1 blockade within the dPAG. The present results suggest that NO production may facilitate CRF release in this midbrain structure. This assumption is consistent with a study (16) demonstrating that CRF is released from cultures of amygdala and hypothalamus cells after incubation with nitroprusside, another type of NO donor. In addition, Lee et al. (26) have demonstrated that *icv* injection of 5-amino-3-3(4-morpholinyl)-1,2,3-oxadiazolium chloride (SIN-1), also an NO donor, increases plasma ACTH, an effect that is abolished by prior treatment with anti-CRF antibody. How NO acts in favor of CRF release is unclear. However, Raber et al. (16) have suggested that NO may exert an excitatory effect directly upon the CRF neuron or indirectly via its action on neighboring glial cells. NO increase may, in turn, enhance intracellular calcium concentration, leading to exocytosis of CRF (16,33). However, it is unlikely that the behavioral effects of intra-dPAG NO donor injection observed in the present study are exclusively related to an increase of CRF release within the dPAG, since intra-dPAG

infusion of CRF does not induce jumping, running or freezing behavior (21,25). Furthermore, it has been reported that NO also increases the release of other neurotransmitters, such as norepinephrine, acetylcholine and glutamate, in various brain areas (10). However, the present results suggest that CRFr1 plays an important role in the mediation of defensive behavior and antinociception induced by the enhancement in the nitrergic tonus within the midbrain dPAG of mice.

On the other hand, inhibition of NO synthesis within the PAG does not modify the anxiogenic and antinociceptive effects provoked by intra-dPAG injection of CRF, suggesting that NO does not play a pivotal role in these aversive effects of CRF. Corroborating previous studies in rats (20,21) and mice (25), intra-dPAG CRF increased anxiety-like behavior in mice exposed to the EPM (Figure 5A), as well as provoking antinociception (25). Importantly, intra-dPAG injection of CRF did not change the number of closed-arm entries, a widely used measure of general activity in the EPM (see, e.g., Ref. 30), suggesting that this CRF-induced profile is behaviorally selective and thus not secondary to changes in general activity levels. Nevertheless, prior local infusion with the nNOS inhibitor, NPLA, neither changed anxiety indices in the EPM nor the antinociceptive effects induced by intra-dPAG CRF. We have observed that intra-PAG NPLA, at a dose similar to that used in the present study, completely blocked the defensive-like behavior and antinociception produced by intra-PAG injection of the glutamate NMDAr agonist (1,27), suggesting that 0.4 nmol NPLA is quite enough to attenuate these pro-aversive effects induced by NO production. In other words, NPLA did not change the effects of CRF, suggesting that NO production may not be involved in the CRFr-triggered intracellular cascade.

Bowers et al. (34) have shown that CRF has excitatory effects on neurons of the PAG in rats, an effect that has also been observed in other brain structures, such as locus coeruleus, hippocampus and cerebellum (35-37). The present results suggest that it is unlikely that CRF-induced anxiogenic and antinociceptive effects are mediated by NO in the mouse PAG. These results contrast with the complete blockade of the aversive effects induced by NMDAr activation observed in animals pretreated with NPLA (1,27). Glutamate NMDAr activation leads to cellular calcium influx, which triggers a cascade of intracellular events including activation of NOS (7,9), an NO synthesis enzyme. Although CRF also leads to cellular calcium influx, it is unlikely that the amount of this ion is enough to activate nNOS, since NPLA failed to block the anxiogenic and antinociceptive effects produced by intra-dPAG CRF. In other words, while the ionotropic NMDAr activation leads to an enhancement of intracellular calcium concentration, the stimulation of CRF receptors activates G-protein-coupled pathways, indicating two distinct mechanisms.

Furthermore, it is important to point out that CRF can induce glutamate release (38), so that the failure of NO inhibition to prevent the action of CRF does not rule out the possibility of glutamatergic mediation of the CRF anxiogenic and antinociceptive effects. This issue needs more clarification and further experiments, such as combined injection of CRF and glutamate receptor antagonists, need to be carried out in the future.

The results of the present study indicate that CRFr1 located within the dPAG plays an important role in the proaversive effects (defensive behavior and pain inhibition) induced by NO synthesis. In contrast, the anxiogenic and antinociceptive effects produced by the neuropeptide CRF do not depend on NO synthesis within this limbic midbrain structure in mice.

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