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Review

Sensing danger through the olfactory system: The role of the hypothalamic dorsal premammillary nucleus

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ABSTRACT

The dorsal premammillary nucleus (PMd) has a critical role on the expression of defensive responses to predator odor. Anatomical evidence suggests that the PMd should also modulate memory processing through a projecting branch to the anterior thalamus. By using a pharmacological blockade of the PMd with the NMDA-receptor antagonist 2-amino-5-phosphonopentanoic acid (AP5), we were able to confirm its role in the expression of unconditioned defensive responses, and further revealed that the nucleus is also involved in influencing associative mechanisms linking predatory threats to the related context. We have also tested whether olfactory fear conditioning, using coffee odor as CS, would be useful to model predator odor. Similar to cat odor, shock-paired coffee odor produced robust defensive behavior during exposure to the odor and to the associated context. Shock-paired coffee odor also up-regulated Fos expression in the PMd, and, as with cat odor, we showed that this nucleus is involved in the conditioned defensive responses to the shock-paired coffee odor and the contextual responses to the associated environment.

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1. Introduction

Predator-derived odors can be highly effective stimuli for eliciting defensive behaviors in rodents, and this has led to their increasing use in neurobiological and behavioral studies of unconditioned and conditioned fear (for review see Apfelbach et al., 2005). Two of the most widely used predator odors in recent research are cat fur/skin odor and trimethylthiazoline (TMT).

Cat odor is a natural odor obtained from domestic cats, and is usually presented to rats in the form of a worn collar or a cloth that has been rubbed against cat fur/skin (Blanchard et al., 1990; Zangrossi and File, 1992; Dielenberg et al., 1999). Acute exposure of

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rats to cat odor causes significant and relatively long-term reduction in overall locomotor activity, reduces non-defensive behaviors, such as grooming and reproduction, and produces robust defensive responses, despite the animals having never previously encountered a cat (Blanchard et al., 1990; Zangrossi and File, 1992; Dielenberg et al., 1999; McGregor et al., 2002). When exposed to the cat odor, these rats exhibit a variety of risk assessment behaviors directed towards the predatory stimulus (Blanchard et al., 1990; Zangrossi and File, 1992; Dielenberg et al., 1999; McGregor et al., 2002). Rats will also readily learn to avoid stimuli and places that are associated with the odor (Blanchard et al., 2001; Hubbard et al., 2004; Staples et al., 2005; Staples and McGregor, 2006).

Over the last years, a great deal has been learned about the neural system involved in processing innate defensive behaviors to a predator or its odor. Predator odors may in fact be processed by prey species in the Accessory Olfactory Bulb (AOB), rather than the Main Olfactory Bulb (MOB) (McGregor et al., 2004). This suggests that cat odor is processed by rats more as a pheromone than a conventional odor, and the authors suggested that cat odor may be an example of a 'kairomone'—a semiochemical released by one species that has a favorable adaptive effect on a different 'receiving' species (Dicke and Grostal, 2001). The AOB projects principally to the medial amygdala, and rats exposed to cat odor also show substantial activation in this nucleus, particularly in its posteroventral part (Dielenberg et al., 2001; McGregor et al., 2004). In line with this view, rats with cytotoxic lesions in the medial nucleus, but not in the central nucleus, exhibited a significant reduction in unconditioned fear responses to cat odor (Li et al., 2004). During exposure to a live predator, in addition to activation of the posteroventral part of the medial amygdalar nucleus, we have also observed a distinct Fos increase in two other amygdalar sites, namely, the posterior basomedial amygdalar nucleus and caudal levels of the lateral amygdalar nucleus (Canteras et al., 2001). Importantly, these amygdalar nuclei receive inputs from visual and auditory association areas, and are likely to integrate predator-derived sensory clues, other than olfactory ones (McDonald, 1998). The amygdalar sites related to predator detection project either directly or indirectly, via the transverse nucleus of the bed nuclei of the stria terminalis, to the ventromedial nucleus of the hypothalamus, where its dorsomedial part receives most of the direct projections from the amygdala and is particularly mobilized during exposure to a live predator or its odor (Canteras et al., 1997, 2001; Dielenberg et al., 2001).

