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Blockade of NMDA Receptors and Nitric Oxide Synthesis in the Dorsolateral Periaqueductal Gray Attenuates Behavioral and Cellular Responses of Rats Exposed to a Live Predator

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Innate fear stimulus induces activation of neurons containing the neuronal nitric oxide synthase enzyme (nNOS) in defensive-related brain regions such as the dorsolateral periaqueductal gray (dlPAG). Intra-dlPAG administration of nitric oxide synthase (NOS) inhibitors and glutamate antagonists induce anxiolytic-like responses. We investigated the involvement of nitric oxide (NO) and glutamate neurotransmission in defensive reactions modulated by dlPAG. We tested if intra-dlPAG injections of the selective nNOS inhibitor, N-propyl-L-arginine (NP), or the glutamate antagonist, AP7 (2-amino-7-phosphonoheptanoic acid), would attenuate behavioral responses and cellular activation induced by predator exposure (cat). Fos-like immunoreactivity (FLI) was used as a marker of neuronal functional activation, whereas nNOS immunohistochemistry was used to identify NOS neurons. Cat exposure induced fear responses and an increase of FLI in the dlPAG and dorsal premammillary nucleus (PMd). NP and AP7 attenuated the cat-induced behavioral responses. Whereas NP tended to attenuate FLI in the dlPAG, AP7 induced a significant reduction in cellular activation of this region. The latter drug, however, increased FLI and double-labeled cells in the PMd. Cellular activation of this region was significantly correlated with time spent near the cat (r = 0.7597 and 0.6057 for FLI and double-labeled cells). These results suggest that glutamate/NO-mediated neurotransmission in the dlPAG plays an important role in responses elicit by predator exposure. Blocking these neurotransmitter systems in this brain area impairs defensive responses. The longer time spent near the predator that follows AP7 effect could lead to an increased cellular activation of the PMd, a more rostral brain area that has also been related to defensive responses.

Key words: defensive behavior; cat exposure; glutamate; nitric oxide

Rats exposed to a predator exhibit unconditioned behaviors characterized by alertness, freezing, fight/flight reactions, analgesia, and autonomic changes (Blanchard and Blanchard, 2008; Blanchard et al., 1990). Because these behaviors are innate and easily evoked in the laboratory, they have been extensively used to investigate the neural substrate mediating emotional responses to threatening stimuli (Blanchard et al., 2003; Hendrie et al., 1996; Rodgers, 1997). Experiments that used Fos protein as a marker of neural activation has unveiled a network of interconnected brain regions comprising the medial amygdala (MeA), various medial hypothalamic structures, and the periaqueductal gray (PAG) (Beijamini and Guimaraes, 2006b; Canteras et al., 1997; Canteras and Goto, 1999) activated by predator exposure (Beijamini and Guimaraes, 2006b; Canteras and Goto, 1999; Dielenberg et al., 2001, 2004). This neural system may constitute the neural basis of psychiatric disorders such as anxiety, panic, and posttraumatic stress (McNaughton and Corr, 2004; Sewards and Sewards, 2002). Several studies have also suggested that these defensive responses are mediated or modulated by neurotransmitters such as glutamate, serotonin, gamma-aminobutyric acid, and neuropeptides (Adamec et al., 1999; Blanchard et al., 1992; McGregor et al., 2004; Moreira and Guimaraes, 2008). More recently, nitric oxide (NO) has also been proposed to modulate these behaviors. In the central nervous system, NO is synthesized by the neuronal nitric oxide synthase (nNOS) enzyme (Bredt and Snyder, 1994). This enzyme is activated by calcium influx trough glutamate N-methyl-D-aspartate receptors (NMDAr), indicating a close relationship between NO and gluta-

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mate-mediated neurotransmission (Bredt and Snyder, 1994).

nNOS neurons are located in several regions related to defensive behaviors, including the dorsolateral columns of PAG (dlPAG) (Onstott et al., 1993; Vincent and Kimura, 1992). Intra-dlPAG injection of NO donors or glutamate agonists induces flight reactions similar to unconditioned fear responses to proximal danger (Aguiar et al., 2006; Bittencourt et al., 2004; Krieger and Graeff, 1985). Moreover, predator exposure increases NO production (Chiavegatto et al., 1998) and induces Fos expression in NADPH-D-positive neurons (a marker of nitric oxide synthase [NOS] presence) in this region, an effect prevented by intracerebroventricular (i.c.v.) injection of a glutamate NMDAr antagonist (Beijamini and Guimaraes, 2006b). These results suggest that NO and glutamate play an important role in aversive behaviors that involve the dlPAG. Corroborating this proposal, local injection of NOS inhibitor and glutamate antagonists induces anxiolytic effects in animal models of anxiety such as the elevated plus maze (Guimaraes et al., 1991, 1994). However, the effects of local dlPAG blockade of NMDA (N-methyl-D-aspartic acid)- or NO-mediated neurotransmission on the behavior responses and cellular activation induced by predator exposure has not yet been tested. The aim of the present study, therefore, was to test the hypothesis that the injection of N-propyl-L-arginine, a selective nNOS inhibitor, or AP7, a glutamate NMDAr antagonist, into the dlPAG would attenuate defensive responses induced by exposure to a live cat and modify c-Fos expression and activation of nNOS containing neurons in regions related to defensive behavior.

MATERIALS AND METHODS

Subjects

Subjects were adult male Wistar rats (220–240 g) obtained from the colony of pathogen-free rats maintained by the Pharmacy School of Ribeirão Preto, University of Sao Paulo. The animals were housed in groups of four with free access to food and water in a temperature-controlled room (24°C) with a 12 hr light/dark cycle. An adult male cat (3 kg), kept at the animal farm of our university campus with free access to food and water, was used throughout the study. A white dummy cat, approximately the same size as the live cat, was used as control. The experiments were carried out according to the Brazilian Society of Neuroscience and Behavior guidelines for care and use of laboratory animals, and all efforts were made to minimize animal suffering. The experiment protocol was approved by the local ethical committee (062-2004).

Drugs

2-Amino-7-phosphonoheptanoic acid (AP7; 2 nmol/0.2 μl; Tocris, Ellisville, MO), a glutamate NMDA-receptor antagonist, and N-propyl-L-arginine (NP; 1, 10 and 100 nmol/0.2 μl; Tocris), a selective nNOS inhibitor, were used. The drugs were dissolved in sterile isotonic saline. The dose of AP7 was chosen on the basis of a previous studies showing anxiolytic-like effects in the elevated plus maze and Vogel tests (Molchanov and Guimaraes, 2002; Resstel et al., 2008). Although several reports have investigated the effects of NOS inhibitors in the dlPAG (Calixto et al., 2008; Guimaraes et al., 1994; Wang et al., 2001), neither used a selective nNOS inhibitor such as NP. To find the best dose to be used in the subsequent experiment, we initially tested several doses of NP (experiment 1). These doses were based on previous results obtained after intramedial prefrontal cortex administration (Resstel et al., 2008).

