EJN
European Journal of Neuroscience

European Journal of Neuroscience, Vol. 28, pp. 1003–1015, 2008 doi:10.1111/j.1460-9568.2008.06392.x

Hypothalamic sites responding to predator threats – the role of the dorsal premammillary nucleus in unconditioned and conditioned antipredatory defensive behavior

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Keywords: amygdala, fear conditioning, hypothalamus, innate fear, periaqueductal gray, rat

Abstract

In this study we provide a comprehensive analysis of the hypothalamic activation pattern during exposure to a live predator or an environment previously associated with a predator. Our results support the view that hypothalamic processing of the actual and the contextual predatory threats share the same circuit, in which the dorsal premammillary nucleus (PMd) plays a pivotal role in amplifying this processing. To further understand the role of the PMd in the circuit organizing antipredatory defensive behaviors, we studied rats with cytotoxic PMd lesions during cat exposure and examined the pattern of behavioral responses as well as how PMd lesions affect the neuronal activation of the systems engaged in predator detection, in contextual memory formation and in defensive behavioral responses. Next, we investigated how pharmacological blockade of the PMd interferes with the conditioned behavioral responses to a context previously associated with a predator, and how this blockade affects the activation pattern of periaqueductal gray (PAG) sites likely to organize the conditioned behavioral responses to the predatory context. Behavioral observations indicate that the PMd interferes with both unconditioned and conditioned antipredatory defensive behavior. Moreover, we have shown that the PMd influences the activation of its major projecting targets, i.e. the ventral part of the anteromedial thalamic nucleus which is likely to influence mnemonic processing, and PAG sites involved in the expression of antipredatory unconditioned and conditioned behavioral responses. Of particular relevance, this work provides evidence to elucidate the basic organization of the neural circuits integrating unconditioned and contextual conditioned responses to predatory threats.

Introduction

The seminal work of Bard (1928) and Hess & Brugger (1943) led to the view that the hypothalamus plays an especially important role in the expression of defensive behavior. In the 1980s, however, this idea was challenged by Bandler (1982), who found that excitatory amino acid (glutamate) injection into the hypothalamus failed to elicit any defensive response, implying that the effects observed with electrical stimulation in the hypothalamus depended upon the activation of fibers whose cell bodies are located elsewhere. This issue was later clarified by studies showing that either subtoxic doses of kainic acid or drugs impairing GABAergic neurotransmission, when delivered into the medial hypothalamus, were able to evoke a pattern of responses resembling the behavior of animals facing natural threats (Di Scala *et al.*, 1984; Schmitt *et al.*, 1985; Brandão *et al.*, 1986; Milani & Graeff, 1987; Silveira & Graeff, 1992).

Rats exposed to a live cat or to its odor up-regulated Fos expression in a distinct medial hypothalamic circuit (Canteras *et al.*, 1997; Dielenberg *et al.*, 2001). Unfortunately, these studies presented a rather fragmentary view of how the different hypothalamic systems respond to predatory threats. In the former study (Canteras *et al.*, 1997), the authors simply presented a qualitative analysis of the hypothalamic pattern of Fos expression in response to cat exposure and, in the in the latter study (Dielenberg *et al.*, 2001), only a relatively small number of hypothalamic sites (ten sites) was analyzed. Nevertheless, these studies support the view that the hypothalamus plays a key role in processing predator-associated cues. In this regard, our first aim in the present study was to provide a comprehensive analysis of the hypothalamic activation pattern during exposure to a live cat and to a predator-associated context. Of particular relevance, we confirmed that the dorsal premammillary nucleus (PMd) is one of the most responsive hypothalamic sites during exposure to the cat and to the predatory context.

Previous studies have shown that lesions centered in the PMd severely reduce the defensive response to both a live cat and its odor (Canteras *et al.*, 1997; Blanchard *et al.*, 2003). Therefore, to have a better understanding of the neural processing of predator-related fear responses, we have further investigated how the PMd interacts with other parts of the system underlying antipredatory behaviors. First, we studied animals with cytotoxic PMd lesions during cat exposure; we examined the pattern of behavioral responses and how PMd lesions affected the neuronal activation of the systems engaged in predator detection, in antipredatory behavioral responses and in contextual memory formation. Next, we also investigated how pharmacological blockade of the PMd interfered with the conditioned behavioral

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Received 11 March 2008, revised 9 June 2008, accepted 2 July 2008

responses to a context previously associated with a predator, and how this blockade affected the activation pattern of neural sites likely to organize the conditioned behavioral responses to the predatory context.

Overall, the present results provide evidence elucidating the basic organization of the neural circuits underlying unconditioned and contextual conditioned responses to predatory threat, and reveal that they share the same neural circuits.

Materials and methods

Animals and housing

Adult male Wistar rats (n = 45), weighing ~250 g and obtained from the local breeding facilities, were used in the present study. The animals were kept under controlled temperature (23 °C) and illumination (12-h cycle) in the animal quarters, and had free access to water and standard laboratory diet. Animals were maintained in accordance with the guidelines of the Committee on Animals of the Colégio Brasileiro de Experimentação Animal (COBEA) and the Committee on Care and Use of Laboratory Animal Resources, National Research Council, USA. All experimental procedures had been previously approved by the Committee on Care and Use of Laboratory Animals of the Institute of Biomedical Sciences, University of São Paulo, Brazil (Protocol number 084/2005).

Experimental apparatus and procedure

The experimental apparatus was made of clear Plexiglas. Each consisted of a 25 × 25 × 25 cm home cage connected to another 25 × 25 × 25 cm chamber (the food compartment) by a hallway 12.5 cm wide and 100 cm long, with 25-cm-high walls. Between the home cage and the hallway there was a sliding door (12.5 cm wide and 26 cm high), which was opened when the animals were allowed to explore the rest of the apparatus. For 9 days each animal was isolated in the home cage and, at the beginning of the dark phase, the animals were allowed to explore the rest of the apparatus and obtain food pellets stored in the food compartment.

The testing procedure consisted of three phases of a 10-min observation period, during the beginning of the dark phase of the light–dark cycle.

Phase 1

After the habituation period, on the 10th day, animals were allowed to explore the familiar environment, providing a low-defense baseline.

Phase 2

On the 11th day, an adult male cat was placed and held in the food compartment by an experimenter and, as the rat's home cage door was opened, it was exposed to a live cat for a 10-min period. After the cat was removed at the end of the 10-min period, the hallway and food compartment were cleaned with 5% alcohol and dried with paper towels.

Phase 3

On the day after the cat exposure, animals were exposed to the environment in which the predator had been previously encountered, providing high levels of contextual conditioned fear responses.

For all testing periods, the pellets in the home cage were removed 3 h before the beginning of the dark phase and, after the testing periods, placed back into the home cage only for the animals that were not going to be killed for histological analysis. During the tests, the animals were recorded using a horizontally mounted video camera, under 50-W red light illumination.

Behavior analysis

Behaviors were scored by a trained observer using the ethological analysis software 'The Observer' (version 5.0; Noldus Information Technology, Wageningen, The Netherlands). The analysis comprised spatiotemporal and behavioral measurements. The spatiotemporal measurements were the time spent in the home cage, the home cage doorway (i.e., with the head or part of the body in the doorway), the hallway or the food compartment. The behavioral data were processed in terms of duration (cumulated duration per session). The following behavioral items were individually encoded: locomotion, grooming, exploratory upright position (i.e., animals actively exploring the environment standing over the hind paws and leaning on the walls with the fore paws), freezing (cessation of all movements except for those associated with breathing), crouch–sniff (animal immobile with the back arched but actively sniffing and scanning the environment), rearing (without wall contact), stretch–attend posture (a posture in which the body is stretched forward and the animal is motionless) and stretch–approach (movement toward the food compartment with the animal's body in a stretched position).

Experiment 1

In experiment 1, we made a systematic analysis of the hypothalamic expression of Fos immunoreactivity in animals after each one of the three different phases of the testing procedure, i.e., exposure to a safe environment (Phase 1), direct exposure to a predator (Phase 2) and exposure to a predator-associated context (Phase 3).

Ninety minutes after the testing procedures, five animals in each different phase of the testing procedure were deeply anesthetized with sodium pentobarbital (Cristália, Itapira, SP, Brazil; 40 mg/kg, i.p.) and perfused transcardially with a solution of 4.0% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4; the brains were removed and left overnight in a solution of 20% sucrose in 0.1 M phosphate buffer at 4ºC. The brains were then frozen and four series of 30-μm sections were cut with a sliding microtome in the frontal plane. One series of sections was processed for immunohistochemistry with anti-Fos antiserum raised in rabbit (Ab-5; Calbiochem, San Diego, CA, USA; lot # D09803) at a dilution of 1 : 10 000. The primary antiserum was localized using a variation of the avidin–biotin complex system (ABC; Hsu & Raine, 1981). In brief, sections were incubated for 90 min at room temperature in a solution of biotinylated goat antirabbit IgG (Vector Laboratories, Burlingame, CA, USA), and then placed in the mixed avidin–biotin horseradish peroxidase complex solution (ABC Elite Kit; Vector Laboratories) for the same period of time. The peroxidase complex was visualized by a 10-min exposure to a chromogen solution containing 0.02% 3,30-diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO, USA) with 0.3% nickel ammonium sulfate in 0.05 M Tris-buffer (pH 7.6), followed by incubation for 10 min, in chromogen solution with hydrogen peroxide (1 : 3000) to produce a blue-black product. The reaction was stopped by extensive washing in potassium phosphate-buffered saline (pH 7.4). Sections were mounted on gelatin-coated slides and then dehydrated and coverslipped with DPX (Sigma). An adjacent series was always stained with thionin to serve as a reference series for cytoarchitectonic purposes.

