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ORIGINAL ARTICLE

The rostrodorsal periaqueductal gray influences both innate fear responses and acquisition of fear memory in animals exposed to a live predator

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Abstract

A few studies have evaluated the behavioral roles of the periaqueductal gray (PAG) in animals facing ethologically relevant threats. Exposure to a live cat induces striking activation in the rostrodorsal and caudal ventral PAG. In the present investigation, we first showed that cytotoxic lesions of the rostrodorsal and caudal ventral PAG had similar effects on innate fear responses during cat exposure, practically abolishing freezing and increasing risk assessment responses. Conversely, rostrodorsal PAG lesions but not caudal ventral lesions disrupted learned contextual fear responses to cat exposure. Next, we examined how muscimol inactivation of the rostrodorsal PAG at different times (i.e., during, immediately after and 20 min after cat exposure) influences learned contextual fear responses, and we found that inactivation of the rostrodorsal PAG during or immediately after cat exposure but not 20 min later impaired contextual fear learning. Thus, suggesting that the rostrodorsal PAG is involved in the acquisition, but not the consolidation, of contextual fear memory to predatory threat. Notably, the dosolateral PAG contains a distinct population of neurons containing the neuronal nitric oxide synthase (nNOS) enzyme, and in the last experiment, we investigated how nitric oxide released in rostrodorsal PAG influences contextual fear memory processing. Accordingly, injection of a selective nNOS inhibitor into the rostrodorsal PAG immediately after cat exposure disrupted learned contextual responses. Overall, the present findings suggest that the acquisition of contextual fear learning is influenced by an optimum level of dorsal PAG activation, which extends from during to shortly after predator exposure and depends on local NO release.

Keywords Defensive behavior · Fear memory · Innate fear · Nitric oxide

Introduction

The PAG has been thought to influence a wealth of behavioral responses to predatory threats, ranging from innate defensive responses to fear memory processing (Motta et al. 2017). A number of studies have shown that stimulation of the PAG induces a pattern of responses that resembles the behavior of animals facing a natural predator. After a predatory encounter, rodents express the species-typical defense response of freezing (postencounter defense), and as predatory imminence and the potential of attack increases, rodents switch from freezing to a circa-strike defense, including escape attempts and jumps (Blanchard et al. 1989; Fanselow 1991). Accordingly, electrical, pharmacological and optogenetic stimulation of the dorsomedial, dorsolateral, and lateral columns of the PAG have been shown to produce both postencounter (i.e.,

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freezing) and circa-strike defensive behaviors (i.e., escape and flight behavior) in the absence of a predatory threat (Carrive 1993; Bandler and Keay 1996; Bittencourt et al. 2004; Assareh et al. 2016; Deng et al. 2016). Conversely, the ventrolateral PAG has been shown to influence freezing responses in fear conditioning to physically aversive stimuli (LeDoux et al. 1988; Tovote et al. 2016) and to mediate immobility during the post defense recuperative-like quiescent behavior (Carrive 1993; Bandler and Keay 1996; Bittencourt et al. 2004; Assareh et al. 2016).

Apart from organizing innate fear responses to predatory threats, the PAG has also been shown to influence fear learning. A number of studies using classical fear conditioning to sound-, light- or odor-conditioned stimuli (CSs) have shown that electrical, chemical or optogenetic stimulation of the dorsal PAG may be used as a useful unconditioned stimulus (US) to support associative learning (Di Scala et al. 1987; Di Scala and Sandner 1989; Kincheski et al. 2012; Kim et al. 2013; Deng et al. 2016).

However, only a few studies have evaluated the behavioral roles of the PAG in the face of ethologically relevant threats. In this regard, NMDA antagonist injection into the dorsal PAG has been shown to reduce innate defensive responses and impair the acquisition of contextual fear to cat odor (Souza and Carobrez 2016). In the present study, we examined the general roles of the PAG in unconditioned and learned contextual fear responses in animals exposed to a live predator.

The PAG is organized in longitudinally organized functional columns, namely, the dorsomedial, dorsolateral, lateral, and ventrolateral columns (Carrive 1993; Bandler and Shipley 1994; Bandler and Keay 1996), and exposure to a live cat induces striking activation in the dorsomedial and dorsolateral columns at the levels of the nucleus of Darkschewitsch and oculomotor nucleus (rostrodorsal PAG) and in the lateral and ventrolateral columns at the levels of the dorsal raphe nucleus (caudal ventral PAG) (Cezário et al. 2008). Thus, in the present study, the rostrodorsal and caudal ventral PAG were specifically targeted to study the role of the PAG in innate and learned contextual responses to predatory threat. First, we applied cytotoxic lesions in the rostrodorsal or caudal ventral PAG and examined the innate fear responses during cat exposure. The results of the cytotoxic PAG lesions also suggested that the rostrodorsal but not the caudal ventral PAG influence contextual fear learning. Next, we examined how muscimol inactivation of the rostrodorsal PAG at different times (i.e., during, immediately after or 20 min after cat exposure) influences learned contextual fear responses. In the last experiment, using a selective neuronal nitric oxide synthase inhibitor (*N*-propyl-L-arginine), we investigated how nitric oxide released in the rostrodorsal PAG immediately after cat exposure may influence contextual fear memory processing.

Overall, this study provides important information that enhances the understanding of the role of the PAG in innate and contextual fear to ethologically relevant threats and should be taken into account in the studies of pathologic fear and fear learning conditions, such as panic and post-traumatic stress disorders.

Materials and methods

Animals

Adult male Wistar rats ($n = 82$), weighing approximately 250 g and obtained from the local breeding facilities, were used in the present study. The animals were maintained under controlled temperature (23 ± 2 °C) and illumination (12 h cycle; lights on—10 a.m./lights off—10 p.m.) in the animal quarters, and, before the animals were placed in the experimental apparatus, they had free access to water and standard laboratory diet.

Ethics

Experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, 1996). All experimental procedures had been previously approved by the Committee on The Care and Use of Laboratory Animals of the Institute of Biomedical Sciences, University of São Paulo, Brazil (Protocol number 085/2012). In the present study, the experiments were planned to minimize the number of animals used and their suffering. In addition, all surgical procedures were performed under deep anesthesia, and analgesic and antibiotic medication were given postoperatively.