Apparatus

The observation box consisted of a rectangular arena (80 × 22 × 50 cm) with Plexiglas walls and a metal grid floor. The apparatus was located in a sound-attenuated 40-lx illuminated room. It was divided into two compartments by a metal grid wall. In the experimental session, each rat was placed in the middle of the compartment opposite to the one containing the live or dummy cat, facing this latter compartment. The box was designed to comfortably contain the cat and to provide enough space for measuring the rat presence proximal or distal to the cat compartment. The rat compartment was divided into two equal parts (near and distant to the cat compartment) by an imaginary line. The rationale is that when the live cat was not present, the rats have no preference for any part of the box, whereas they escape to the cat-distant part and tend to remain there when the predator is present (Moreira and Guimaraes, 2008).

Surgery and Intra-dlPAG Injection

The rats were anesthetized with 2.5% 2,2,2-tribromoethanol (Sigma-Aldrich, St. Louis, MO, 10 ml/kg, i.p.), placed in a stereotaxic frame (David Kopf), and unilaterally implanted with a stainless steel guide cannula (11 mm, length) aimed at the dlPAG (coordinates: AP = 0 from lambda, L = 1.9 mm, D = 4.0 mm below the surface of the skull) (Paxinos and Watson, 1997). The cannula was attached to the bones with stainless steel screws and acrylic cement. An obturator inside the guide cannula prevented obstruction.

Seven days after surgery, intra-dlPAG injections were performed with a thin dental needle (0.3 mm, o.d.) introduced through the guide cannula until its tip was 1 mm below the cannula end. A volume of 0.2 μl was injected during 30 sec using an infusion pump (KD Scientific, Holliston, MA). The movement of an air bubble inside the PE 10 polyethylene tubing connecting the pump to the dental needle confirmed drug flow. The drugs were injected 10 min before the exposure to the observation box.

Behavioral Procedure

Experiment 1. This first behavioral experiment was performed to choose the best dose of NP to be used in experiment 2. The animals were divided into two experimental groups: exposed to the dummy or to the live cat. During three consecutive days, the animals were handled daily by the experimenter for 5 min and habituated to the observation box for 10 min. On the fourth day, the animals received intra-

dlPAG injections of the vehicle or NP (1, 10, or 100 nmol/0.2 μl), and 10 min later, they were placed into the observation box. They were exposed to the dummy or to the live cat for 10 min. After each trial, the observation box was carefully cleaned with an alcohol solution. To prevent cat smell interference, exposure to the live cat always followed that of the dummy cat.

Experiment 2. In this experiment, in addition to the behavioral test, the animals were also used for immunohistochemistry analysis. The single dose of NP (100 nmol) was based on the results of experiment 1. The procedure was similar to this latter experiment except that one additional group (naive group) was included. Thus, the animals were divided into five experimental groups: 1) naive, 2) saline + exposure to the dummy cat, 3) NP + exposure to the dummy cat, 4) saline + exposure to the live cat, and 5) NP + exposure to the live cat. The animals from the naive group remained undisturbed in their home cages until the perfusion for the immunohistochemistry assay.

Experiment 3. In this experiment the effects of intra-dlPAG injection of the NMDAr antagonist AP7 were verified. The procedure was similar to experiment 2, and the experimental groups were as follows: 1) naive, 2) saline + exposure the dummy cat, 3) AP7 + exposure the dummy cat, 4) saline + exposure the cat, and 5) AP7 + exposure the live cat.

All sessions were videotaped and later analyzed by Ethovision software, version 1.9 (Noldus, Wageningen, The Netherlands). The program detected the animal position in the observation box and calculated the time spent and the distance moved in the area near to the cat compartment. The immobility time (freezing) was considered as the cessation of the movements except those associated with breathing and was analyzed manually by an observer who was blinded to the animal's treatment.

Histology

After the behavioral tests, rats from experiment 1 were killed under deep urethane (Sigma-Aldrich, 5 ml/kg, i.p.) anesthesia and perfused through the left ventricle of the heart with isotonic saline followed by 10% formalin solution. A dental needle was then inserted through the guide cannula, and 0.2 μl of fast green was injected. The brains were removed, and after a minimum period of 3 days, they were immersed in a 10% formalin solution; 50-μm sections were obtained with a cryostat (Cryocut 1800). The injection sites were identified in diagrams from the atlas of Paxinos and Watson (1997). The injection sites can be seen in Figure 1. Rats that received injections outside the targeted area were excluded from analysis.

c-Fos and nNOS Double Immunohistochemistry

Two hours after exposure to the observation box, the animals from experiments 2 and 3 were anesthetized with an overdose of urethane and perfused transcardially with saline followed by 4% paraformaldehyde in 0.05 M Tris-phosphate buffer (TBS, pH 7.4). Brains were removed and postfixed over 2 hr in paraformaldehyde and stored for at least 30 hr in 30% sucrose for cryoprotection. Coronal sections (40 μm) were obtained in duplicate in a cryostat. The sections were first processed for c-Fos immunohistochemistry as previously described (Beijamini and Guimaraes, 2006a,b; de Oliveira et al., 2000). Briefly, tissue sections were washed and incubated overnight at room temperature with rabbit IgG (1/1,000 into TBS, sc 52, Santa Cruz Biotechnology Inc., Santa Cruz, CA), which was raised against an amino acid sequence of the N-terminal region of the peptide and specifically recognizes c-Fos. After incubation in the primary antiserum, the tissue sections were washed in TBS and sequentially incubated with a biotinylated goat anti-rabbit IgG (1:1,000 into TBS). Sections were then processed by the avidin–biotin immunoperoxidase method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). c-Fos immunoreactivity (FLI) was revealed by the addition of the chromogen diaminobenzidine (Sigma-Aldrich) into TBS, H_2O_2 0.02%, and nickel ammonium sulfate 1%) and visualized as a black reaction product inside the neuronal nuclei. After the c-Fos immunohistochemistry assay, the same tissue sections were processed for nNOS immunohistochemistry. The sections were incubated as mentioned before, except that the primary antiserum was rabbit IgG (1/1,000 into TBS, Santa Cruz Biotechnology) that was raised against C-terminus of nNOS. nNOS immunoreactivity (nNOSIR) was revealed by the addition of the chromogen diaminobenzidine (Sigma-Aldrich; into TBS, H_2O_2 0.02%) and visualized as a brown reaction product inside the neuronal cytoplasm.

Analysis

Because the variances between groups were not homogenous, the behavioral data were analyzed by the nonparametric Kruskal-Wallis test followed by the Mann-Whitney *U*-test. The significance level was set at $P < 0.05$. Only animals with cannula located inside the dlPAG were considered in the analysis.