Experiment 2

In experiment 2 we examined the behavioral changes in PMd-lesioned animals during cat exposure, and how *N*-methyl-D-aspartate (NMDA) lesions in this nucleus interfered with the neuronal activation of systems engaged in predator detection and those involved in antipredatory defensive behavior.

NMDA lesions were made prior to the testing, and a 2-week postsurgery period elapsed before animals were placed in the experimental apparatus. For the lesion procedure, rats were deeply anesthetized with sodium pentobarbital (Cristália; 40 mg/kg, i.p.), and positioned in a stereotaxic frame. Xylocaine (Probem, Catanduva, SP, Brazil; 0.1 mL; 2 mg/mL) was subcutaneously injected into the scalp and a longitudinal incision was made. The bone was then exposed and a small window was opened, exposing the dura mater and the sagital sinus. Iontophoretic deposits were made through glass micropipettes (tip diameter 20 μm), filled with a 0.15 M solution of NMDA (Sigma, St Louis, MO, USA) or saline. The glass micropipettes were placed stereotaxically into the PMd (coordinates: anteroposterior, −4.16 mm from bregma; laterolateral, ±0.4 mm from midline of the sagital sinus; dorsoventral, −8.4 mm from the surface of the brain), and iontophoretic deposits of NMDA (n = 9) or saline (n = 6) were bilaterally placed. Iontophoretic deposits were made over 10 min, using a constant-current device (model CS3; Midgard Electronics, Canton, MA, USA) set to deliver −8 μA, with 7-s pulse and interpulse durations.

After a 2-week postsurgery period, the animals were placed in the experimental apparatus following the experimental procedure previously described, and were tested during a 10-min exposure to a live cat. Ninety minutes after the test, animals were deeply anesthetized with sodium pentobarbital (Cristália; 40 mg/kg, i.p.) and the brains were processed for Fos immunohistochemistry, as described in experiment 1.

Experiment 3

In experiment 3 we investigated how pharmacological blockade of the PMd with muscimol interfered with the behavioral responses to a context previously associated with a predator, and how this blockade affected the activation pattern of neural sites likely to organize the conditioned behavioral responses to a predatory context.

Animals were anaesthetized with a mixture of ketamine (Vetaset, Fort Dodge Laboratory, Campinas, SP, Brazil) and xylazine (Rompum, Bayer, São Paulo, SP, Brazil; 1: 2 v/v; 1 mL/kg body weight) and positioned in a stereotaxic frame. Xylocaine (Probem; 0.1 mL; 2 mg/mL) was subcutaneously injected into the scalp and a longitudinal incision was made. The bone was then exposed and a small window was opened, exposing the dura mater and the sagital sinus. A stainless-steel guide cannula (cat # C313GS-5; Plastics One Inc., Roanoke, VA, USA) was stereotaxically implanted, in a median position, with the cannula tip 1 mm above the PMd (coordinates: anteroposterior, −4.16 mm from bregma; laterolateral, 0 mm from midline of the sagital sinus; dorsoventral, −7.4 mm from the surface of the brain) or the medial mammillary nucleus PMd (coordinates: anteroposterior, −5.0 mm from bregma; laterolateral, 0 mm from midline of the sagital sinus; dorsoventral, −7.8 mm from the surface of the brain). To avoid bleeding while the guide cannula was being implanted through the midline, the sagital sinus was gently pulled laterally. The cannula was fixed with polyacrylic cement and anchored to the skull with stainless-steel screws.

After a 2-week recovery period, the animals were placed in the experimental apparatus following the experimental procedure previously described, and were tested during a 10-min exposure to the predatory context. Fifteen minutes prior to the testing period, two groups of animals received a single muscimol (Tocris, Ellisville, MO, USA; 0.5 μg/μL) injection, one group into the PMd region (n = 5) and the other group into the medial mammillary nucleus (n = 5); a third group received saline injections into the PMd region (n = 5). For drug administration the animals were gently held, and a removable injector cannula (cat # C313IS-5, Plastics One Inc.) was inserted into the guide cannula, extending 1 mm beyond the guide tip. The injector was linked to a 1-μL Hamilton syringe attached to an infusion pump (model 11; Harvard Apparatus, Holliston, MA, USA), and a volume of 0.1 μL was injected over a 2-min period. The injector remained in the guide cannula for an additional 2-min period after infusion. Ninety minutes after ending the behavioral testing the animals were deeply anesthetized with sodium pentobarbital (Cristália; 40 mg/kg, i.p.) and the brains were processed for Fos immunohistochemistry, as described for experiment 1.

Quantification of Fos-labeled cells

For all experiments, counts of the number of Fos-immunoreactive neurons were evaluated by an observer without knowledge of the animal's experimental status and were generated for selected brain regions by using the 10× objective of a Nikon Eclipse 80i (Nikon Corporation, Chiyoda-Ku, Tokyo-To, Japan) microscope equipped with a Nikon digital camera DXM1200F (Nikon Corporation). For the quantification of Fos labeling we first delineated, in a given section, the borders of a region of interest, as defined in adjoining Nissl-stained sections, and Fos-labeled cells were counted therein. Only darkly labeled oval nuclei that fell within the borders of a region of interest were counted. The density of Fos labeling was determined by dividing the number of Fos-immunoreactive cells by the area of the region of interest. Both cell counting and area measurements were done with the aid of a computer program (Image-Pro Plus, version 4.5.1; Media Cybernetics, Silver Spring, MD, USA). The parcellation of the hypothalamic, thalamic and amygdalar regions examined in the present investigation followed The Brain Maps: structure of the rat brain (Swanson, 2004). In the tables, for each brain site we indicate the corresponding atlas levels where the cell density was measured. For

TABLE 1. Experiment 1: behavioral and spatiotemporal measurements

	Experimental groups		
	Phase 1 Safe environment (n = 5)	Phase 2 Cat exposure (n = 5)	Phase 3 Predatory context (n = 5)
Behavioral items			
Locomotion	361.0 ± 18.6	0.5 ± 0.5*	30.7 ± 7.5*,#
Grooming	18.9 ± 12.0	0 ± 0*	42.3 ± 16.9ns,#
Upright position	187.5 ± 26.7	1.2 ± 1.2*	24.8 ± 3.8*,#
Rearing	18.8 ± 5.8	0 ± 0*	50.5 ± 22.9ns,#
Freezing	0 ± 0	555.6 ± 7.8*	34.3 ± 12.3*,#
Crouch–sniff	6.2 ± 0.5	7.9 ± 5.2ns	392.1 ± 22.2*,#
Stretch–attend	0 ± 0	0.4 ± 0.4ns	22.4 ± 8.5*,#
Stretch–approach	0 ± 0	4.4 ± 3.1ns	1.0 ± 1.0ns,ns
Spatiotemporal measurements			
Home cage	101.1 ± 26.5	583.1 ± 4.9*	452.3 ± 37.5*,#
Home-cage doorway	21.9 ± 4.1	8.3 ± 5.1ns	146.7 ± 36.9*,#
Hallway	175.2 ± 16.6	8.6 ± 5.6*	1.0 ± 1.0*,ns
Food compartment	300.3 ± 37.8	0 ± 0*	0 ± 0*,ns

Values are mean ± SEM of the time in s during a 10-min observation period. *P < 0.05 vs. animals exposed to the safe environment; #P < 0.05 vs. animals exposed to the cat; ns$P$ > 0.05.

the periaqueductal gray (PAG) we followed the parcellation originally proposed by Bandler *et al.* (1991) and revised by Carrive *et al.* (1997). This parcellation divides the PAG into dorsomedial, dorsolateral, lateral and ventrolateral columns, in addition to a ventromedial portion containing the supraoculomotor region, as well as the oculomotor, dorsal raphe and laterodorsal tegmental nuclei, usually considered, on functional and anatomical grounds, as separable from the rest of the PAG. Similarly to what had been done in a previous investigation (Comoli *et al.*, 2003), cell density was examined at six different rostrocaudal PAG segments (levels 1–6), namely, level of the nucleus of Darkschewitsch (level 1), rostral and caudal oculomotor nucleus levels (levels 2 and 3, respectively), and rostral, middle and caudal dorsal raphe nucleus levels (levels 4, 5 and 6, respectively).

Statistical analysis

After having tested for homogeneity of variances, the data on the density of Fos-labeled cells were analyzed using a parametric multivariate analysis of variance (MANOVA). As a significant multivariate test had been obtained, we performed a univariate analysis of variance (ANOVA) for each dependent variable, followed by a *post hoc* analysis (Tukey's HSD test) to isolate the respective effect. In order to keep the overall type I error at 5%, the significance level employed in the univariate ANOVAs and respective *post hoc* tests was adjusted downward (Sidak's correction) according to the respective number of dependent variables in each experiment.

In experiments 1, 2 and 3, for the spatiotemporal and behavioral measurements, nonparametric methods were chosen due to severe violations of the homogeneity of variances assumption by the recorded data. Due to the different number of experimental groups, the Mann–Whitney *U*-test was employed for experiment 2 and the Kruskal–Wallis test was used in experiments 1 and 3. The significance level was set at $\alpha = 5\%$. Average results are expressed as mean ± SEM throughout the text.