Experimental apparatus and procedure

The experimental protocol currently used to investigate innate and learned contextual fear related to predator exposure followed Ribeiro-Barbosa et al. (2005). The experimental apparatuses were made of clear Plexiglas. Each consisted of a $25 \times 25 \times 25$ cm^3 home cage connected to another $25 \times 25 \times 25$ cm^3 chamber (the food compartment) by a hallway 12.5 cm wide and 80 cm long, with walls 25 cm high. 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. During 10 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 two phases—cat and context

exposure tests—which consisted of a 10-min observation period during the beginning of the dark phase of the light/dark cycle. During the tests, the animals were recorded using a horizontally mounted video camera under 50-W red light illumination.

Phase 1: cat exposure test

On the 11th day, an adult male cat was placed and held in the food compartment by an experimenter as the rat's home cage door was opened. The first phase of the testing procedure involved observation of the rat's behavior during a 10-min exposure to a live cat. 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 2: context exposure test

On the day after cat exposure, the 10-min test period involved the observation of the rats in the environment where the predator had been previously encountered (i.e., the predator context). Note that the animals were living in the home cage and were exposed to the predator context only after the home cage door had been opened, when the animals were exposed to the part of the apparatus where the predator had been previously encountered. No food pellets were offered during the test periods. These two phases are expected to elicit high levels of freezing during cat exposure and high levels of risk assessment during exposure to the predator context. Ninety minutes after ending the behavioral testing, the animals were deeply anesthetized with sodium pentobarbital (Cristália; Itapira, SP, Brazil; 40 mg/kg, i.p.), and the brains were processed for histology.

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). As for the experimental protocol, the behavioral analysis currently used to investigate innate and contextual fear related to predator exposure followed Ribeiro-Barbosa et al. (2005). The analysis comprised of spatiotemporal and behavioral measurements. The spatiotemporal measurements were the amounts of time spent in the home cage, the hallway, or the food compartment. The behavioral data were processed in terms of duration (total duration per session). The following behavioral items were encoded:

- Freezing: cessation of all movements, except for those associated with breathing.
- Risk-assessment behaviors: comprising crouch sniff (animal immobile with the back arched, but actively sniffing and scanning the environment) and stretch postures (consisting of both stretch attend posture, during which the body is stretched forward and the animal is motionless, and stretch approach, consisting of movement directed toward the food compartment with the animal's body in a stretched position).
- Fearless exploration: including nondefensive locomotion (locomotion with arched back) and exploratory up-right position (i.e., animals actively exploring the environment, standing over the rear paws and leaning on the walls with the forepaws). All behavioral scoring was conducted by an observer who was blind to the rat's condition.

Histology

Upon completion of behavioral testing, all rats were injected 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 placed 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 mounted on gelatin-coated slides and stained with thionin to serve as the reference series for cytoarchitectonic purposes. Parcellation of the periaqueductal gray in the present investigation followed Comoli et al. (2003), and for the rest of the brain, followed *The Brain Maps: structure of the rat brain* (Swanson 2004).

Experiment 1: Behavioral analysis of innate fear and learned contextual fear responses to cat exposure in animals bearing cytotoxic NMDA lesions in the rostrodorsal and caudal ventral PAG regions

In this experiment, we explored how bilateral NMDA cytotoxic lesions in the rostrodorsal or caudal ventral PAG interfere with innate fear and learned contextual fear responses to predator threat.

NMDA lesions

For the lesion procedure, rats were deeply anesthetized with sodium pentobarbital (Cristália; Itapira, SP, Brazil; 40 mg/kg, i.p.) and were placed in a stereotaxic apparatus. Bilateral iontophoretic deposits of a 0.15 M solution of *N*-methyl-D-aspartate (NMDA, Sigma, St. Louis, MO, USA) were bilaterally centered in the rostrodorsal PAG ($n = 8$; coordinates: anteroposterior, 6.2 mm from bregma; lateral, 0.3 mm from the midline of the sagittal sinus; dorsoventral, 4.0 mm from the surface of the brain) or in the caudal ventral PAG ($n = 7$; coordinates: anteroposterior, 7.6 mm from bregma;

lateral, 0.7 mm from the midline of the sagittal sinus; dorsoventral, 4.8 mm from the surface of the brain). In addition, in five other animals, control saline injections (sham group) were performed bilaterally at the same coordinates used for the rostrodorsal PAG. NMDA deposits were performed over a 15-min period through a glass micropipette (30-µm tip diameter) using a constant-current device (model CS3, Midgard Electronics, Canton, MA, USA) set to deliver − 15 µA, with 7-s pulse and interpulse durations. Animals received postoperative analgesics (Ibuprofen; Medley; Campinas, SP, Brazil; 30 mg/kg in drinking water) and antibiotics (Pentabiótico©; Zoetis; Campinas, SP, Brazil; 0.1 ml/100 g, i.p.). After a 1-week postsurgical period, the animals were placed in the experimental apparatus, and the behaviors were scored during the two phases of behavioral testing.

Experiment 2: Behavioral analysis of innate fear and learned contextual fear responses to cat exposure in animals receiving muscimol inactivation in the rostrodorsal PAG

In this experiment, we explored how inactivation of the rostrodorsal PAG with muscimol injection at different time points (i.e., prior to cat exposure, immediately after cat exposure or 20 min after cat exposure) interfered with innate fear and learned contextual fear responses to predator threat.

Cannula implants

The animals were anesthetized with a mixture of ketamine (Vetaset, Fort Dodge Laboratory, Campinas, SP, Brazil) and xylazine (Rompum, Bayer, Sao Paulo, SP, Brazil; 1:2 v/v; 1 ml/kg body weight) and positioned in a stereotaxic frame. In Experiment 2, 38 animals received guide cannula implants. For the guide cannula implants, a single stainless-steel guide cannula (cat# C313GS-5; Plastics One Inc., Roanoke, VA, USA) was stereotaxically implanted in the midline with the guide cannula tip 0.5 mm above the dorsal border of the dorsal PAG (coordinates: anteroposterior, 6.2 mm from bregma; lateral, 0.3 mm from the midline of the sagittal sinus; dorsoventral, 3.5 mm from the surface of the brain). The guide cannula was implanted at a 10° angle from the vertical axis to avoid the sagittal sinus. The cannula was fixed with polyacrylic cement and anchored to the skull with stainless-steel screws. Animals received postoperative analgesics (Ibuprofen; Medley; Campinas, SP, Brazil; 30 mg/kg in drinking water) and antibiotics (Pentabiótico©; Zoetis; Campinas, SP, Brazil; 0.1 ml/100 g, i.p.). After a 1-week postsurgical period, the animals were placed in the experimental apparatus. A group of intact animals ($n = 5$) was also used as a control in this experiment.