The identification method of double-stained cells was similar to that described in previous works (Beijamini and Guimaraes, 2006a; De Oliveira et al., 2001). The number of FLI, nNOSIR, and doubled-stained cells were manually counted with the help of a computerized image analysis system (Image Pro-Plus 4.0, Media Cybernetics) to capture the images. An observer blind to group assignment performed the analysis. For each group, one section from each animal was evaluated. All stained cells in the whole area of each brain region of interested were recorded. The areas of the analyzed regions were calculated and the results expressed as the number of positive cells/0.1 mm^2. Double-stained cells were represented as percentage of nNOSIR cells. Neuroanatomical sites were identified with the help of the atlas of Paxinos and Watson (1997). The anteroposterior (AP) localization from bregma of the analyzed regions were as follows: cingulated cortex 1 (AP: 2.7 mm), infralimbic cortex (IL, AP: 2.7 mm), prelimbic cortex (PrL, AP: 2.7 mm), medial amygdaloid nucleus (MeA, AP: −2.80 mm), lateral magnocellular part (PVNm, AP: −1.80 mm), and ventral part (PVNp, AP: −1.80 mm) of the paraventricular hypothalamic nucleus, dorsal premammillary nucleus of hypothalamus (PMd, AP: −4.16 mm), and dorsolateral periaqueductal gray (dlPAG, AP: −7.04 mm). The immunohistochemistry data were analyzed by the

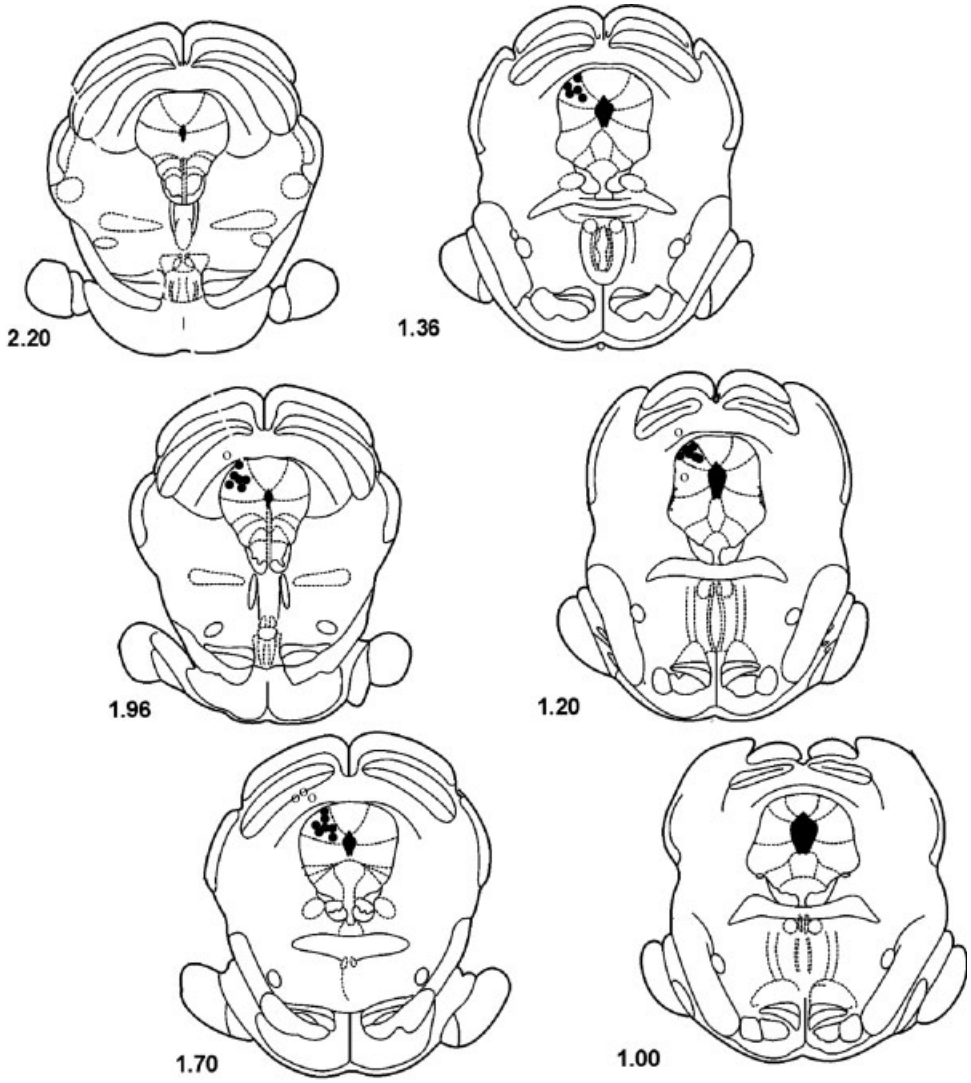

Fig. 1. Histological localization of injection sites from experiment 1 in diagrams based on the atlas of Paxinos and Watson (1997). Solid and open circles represent the injection sites inside and outside the dlPAG, respectively.

nonparametric Kruskal-Wallis test followed by the Mann-Whitney *U*-test. The significance level was set at $P < 0.05$.

In addition, to investigate whether cat closeness was associated with neural activation in the PMd, a Pearson's analysis was performed with the rats exposed to the cat correlating the density of FLI cells and double-stained cells with the time spent near the cat.

RESULTS

Experiment 1

Exposure to a live cat induced a significant fear reaction reflected by a reduction in the distance moved in the compartment near the cat ($H_4 = 11.11$, Kruskal-Wallis $P = 0.0253$; Fig. 2A) and by an increase in freezing behavior ($H_4 = 13.03$, Kruskal-Wallis $P = 0.011$; Fig. 2C) compared with animals exposed to the dummy cat. The highest dose of NP (100 nmol/0.2 μl) attenuated the behavioral consequences of predator exposure. It increased the distance moved in the compartment near the cat (Mann-Whitney $P < 0.05$ compared with saline/cat group Fig. 2A) and reduced freezing behavior (Mann-Whitney $P < 0.05$ compared with saline/cat group; Fig. 2C). Although the smallest dose of NP (1 nmol/0.2 μl) also reduced freezing behavior (Mann-Whitney $P < 0.05$ compared with saline/cat group; Fig. 2C), it did not prevent the decrease in the distance moved in the compartment near the cat. For this reason, we chose a dose of 100 nmol for use in experiment 2.

Experiment 2

As in the previous experiment, the presence of a live cat induced a significant fear reaction in the rats, reflected by a reduction in the distance moved in the compartment near the cat ($H_3 = 12.33$, Kruskal-Wallis $P = 0.006$; Fig. 3A) and in the time spent in this compartment ($H_3 = 10.32$, Kruskal-Wallis $P = 0.016$; Fig. 3B) compared with animals exposed to dummy cat.