Results

Experiment 1

As shown in Table 1, the three different phases of the experimental procedure provide, respectively, a low defensive

TABLE 2. Experiment 1: density of Fos-labeled cells in hypothalamic regions of animals in response to a safe environment, cat exposure and predatory context

		Experimental groups		
	LEV	Safe environment ($n = 5$)	Cat exposure ($n = 5$)	Predatory context ($n = 5$)
Periventricular hypothalamic zone				
Arcuate nucleus	29	644.2 ± 72.2	253.3 ± 26.6*	308.9 ± 15.3^{*,ns}
Anteroventral periventricular nucleus	19	165.0 ± 18.7	94.3 ± 26.6^{ns}	170.8 ± 25.3^{ns,ns}
Median preoptic nucleus	19	240.0 ± 23.9	511.4 ± 49.9^{ns}	950.0 ± 63.9$^{*,\#}$
Paraventricular nucleus				
Autonomic part†	27	589.9 ± 32.6	748.1 ± 68.6^{ns}	587.5 ± 32.2^{ns,ns}
Magnocellular part‡	26	244.1 ± 14.7	429.3 ± 68.6^{ns}	520.6 ± 59.1^{ns,ns}
Parvicellular part§	26	964.4 ± 125.5	694.2 ± 69.0^{ns}	893.1 ± 142.4^{ns,ns}
Supraoptic nucleus	23	72.7 ± 39.6	174.9 ± 35.4^{ns}	427.6 ± 46.3$^{*,\#}$
Suprachiasmatic nucleus	22	1359.4 ± 116.6	1479.9 ± 33.0^{ns}	1462.9 ± 73.8^{ns,ns}
Medial hypothalamic zone				
Anterodorsal preoptic nucleus	19	767.8 ± 38.7	647.7 ± 25.1^{ns}	636.9 ± 36.9^{ns,ns}
Medial preoptic nucleus	21	64.2 ± 4.8	82.0 ± 4.6^{ns}	72.1 ± 7.4^{ns,ns}
Anterior hypothalamic nucleus (AHN)				
Rostral level	24	99.9 ± 7.9	663.0 ± 56.7*	336.1 ± 25.1$^{*,\#}$
Intermediate level	25	123.2 ± 5.9	948.2 ± 71.8*	398.0 ± 33.2$^{*,\#}$
Caudal level	26	182.0 ± 8.0	1325.3 ± 48.5*	506.7 ± 37.7$^{*,\#}$
Ventromedial hypothalamic nucleus (VMH)				
Dorsomedial part	29	40.0 ± 8.3	1512.0 ± 88.9*	170.8 ± 15.4$^{ns,\#}$
Ventrolateral part	29	46.0 ± 12.5	77.6 ± 11.3^{ns}	61.7 ± 7.5^{ns,ns}
Dorsomedial hypothalamic nucleus	30	338.4 ± 27.1	620.0 ± 26.5*	393.8 ± 26.9$^{ns,\#}$
Dorsal premammilary nucleus	33	169.6 ± 24.5	1371.9 ± 51.3*	936.0 ± 28.5$^{*,\#}$
Ventral premammillary nucleus	32	27.3 ± 4.1	38.2 ± 5.3^{ns}	20.8 ± 4.2^{ns,ns}
Supramammillary nucleus	35	399.3 ± 12.3	529.0 ± 35.3^{ns}	507.2 ± 19.0^{ns,ns}
Medial mammillary nucleus	35	24.5 ± 2.3	38.0 ± 5.1^{ns}	27.5 ± 1.2^{ns,ns}
Lateral mammillary nucleus	35	70.8 ± 9.5	86.8 ± 4.2^{ns}	74.4 ± 9.4^{ns,ns}
Posterior hypothalamic nucleus	33	244.5 ± 14.7	255.5 ± 11.2^{ns}	300.3 ± 11.1^{ns,ns}
Lateral hypothalamic zone				
Lateral preoptic area	20	177.6 ± 26.2	748.8 ± 64.6*	184.3 ± 15.2$^{ns,\#}$
Anterior region¶	24	145.4 ± 24.5	177.2 ± 19.0^{ns}	144.8 ± 14.8^{ns,ns}
Juxtaparaventricular region	25	208.3 ± 22.9	498.3 ± 47.1*	249.2 ± 29.7$^{ns,\#}$
Subfornical region				
Anterior zone	27	138.6 ± 17.5	734.6 ± 49.1*	188.8 ± 13.2$^{ns,\#}$
Posterior zone	28	146.6 ± 6.6	626.6 ± 48.8*	151.1 ± 10.8$^{ns,\#}$
Tuberal region**	29	298.1 ± 12.7	335.9 ± 33.6^{ns}	318.9 ± 9.6^{ns,ns}

Values represent the number of Fos-labeled cells per mm^2. Data are expressed as mean ± SEM. The table column entitled 'LEV' refers to plate numbers in Brain Maps (Swanson, 2004), to indicate the approximate rostrocaudal levels at which the measurements were taken. †Measurements from the lateral and medial parvicellular parts. ‡Measurements from the posterior magnocellular part. §Measurements from the dorsal zone of the medial parvicellular part. ¶Encompassing the intermediate and dorsal zones. **Encompassing the juxtadorsomedial, suprafornical and dorsal regions of the lateral hypothalamic area. $^{*}P < 0.002$ vs. the experimental group of animals exposed to a safe environment; $^{\#}P < 0.002$ vs. the experimental group of animals exposed to the cat; $^{ns}P > 0.002$.

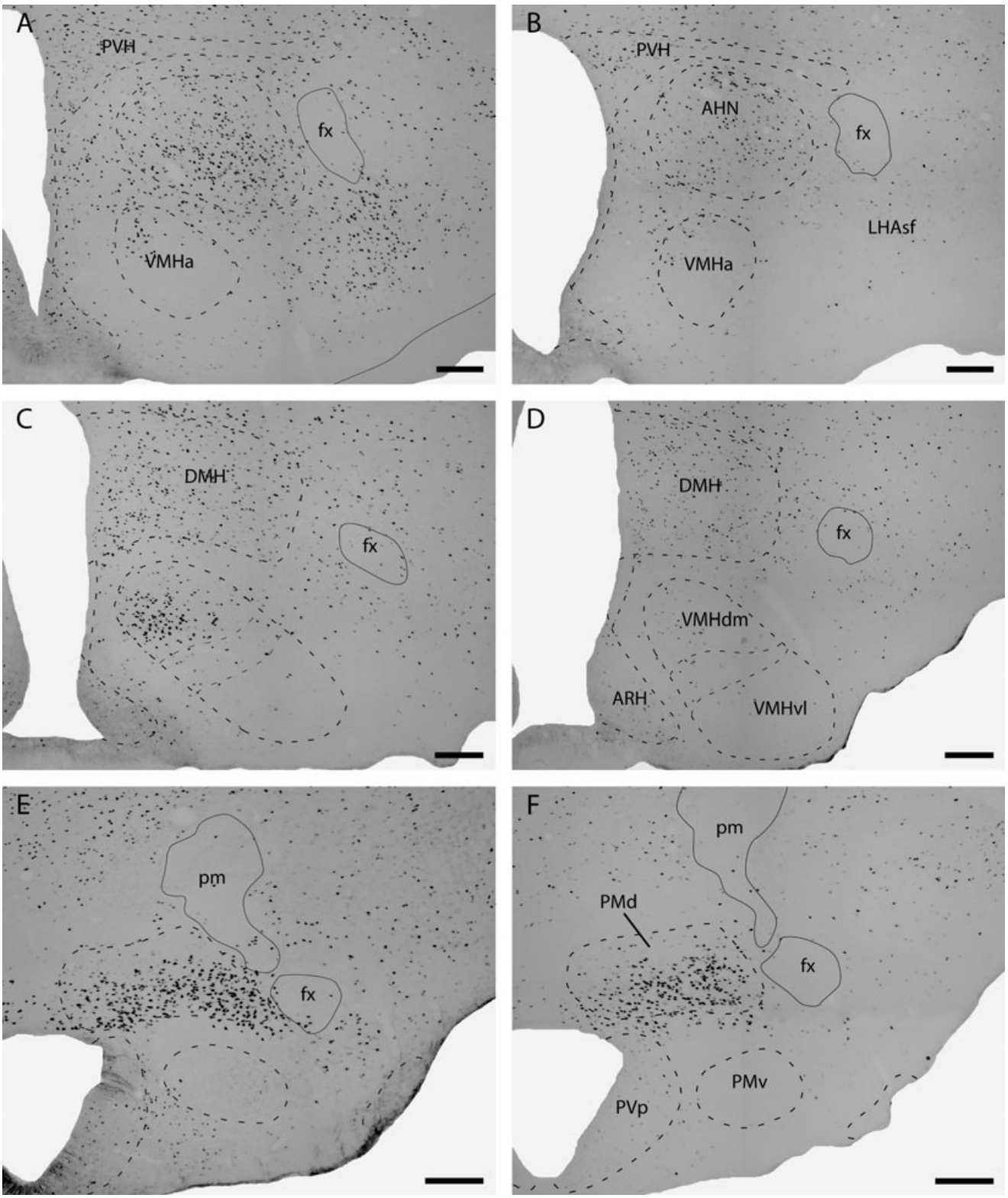

FIG. 1. Experiment 1: photomicrographs of transverse Fos-stained sections of (A and B) the AHN and LAHsf, (C and D) VMH and (E and F) PMd from (left-hand column, A, C and E) a rat exposed to a cat and from (right-hand column, B, D and F) an animal exposed to the predatory context. Abbreviations: see list. Scale bars, 200 μm.

baseline (Phase 1), a high level of freezing during cat exposure (Phase 2), and a high level of risk assessment of the environment where the predator had been previously found (Phase 3).