The amygdalar sites involved in detecting predator cues, in particular the lateral and posterior basomedial nuclei, also provide inputs to hippocampal field CA1 and subiculum, which are likely to be involved in some aspects of the association between these predator threats and the environment where the animal encounters this stimulus (Petrovich et al., 2001). Hippocampal processing, via projections to the lateral septal nucleus, may influence the anterior hypothalamic nucleus, which also up-regulates Fos expression during predator exposure (Canteras et al., 1997). Both the anterior hypothalamic nucleus and the dorsomedial part of the ventromedial nucleus project to the dorsal premammillary nucleus (PMd) (Canteras et al., 1994; Risold et al., 1994), one of the brain regions mostly responsive to a predator or its cues, and where lesions have been most effective in reducing anti-predator defensive responses (Canteras et al., 1997; Blanchard et al., 2003a, 2003b, 2005; Markham et al., 2004). The anterior hypothalamic nucleus, the dorsomedial part of the ventromedial hypothalamic nucleus, and the PMd nucleus are particularly interconnected, forming a partially segregated circuit in the medial zone of the hypothalamus, the so-called medial hypothalamic defensive circuit (Canteras, 2002). Notably, the PMd appears to work as an amplifier for the neural processing in the medial hypothalamic defensive circuit. This would explain why this region is so responsive to predator threats, and why lesions therein are able to reduce defensive responses so drastically (Canteras et al., 1997; Blanchard et al., 2003a, 2003b, 2005; Markham et al., 2004).

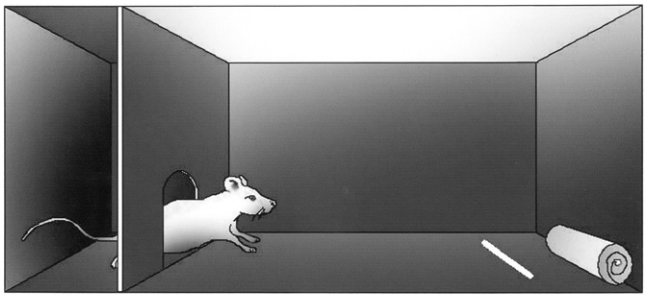
Given the pivotal role of the PMd in the neural processing of anti-predatory defensive responses, we presently investigated how the pharmacological blockade of the nucleus interferes with unconditioned and contextual conditioned responses to cat odor.

In addition to cat odor, TMT, a synthetic compound isolated from fox feces, has been used to mimic predator odor. TMT has the advantages of being commercially available and easily quantifiable (for a review see Fendt et al., 2005), and elicits avoidance and freezing in rats (Wallace and Rosen, 2000; Fendt et al., 2005). However, in contrast to cat odor, it does not elicit characteristic defensive responses such as risk assessment or fear conditioning (McGregor et al., 2002; Blanchard et al., 2003a, 2003b; Staples and McGregor, 2006). The lack of conditioning to TMT may be related to the fact that predator feces, or its compounds, are poorly predictors of the actual predator presence. In fact, as TMT has a strong aversive odor, its limited behavioral effects may be due more to its noxious qualities, rather than its abilities to signal a predator threat. Moreover, this compound lacks the "pheromone-like" quality that engages key hypothalamic sites involved in defensive behavior (Day et al., 2004).

By and large, shock-based Pavlovian conditioning is the most common experimental approach used to investigate the neural basis of fear. The phenomenon of Pavlovian fear conditioning is highly reproducible, and it generates clearly measurable responses, such as freezing and startle, both of which seen in the repertoire of animals confronting predator threats. However, shock-based fear conditioning to non-olfactory cues does not appear to engage elements of the hypothalamic defensive system, and, therefore, seems inadequate to predict the neural processing engaged on natural fear responses observed during cat odor exposure (see Canteras and Blanchard, 2008). In this regard, the present study was outlined to verify whether the shock-based fear conditioning to olfactory cues involves the same hypothalamic structures, particularly the PMd, known to be activated during anti-predator fear responses (Canteras et al., 1997; Dielenberg et al., 2001). For this purpose, we investigated through immunohistochemical studies the activation of the PMd during exposure to an olfactory stimulus previously paired with electrical footshocks. Next, we examined if pharmacological blockade of the PMd during the conditioned olfactory cue exposure affects the conditioned defensive responses to both the odor and the context where the odor had been presented. In this way, we were able to verify to what extent the neural processing of a footshock-paired odor and cat odor share similar neural circuits.

2. Does the pharmacological blockade of the PMd during exposure to cat odor interfere with unconditioned and contextual conditioned responses?

As previously mentioned, the PMd is one of the hypothalamic sites most responsive to cat odor, and electrolytic and neurotoxic lesions therein produced a robust reduction in defensive behaviors to a live cat or its odors, but had minimal effects on nonpredator threat stimuli, such as an elevated plus maze and postshock contextual cues (Canteras et al., 1997; Blanchard et al., 2003a, 2003b, 2005; Markham et al., 2004). Curiously, the PMd gives rise to a branched pathway ending in the anterior thalamic group or the brainstem (Canteras and Swanson, 1992). In the brainstem, the nucleus provides heavy inputs to the periaqueductal gray, which seems critical for the expression of defensive responses. In the anterior thalamus, the PMd projects massively to the ventral part of the anteromedial nucleus. Previous studies have shown that the



Cat Odor Exposure Procedure

Day 1 – Familiarization – neutral odor exposure – 10 min session
Day 2 – Test Session – cat odor exposure – 10 min session
Day 3 – Context Session – neutral odor exposure – 10 min session