Drug administration

In Experiment 2, the animals received either muscimol (Tocris, Ellisville, MO, USA; 0.5 µg/µL) or saline injections at different time points (i.e., 10 min prior to cat exposure, immediately after cat exposure or 20 min after cat exposure). 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, which extended 0.5 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 0.4 µl was injected over a 5-min period. The injector remained in the guide cannula for an additional 3 min after infusion. Local electrophysiological recordings and autoradiography results on [^{3}H] muscimol spread and local change in [^{14}C] glucose uptake indicate that muscimol diffusion is larger than usually supposed (Martin 1991; Edeline et al. 2002). Edeline et al. (2002) showed that 0.4 µl muscimol injection may decrease neuronal activity and spread up to 3 mm from the injection site. These findings support the use of a 0.4 µl midline muscimol injection close to the border of the dorsal PAG to diffuse over a 1.5 mm radius and cover bilaterally the dorsomedial and dorolateral PAG.

Experiment 3: Analysis of learned contextual fear behaviors to cat exposure in response to the inhibition of neuronal nitric oxide synthase (nNOS) in the rostrodorsal PAG immediately after cat exposure

In this experiment, we explored how the inhibition of neuronal nitric oxide synthase (nNOS) in the rostrodorsal PAG immediately after cat exposure interfered with learned contextual fear responses to predator threat. To this end, animals received an injection of a selective nNOS inhibitor ($n = 8$), *N*-propyl-L-arginine (NP; Tocris, Ellisville, MO, USA; 200 nmol/0.4 µl), or saline ($n = 6$) in the rostrodorsal PAG immediately after cat exposure and were compared to a group of intact animals ($n = 5$). Cannula implants and drug administration followed the same procedures previously described in Experiment 2.

Statistical analysis

After testing for homogeneity of variance (Levene's test), spatiotemporal and behavioral data were square-root transformed. For all three experiments, the analysis was performed by means of a parametric multivariate analysis of variance (MANOVA). When the multivariate test was significant, we performed a univariate analysis of variance (ANOVA) for each dependent variable (spatiotemporal and behavioral items), followed by a post hoc analysis (Tukey's

HSD test) to isolate the respective effects (Tabachnick and Fidell 2007). Experiments 1 and 3 had a similar statistical design (a 3 × 2 factorial MANOVA with six dependent variables). In Experiment 1, the factors were site of lesion (rostrodorsal PAG, caudal ventral PAG or sham) and condition (cat exposure or context exposure). For Experiment 3, the factors were type of injection (NP, saline, intact) and condition (cat exposure or context exposure). For Experiment 2, a preliminary 4 × 2 MANOVA was employed to compare animals among the four control groups (intact animals and those that received saline injections prior to cat exposure, immediately after cat exposure or 20 min after cat exposure). Because there were no significant differences among the intact and saline groups, we used the animals that received saline injections (prior to cat exposure, immediately after cat exposure or 20 min after cat exposure) as the control groups, which were compared to the animals that received muscimol injections at those same times. A three-way (2 × 3 × 2) MANOVA was employed in the main analysis of Experiment 2 with the following factors: drug treatment (muscimol or saline), time of injection (prior to cat exposure, immediately after cat exposure or 20 min after cat exposure), and condition (cat exposure or context exposure). In all analyses, due to the number of dependent variables involved in the behavioral testing, the significance level employed in the univariate ANOVAs that followed the significant MANOVAs was adjusted downward by Bonferroni's correction ($\alpha = 0.0083$).

Results

Experiment 1

The parameters described above for the NMDA iontophoretic injections resulted in significant periaqueductal lesions that were characterized by neuronal cell loss filled with gliosis (Fig. 1a, b). From the eight bilateral NMDA injections in the rostral part of the dorsal PAG, we obtained good dorsal PAG bilateral lesions in five injected animals; in the remaining three rostrodorsal PAG-injected rats, we did not observe clear bilateral lesions. As shown in Fig. 2, these lesions were centered in the dorsomedial and dorsolateral PAG, extending a variable degree to the lateral PAG and adjacent parts of the deep layers of the superior colliculus. From the seven bilateral NMDA iontophoretic injections in the caudal ventral PAG, we obtained good bilateral lesions in five injected animals, and in the other two injected animals, the lesions were restricted to one side of the brain. As shown in Fig. 3, the caudal ventral PAG lesions mostly included the lateral and ventrolateral PAG and extended to a small degree to adjacent parts of deep layers of the superior colliculus, external nucleus of the inferior colliculus and cuneiform nucleus. A 3 × 2 factorial MANOVA revealed a main effect for both factors (i.e., group—rostrodorsal PAG, caudal ventral PAG or sham lesion groups [Wilks lambda = 0.000032, $F(12, 16) = 234.1$, $p < 0.0001$, partial eta-squared = 0.9943] and condition—cat exposure or context exposure [Wilks lambda = 0.0024, $F(6, 8) = 561.3$, $p < 0.0001$, partial eta-squared = 0.9976] and a significant interaction between the factors [Wilks lambda = 0.00002, $F(12, 16) = 310.8$, $p < 0.0001$, partial eta-squared = 0.9957]. For the spatiotemporal measurement, univariate ANOVAs revealed a significant interaction between the factors group and condition for the time spent in the home cage [$F(2, 13) = 30.6$, $p < 0.0001$] and food compartment [$F(2, 13) = 191.1$, $p < 0.0001$]; after applying Bonferroni's correction ($\alpha = 0.0083$), the interaction between the factors group and condition did not reach significance for the time spent in the hallway [$F(2, 13) = 5.6$, $p < 0.0178$]. For the behavioral measurement, univariate ANOVAs revealed a significant interaction between the