Phase 1

The animals were fully adapted to the apparatus, showing no avoidance of the food compartment, and <7 s of crouch–sniff (Table 1). Their major activity was locomotion (361.0 ± 18.6 s)

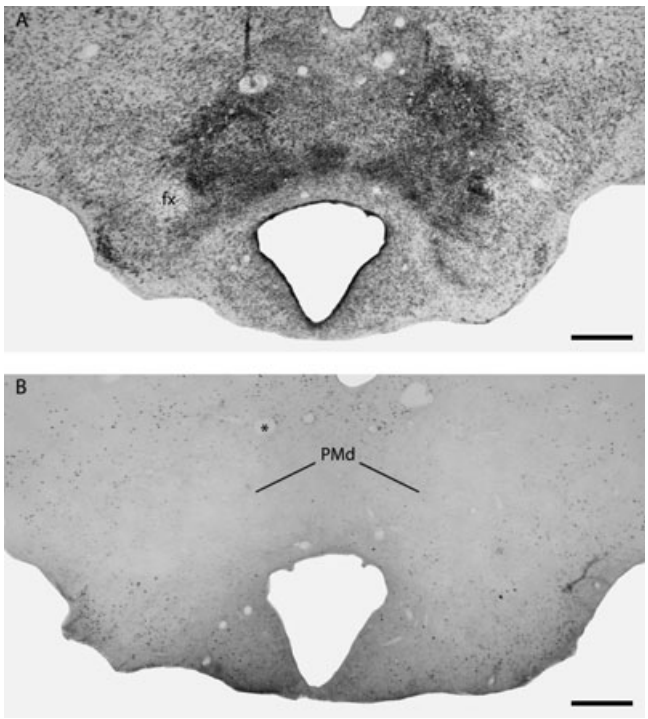

FIG. 2. Experiment 2: photomicrographs of transverse (A) Nissl-stained and (B) adjacent Fos-stained sections through a bilateral NMDA lesion centered in the PMd, to illustrate the appearance of an NMDA lesion, characterized by (A) neuronal cell loss filled with gliosis and (B) lack of Fos immunoreactivity in the lesioned area. Abbreviations: see list. Scale bars, 400 μm.

TABLE 3. Experiment 2: behavioral and spatiotemporal measurements

	Experimental groups	
	Saline PMd (n = 6)	NMDA lesion PMd (n = 7)
Behavioral items		
Locomotion	0.7 ± 0.5	27.1 ± 1.9*
Grooming	0 ± 0	2.1 ± 1.9^{ns}
Upright position	1.0 ± 1.0	27.6 ± 6.3*
Rearing	0 ± 0	20.9 ± 5.0*
Freezing	552.9 ± 7.2	65.4 ± 6.9*
Crouch–sniff	9.8 ± 4.1	305.7 ± 27.3*
Stretch–attend	0 ± 0	110.2 ± 9.3*
Stretch–approach	4.6 ± 0.9	13.4 ± 1.3*
Spatiotemporal measurements		
Home cage	563.8 ± 7.7	422.5 ± 30.7*
Home-cage doorway	10.0 ± 4.7	123.8 ± 18.7*
Hallway	5.4 ± 2.7	31.8 ± 10.1*

Values are mean ± SEM time in s during a 10-min observation period. *$P < 0.05$ vs. animals of the saline-injected group; $^{ns}P > 0.05$.

throughout the apparatus. They also frequently assumed an upright position, leaning on the apparatus walls while actively investigating the environment (exploratory upright position, 187.5 ± 26.7 s). In terms of spatiotemporal measurements, rats in Phase 1 spent about half their time (300.3 ± 37.8 s) in the food compartment.

Phase 2

During the cat-exposure test, animals remained frozen in the home cage for almost the entire duration of the test (freezing duration 555.6 ± 7.8 s), a significantly longer period than during Phase 1 ($P = 0.0012$). Although some risk assessment responses, such as crouch–sniff and even flat-back approaches, were seen as animals left the home cage and approached the cat, these were not significantly different in duration from those made during Phase 1 ($P > 0.90$). In Phase 2, animals spent >97% of their time (583.1 ± 4.9 s) in the home cage.

Phase 3

Animals exposed to the environment where the cat had been previously encountered showed a drop in freezing (34.3 ± 12.3 s, $P = 0.0080$) but significantly more crouch–sniff (392.1 ± 22.2 s, $P = 0.0140$) and stretch–attend posture (22.4 ± 8.5 s, $P = 0.0399$). Stretch–approaches were uncommon, and not significantly different in duration from those of Phases 1 and 2 ($P > 0.90$). Locomotion and the upright position were significantly reduced in comparison to Phase 1 ($P = 0.0079$) and significantly increased in comparison to Phase 2 ($P < 0.0079$). Animals remained most of the time in the home cage (452.3 ± 37.5 s) or in the home-cage doorway (146.7 ± 36.9 s). The home cage times were significantly higher than those of Phase 1 and significantly lower than those of Phase 2, and the doorway times were significantly higher than those of either of the two initial phases ($P < 0.0080$).

As shown in Table 2, for all three test situations we provided a comprehensive analysis of Fos expression in the different hypothalamic zones.

Cat exposure induced a significant increase in Fos levels in distinct elements of the medial and lateral hypothalamic zones ($F_{26,2} = 45.9$, $P = 0.02$). Due to the large number of dependent variables, a stricter significance level ($\alpha = 0.002$) was applied in the *post hoc* pairwise comparisons. In the medial hypothalamic zone, the intermediate and caudal levels of the anterior hypothalamic nucleus (AHN), the dorsomedial part of the ventromedial hypothalamic nucleus (VMHdm) and the PMd presented the most striking Fos up-regulation ($P < 0.0002$; Table 2; Fig. 1A, C and E). In addition, in the medial hypothalamic zone cat exposure also induced a significant, but much less intense, Fos increase in the dorsomedial hypothalamic nucleus (DMH; $P < 0.0002$; Table 2). In the lateral hypothalamic zone, the lateral preoptic area and the subfornical region (Fig. 1A) responded with a significant Fos increase to cat exposure ($P < 0.0002$), followed by a less intense, but still significant, activation of the juxtaparaventricular region ($P < 0.0004$; Table 2).

Compared to the baseline situation (Phase 1), exposure to the environment previously associated with a cat (Phase 3) also yielded a significant activation in the AHN ($P < 0.002$) and the PMd ($P < 0.0002$), but not in the VMHdm ($P > 0.1755$; Table 2; Fig. 1B, D and F). Notably, the PMd represented the most responsive hypothalamic site during exposure to the predatory context (Table 2; Fig. 1F). In the periventricular zone, as compared to the other experimental groups, animals tested in Phase 3 of the experimental procedure presented a significant increase in Fos levels in the median preoptic nucleus and supraoptic nucleus ($P < 0.0024$), as well as an apparent, but not significant ($P > 0.005$), increase in Fos levels in the magnocellular part of the paraventricular nucleus (Table 2). Both cat exposure and exposure to a hostile environment yielded clear increases in the supramammillary nucleus Fos labeling, but did not reach statistical significance ($P > 0.005$). Finally, compared to animals tested in Phase 1, when the animals search for food showing clear foraging activities, exposure to the predator or the predatory context resulted in a significant decrease in Fos expression in the arcuate nucleus ($P < 0.0004$; Table 2).