Fig. 1. Representation of the Odor Box (40 cm × 26 cm × 40 cm modified from Dielenberg and McGregor, 1999) composed of a roofed-enclosed compartment (left side) and an open compartment (right side). On the opposite side of the enclosed compartment, a cloth, working as neutral or cat odor source, was fixed within a 7 cm extension region limited by a white stripe. In the box above, it is outlined the cat odor exposure procedure testing direct exposure and exposure to the cat odor-associated context.

ventral part of the anteromedial nucleus projects to the lateral retrosplenial area, thought to be involved in modulating the eye and head movements associated with attentional processes (Risold and Swanson, 1995). In addition, the retrosplenial area is also associated with the hippocampal formation, known to be critically involved in contextual memory processing, and a growing body of evidence has also suggested a key role for the anterior thalamic nuclei in contextual memory mechanisms (Vann and Aggleton, 2004). Taking these findings together, it is tempting to suggest that the path comprising the PMd and ventral anteromedial thalamic nucleus would play an important role in the emotional memory possessing to predator threats, perhaps influencing contextual conditioning to predator cues. Therefore, the PMd seems to occupy a strategic position to control both emotional mnemonic processing and anti-predatory defensive behavior. To test this hypothesis, we examined how the pharmacological blockade of the PMd during cat odor exposure interferes with the unconditioned responses to predator odor, as well as the contextual conditioned responses to the environment previously associated with cat odor.

Knowledge about cellular communications within the PMd is very poor, however previous studies have suggested increased glutamatergic transmission in the PMd during exposure to a live cat (Beijamini and Guimarães, 2006). In the present study, we worked with the hypothesis of a glutamatergic mediation in the PMd, and tested 3-month-old Wistar rats that received an NMDA-receptor antagonist within the PMd before being exposed to a cat odor stimulus. The task was performed in a box made up of black Plexiglas (Fig. 1-Odor Box) comprising an open and an enclosed (roofed) compartment. A 6 cm × 6 cm open door allowed the rat to move both ways through the compartments. During test session, a cloth that had been rubbed against a cat's back fur/skin was used as the odor source and placed against the wall opposite to the transition door. The following defensive responses were measured during the exposure to the odor box: the amount of time the rats spent near (within 7 cm) of the odor source (approach time); the amount of time spent in the enclosed compartment (hide time); and the amount of time spent stretching out from the enclosed compartment towards the open compartment (head-out). The sessions lasted 10 min and were carried out in a low illumination room (4 lux).

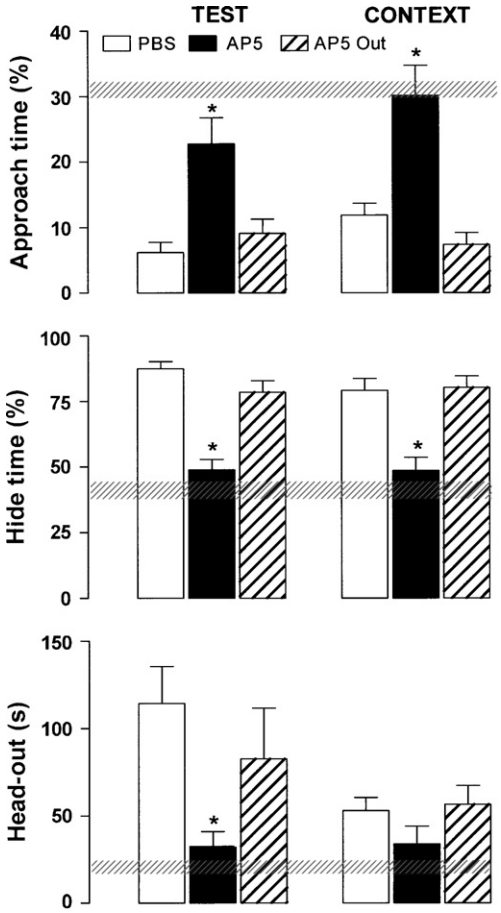


Fig. 2. Behavioral data on the cat exposure procedure. The parameters analyzed were the percentage of approach and hide times, and the total amount of time the animals spent stretching out from the enclosed compartment towards the open compartment (head-out time). The familiarization, the test, and the context sessions were conducted during three consecutive days. The hatched horizontal bars represent the mean and the confidence limits (within 95%) for the familiarization session. Subjects received AP5 (6 nmol/0.2 μl; $n = 7$) or PBS ($n = 9$) bilateral injections into the PMd 10 min before the test session. Histological results revealed a group of animals ($n = 10$) with AP5 injections outside the PMd—the AP5-out group. Histograms represent the test and the context data, and were expressed as mean + S.E.M. $^*P < 0.05$ compared to the PBS control group (repeated measures ANOVA; Duncan *post hoc* test).