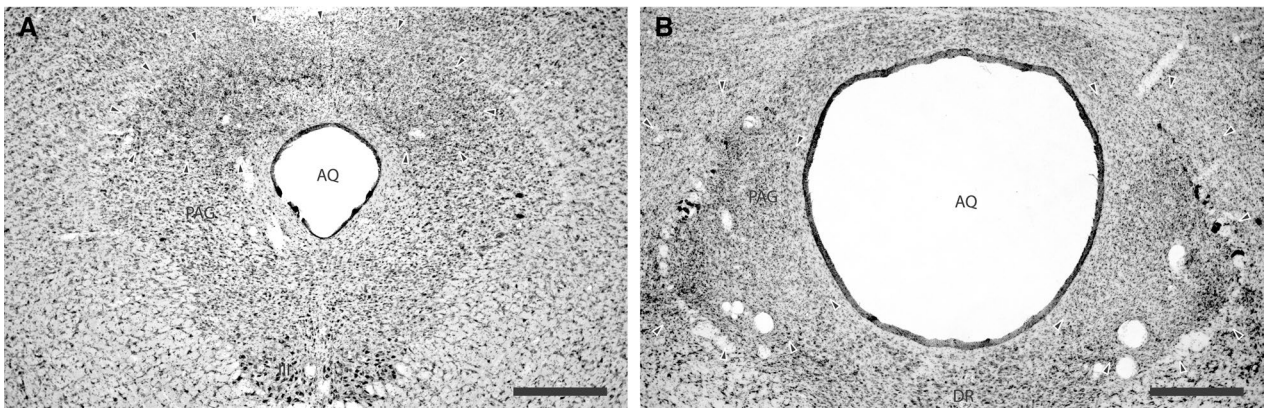

Fig. 1 Experiment 1: NMDA lesion appearance. Photomicrographs of transverse thionin-stained sections illustrating the extent and appearance of lesions in the rostrodorsal (**a**) and caudal ventral (**b**) PAG, delineated by arrowheads, from representative cases in Experiment 1. *AQ* cerebral aqueduct, *DR* dorsal raphe nucleus, *III* oculomotor nucleus, *PAG* periaqueductal gray. Scale bars = 500 µm

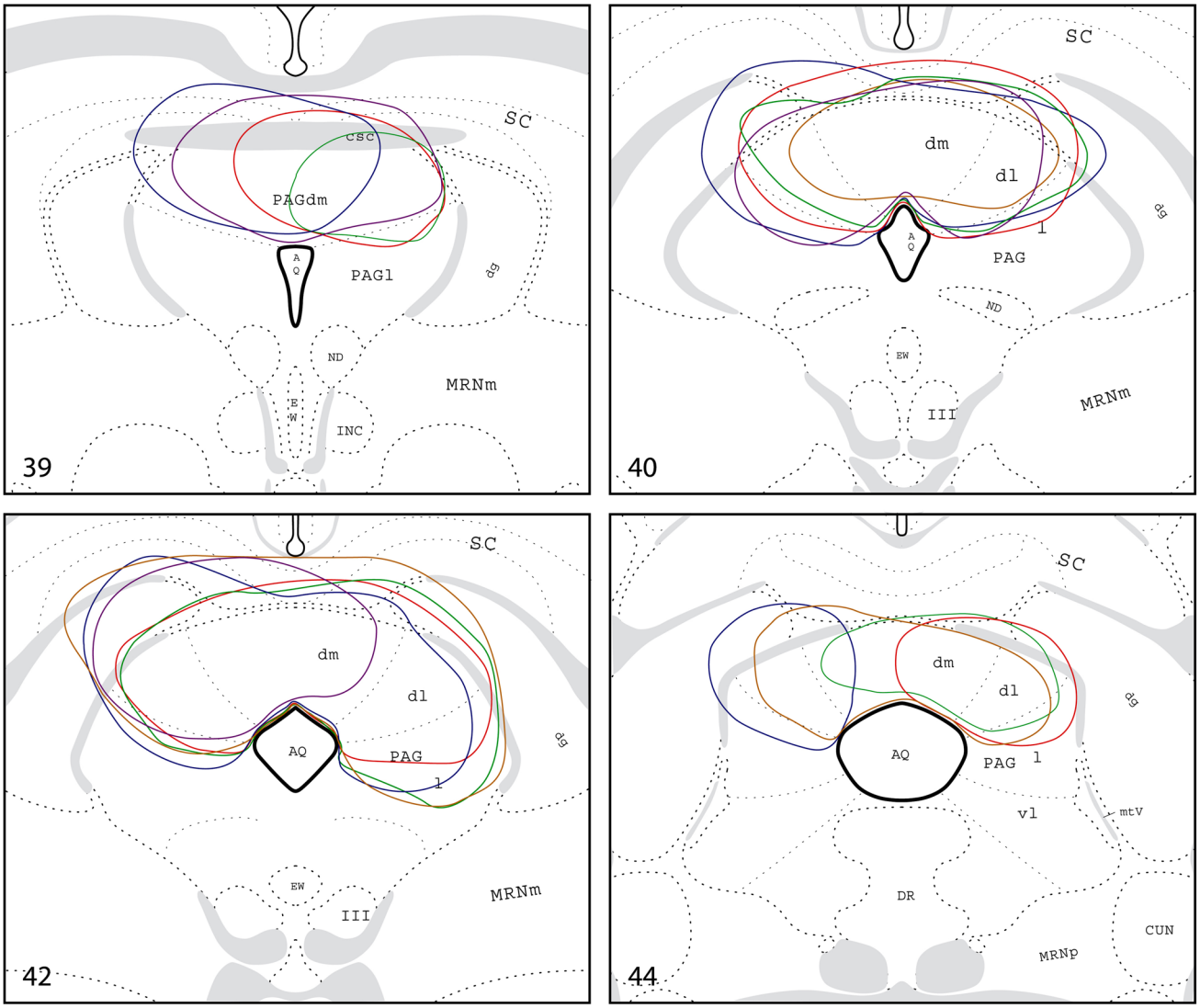

Fig. 2 Experiment 1: Location and extent of NMDA lesions including the rostrodorsal PAG that were used for behavioral analysis. The approximate location and extent of each lesion were determined by analysis of Nissl-stained cytoarchitecture, and for comparison, the data are plotted on a reference rat brain atlas (Swanson 2004). Numbers on the left lower corner indicate the plate number from the reference rat brain atlas (Swanson 2004). *AQ* cerebral aqueduct, *CUN* cuneiform nucleus, *DR* dorsal raphe nucleus, *EW* Edinger–Westphal nucleus, *III* oculomotor nucleus, *INC* intersticial nucleus of Cajal, *MRN* mesencephalic reticular nucleus, *mtV* mesencephalic tract of the trigeminal nerve, *ND* nucleus of Darkchewitsch, *PAG dl, dm, l* periaqueductal gray, dorsolateral, dorsomedial, lateral parts, *SCdg* superior colliculus, deep gray layer

factors group and condition for freezing [$F(2, 13) = 2868.0$, $p < 0.0001$], risk assessment [$F(2, 13) = 533.0$, $p < 0.0001$] and exploration [$F(2, 13) = 17.7$, $p < 0.0019$]. As shown in Fig. 4, post hoc analysis (Tukey's HSD test) revealed that during cat exposure, compared to the sham-lesioned group, the rostrodorsal PAG-lesioned and caudal ventral PAG-lesioned groups exhibited significantly less freezing ($p = 0.0001$), whereas both the rostrodorsal PAG-lesioned and caudal ventral PAG-lesioned animals exhibited significantly increased risk assessment ($p = 0.0002$). Conversely, during exposure to the context (see Fig. 4), compared to the other groups, the rostrodorsal PAG-lesioned group spent significantly less time in the home cage ($p = 0.0001$), visited the food compartment more often ($p = 0.0001$), spent significantly less time in risk assessment of the environment ($p < 0.0008$) and spent more time in fearless exploration ($p < 0.0021$).