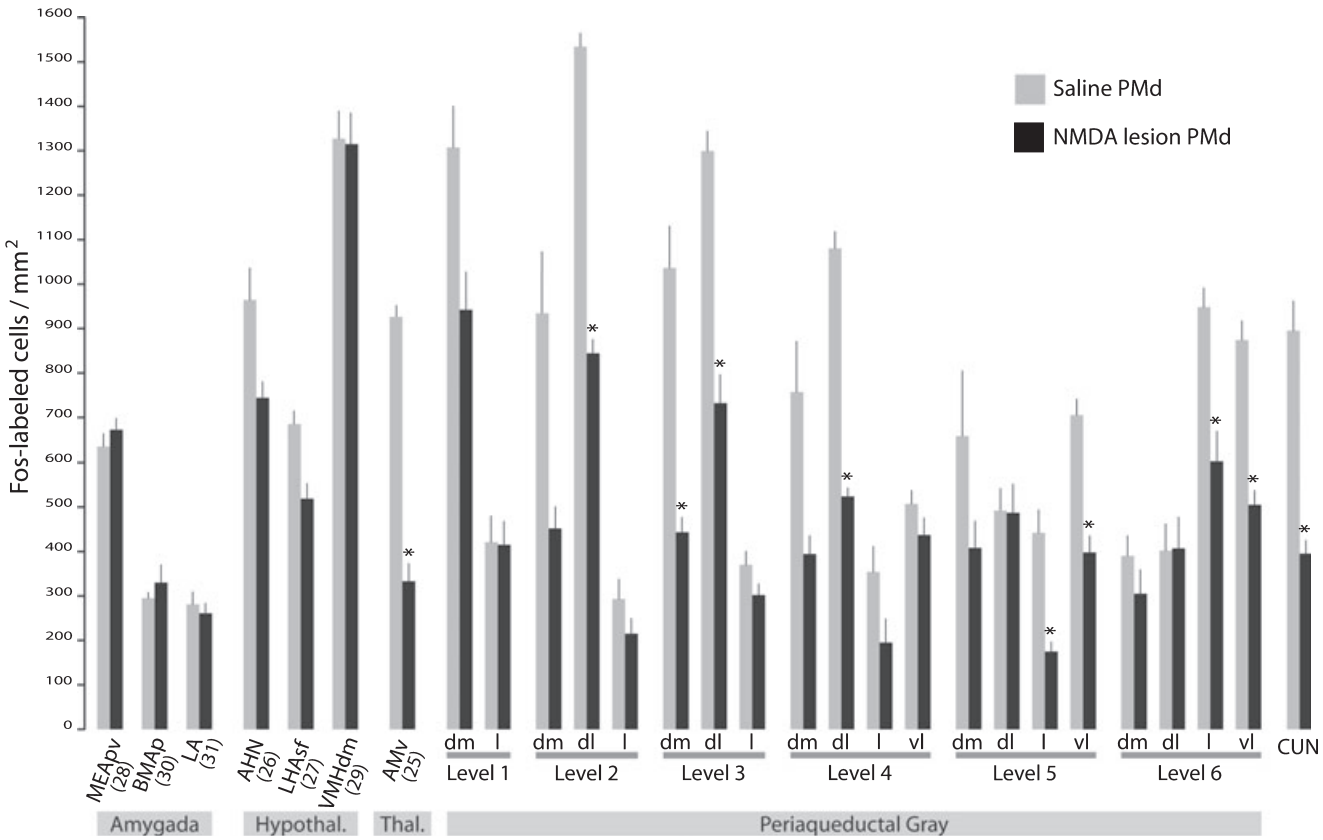

FIG. 3. Experiment 2: frequency histograms show the density of Fos-immunoreactive cells in selected brain regions of the amygdala, hypothalamus, thalamus and brain stem (PAG and cuneiform nucleus) from the saline-treated animals (Saline PMd, $n = 6$) and the PMd NMDA-lesioned rats (NMDA lesion PMd, $n = 7$) in response to cat exposure. Levels 1–6 correspond to the rostrocaudal levels described in the text (i.e., level 1, level of the nucleus of Darkschewitsch; levels 2 and 3, rostral and caudal oculomotor nucleus levels, respectively; levels 4, 5 and 6, rostal, middle and caudal dorsal raphe nucleus levels, respectively). In each PAG level, the density of Fos-immunoreactive cells was individually measured for each PAG column. Values represent the number of Fos-labeled cells per mm^2. Data are expressed as mean + SEM. The numbers in parentheses for each amygdalar, hypothalamic and thalamic nucleus refer to Brain Maps (Swanson, 2004) plate numbers, to indicate the approximate rostrocaudal levels at which the measurements were taken. *$P < 0.002$ vs. animals of the saline injected group. Abbreviations: see list.

Experiment 2

In experiment 2, animals with NMDA or sham lesions in the PMd were tested during cat exposure. The parameters described above for NMDA iontophoretic injections resulted in relatively small hypothalamic lesions, characterized by neuronal cell loss filled with gliosis (Fig. 2). In seven animals, the lesions were centered bilaterally in the PMd and also spread to parts of adjacent nuclei, such as the medial mammillary nucleus (five animals), the posterior hypothalamic nucleus (seven animals) and the ventral premammillary nucleus (four animals).

Behavioral analysis revealed that, during cat exposure, PMd lesions significantly reduced freezing ($P < 0.0027$) and, at the same time, significantly increased risk assessment activities, such as crouch–sniff ($P < 0.0027$), stretch–attend postures ($P < 0.0016$) and, to a lesser degree, stretch–approaches ($P < 0.0042$; Table 3). During cat exposure, PMd-lesioned rats also presented a significant increased locomotion ($P < 0.0024$), exploratory upright position with the forepaws leaning on the walls of the apparatus ($P < 0.0020$), and rearing ($P < 0.0016$; Table 3). The spatiotemporal measurements also revealed increased mobility in the experimental apparatus for the PMd-lesioned animals, which spent significantly more time in the home-cage doorway ($P < 0.0026$) and in the hallway ($P < 0.0096$; Table 3). By and large, PMd lesions sharply reduced defensiveness during cat exposure, reducing freezing and, at the same time, increasing risk assessment and mobility in the experimental apparatus.

In this experiment, we further examined how PMd lesions interfered with the pattern of activation of amygdalar sites involved in the perception of the predator (i.e., the posteroventral part of the medial amygdalar nucleus, the posterior basomedial amygdalar nucleus and caudal levels of the lateral amygdalar nucleus), hypothalamic sites previously found to be particularly activated during predator exposure, the main PMd thalamic target likely to influence attentional and mnemonic processing (i.e., the ventral part of the anteromedial thalamic nucleus), and PAG sites critical for unconditioned defensive responses to the predator. A MANOVA showed a significant treatment effect: $F_{11,1} = 186.1$, $P = 0.05$. The MANOVA was followed by univariate ANOVA of each dependent variable, and *post hoc* analyses when appropriate (Tukey's HSD test). Due to the large number of dependent variables, a stricter significance level ($\alpha = 0.002$) was applied to the set of univariate ANOVAs and to the *post hoc* tests as well. Importantly, PMd lesions did not seem to affect the level of Fos expression in the amygdalar sites involved in the perception of the predator ($P > 0.3499$; Figs 3, and 4A and B) and in the dorsomedial part of the ventromedial nucleus ($P > 0.8971$; Figs 3, and 4C and D). Conversely, PMd lesions led to an apparent, but not significant, Fos expression decrease in other hypothalamic elements largely activated during cat exposure, namely, the AHN and the subfornical region of the lateral hypothalamus (LHAsf; $P > 0.004$; Fig. 3), both of which

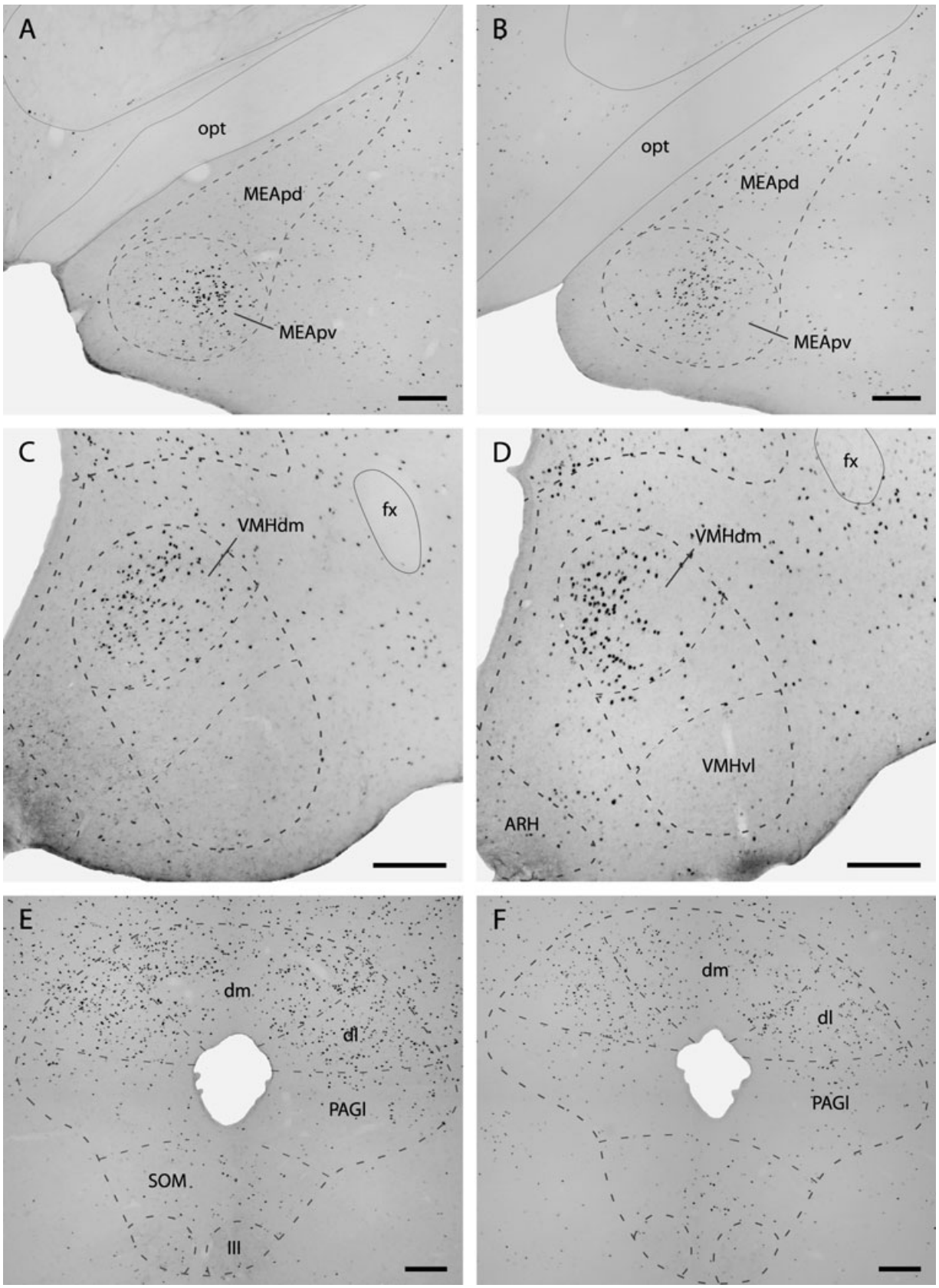

FIG. 4. Experiment 2: photomicrographs of transverse Fos-stained sections of (A and B) the medial amygdalar nucleus, (C and D) VMH and (E and F) PAG from (left-hand column, A, C and E) a saline-treated rat and (right-hand column, B, D and F) a PMd NMDA-lesioned rat, both in response to cat exposure. Abbreviations: see list. Scale bars, 200 μm.