The experimental procedure used consisted of three sessions, each spaced 24 h apart: familiarization, cat odor exposure and context. In the familiarization sessions, all rats were allowed to habituate to the apparatus, and baseline levels of behavioral parameters were measured in the presence of a neutral odor cloth. Prior to the cat odor exposure (test session), rats were divided into two groups: the control group (PBS) and the, *N*-methyl-D-aspartate (NMDA) receptor antagonist, (±)-2-amino-5-phosphonopentanoic acid (AP5) group. Rats received bilateral microinjections (0.2 μl) of AP5 (6 nmol) or phosphate buffered saline (PBS) into the PMd 10 min before being placed in the chamber in the presence of cat odor. The AP5 dose was chosen based on previous studies (Carobrez and Kincheski, 2005; Nascimento Häckl and Carobrez, 2007). During the context session, all rats were placed in the odor box in the presence of a neutral odor cloth. This latter phase allowed the determination of any conditioned avoidance occurring as a result of the pairing of cat odor with the environment during its exposure. All the sessions were recorded on a DVD system and the behavioral measures were further analyzed by an experimenter blind to the treatment groups.

As shown in Fig. 2, PBS-group subjects confronted with the cat odor were able to show a full range of defensive behavior

characterized by a reduced approach time, and an increased hide-time and head-out time. Subjects receiving AP5 in the PMd presented a significantly reduced defensive behavior, spending more time approaching the cat odor source and less time hiding or stretching out from the enclosed compartment than its counterpart control PBS-group or subjects receiving AP5 bilateral injections in sites positioned outside the PMd, mostly in the posterior hypothalamic area (AP5-out group). Subjects from the PBS-group also displayed defensive responses when re-exposed to the same chamber during a context session. Although none of the subjects received further treatment after the cat odor exposure, during the context session 24 h later, rats from the PBS and AP5-out groups continued to exhibit high levels of defensive behavior to the contextual environment, while the AP5-group did not. No differences were detected in the defensive responses elicited in the PBS when compared to the AP5-out group, suggesting that the reduction of fear responses toward cat odor or its context is due to a specific blockade in NMDA receptors restricted to the PMd.

Corroborating previous findings with electrolytic and neurotoxic lesions in the PMd (Blanchard et al., 2003a, 2003b, 2005; Markham et al., 2004), these data show that blocking the NMDA mediated neurotransmission of the nucleus drastically reduced unconditioned defensive responses to cat odor. Of particular relevance, we were also able to confirm that the PMd seems to be a critical site to influence associative mechanisms linking predatory threats to the related context. One could argue that by blocking the aversive response there would be no aversive experienced to be remembered. However, previous studies from our lab have shown that PMd lesions do not seem to affect predator detection, and therefore, PMd-lesioned animals certainly have the aversive experience to the predator presence (Cezario et al., submitted). As previously discussed, the most likely pathway influencing mnemonic mechanisms linking predatory threats to the associated context is the PMd projecting branch to the ventral anteromedial thalamic nucleus, but further studies are obviously needed to investigate this hypothesis. This relationship between the PMd and memory processing perhaps helps to explain why cat odor, which induces a striking PMd activation, produces such a robust conditioned defensive behavior, while TMT, which does not activate the PMd, fails to elicit conditioned responses (McGregor et al., 2002; Blanchard et al., 2003a,b; Staples and McGregor, 2006).

3. Does the hypothalamic defensive system support fear conditioning using a neutral stimulus as CS?

3.1. Olfactory fear conditioning

Fear conditioning acquisition occurs when an initially neutral stimulus is paired with a biologically significant aversive event, the unconditioned stimulus (US). After one or a few pairings, the neutral stimulus (CS) can acquire affective properties eliciting defensive responses typically occurring in the presence of the US (LeDoux, 2000). Investigations of fear conditioning in rodents usually employ auditory or visual CSs paired with electric footshock. However, recent studies have used odor as a conditioned stimulus, since rodents rely essentially on olfaction for feeding, social recognition, reproduction, and predator detection (Restrepo et al., 2004).

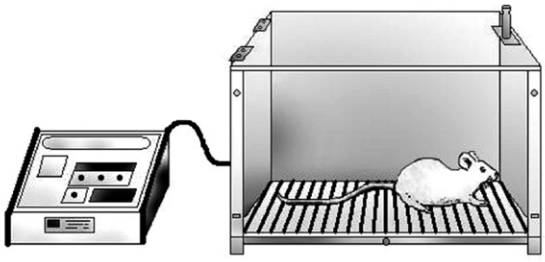
3.2. The acquisition and expression of olfactory fear conditioning: behavioral studies

The usefulness of odors as a conditioned stimulus for producing conditioned fear is already well established. Otto et al. (1997, 2000) reported that rats exhibit robust and long-lasting freezing responses to an odorant that had previously been paired with a brief, mild footshock. In addition, it has been shown that odor cues serve as effective conditioned stimuli (CSs) for potentiating the acoustic startle response (Richardson et al., 1999; Paschall and Davis, 2002).