Experiment 2

The tips of the injector cannulae were aimed at the midline close to the border of the dorsal PAG (Figs. 5, 6). As

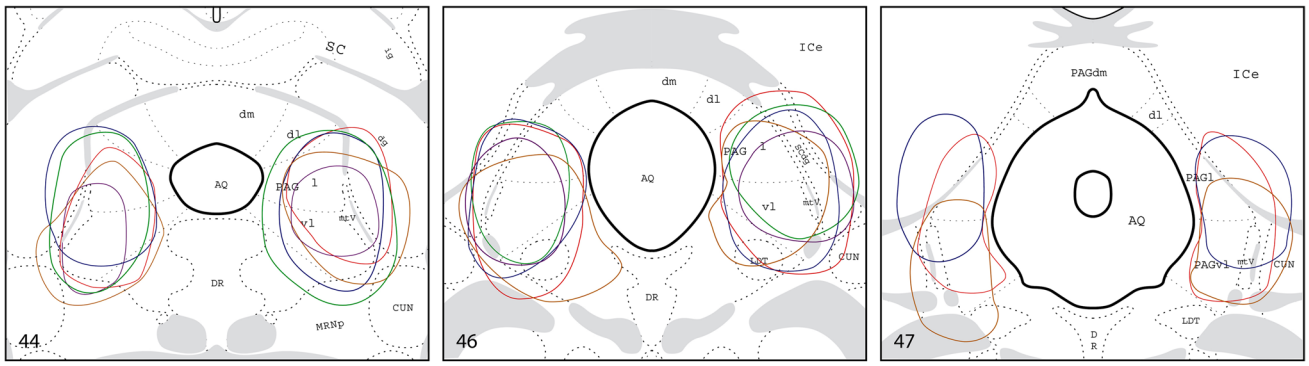

Fig. 3 Experiment 1: Location and extent of NMDA lesions including the caudal ventral PAG that were used for behavioral analysis. The approximate location and extent of each lesion were determined by analysis of Nissl-stained cytoarchitecture, and for comparison, the data are plotted on a reference rat brain atlas (Swanson 2004). Numbers on the left lower corner indicate the plate number from the reference rat brain atlas (Swanson 2004). *AQ* cerebral aqueduct, *CUN* cuneiform nucleus, *DR* dorsal raphe nucleus, *ICe* inferior colliculus, external nucleus, *LDT* laterodorsal tegamentl nucleus, *MRN* mesencephalic reticular nucleus, *mtV* mesencephalic tract of the trigeminal nerve, *ND* nucleus of Darkchewitsch, *PAG dl, dm, l, vl* periaqueductal gray, dorsolateral, dorsomedial, ventrolateral lateral parts, *SCdg, ig* superior colliculus, deep gray layer, intermediate gray layer

shown in Supplementary Material, a 0.4-µl muscimol injection in the midline close to the border of the dorsal PAG during cat exposure yielded a clear inactivation spreading throughout the dorsomedial and dorsolateral PAG bilaterally (Supplementary Material Fig. 1B). Only those with correct placement were considered in the present analysis. From the 38 animals injected, 30 had correct cannula placement [animals that received saline injection in the dorsal PAG prior to cat exposure ($n=5$), immediately after cat exposure ($n=5$) and 20 min after cat exposure ($n=5$); animals that received muscimol injection in the dorsal PAG prior to cat exposure ($n=5$), immediately after cat exposure ($n=5$) and 20 min after cat exposure ($n=5$); see Fig. 6]. First, we examined whether the cannula implants and injection procedures affected the innate and contextual fear responses to cat exposure. To this end, a 4×2 MANOVA was employed to compare animals among the four control groups (intact animals and those that received saline injections prior to cat exposure, immediately after cat exposure and 20 min after cat exposure). Accordingly, 4×2 MANOVA revealed no main effect for group [Wilks lambda = 0.5404, $F(18, 42)=0.6$, $p<0.8948$, partial eta-squared = 0.1955] and a non-significant interaction between group and condition [Wilks lambda = 0.4819, $F(18, 42)=0.7$, $p=0.7890$, partial eta-squared = 0.2274]. Therefore, animals that received saline injections (prior to cat exposure, immediately after cat exposure and 20 min after cat exposure) were used as control groups and compared to the animals that received muscimol injections at those same times. A three-way ($2\times3\times2$) MANOVA revealed a significant three-way interaction among the factors—drug treatment, time of injection and condition [Wilks lambda = 0.0033, $F(12, 46)=63.3$, $p<0.0001$, partial eta-squared = 0.9428]. For the spatiotemporal measurement, univariate ANOVAs revealed a significant three-way interaction among the factors (drug treatment, time of the injection and condition) only for the time spent in the food compartment [$F(2, 28)=34.8$, $p<0.0001$] but not for the time spent in the home cage [$F(2, 28)=2.7$, $p=0.0849$] or in the hallway [$F(2, 28)=0.08$, $p=0.926$]. For the behavioral measurements, univariate ANOVAs revealed a significant three-way interaction among the factors for freezing [$F(2, 28)=84.5$, $p<0.0001$], risk assessment [$F(2, 28)=210.4$, $p<0.0001$] and exploration [$F(2, 28)=7.2$, $p=0.0030$]. As shown in Fig. 7, during cat exposure, post hoc analysis (Tukey's HSD test) revealed that the group of animals that received muscimol prior to the cat exposure, compared to its respective saline-injected control group, presented a significant decrease in the time spent freezing ($p=0.0001$) and a significant increase in risk assessment ($p=0.0001$), and the animals that received muscimol either immediately after cat exposure or 20 min after cat exposure did not differ from their respective saline control group ($p>0.1068$; Fig. 7). During exposure to the context, the groups that received muscimol either prior to or immediately after the cat exposure, compared to their respective saline-injected controls, presented a significant increase in the time spent in the food compartment ($p=0.0001$) and the time spent fearlessly exploring the apparatus ($p=0.0001$) but a significant decrease in the time spent in risk assessment of the environment ($p=0.0001$). Although univariate ANOVA did not reveal a significant three-way interaction among the factors for the time spent in the home cage, visual inspection of the data suggests that the groups that received muscimol either prior to or immediately after the cat exposure spent less time in the home cage during exposure to the predatory context when compared to