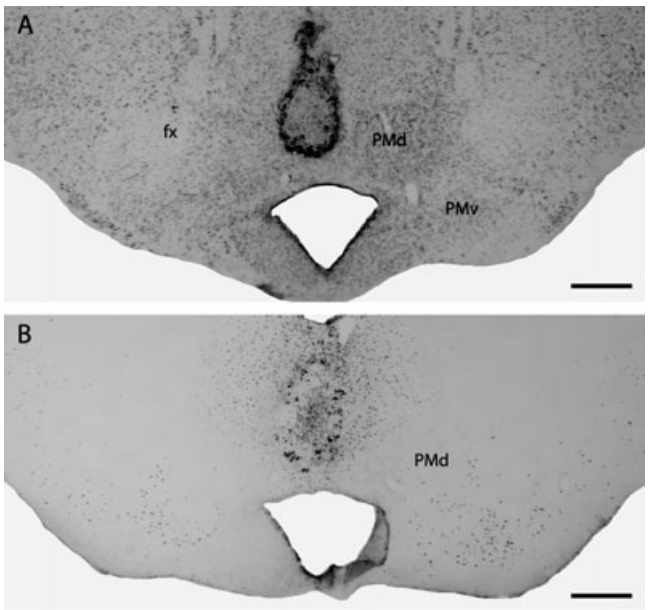

FIG. 5. Experiment 3: bright-field photomicrographs of (A) thionin-stained and (B) adjacent Fos-stained sections showing the injection cannula placement in a representative animal that received muscimol into the PMd region. Notice the lack of Fos staining in the PMd and immediate adjacent regions. Abbreviations: see list. Scale bars, 400 μm.

TABLE 4. Experiment 3: behavioral and spatiotemporal measurements

	Experimental groups		
	Saline PMd (n = 5)	Muscimol MM (n = 5)	Muscimol PMd (n = 5)
Behavioral items			
Locomotion	64.1 ± 13.0	65.8 ± 11.6^{ns}	332.5 ± 35.0$^{*,\#}$
Grooming	33.0 ± 13.3	25.2 ± 11.5^{ns}	43.0 ± 19.7^{ns,ns}
Upright position	36.4 ± 1.0	41.3 ± 2.4^{ns}	67.6 ± 7.6$^{*,\#}$
Rearing	22.1 ± 3.5	20.7 ± 2.4^{ns}	16.2 ± 2.8^{ns,ns}
Freezing	19.8 ± 3.7	18.7 ± 4.2^{ns}	0 ± 0$^{*,\#}$
Crouch–sniff	384.1 ± 14.2	337.8 ± 34.9^{ns}	59.6 ± 25.0$^{*,\#}$
Stretch–attend	31.8 ± 9.4	46.8 ± 9.4^{ns}	6.2 ± 1.8$^{*,\#}$
Stretch–approach	18.7 ± 3.6	21.4 ± 4.3^{ns}	7.0 ± 2.2^{ns,ns}
Spatiotemporal measurements			
Home cage	453.3 ± 16.3	396.0 ± 20.1^{ns}	143.8 ± 34.9^{*,ns}
Home-cage doorway	109.1 ± 31.5	135.7 ± 28.7^{ns}	74.0 ± 24.9^{ns,ns}
Hallway	31.6 ± 5.6	48.8 ± 8.1^{ns}	237.6 ± 43.6$^{*,\#}$
Food compartment	0 ± 0	0 ± 0^{ns}	96.4 ± 34.5$^{*,\#}$

Values are mean ± SEM time in s during a 10-min observation period. $^{*}P < 0.05$ vs. animals of the saline-injected group (saline PMd group); $^{\#}P < 0.05$ vs. muscimol-injected animals in the medial mammillary nucleus (muscimol MM group); $^{ns}P > 0.05$.

are heavily targeted by the PMd. In sharp contrast, during cat exposure, PMd lesions resulted in a large reduction in Fos expression in the thalamic and PAG targets of the nucleus. In the thalamus, PMd lesions yielded a significant reduction in Fos levels in the ventral part of the anteromedial nucleus ($P < 0.0002$; Fig.3). In the PAG, during cat exposure, PMd-lesioned animals presented a significant reduction in Fos levels in the dorsomedial and dorsolateral columns at the levels of the oculomotor (Fig. 4E and F) and trochlear nuclei (levels 2–4; $P < 0.002$) whereas, at caudal PAG levels (levels 5 and 6), a significant decrease in Fos expression was found in the lateral and ventrolateral columns ($P < 0.002$; Fig. 3). In PMd-lesioned animals, the cuneiform nucleus also presented a significant decrease in Fos levels in response to cat exposure ($P < 0.0002$; Fig. 3).

Experiment 3

In experiment 3, muscimol or saline injections were placed into the PMd or into the nearby medial mammillary nucleus 15 min prior to the exposure to the environment where the cat had been previously found (predatory context). In five experiments, the muscimol injection site was placed in a median position between the two dorsal premammillary nuclei on each side of the brain and provided a clear inactivation of the nucleus, as could be evaluated by the lack of Fos staining in the nucleus and immediate surroundings (Fig. 5). In contrast, animals that received muscimol injection into the medial mammillary nucleus presented a robust Fos staining in the PMd. Notably, all animals, injected either with muscimol or saline, presented a clear Fos upregulation in ependimal cells surrounding the third ventricle and the cerebral aqueduct (Fig. 5).

Muscimol injection into the PMd yielded significant changes in most behavioral measurements ($P < 0.05$) and, also, a marginally significant overall effect on the pattern of PAG Fos expression ($F_{24,2} = 14.53$, $P = 0.0663$), strengthened by statistically significant pairwise comparisons found in the *post hoc* analysis (see below). These findings contrast with those found in the other experimental groups (animals that received saline into the PMd or muscimol into the medial mammillary nucleus), which presented similar levels of risk assessment behavior ($P > 0.5961$) and also equivalent pattern and levels of activation in the different PAG columns ($P > 0.1581$) when compared to each other (Table 4; Fig. 6).

Behavioral measurements revealed that pharmacological PMd blockade significantly increased relaxed locomotion ($P < 0.0053$) and fearless exploratory behavior (i.e., exploratory upright position; $P < 0.0015$) and, at the same time, significantly reduced freezing ($P < 0.0073$) and risk assessment activities such as crouch–sniff ($P < 0.0029$) and stretch–attend ($P < 0.0311$; Table 4) postures. In this experimental group, the spatiotemporal measurements also corroborated the idea of increased mobility in the apparatus during exposure to the predatory context, when the animals spent significantly more time in the hallway ($P < 0.0015$) and in the food compartment ($P < 0.0009$; Table 3).

Considering this evident reduction in contextual predatory defense after the muscimol injection into the PMd, we further examined how this pharmacological blockade interfered with the pattern of PAG activation, to investigate which columns would be more critically involved in the contextual conditioned responses to the predatory environment. As mentioned above, a MANOVA showed a marginally significant treatment effect ($F_{24,2} = 14.53$, $P = 0.0663$). The MANOVA was followed by ANOVA of each dependent variable and *post hoc* analyses when appropriate (Tukey HSD test). Due to the large number of dependent variables, a stricter significance level ($\alpha = 0.002$) was applied to the set of ANOVAS and to the *post hoc* tests.