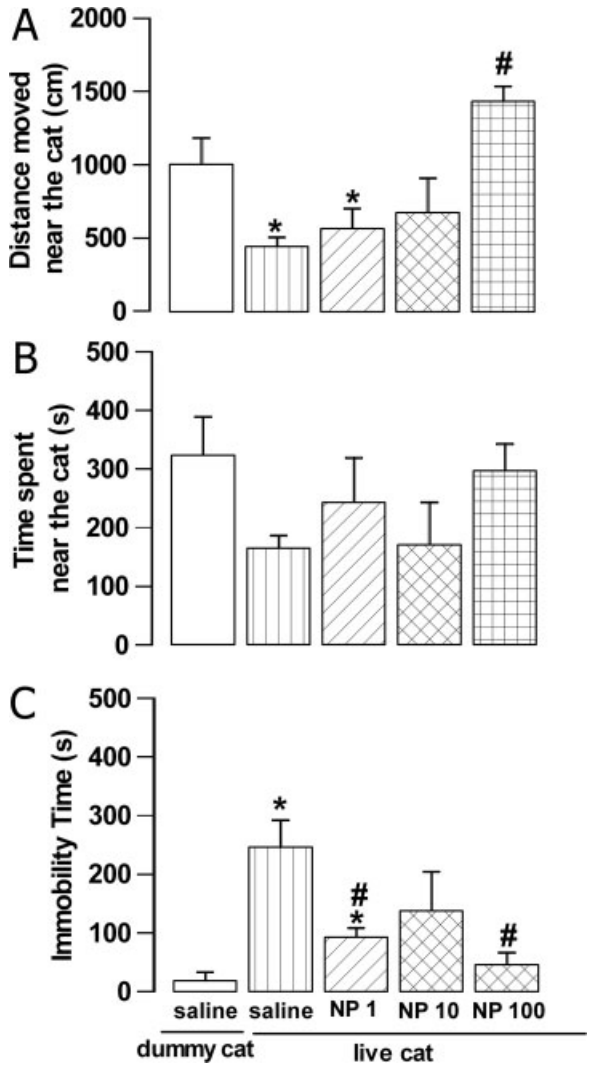

Fig. 2. Effects of NP (1, 10, or 100 nmol/0.2 μl) injected into the dlPAG in the distance moved (cm, **A**) and time spent (sec, **B**) in the compartment near the cat and in immobility time (sec, **C**) after exposure to a live cat for 10 min. Data represented the means ± SEM of 3–5 animals per group (⋆$P < 0.05$ compared with dummy cat and #$P < 0.05$ compared with live cat, Kruskal-Wallis followed by Mann-Whitney *U*-test).

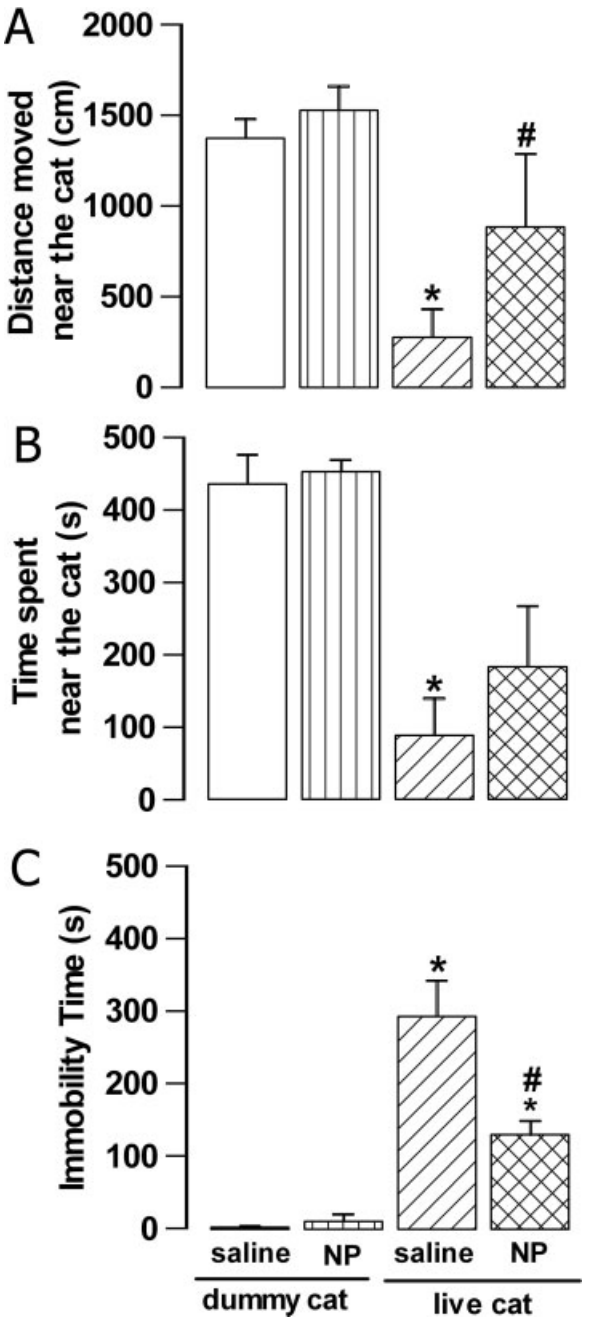

Fig. 3. NP (100 nmol/0.2 μl) injected into the dlPAG increased the distance moved (cm, **A**) and time spent (sec, **B**) in the compartment near the cat, and immobility time (sec, **C**) after exposure to a live cat. Data represented the means ± SEM of 4–6 animals per group (⋆$P < 0.05$ compared with dummy cat and #$P < 0.05$ compared with live cat, Kruskal-Wallis followed by Mann-Whitney *U*-test).

These animals also displayed increased freezing behavior compared with those exposed to the dummy cat (H_3 = 16.451, Kruskal-Wallis $P = 0.0009$; Fig. 3C). NP (100 nmol/0.2 μl) pretreatment in the dlPAG significantly reduced freezing behavior induced by predator exposure compared with saline/cat group (Mann-Whitney $P < 0.05$) and attenuated the reduction in distance moved by the animals in the compartment near the cat (Fig. 3A,C).

The immunohistochemistry results are provided in Table I and Figure 4. Exposure to the live cat induced a significant increase in FLI cells compared with both naive and saline/dummy cat groups in the ipsilateral (H_4 = 15.43, Kruskal-Wallis $P = 0.0039$) and contralateral (H_4 = 10.32, Kruskal-Wallis $P = 0.0354$) sides of the dlPAG and in the PMd (H_4 = 9.60, Kruskal-Wallis $P = 0.048$). The difference against the saline/dummy group

TABLE I. Effects of Intra-dlPAG Injection of NP (100 nmol/0.2 µl) in the Number of c-FOS-positive Cells and nNOS-positive Cells, and Percentage of Double-stained Cells in Brain Regions of Rats Exposed to a Toy or a Live Cat[†]