Data from our laboratory confirmed that an olfactory stimulus, such as coffee odor, can effectively serve as a CS in a fear conditioning paradigm. The experimental paradigm used consisted of two consecutive phases: the acquisition of olfactory fear conditioning (days 1 and 2) and the expression of olfactory fear conditioning (days 3–5). All sessions were spaced 24 h apart.

The acquisition of olfactory fear conditioning (first phase) was performed in a conditioning box (Fig. 3) and consisted of two sessions (24 h apart), each lasting 3 min 20 s in total duration. The conditioning box (Fig. 3) was constructed with stainless steel walls and a grid floor composed of 1 cm spaced stainless steel bars connected to a shock generator (Insight, Ribeirão Preto, SP, Brazil). A 15 g amount of coffee powder (Melitta Tradicional®, Brazil) was uniformly distributed in a compartment under the grid floor which served as the olfactory stimulus. Three training conditions were used in this phase: (1) coffee odor alone; (2) non-paired group (coffee and footshock in two alternate days); and (3) paired group (coincident coffee odor + shock; 5 trials; 40 s inter-trial period; 0.4 mA/2 s footshock). Subjects in the coffee odor alone and in the paired groups were placed in the conditioning box and allowed to explore freely on day 1, during a familiarization session. On the following trial, the paired group returned to the conditioning box, where it received five pairings of coffee odor and footshock (US). The coffee odor alone group was placed in the conditioning box, where olfactory stimulus was presented without the footshock. The subjects in the non-paired group were first placed in the conditioning box, where they received five brief footshocks on the same schedule as the paired group, but without odor presentation. On the next day, these subjects returned to the conditioning box, where coffee odor was presented without the footshock.

The expression of olfactory fear conditioning (second phase) was performed in the Odor Box (Fig. 1), which was previously described, and consisted of three sessions: familiarization (day 3), coffee odor exposure (day 4; test session) and context (day 5), as outlined in Fig. 3. The same procedures, behavioral measures and scores previously described for the cat odor experiment were applied in this phase.



Olfactory Fear Conditioning Schema

Phase A: Acquisition in the Conditioning Box
Day 1 – Familiarization – 3.20 min, spontaneous exploration
Day 2 – Conditioning – 5 trials; 40 s inter-trial period; 0.5 mA/2 s footshock

Phase B: Expression in the Odor Box (Fig 1)
Day 3 – Familiarization – 10 min spontaneous exploration neutral odor exposure
Day 4 – Context Conditioning – 10min spontaneous exploration CS-odor exposure
Day 5 – Context Expression- 10min spontaneous exploration neutral odor exposure

Fig. 3. Representation of the Conditioning Box (50 cm × 26 cm × 35 cm). In the box above, it is outlined the olfactory conditioning fear procedure with the acquisition and the expression phases.

As illustrated in Fig. 4, none of the groups exhibited fear responses to the Odor Box during the familiarization session, suggesting that subjects did not generalize the fear conditioning to a different context. When re-exposed to the coffee odor, in the Odor Box, only those rats who had previously received pairings of coffee odor and electrical footshock (paired group) exhibited a full range defensive behavior, and spent most of the time either hiding or engaged in 'head-out' behavior, and presented a significant reduction in the time approaching the odor source (less than 15% of the entire observation period). Neither the non-paired group nor the coffee odor alone group showed significant different values between the familiarization and test sessions.

These results showed a robust expression of the fear conditioning, which is selective to subjects receiving pairings of the odorant and the electrical footshock, reflecting an effective CS–US association. When returned to the same context in the absence of coffee odor, the paired group showed similar patterns of defensive behavior, as on the previous day, when coffee odor had been presented in that context, with increased hiding and stretching head-out, and reduced approach toward the neutral cloth (Fig. 4). These results confirm the biological relevance acquired by the CS (coffee odor), which, similarly to cat odor, was able to produce a conspicuous contextual conditioned defensive behavior.