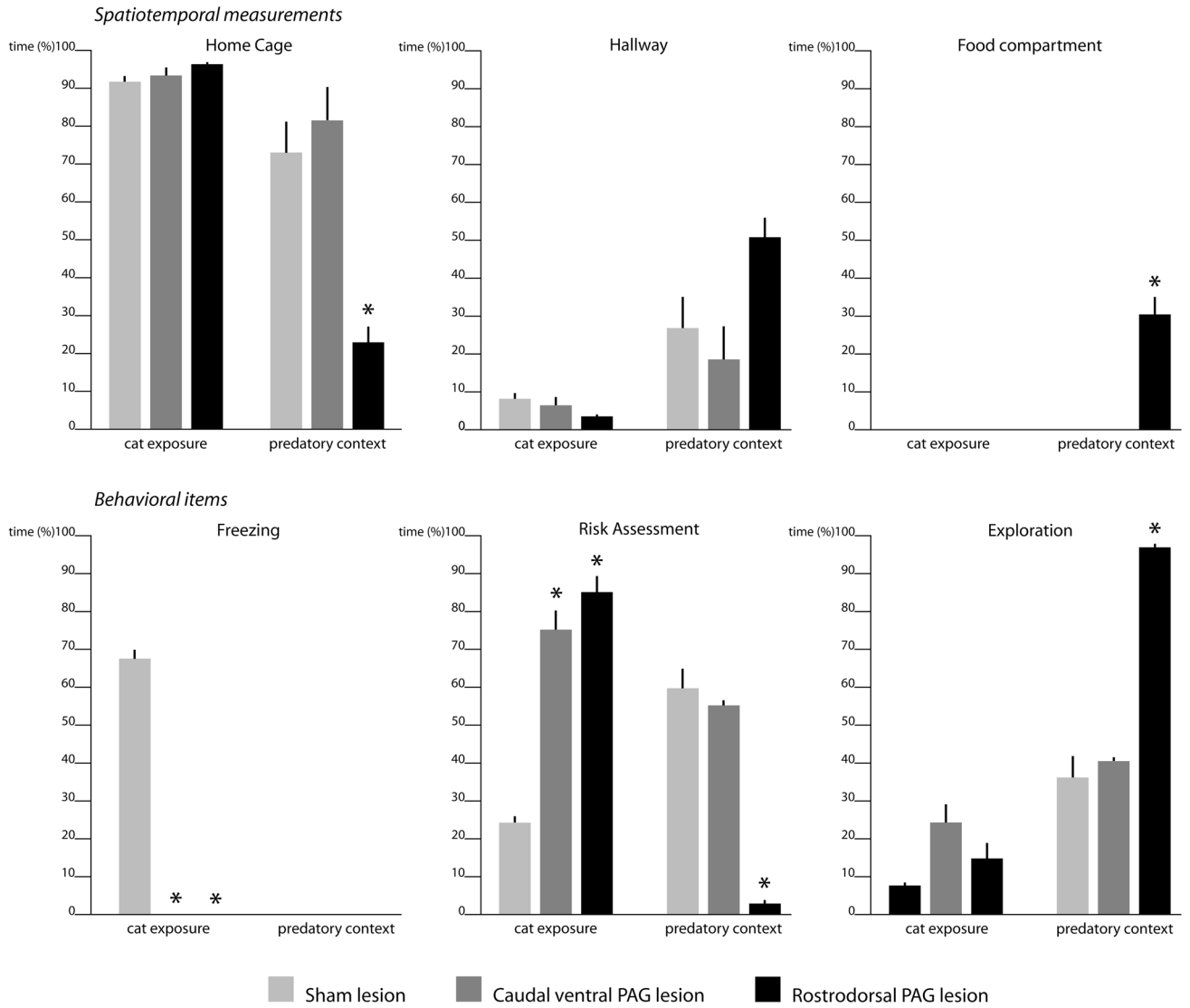

Fig. 4 Experiment 1: Behavioral analysis. Bar graphs representing the spatiotemporal and behavioral measurements during cat exposure and predatory context; for the sham—($n=5$) and caudal ventral—($n=5$) and rostrodorsal PAG lesions. Values are mean ± SEM of the percentage of the time during a 10 min observation period. *$p<0.05$ vs. sham group (Tukey's HSD post hoc test)

their respective control saline-injected groups (Fig. 7). In contrast to animals that received muscimol either prior to or immediately after the cat exposure, animals that received muscimol injection 20 min after cat exposure did not differ from their respective control saline-injected group in regard to either spatiotemporal or behavioral measurements during exposure to the predatory context ($p>0.626$; Fig. 7).

Experiment 3

As shown in Fig. 8, the tips of the injector cannulae were aimed at the midline, close to the border of the dorsal PAG. Although we have not specifically tested the diffusion for the NP injections and this issue requires caution, our findings on muscimol injections indicate that a 0.4-µl volume injection in the midline close to the border of dorsal PAG is expected to extend over a 1.5-mm radius and cover the dorsomedial and dorsolateral PAG bilaterally (see Supplementary Material). Of the 14 animals injected, only 10 had correct cannula placement [5 animals that received saline injection and 5 animals that received *N*-propyl-L-arginine (NP) injection].