Structure	Immunohistochemistry	Naive	Saline/dummy	NP/dummy	Saline/cat	NP/cat
Cg1	FOS	4.07 ± 2.86	9.13 ± 3.13	2.34 ± 1.91	11.72 ± 4.06	3.24 ± 1.54
	nNOS	8.67 ± 1.56	14.47 ± 3.96	8.08 ± 0.97	16.00 ± 5.24	8.57 ± 1.25
	% Double stained	0.5 ± 0.5	1.69 ± 1.0	0	0.81 ± 0.8	0
IL	FOS	4.65 ± 3.43	5.49 ± 3.10	4.10 ± 0.54	2.34 ± 1.94	0.65 ± 3.43
	nNOS	7.63 ± 1.8	10.6 ± 3.6	9.38 ± 2.24	5.90 ± 1.7	5.12 ± 1.1
	% Double stained	1.05 ± 1.1	2.46 ± 2.5	1.15 ± 1.2	0	0
PrL	FOS	5.56 ± 5.54	6.39 ± 2.55	6.47 ± 4.94	5.01 ± 2.86	0.70 ± 0.46
	nNOS	7.83 ± 3.3	12.3 ± 3.4	15.5 ± 4.7	7.03 ± 1.9	5.55 ± 1.1
	% Double stained	0.32 ± 0.32	0	0.83 ± 0.83	2.40 ± 1.6	0
PVNm	FOS	0	6.81 ± 4.78★	1.95 ± 1.12★	7.14 ± 2.82★	2.59 ± 1.42★
	nNOS	89.33 ± 5.57	102.52 ± 1.18	75.92 ± 29.83	115.07 ± 9.13	78.95 ± 16.78
	% Double stained	0.37 ± 0.24	0.64 ± 0.44	1.19 ± 0.73	1.44 ± 0.98	2.32 ± 1.70
PVNp	FOS	5.62 ± 2.31	9.08 ± 5.21	10.38 ± 4.56	15.57 ± 7.99	14.53 ± 7.72
	nNOS	199.86 ± 15.74	260.2 ± 23.83	162.21 ± 8.85	227.1 ± 29.03	214.65 ± 20.09
	% Double stained	0.37 ± 0.24	0.64 ± 0.44	1.19 ± 0.73	1.44 ± 0.98	2.32 ± 1.70
MeA	FOS	5.84 ± 4.88	2.27 ± 2.27	0.43 ± 0.43	22.06 ± 17.76	12.71 ± 9.46
	Double staineda	1.29 ± 1.1	0.65 ± 0.65	0	3.6 ± 2.26	4.11 ± 2.61
PMd	FOS	1.29 ± 1.29	12.95 ± 6.52	16.44 ± 9.75	73.54 ± 17.32★,+	126.74 ± 60.52★
	nNOS	297.83 ± 25.71	341.74 ± 29.30	360.78 ± 12.52	354.73 ± 12.41	319.23 ± 36.56
	% Double stained	0.1 ± 0.1	1.18 ± 0.64	1.23 ± 0.62	6.35 ± 1.54★,+	7.38 ± 3.26★
dlPAG contralateral	FOS	0.78 ± 0.52	12.03 ± 3.73★	8.13 ± 4.7	20.03 ± 5.77★,+	12.14 ± 2.99★
	nNOS	161.04 ± 9.11	179.46 ± 18.73	140.45 ± 6.65	166.98 ± 13.94	164.07 ± 12.05
	% Double stained	0	1.07 ± 0.44★	0.99 ± 0.69	2.54 ± 0.48★	0.24 ± 0.74#
dlPAG ipsilateral	FOS	1.08 ± 0.39	5.2 ± 1.68★	8.45 ± 5.11★	35.89 ± 22.96★,+	21.89 ± 10.04★
	nNOS	168.63 ± 10.8	159.31 ± 18.06	172.31 ± 15.9	152.67 ± 10.03	166.02 ± 9.82
	% Double stained	0	0	0.81 ± 0.81	0.72 ± 0.53	1.36 ± 0.74

[†]Data are represented as means ± SEM from 4–6 animals/group. Animals were killed 2 hr after exposure to a toy or live cat. Naive animals remained undisturbed in their home cages. Gg1, cingulated cortex 1; IL, infralimbic cortex; PrL, prelimbic cortex; PVNm, lateral magnocellular part of paraventricular hypothalamic nucleus; PVNv, ventral part of paraventricular hypothalamic nucleus; MeA, medial amygdala; PMd, dorsal premammillary nucleus of hypothalamus; dlPAG, dorsolateral periaqueductal gray. Double-stained cells are expressed as percentage of nNOS-positive cells except in the MeA (a), where the results show the absolute number of double-stained cells.

★$P < 0.05$ vs. naive.

+$P < 0.05$ vs. saline/dummy cat.

#$P < 0.05$ vs. saline/cat (Kruskal-Wallis followed by Mann-Whitney test).

was not present in the NP/cat group, suggesting that in the dlPAG the drug attenuated FLI (Table I). In the PMd, on the other hand, NP treatment failed to change FLI compared with the saline/cat group (Table I). FLI also increased in the PVNm of all groups compared with the naive group ($H_4 = 12.20$, Kruskal-Wallis $P = 0.016$). No change in FLI was found in the PVNp, MeA, PrL, IL, and Cg1. Additionally, there was no difference in the number of nNOS-positive cells in any of the analyzed structures.

Predator exposure induced a significant increase in the percentage of double-stained cells in the PMd ($H_4 = 9.60$, $P = 0.048$) compared with naive and saline/dummy animals, respectively (Table I). A similar effect was found in the contralateral side of the dlPAG ($H_4 = 12.43$, $P = 0.0145$) compared with naive animals. In this case, however, the saline/dummy group was also different from the naive group (Table I). NP treatment decreased the percentage of double-stained cells in the contralateral dlPAG in cat exposed animals compared with the saline/cat group (Table I). No differences for double-stained cells were found in the PrL, IL, Cg1, PVNp, and PVNm as well as in the ipsilateral dlPAG. As a result of the intense packing of nNOS cells and fibers, processes in the MeA quantification of these cells were not possible. As a consequence, the number of double-stained cells in this area was represented as an absolute number. No difference was found in this region.

Experiment 3

Rats exposed to the live cat exhibited a significant fear reaction reflected by increased freezing behavior ($H_3 = 27.37$, Kruskal-Wallis $P = 0.0001$; Fig. 5C) and a reduction in the distance moved and time spent in the compartment near the cat ($H_3 = 27.31$, Kruskal-Wallis $P = 0.0001$; Fig. 5A, $H_3 = 17.64$, Kruskal-Wallis $P = 0.0005$; Fig. 5B, respectively) compared with animals exposed to dummy cat. AP7 pretreatment in the dlPAG decreased the behavioral consequences of predator exposure (Fig. 5).

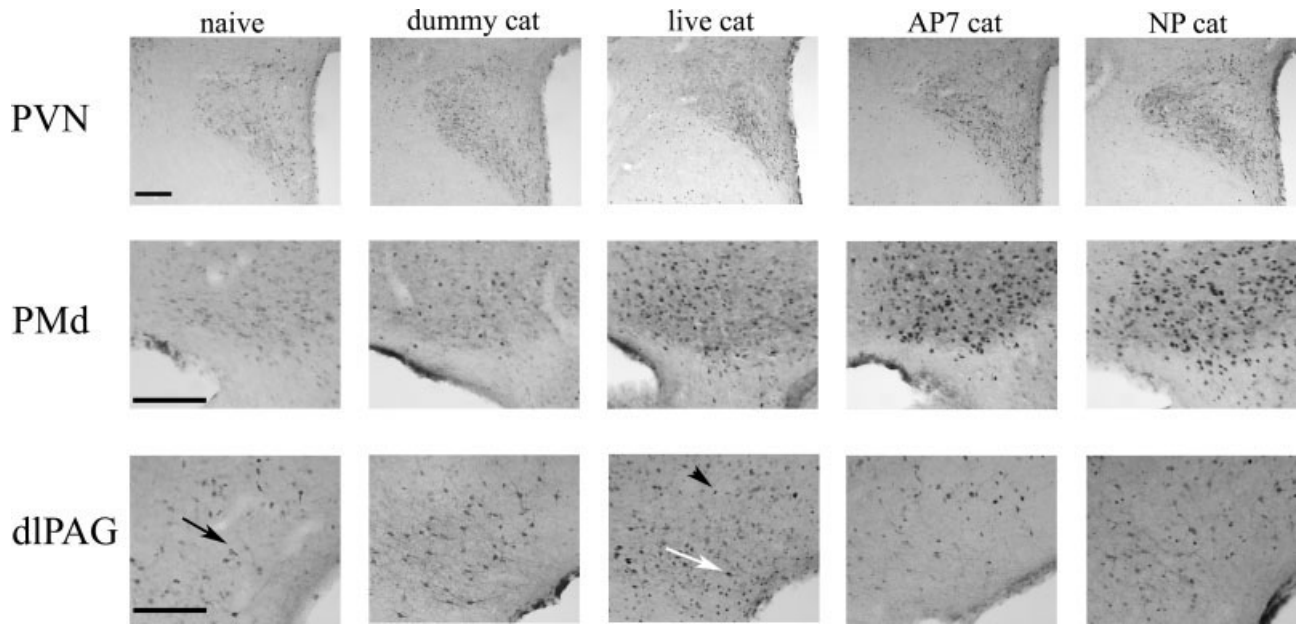

Fig. 4. Photomicrographs showing c-Fos immunohistochemistry (arrowhead), nNOS immunohistochemistry (black arrow), and double-stained neurons (white arrow) in the paraventricular nucleus of hypothalamus (PVN), PMd, and dlPAG of naive rats (**A**), rats exposed to a dummy cat (**B**), rats exposed to a live cat (**C**), rats treated with intra-dlPAG AP7 exposed to a live cat (**D**), and rats treated with intra-dlPAG NP exposed to a live cat (**E**). Scale bar = 150 μm.