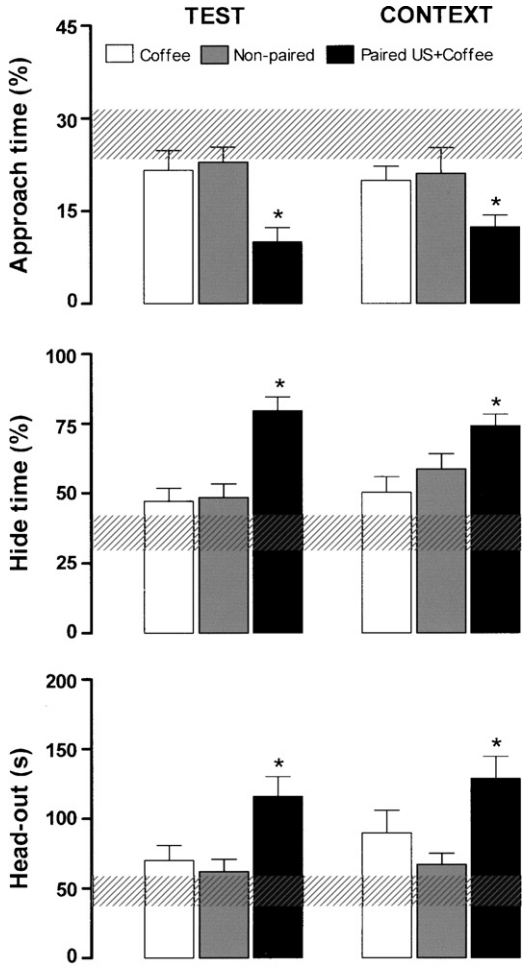


Fig. 4. Behavioral data on the olfactory fear conditioning procedure. The parameters analyzed were the percentage of approach and hide times, and the total amount of time the animals spent stretching out from the enclosed compartment towards the open compartment (head-out time). The familiarization, the test, and the context sessions were conducted during three consecutive days. The hatched horizontal bars represent the mean and the confidence limits (within 95%) for the familiarization sessions. Subjects were grouped according to the training conditions during the acquisition phase—(1) coffee odor alone (clear histogram; $n = 10$); (2) non-paired group (grey histogram; $n = 12$; coffee and footshock in two alternate days); and (3) paired group (black histogram; $n = 12$; coincident coffee odor + shock; 5 trials; 40 s inter-trial period; 0.4 mA/2 s footshock). Histograms represent the test and context session data during the expression phase of the experimental procedure, and were expressed as mean + S.E.M. $^*P < 0.05$ compared to the PBS control group (repeated measures ANOVA; Duncan *post hoc* test).

3.3. Participation of the dorsal premammillary nucleus (PMd) in the olfactory fear conditioning paradigm: immunohistochemical and pharmacological studies

Considering that rodents use odors better than any other sensory cues to predict the predator presence, we decided to examine whether the medial hypothalamic defensive system, in particular the PMd, would be involved in learned fear responses to a neutral odor before and after it was paired with an unconditioned aversive stimulus. In order to investigate whether the olfactory fear conditioning engages elements of the medial hypothalamic defensive system, we first examined the PMd Fos immunoreactivity in response to coffee odor alone, and in response to coffee odor previously paired to a footshock (five trials; 40 s inter-trial; 0.4 mA; 2 s). In this experiment, we used the same schedule as previously described for the paired group and the coffee-odor alone group.

Ninety minutes after the test session, each animal was deeply anesthetized with sodium pentobarbital (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 4 °C. 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 and Raine, 1981). In brief, sections were incubated for 90 min at room temperature in a solution of biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA), and then placed in the mixed avidin–biotin horseradish peroxidase (HRP) 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 (DAB, 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 (KPBS; 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. Counts of the number of Fos immunoreactive (Fos-ir) neurons as a function of experimental status were generated for the PMd by using the 10× objective of a Nikon Eclipse E600 microscope equipped with a camera lucida. For a cell to be considered as expressing Fos-like immunoreactivity, the nucleus of the neurons had to be of appropriate size (ranging approximately from 8 to 15 mm) and shape (oval or round), show the characteristic blue-black staining of oxidized DAB-Ni, and be distinct from the background at magnification of 10×. For each animal, Fos-positive cells were plotted and counted at three distinct rostrocaudal levels of the PMd (120 μm apart).

Animals exposed to coffee odor previously associated with footshocks exhibited clear defensive responses, and as illustrated