Compared to the other experimental groups, the PMd pharmacological blockade group showed a significant Fos level reduction in the dorsolateral column, at the levels of the oculomotor and trochlear nuclei: levels 2, 3 and 4 ($P < 0.0012$; Figs 6 and 7). In this experimental group, a significant decrease in Fos expression was also found in the dorsomedial column, extending from the rostral to the oculomotor nucleus levels: levels 1, 2 and 3 ($P < 0.0074$; Figs 6 and 7). In addition, we have found that the cuneiform nucleus also

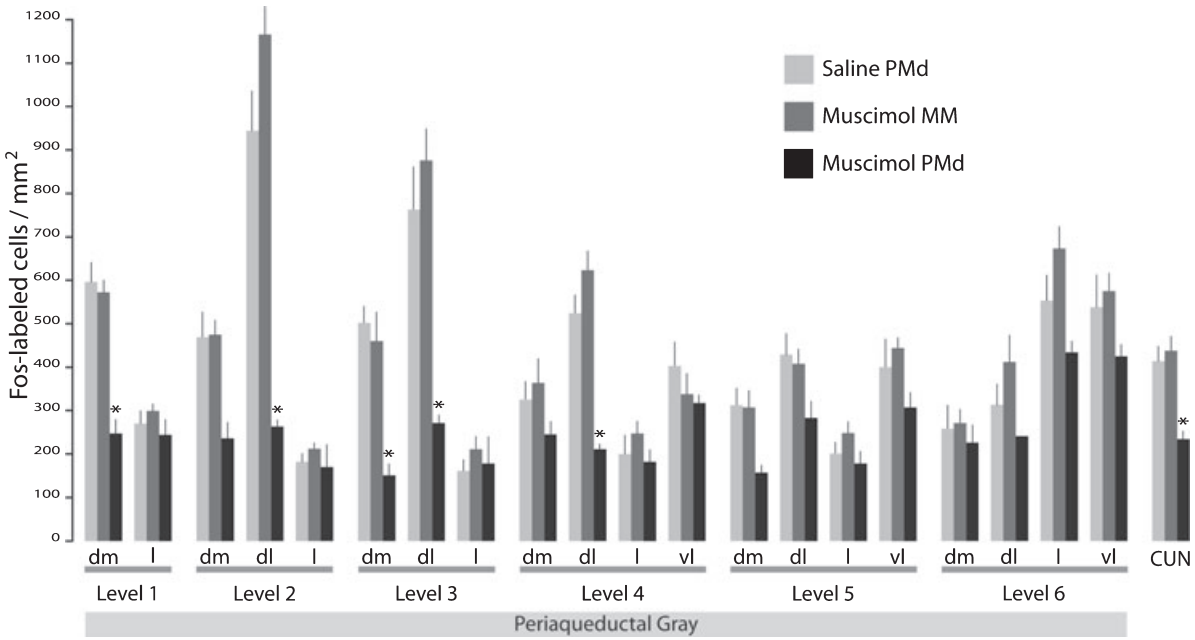

FIG. 6. Experiment 3: frequency histograms show the density of Fos-immunoreactive cells in the PAG and cuneiform nucleus from the saline-treated animals (Saline PMd, $n = 5$), the animals injected with muscimol in the medial mammillary nucleus (Muscimol MM, $n = 5$) and the animals injected with muscimol in the PMd (Muscimol PMd, $n = 5$) in response to the predatory context. Levels 1–6 correspond to the rostrocaudal levels described in the text (i.e., level 1, level of the nucleus of Darkschewitsch; levels 2 and 3, rostral and caudal oculomotor nucleus levels, respectively; levels 4, 5 and 6, rostal, middle and caudal dorsal raphe nucleus levels, respectively). In each PAG level, the density of Fos-immunoreactive cells was individually measured for each PAG column. Values represent the number of Fos-labeled cells per mm^2. Data are expressed as mean + SEM. $*P < 0.002$ vs. animals of the saline-injected group and the animals injected with muscimol in the medial mammillary nucleus. Abbreviations: see list.

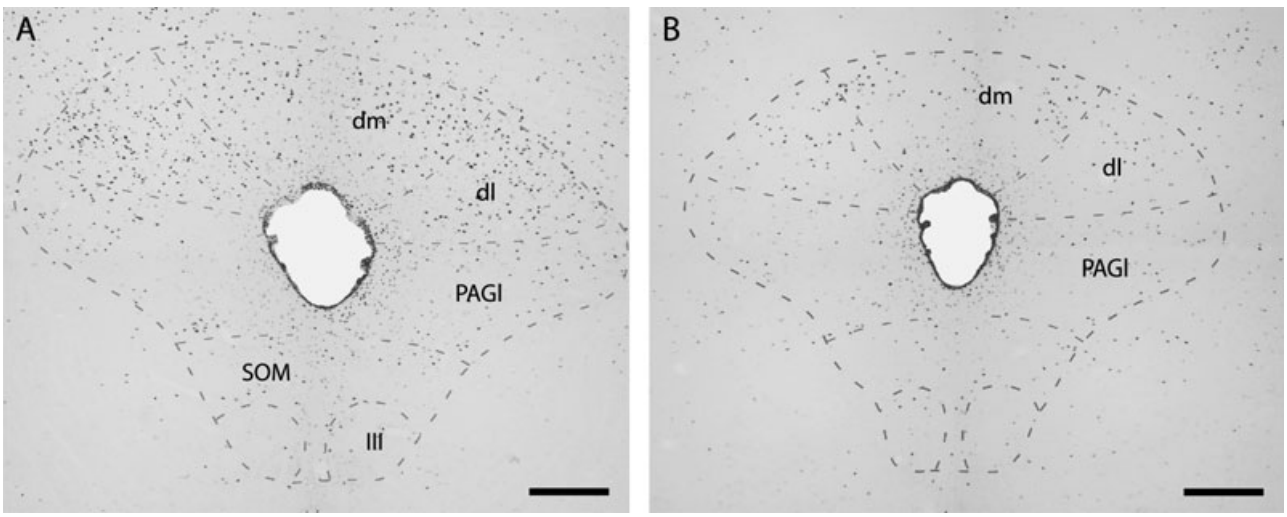

FIG. 7. Experiment 3: photomicrographs of transverse Fos-stained sections of the PAG from (A) an animal injected with muscimol in the medial mammillary nucleus and (B) an animal injected with muscimol in the PMd, both in response to a context previously associated with a cat. Abbreviations: see list. Scale bars, 300 μm.

presented a significant reduction in Fos levels after the muscimol injection into the PMd ($P < 0.0028$; Fig. 6).

Discussion

In the present study, we made a systematic investigation into how the different hypothalamic regions respond to a live predator or an environment previously associated with a predator, and tested how one of the hypothalamic sites most responsive to these threats (i.e., the PMd) interferes with both unconditioned and conditioned antipredatory defensive behavior. We have also analyzed how this hypothalamic site influences the systems engaged in predator detection, in contextual memory formation and in unconditioned and conditioned antipredatory behavior.

Cat exposure induced a significant increase in Fos levels in distinct elements of the medial and lateral hypothalamic zones. In the medial hypothalamic zone, the AHN, the dorsomedial part of the ventrome-

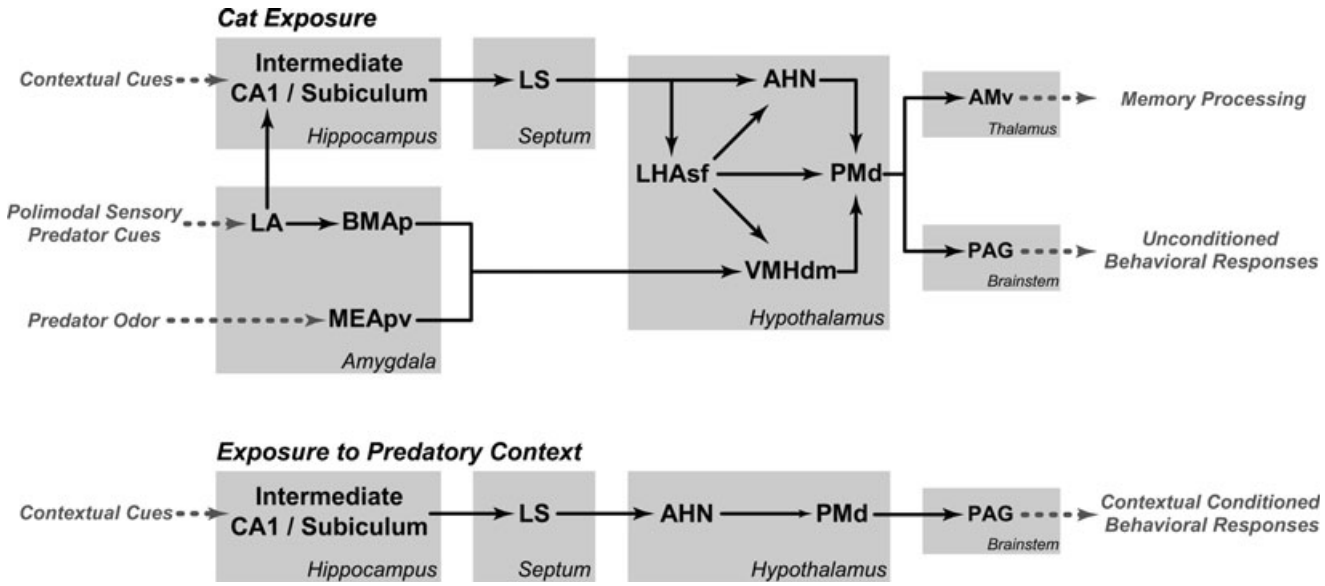

FIG. 8. Schematic diagrams showing the putative brain systems involved in processing unconditioned and contextual-conditioned predatory threats as well as in organizing unconditioned and contextual conditioned behavioral responses. Abbreviations: see list.

dial hypothalamic nucleus (VMHdm) and the PMd presented a particularly prominent Fos up-regulation. The VMHdm is known to integrate amygdalar information critical for predator perception arising from the posteroventral part of the medial amygdalar nucleus and from the posterior part of the basomedial nucleus (see Canteras *et al.*, 2001). The posteroventral part of the medial amygdalar nucleus has its role in pheromone-like processing of predator odor (McGregor *et al.*, 2004). Conversely, the posterior basomedial amygdalar nucleus and caudal levels of the lateral amygdalar nucleus respond to the totality of predator stimuli rather than the odor alone (Canteras *et al.*, 2001), and are likely to integrate predator-derived sensory cues other than olfactory ones (McDonald, 1998). The AHN, in its turn, is densely targeted by distinct septal regions known to receive inputs from intermediate regions of field CA1 and the subiculum, and integrates information from the septohippocampal system (Risold & Swanson, 1997).