The immunohistochemistry results can be seen in Table II and Figure 4. Exposure to the live cat induced a significant increase in FLI cells compared with naive and saline/dummy cat groups in the ipsilateral (H_4 = 21.46, Kruskal-Wallis P = 0.0003) and contralateral (H_4 = 9.53, Kruskal-Wallis P = 0.049) side of the dlPAG and in the PVNp (H_4 = 9.19, Kruskal-Wallis P = 0.056). The increase was also significant compared with naive group in the PMd (H_4 = 11.87, P = 0.018) (Table II). AP7 pretreatment attenuated FLI increase in the dlPAG and PVNp. The drug, however, induced a significant increase in FLI in the PMd of cat-exposed animals compared with saline/dummy cat and naive groups. AP7/dummy cat animals were also different from animals in the naive group, suggesting that the drug, by itself, also increased FLI in the PMd (Table II). There were no differences for FLI cells in the MeA, PVNm, PrL, IL, and Cg1. Additionally, no effect was found for the number of nNOS-positive cells.

In the ipsilateral dlPAG, all experimental groups presented a significant increase in the percentage of double-stained cells compared with the naive group (H_4 = 17.14, Kruskal-Wallis P = 0.0018). There were no differences for double-stained cells in the PrL, IL, Cg1, PVNp, and PVNm and in the contralateral side of the dlPAG. In the PMd, AP7 induced a significant increase in the percentage of double-stained cells (Mann-Whitney $P < 0.05$) compared with naive and saline/cat groups (Table II).

A Pearson's correlation analysis including all the 15 animals that were exposed to the cat and had brain sections containing the PMd showed that the time spent near the cat correlated with both the density of FLI cells (r = 0.7597, P = 0.001; Fig. 6, top) and the percentage of double-stained cells (r = 0.6057, P = 0.017; Fig. 6, bottom), indicating that the nearer the rat gets to the predator, the higher the neural activation of the PMd.

DISCUSSION

Cat exposure produced a fear reaction characterized by freezing behavior and avoidance of the area near the predator compartment. These results are in agreement with previous data reporting reduction in overall locomotor activity after live cat exposure (Beijamini and Guimaraes, 2006b; Blanchard and Blanchard, 1989; Canteras et al., 1997; Moreira and Guimaraes, 2008). In accordance with our hypothesis, microinjection of NP or AP7 into the dlPAG reduced defensive behavior induced by predator exposure. Similar results have been observed after systemic or i.c.v. microinjection of NMDA antagonists (Adamec et al., 1999; Beijamini and Guimaraes, 2006b; Blanchard et al., 1992). The involvement of NMDAr in the defensive behavior modulated by the dlPAG was also observed in guinea pigs (Ramos Coutinho et al., 2008). Although there were no reports showing the effects of NOS inhibitors in this model, several studies have detected anxiolytic-like effects of

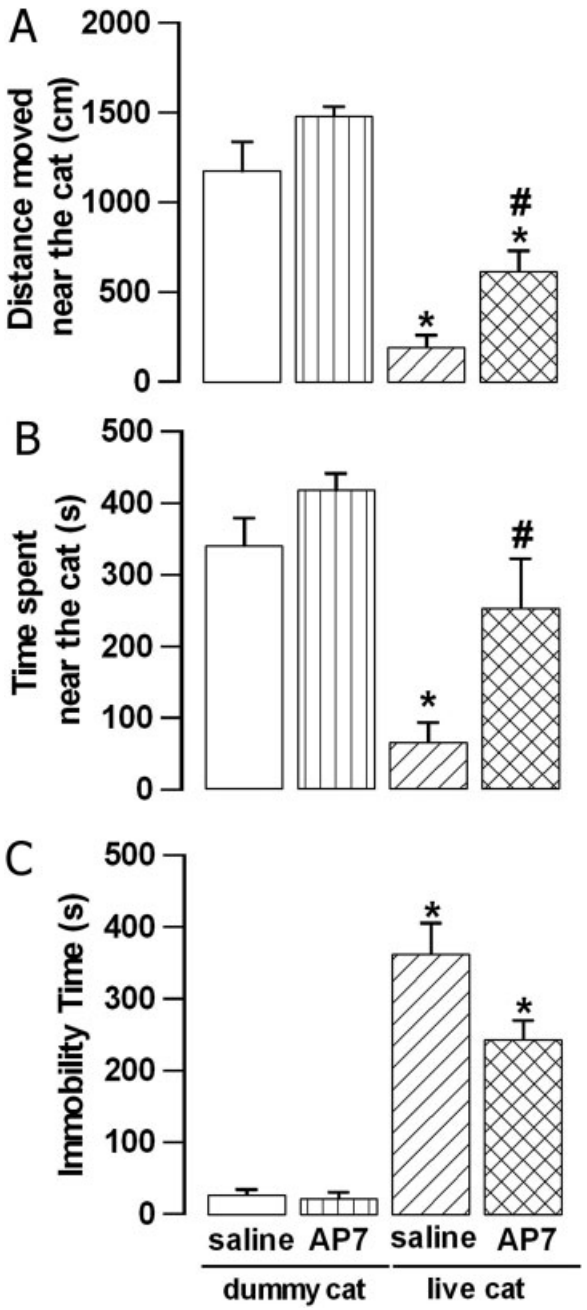

Fig. 5. AP7 (2 nmol/0.2 μl) injected into the dlPAG increased the distance moved (cm, **A**) and time spent (sec, **B**) in the compartment near the cat, and immobility time (sec, **C**) after exposure to a live cat. Data are represented by mean ± SEM of 9–10 animals per group ($^{*}P < 0.05$ compared with dummy cat and $\#P < 0.05$ compared with live cat, Kruskal-Wallis followed by Mann-Whitney *U*-test).

these drugs after systemic or intra-dlPAG administration (for a review, see Guimaraes et al., 2005).

In the present study, cat exposure induced an increase in c-Fos expression in the dlPAG, the PMd, and, in experiment 3, the PVNp. These effects are in agreement with other studies showing that threatening stimuli such as live predator or its odor result in an increase in the number of c-Fos-positive cells in the PAG and hypothalamic nuclei (Beijamini and Guimaraes, 2006b; Blanchard et al., 2005; Canteras et al., 1997; Canteras and Goto, 1999; Dielenberg et al., 2001). These structures, together with the medial amygdala, comprise the circuitry responsible for integrating innate fear responses (Canteras and Blanchard, 2008). In line with this view, the dlPAG is activated by different stressful stimuli, including immobilization (Cullinan et al., 1995), exposure to a novel open field (Nagahara and Handa, 1997), foot shocks (Campeau et al., 1997b), and audiogenic stress (Campeau et al., 1997a).