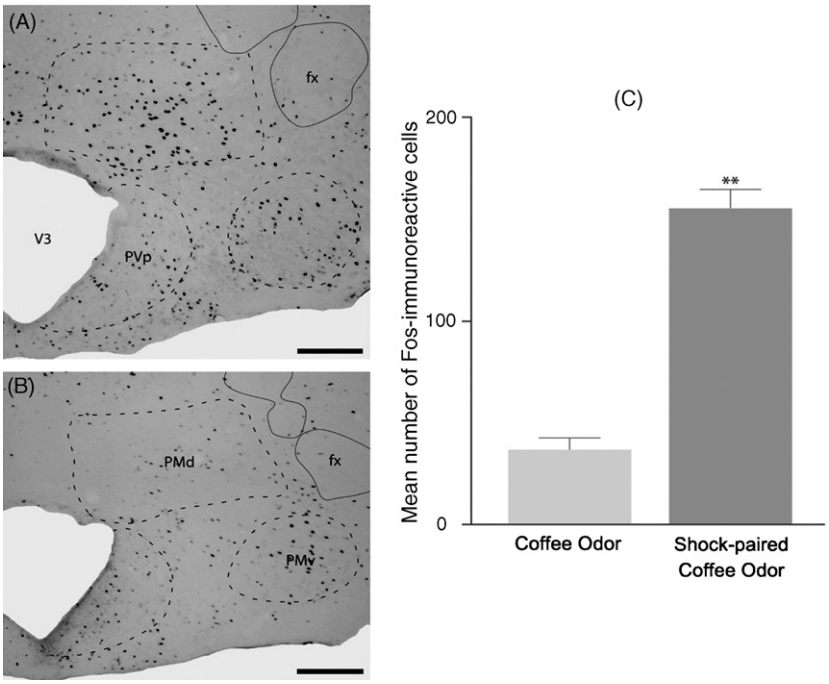


Fig. 5. Photomicrographs of transverse Fos-stained sections, at the level of the dorsal premammillary nucleus, from rats exposed to coffee odor previously paired to footshock (A) or to coffee odor alone (B). (C) Histograms showing the mean number of Fos-immunoreactive cells counted in the PMd from animals exposed to coffee odor alone (coffee odor, $n = 6$, 10 min exposure) and from animals exposed to coffee odor previously paired to footshock (shock-paired coffee odor, $n = 6$, 10 min exposure). Data are expressed as mean + S.E.M. $^{**}P < 0.01$ (two-tailed unpaired t-test). Abbreviations—fx: fornix; PMd: dorsal premammillary nucleus; PMv: ventral premammillary nucleus; PVp: periventricular nucleus, posterior part; V3: third ventricle. Scale bars = 200 μm.

in Fig. 5, up-regulated Fos expression in the PMd. Therefore, similarly to what had been found for predator odor, the PMd also seems to respond to primary neutral odors that had gained an aversive valence by being previously associated with a noxious stimulus.

To further investigate a possible role of the PMd in the olfactory fear conditioning expression, AP5 or vehicle was infused into this nucleus prior to re-exposure to the footshock-paired coffee odor. As previously described, the experimental procedures consisted of two phases: the acquisition of olfactory fear conditioning and the expression of olfactory fear conditioning. Prior to the expression of olfactory fear conditioning, rats were divided into two groups: the control group (PBS) and the AP5 (6 nmol, 0.2 μl) group. Rats were microinjected into the PMd and, 10 min later, placed in the apparatus in the presence of coffee odor. As shown in Fig. 6, in contrast to the other experimental groups (the PBS and the AP5-out groups), animals treated with AP5 into the PMd (AP5 PMd group) showed a significant decrease in the hiding time during exposure to footshock-paired coffee odor, as well as a clear impairment in contextual defensive responses on the following day, when the animals presented a significant increase in the time approaching the neutral cloth, as well as a decreased hide time.

Although less striking than the effects found for cat odor, the NMDA-receptor blockade of the PMd significantly reduced conditioned fear responses to coffee odor. Interestingly, the PMd does not seem to participate in the fear conditioning responses to other sensory modalities, since shock-based fear conditioning to auditory stimulus does not engage the PMd (Pezzone et al., 1992). Therefore, the present finding provides an interesting perspective that, depending on the sensory modality used as a CS, somewhat distinct pathways appear to mediate shock-based fear conditioning. Of particular interest, we have also found that the NMDA-receptor blockade of the PMd was able to block contextual conditioning to footshock-paired coffee odor, giving further support to the idea that the neural processing of an odor that had gained threatening status in, shock-based fear conditioning, and the actual predator odor may share similar neural circuits.

3.4. How does the PMd mediate fear conditioning arising from olfactory stimuli?

An important conclusion to be drawn from the present study is that, among the different approaches using learned fear, olfactory fear conditioning seems to be the only one that actually shares some common paths with those underlying natural anti-predator fear responses. Differently from the predator odor, neutral odors are likely to be processed by the main olfactory bulb, and a key question is to understand how neutral odors previously paired to threatening situations are able to mobilize the PMd.

Studies on the neural basis of Pavlovian conditioned fear indicate the amygdala as a major player in the learning of fear conditioning. As shown in Fig. 7, associative learning between the conditioned and unconditioned stimuli is likely to occur in the lateral nucleus. In fact, both acquisition and retention of fear conditioning occur in the lateral nucleus, where electrolytic and excitotoxic lesions, as well as pharmacological blockade, prevent acquisition and expression of fear conditioning (Campeau and Davis, 1995; Muller et al., 1997; LeDoux, 2000; Gale et al., 2004). In line with this view, Kilpatrick and Cahill (2003) have shown that tetrodotoxin infusion in the region of the lateral nucleus, immediately following odor-shock pairings, impaired the acquisition of odor fear conditioning. Notably, the lateral nucleus may influence the medial hypothalamic defensive system through its dense projections to the posterior part of the basomedial nucleus, which represents an important afferent source to the ventromedial hypothalamic nucleus (Petrovich et al., 1996). Conversely, the lateral nucleus also projects to the hippocampal field CA1 and subiculum (Petrovich et al., 2001), which, via projections to the