A 3 × 2 factorial MANOVA revealed a main effect for both factors (i.e., group—NP injection, saline injection or intact groups [Wilks lambda = 0.0072, $F(12, 16) = 14.4$, $p<0.0001$, partial eta-squared = 0.9153] and condition—cat exposure or context exposure [Wilks lambda = 0.00010, $F(6, 8) = 1483.6$, $p<0.0001$, partial eta-squared = 0.9991)] and a significant interaction between them [Wilks

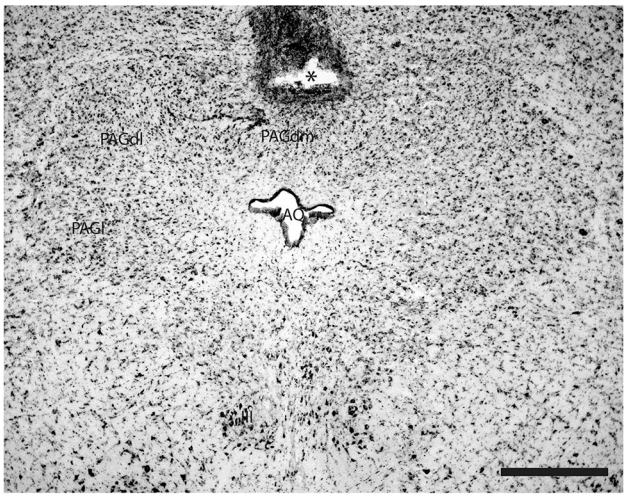

Fig. 5 Experiment 2: Photomicrograph of transverse thionin-stained section illustrating location of the injector cannula tip (labeled with asterisk). *AQ* cerebral aqueduct, *DR* dorsal raphe nucleus, *III* oculomotor nucleus, *PAGdl, dm, l* periaqueductal gray, dorsolateral, dorsomedial, lateral parts. Scale bar = 500 µm

lambda = 0.0167, $F(12, 16) = 9.0$, $p < 0.0001$, partial eta-squared = 0.8708]. For the spatiotemporal measurement, univariate ANOVAs revealed a significant interaction between the factors group and condition for the time spent in the home cage [$F(2, 13) = 15.5$, $p = 0.0004$] and food compartment [$F(2, 13) = 83.0$, $p < 0.0001$] but not for the time spent in the hallway [$F(2, 13) = 3.1$, $p = 0.0819$]. For the behavioral measurement, univariate ANOVAs revealed a significant interaction between the factors group and condition for risk assessment [$F(2, 13) = 37.4$, $p < 0.0001$] and exploration [$F(2, 13) = 12.8$, $p = 0.0008$], but the difference was not significant for freezing [$F(2, 13) = 6.2$, $p = 0.0128$] due to Bonferroni's correction ($\alpha = 0.0083$). As shown in Fig. 9, post hoc analysis (Tukey's HSD test) revealed that during cat exposure, there were no significant differences in the spatiotemporal measurements among the groups, and for the behavioral measurements, the animals in the intact group presented significantly higher times for freezing and lower times for risk assessment compared to the animals that received NP ($p < 0.0263$), which did not differ from the control saline-injected group ($p > 0.7658$). During exposure to the context (see Fig. 9), post hoc analysis (Tukey's HSD test) revealed no significant differences between the intact and saline-injected groups, and compared to these control groups, the animals that received NP injection immediately after cat exposure spent significantly less time in the home cage ($p = 0.0001$), visited the food compartment more often ($p = 0.0001$), spent significantly less time in risk assessment of the environment ($p = 0.0001$) and spent significantly more time in fearless exploration ($p = 0.0005$).

Discussion

The aim of the present investigation was to characterize the differential role of the rostrodorsal and ventral caudal PAG on the acquisition of learned contextual fear responses to predatory threats and help to reveal their roles in the expression of innate defensive responses during predator exposure.

In Experiment 1, lesions of the rostrodorsal and caudal ventral PAG had similar effects on innate fear responses during cat exposure, practically abolishing freezing and increasing risk assessment responses. Thus, an important conclusion that can be drawn from the present findings is that freezing response to a predator threat depends on the integrity of both the rostrodorsal and caudal ventral PAG. The role of the ventral PAG in freezing has been widely explored in fear conditioning studies using footshock and, to a lesser extent, in innate fear (LeDoux et al. 1988; Walker and Carrive 2003; Koutsikou et al. 2014; Tovote et al. 2016).

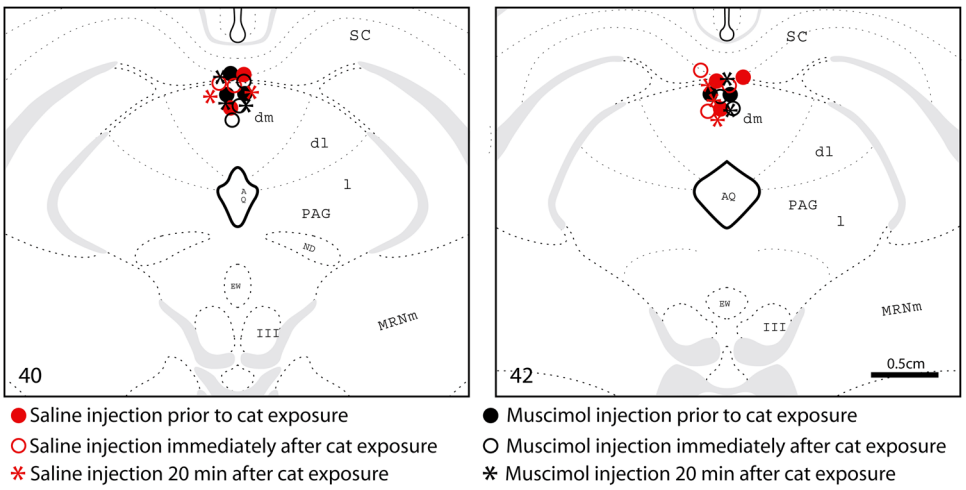

Fig. 6 Experiment 2: Location of the injector cannula tips for saline and muscimol injections plotted on a reference rat brain atlas (Swanson 2004). Numbers on the left lower corner indicate the plate number from the reference rat brain atlas (Swanson 2004). *AQ* cerebral aqueduct, *EW* Edinger–Westphal nucleus, *III* oculomotor nucleus, *MRN* mesencephalic reticular nucleus, *ND* nucleus of Darkchewitsch, *PAG dl, dm, l* periaqueductal gray, dorsolateral, dorsomedial, lateral parts, *SC* superior colliculus. Scale bar = 500 µm