Cat exposure did not induce a significant increase in the percentage of double-stained cells in the dlPAG. Although this result suggests that NO in this region is not essential for behavioral responses to predators, as a diatomic gas, this neurotransmitter is a freely diffusible and can readily enter adjacent neuronal cells on a sphere of approximately 100 μm (Schuman et al., 1994). Thus, even released from a small population of NOS neurons, it can influence a large proportion of neurons in the dlPAG, where it could modulate the release of several neurotransmitters, including glutamate (Guimaraes et al., 2005). Our results, by showing that either blockade of NOS synthesis or NMDA receptor antagonism in the dlPAG is able to reduce defensive behavior responses induced by cat exposure, suggest that the NMDA/NO pathway is indeed important for defensive responses in this brain region. The reduction in cellular activation of this region induced by AP7 and the similar trend observed with NP agree with this suggestion.

Cat exposure also induced a significant increase in FLI in the PMd. A trend in the same direction was found in the percentage of double-stained cells (which reached significance only in experiment 3). The PMd is a component of the medial hypothalamic defense zone proposed to be essential for responses to a predatory threat (Canteras, 2003; Canteras and Blanchard, 2008; Canteras et al., 2001, 2008). In line with this view, this hypothalamic site is activated after predator cues (Beijamini and Guimaraes, 2006b; Canteras et al., 2001; Dielenberg et al., 2001). Interestingly, in the present study, animals that received AP7 into the dlPAG and were exposed to the live cat showed greater c-Fos expression and a moderate increase in the number of activated nitrergic neurons in the PMd. These results contrast with those previously obtained by our group, which showed that i.c.v. administration of AP7 attenuated the cellular consequences of predator exposure in the PMd (Beijamini and Guimaraes, 2006b). The different administration routes could be responsible for this difference because after i.c.v. injection, the drug was able to act directly in several brain areas related to defensive responses including the PMd, whereas in the present study, its action was restricted to the dlPAG. This localized blocked of NMDAr-mediated neurotransmission attenuated the defensive responses and as a consequence increased the time the animal remained near the cat.

TABLE II. Effects of Intra-dlPAG Injection of AP7 (2 nmol/0.2 μl) in the Number of c-Fos-positive Cells and nNOS-positive Cells, and Percentage of Double-stained Cells in Brain Regions of Rats Exposed to a Toy or a Live Cat[†]

Structure	Immunohistochemistry	Naive	Saline/dummy	AP7/dummy	Saline/cat	AP7/cat
Cg1	FOS	0.16 ± 0.11	7.56 ± 6.15	10.54 ± 6.64	1.23 ± 0.31	6.95 ± 6.38
	nNOS	4.42 ± 1.06	13.65 ± 6.88	16.62 ± 3.06	10.35 ± 2.53	10.24 ± 3.02
	% Double stained	0	0	0	0	0
IL	FOS	1.24 ± 0.84	2.65 ± 1.70	9.18 ± 4.68	1.23 ± 0.71	3.88 ± 3.05
	nNOS	5.50 ± 1.17	8.08 ± 2.70	13.65 ± 6.88	7.11 ± 1.82	6.05 ± 1.70
	% Double stained	0	0	0	0	0
PrL	FOS	0.16 ± 0.16	10.54 ± 6.65	1.23 ± 0.32	6.95 ± 6.39	0.16 ± 0.12
	nNOS	4.42 ± 1.06	13.65 ± 6.88	16.62 ± 3.06	10.35 ± 2.53	10.24 ± 3.02
	% Double stained	0	0	0	0	0
PVNm	FOS	0.26 ± 0.26	1.14 ± 0.97	6.65 ± 3.73	7.5 ± 2.48	5.07 ± 2.62
	nNOS	87.91 ± 11.78	120.03 ± 4.88	118.62 ± 12.11	118.52 ± 14.19	129.3 ± 6.74
	% Double stained	0	0.29 ± 0.19	0.98 ± 0.51	1.58 ± 0.77	0.8 ± 0.8
PVNp	FOS	3.14 ± 2.82	9.44 ± 2.82	13.58 ± 7.30	34.68 ± 10.62★,+	22.04 ± 8.48★
	nNOS	193.09 ± 24.31	209.17 ± 16.08	193.08 ± 10.56	207.71 ± 12.17	198.31 ± 10.56
	% Double stained	0.58 ± 0.58	1.09 ± 0.49	1.11 ± 0.55	4.32 ± 1.5	2.54 ± 0.87
MeA	FOS	1.56 ± 0.76	7.95 ± 3.77	11.44 ± 5.79	46.50 ± 28.22	19.88 ± 15.72
	Double stained[a]	4.12 ± 3.13	4.48 ± 2.06	4.16 ± 2.11	10.56 ± 7.2	3.15 ± 1.98
PMd	FOS	1.3 ± 0.92	14.31 ± 9.7	53.65 ± 21.09★	48.4 ± 29.28★	141.92 ± 55.71★,+
	nNOS	355 ± 52.34	317 ± 19.99	335.37 ± 11.25	304.47 ± 48.75	358.33 ± 9.08
	% Double stained	0.22 ± 0.13	1.51 ± 0.91	4.5 ± 1.36	2.69 ± 1.16	7.41 ± 2.19★,+
dlPAG contralateral	FOS	5.69 ± 4.43	9.83 ± 4.91	9.1 ± 3.37	27.23 ± 7.03★,+	14.7 ± 5.99
	nNOS	144.83 ± 10.36	145.43 ± 10.37	152.01 ± 11.07	148.21 ± 11.32	152.43 ± 9.61
	% Double stained	0.28 ± 0.28	0.7 ± 0.33	0.59 ± 0.3	1.81 ± 0.41	1.13 ± 0.58
dlPAG ipsilateral	FOS	3.58 ± 1.85	42.25 ± 16.97★	43.90 ± 35.30★	72.41 ± 10.53★,+	24.34 ± 6.81★,#
	nNOS	149.55 ± 14.80	167.66 ± 14.66	155.03 ± 11.70	150.39 ± 11.79	147.55 ± 14.80
	% Double stained	0	3.23 ± 1.28★	2.30 ± 0.76★	6.7 ± 2.53★	4.6 ± 2.11★

[†]Data are represented as means ± SEM from 8–9 animals/group. Animals were killed 2 hr after exposure to a toy or a live cat. Naive animals remained undisturbed in their home cages. Gg1, cingulated cortex 1; IL, infralimbic cortex; PrL, prelimbic cortex; PVNm, lateral magnocellular part of paraventricular hypothalamic nucleus; PVNv, ventral part of paraventricular hypothalamic nucleus; MeA, medial amygdala; PMd, dorsal premammillary nucleus of hypothalamus; dlPAG, dorsolateral periaqueductal gray. Double-stained cells are expressed as a percentage of nNOS-positive cells.
[a]Absolute number of double-stained cells.
★$P < 0.05$ vs. naive.
+$P < 0.05$ vs. saline/dummy cat.
#$P < 0.05$ vs. saline/cat (Kruskal-Wallis followed by Mann-Whitney test).