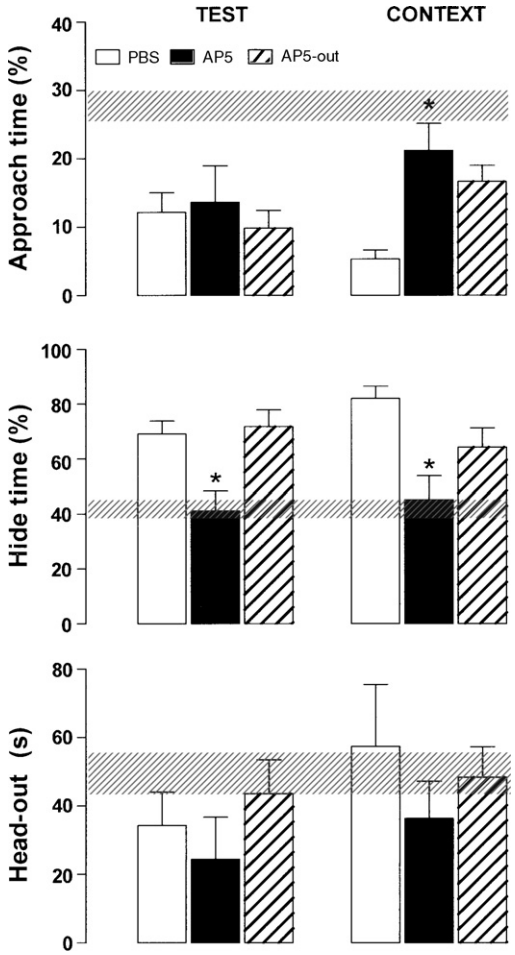


Fig. 6. Behavioral data showing the role of the dorsal premammillary nucleus (PMd) in the expression phase of the olfactory fear conditioning paradigm. The parameters analyzed were the percentage of approach and hide times, and the total amount of time the animals spent stretching out from the enclosed compartment towards the open compartment (head-out time). The familiarization, the test, and the context sessions were conducted during three consecutive days. The hatched horizontal bars represent the mean and the confidence limits (within 95%) for the familiarization session. Subjects received AP5 (6 nmol/0.2 μl; $n = 6$) or PBS ($n = 7$) bilateral injections into the PMd 10 min before the test session. Histological results revealed a group of animals ($n = 6$) with AP5 injections outside the PMd—the AP5-out group. Histograms represent the test and the context data, and were expressed as mean + S.E.M. $^*P < 0.05$ compared to the PBS control group (repeated measures ANOVA; Duncan *post hoc* test).

lateral septal nucleus, may also reach the anterior hypothalamic nucleus (Risold and Swanson, 1996), another component of the medial hypothalamic defensive system. Fig. 7 summarizes the putative links between the systems that provide threatening status to a previously neutral odor and those primarily involved in processing predator odor. At this point, further studies are needed

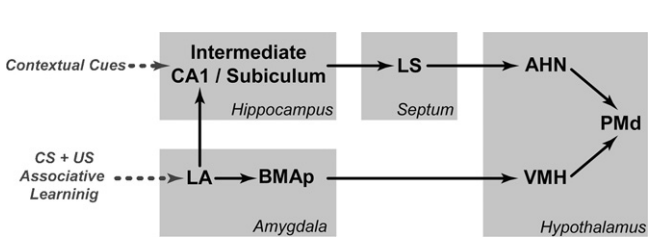


Fig. 7. Schematic diagram showing the putative links between the systems that provide threatening status to a previous neutral odor and the elements of the medial hypothalamic defensive system primarily responsive to predator odor.

to improve the understanding of these paths, especially concerning the PMd outputs to the periaqueductal gray and their relationship with olfactory fear conditioning.

4. Concluding remarks

In the present work, we have confirmed the PMd's role in the expression of unconditioned defensive responses to cat odor, and further revealed that the nucleus is also involved in influencing associative mechanisms linking predatory threats to the related context.

We have also shown that a neutral odor previously associated with a noxious stimulus shares a lot of similarities with cat odor. Similar to cat skin/fur odor, shock-paired neutral odor produced robust defensive behavior during both direct exposure and exposure to the context where this odor had been presented. In addition, as for cat odor, shock-paired neutral odor also up-regulates Fos expression in the PMd, and a pharmacological blockade therein, immediately previous to an exposure to shock-paired neutral odor, interfered with defensive responses observed during direct exposure to the odor and exposure to the associated context, as well.

On the whole, we have confirmed and extended the critical PMd role in anti-predatory defensive behavior, and suggested that the use of olfactory cues as CSs, in shock-based fear conditioning, provides a more realistic model to study natural fear responses observed during cat odor exposure, engaging the medial hypothalamic defensive system.

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