In the lateral hypothalamic zone, the lateral preoptic area and the LHAsf presented a significant Fos increase in response to cat exposure. The lateral preoptic area has been implicated in the modulation of somatomotor responses and general arousal associated with motivated behaviors (see Swanson, 1987). The LHAsf, similar to the AHN, also integrates information from the septohippocampal system, and projects to all three medial hypothalamic sites largely activated during cat exposure (Goto *et al.*, 2005).

Interestingly, the VMHdm, the AHN and the LHAsf provide a unique kind of projection to the PMd, with dense bilateral projection fields (Canteras *et al.*, 1994; Risold *et al.*, 1994; Goto *et al.*, 2005). This kind of synaptic arrangement enables the PMd to work as an amplifier for the hypothalamic neural processing of predator-related cues and, corroborating this view, we have presently shown that the PMd is highly responsive to predatory threats.

In experiment 2, we confirmed previous findings showing that PMd lesions significantly reduce freezing and, at the same time, increase risk assessment activities during cat exposure (Blanchard *et al.*, 2003). We have further examined how the PMd lesions interfered with the neural systems organizing unconditioned antipredatory defensive responses. Of particular relevance, PMd lesions do not seem to affect the amygdalar–hypothalamic paths responding to the predator presence (including the posteroventral part of the medial amygdalar nucleus, the posterior basomedial amygdalar nucleus and caudal levels of the lateral amygdalar nucleus on the amygdalar side, and the dorsomedial part of the ventromedial nucleus on the hypothalamic side). Moreover, hypothalamic PMd targets, such as the AHN and the LHAsf, presented a small but nonsignificant Fos expression decrease in PMd-lesioned animals. In sharp contrast, PMd lesions significantly decreased the activation level of its thalamic and PAG targets during cat exposure.

In the thalamus, the PMd provides a dense projection to the ventral part of the anteromedial thalamic nucleus (Canteras & Swanson, 1992) which, in PMd-lesioned animals, presented a three-fold reduction in activation during cat exposure. Previous studies have shown that the anteromedial thalamic nucleus projects to the lateral retrosplenial area, thought to be involved in modulating the eye and head movements associated with attentional processes (Risold & Swanson, 1995). A growing body of evidence has also suggested a key role for the anterior thalamic nuclei in contextual memory mechanisms (Vann & Aggleton, 2004), and recent findings from our lab have shown that NMDA-receptor antagonist injection in the PMd was able to block contextual fear conditioning to cat odor (Canteras *et al.*, 2008). Taken together, the evidence suggests the path comprising the PMd and ventral anteromedial thalamic nucleus as playing a role in the emotional memory processing to predator threats.

During cat exposure, PMd lesions also significantly reduced the Fos levels in PAG sites heavily targeted by this nucleus, including the PAGdm and PAGdl columns, at the levels of the oculomotor and trochlear nuclei, in addition to other PAG sites such as the caudal PAGl and PAGvl columns, and the cuneiform nucleus. This resulting pattern and levels of activation of the PAG and cuneiform nucleus during cat exposure in PMd-lesioned animals is similar to those found in animals presenting risk assessment activities in a predatory context (see experiment 3). The putative pathway accounting for this pattern of PAG activation is likely to involve the VHMdm, which presents clear activation in response to cat exposure in PMd-lesioned animals and is known to project to the dorsal parts of the PAG, and the caudal PAGl and PAGvl columns (Canteras *et al.*, 1994).

Predatory context yielded over five-fold Fos increase in the PMd, in addition to a less intense, but significant, activation of the AHN. In line with the present observations, Staples *et al.* (2005) have also reported the PMd as the most responsive hypothalamic site to a context previously associated with cat odor. Recent findings from the Blanchards' laboratory indicate that ventral hippocampal lesions (including intermediate regions of field CA1 and subiculum) significantly reduced conditioned defensive behaviors during re-exposure to the context associated with either direct exposure to the cat or exposure to its odor (Pentkowski *et al.*, 2006). As shown in Fig. 8, the septohippocampal system, via the AHN, influences the medial hypothalamic circuit here shown to be activated in response to the predatory context, forming a potential pathway related to the predatory contextual conditioned responses. To test this hypothesis, in experiment 3, we examined how pharmacological blockade of the PMd affected contextual conditioning responses, and how this blockade affected the activation pattern of periaqueductal sites likely to organize the conditioned responses to the predatory context.

Muscimol injection into the PMd induced a clear drop in the nucleus Fos immunoreactivity, thus revealing that the PMd presents an ongoing activation in response to the environment previously associated with a cat. Importantly, animals that received muscimol injection into the medial mammillary nucleus presented a robust Fos expression of the PMd, indicating that these injections did not affect the pattern of activation of the PMd. Moreover, we have found that all animals in this experiment, injected with either muscimol or saline, presented a clear Fos upregulation in ependimal cells surrounding the third ventricle and the cerebral aqueduct, suggesting, for all experimental groups, a certain degree of leaking into the ventricular system. However, given the clear differences found in terms of the behavioral responses and the pattern of Fos labeling among the experimental groups, this possible leaking into the ventricular system did not seem to have any significant impact on the results.

Pharmacological inactivation of the PMd, but not of the nearby mammillary nuclei, was able to practically abolish the contextual conditioned responses. This sharp decline in the contextual conditioned defense was associated with a large decrease in Fos levels in the rostral half of the PAGdl and PAGdm columns and in the cuneiform nucleus, strongly suggesting that the activation seen in the dorsal PAG and the cuneiform nucleus should be related to risk assessment activities during the predatory contextual fear. Staples *et al.* (2005) reported that the context associated with cat odor activates PAGvl but not the PAGdl. A possible explanation for this apparent discrepancy is that cat odor-associated contexts represent much weaker threats than those associated with a live cat and, therefore, are likely to result in relatively small activation of the dorsolateral PAG.

In the medial hypothalamic zone, cat exposure also increased Fos expression in the DMH. A number of studies have implicated the DMH as mediating fear responses (Shekhar *et al.*, 1996). However, compared to the most responsive elements of the medial and lateral hypothalamic zones, the DMH presents a much smaller activation during cat exposure and doesn't seem to up-regulate Fos expression in response to the predatory context.

Inhibition of nondefensive behaviors is also part of the behavioral response to predatory threats (Blanchard & Blanchard, 1989). In this regard, it is noteworthy that animals killed after Phase 3 of the behavioral test presented increased Fos levels in the supraoptic nucleus and the median preoptic nucleus, likely to reflect a rise in plasma osmolarity due to inhibition of drinking behavior after the cat exposure. Behavioral tests were performed during the onset of the dark phase of the light/dark cycle, when the animals naturally present the early surge of food intake, which is normally associated with increased Fos expression in hypothalamic periventricular sites related to the homeostatic control of eating, such as the arcuate nucleus (see Saper *et al.*, 2002). Interestingly, rats exposed to the live predator or to the predatory context showed a significant decrease in Fos expression in the arcuate nucleus.

As summarized in Fig. 8, the present work showed that hypothalamic processing of the actual and the contextual predatory threats share the same circuit, where the PMd plays a pivotal role in amplifying this processing. The PMd, on the other hand, influences key sites underlying attentional and mnemonic processing, as well as unconditioned and conditioned behavioral responses. We have further suggested that both unconditioned and conditioned responses seem to depend upon the PAG, which presented a similar activation pattern in both situations, differing only in the level of activation, ranging from very high activation levels in responses to the actual predator to moderate to low activation levels during exposure to the predatory context.

Acknowledgements

This research was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, no. 01/14039-9 and 05/59286-4) and Conselho Nacional de Desenvolvimento Científico e Tecnologico awarded to N.S.C. A.F.C. and E.R.R.B. were supported by FAPESP fellowships.

Abbreviations

AHN, anterior hypothalamic nucleus; AMv, anteromedial nucleus thalamus, ventral part; ARH, arcuate nucleus; BMAp, basomedial nucleus amygdala, posterior part; CA1, field CA1; CUN, cuneiform nucleus; DMH, dorsomedial hypothalamic nucleus; fx, fornix; III, oculomotor nucleus; LA, lateral nucleus of the amygdala; LHAsf, lateral hypothalamic area, subfornical region; LS, lateral septal nucleus; MANOVA, multivariate ANOVA; MEA(pd or pv), medial nucleus of the amygdala (posterodorsal or posteroventral parts); NMDA, *N*-methyl-D-aspartate; opt, optic tract; PAG(dm, dl, l or vl), periaqueductal gray (dorsomedial, dorsolateral, lateral or ventrolateral columns); pm, principal mammillary tract; PMd, dorsal premammillary nucleus; PMv, ventral premammillary nucleus; PVH, paraventricular hypothalamic nucleus; PVp, periventricular hypothalamic nucleus, posterior part; SOM, supraoculomotor region; VMH(a, dm or vl), ventromedial hypothalamic nucleus (anterior, dorsomedial or ventrolateral).

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