Because the PMd has been proposed as an amplifier of defensive responses to predators (Canteras and Blanchard, 2008; Canteras et al., 2008), this latter effect could help to explain the apparent paradox of a higher activation of a brain structure closely related to defensive responses in animals displaying reduced fear signals. This possibility is reinforced by the positive correlation between proximity to the cat and neural activation of the PMd. The positive correlation between the number of double-labeled cells and time near the cat also suggests that NO-mediated neurotransmission could modulate defensive responses to predators in this nucleus.

Exposure to the cat also induced activation of the PVNp in experiment 3, a complex structure responsible for maintenance of individual homeostasis that integrates behavioral, autonomic, and endocrine responses to stress (Benarroch, 2005; Harbuz and Lightman, 1992; Sawchenko and Swanson, 1983). Rodents exposed to a live cat or to its odor displayed increases in plasmatic levels of corticosterone and increases in corticotrophin-releasing hormone mRNA in the paraventricular nucleus of hypothalamus (PVN) (Morrow et al., 2000; Perrot-Sinal et al., 1999). Despite contradictory results, most studies suggest that NO modulates the activation of the hypothalamic–pituitary–adrenal axis by stressors. Moreover, restraint stress induces an increase in NOS expression (De Oliveira et al., 2001) and activates NOS neurons in the PVN (Amir et al., 1997). This activation was blocked by pretreatment with a NOS inhibitor, 7-nitroindazole (Amir et al., 1997). Probably reflecting a decreased stress response, in our work, the cellular activation of the PVN induced by cat exposure was attenuated by intra-dlPAG administration of AP7.

Among other structures related to defensive reactions, the medial prefrontal cortex (mPFC) has been also implicated in fear- and anxiety-related processes (Resstel et al., 2006b; Vertes, 2006). The ventral regions of the mPFC (PL/IL) is functionally linked to subcortical structures related to defensive behavior such as the amygdala, hippocampus, hypothalamus, and PAG (Floyd et al., 2000; Gabbott et al., 2005; Price, 2007). In the present study, no cellular effects were detected in the mPFC.

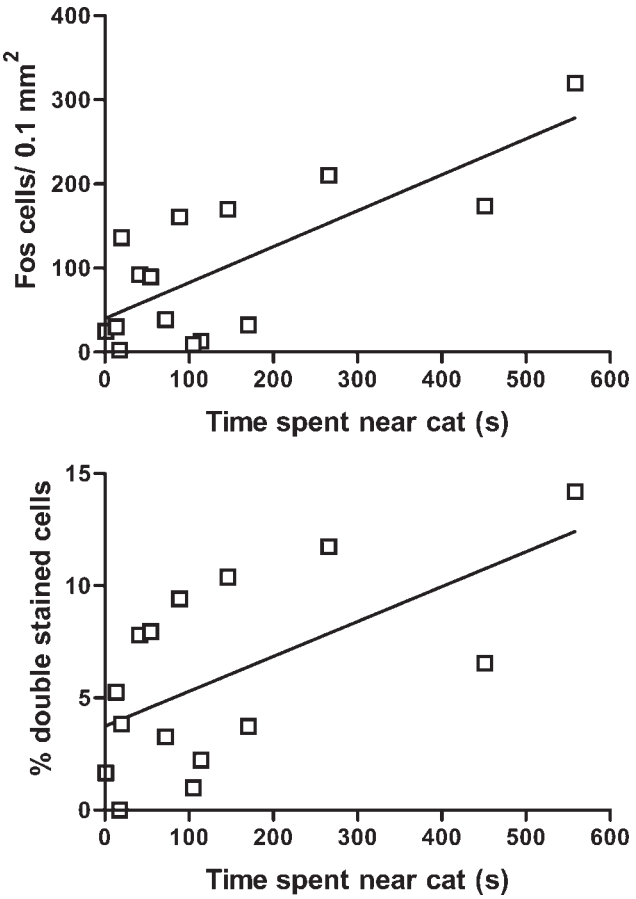

Fig. 6. Correlations between time spent near the live cat and the densities of FLI (top) and percentage of double-stained cells (bottom) in the PMd of rats ($n = 15$) exposed to the cat. The data were analyzed by Pearson's correlation ($r = 0.7597$ and $P = 0.001$ for the FLI cells; $r = 0.6057$ and $P = 0.017$ for the percentage of double-stained cells).

Although several pieces of evidence indicate the involvement of this region in the modulation of defensive responses (Canteras et al., 1997; Dielenberg et al., 2001; Duncan et al., 1996), its precise role is still controversial. For example, studies that used lesions of the mPFC have shown increase, decrease, or no change in anxiety-like responses (Burns et al., 1996; Heidbreder and Groenewegen, 2003; Jinks and McGregor, 1997; Lacroix et al., 1998; Sah et al., 2003; Shah and Treit, 2003; Wall et al., 2004). The mPFC has been proposed to be particularly important for the expression of contextual fear (Corcoran and Quirk, 2007; Resstel et al., 2006a, 2008) but not for the expression of innate fear (Corcoran and Quirk, 2007).

One possibility to explain these findings is that under high-threat situations, where avoidance is impossible, a shift from cortical to subcortical processing during danger occurs. In support of this possibility, several studies have shown that fight or flight behaviors are essentially mediated by phylogenetically old subcortical structures such as the dorsal PAG (Mobbs et al., 2007; Sewards and Sewards, 2002). Corroborating this possibility, Mobbs et al. (2007), by means of a virtual predator model in volunteers, showed that brain activation shifts from the ventromedial prefrontal cortex to the central amygdala and PAG as the predator gets closer to the subject. These results reinforce the proposal that cortical structures such as the mPFC organize defensive behaviors when the threat is remote. Proximity to the threat, on the other hand, would engage the dorsal PAG and inhibit cortical structures (Mobbs et al., 2007). The present results, by showing that the injection of a glutamate antagonist or a NOS inhibitor into the dlPAG attenuates the defensive behaviors induced by predator exposure, are in agreement with this hypothesis.

Several studies have suggested that the MeA is involved in innate fear responses during encounters with a predator (Blanchard et al., 2005; Canteras and Blanchard, 2008; Dielenberg et al., 2001; McGregor et al., 2004; Muller and Fendt, 2006). The posteroventral part of the MeA, which is a component of the vomeronasal pathway, seems to be particularly related to the processing pheromone-like of predator odor (Canteras and Blanchard, 2008; McGregor et al., 2004). In the present study, however, we failed to replicate the increase in MeA activation found after predator exposure. Although we have no explanation for this lack of activation, there was a clear trend in this direction, suggesting that the higher data variability observed in cat-exposed animals could have prevented the differences to become significant. Before the experiment, our animals were handled daily—a procedure that has been described to attenuate *cfos* expression (Asanuma et al., 1992). In addition, another factor that could have increased data variability in the MeA and other regions is that the animals, except for those in the naive group, have been submitted to a mechanical stimulation of the dlPAG by the injection procedure.

In conclusion, our results suggest that glutamate- and NO-mediated neurotransmission in the dlPAG play an important role in defensive responses elicit by predator exposure. Blocking these neurotransmitter systems in this brain area impairs these responses. The longer time spent near the predator that follows this effect could increase cellular activation of the PMd, a more rostral brain area proposed to play a role in the processing of predator cues.

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