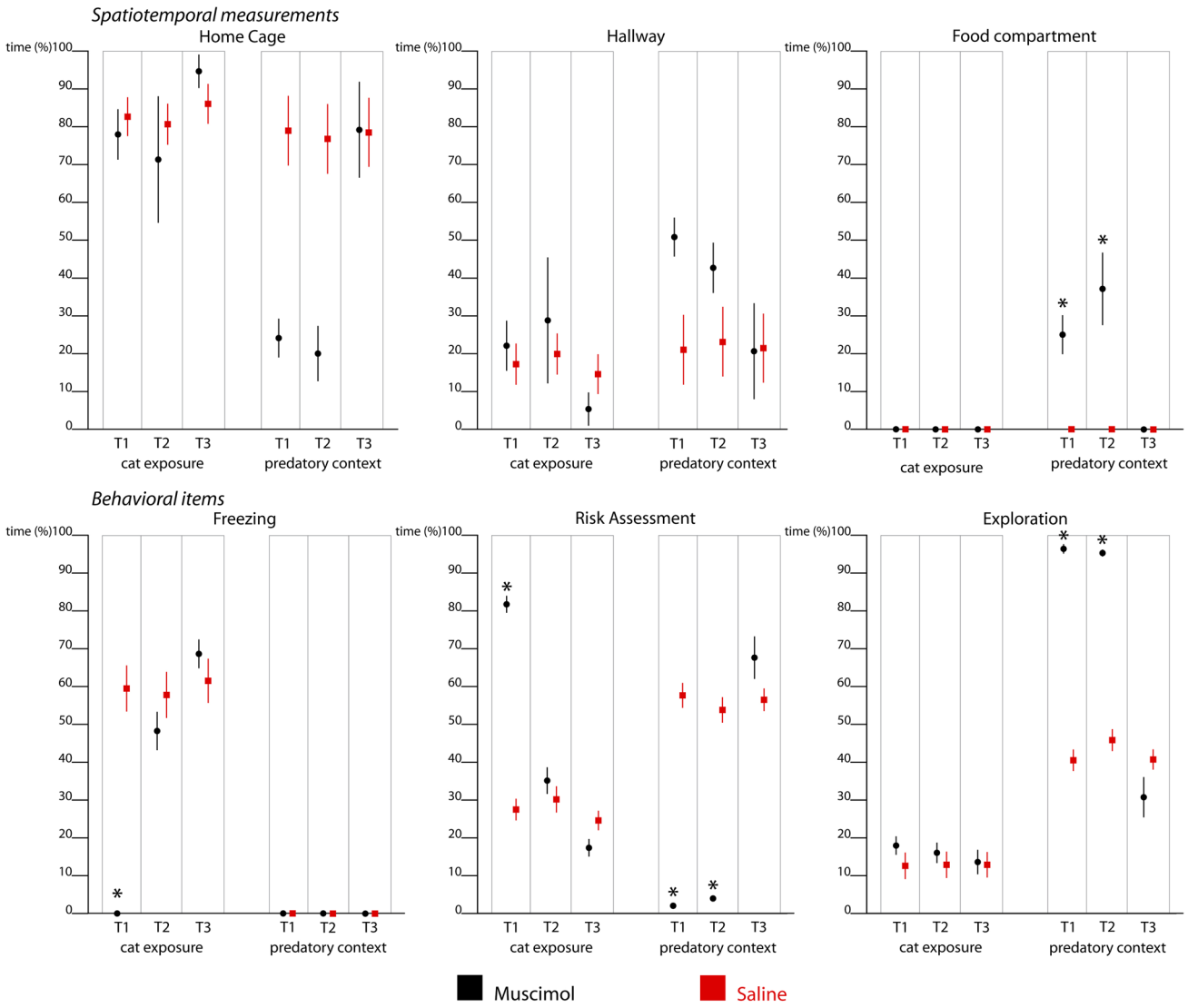

Fig. 7 Experiment 2: Behavioral analysis. Histograms representing the spatiotemporal and behavioral measurements for animals injected with saline or muscimol prior to cat exposure (T1), immediately after cat exposure (T2) or 20 min after cat exposure (T3). Values are mean ± SEM of the percentage of the time during a 10 min observation period. *$p < 0.05$ vs. respective saline-injected control (Tukey's HSD post hoc test)

At least two pathways from the ventrolateral PAG (vlPAG) have been identified to mediate freezing responses. The first involves a direct glutamatergic projection to premotor cells in the magnocellular nucleus (Tovote et al. 2016), and the other path links the vlPAG to the cerebellar vermal cortex, engaging an olivocerebellar circuit terminating as climbing fibers in the lateral vermal lobule VIII, which are likely to influence motor pathways known to regulate muscle tone (Koutsikou et al. 2014, 2015). Apart from lesions of the ventral PAG, lesions of the rostrodorsal PAG also abolished freezing during cat exposure. This finding is at odds with previous observations from De Oca et al. (1998), who reported that rostrodorsal PAG electrolytic lesions increase freezing during cat exposure. The reasons for this discrepancy are not entirely clear; however, notably, the levels of freezing during cat exposure in sham-lesioned animals differed markedly between De Oca's work (less than 20% of the observation period) and the present study (close to 67% of the observation period), suggesting important differences in the experimental conditions between the studies. Moreover, we may also speculate that particularly crouch sniff, presently considered as a risk-assessment response increasing in PAG lesions during cat exposure, may have been regarded as freezing in De Oca's work. In line with the present observations, electrical, pharmacological and optogenetic stimulation of the rostrodorsal PAG has been shown to produce freezing as well as escape and flight behavior in the absence of a predatory threat (Carrive 1993; Bandler

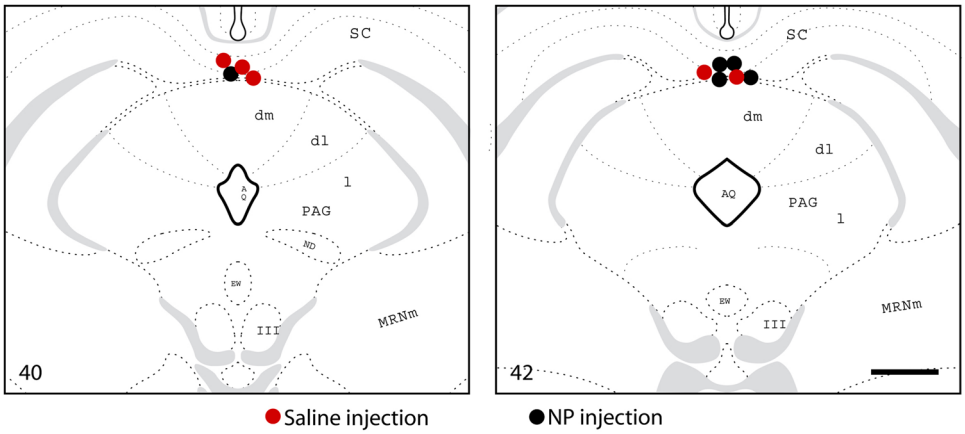

Fig. 8 Experiment 3: Location of the injector cannula tips for saline (red dots) and *N*-propyl-L-arginine (NP) injections plotted on a reference rat brain atlas (Swanson 2004). Numbers on the left lower corner indicate the plate number from the reference rat brain atlas (Swanson 2004). *AQ* cerebral aqueduct, *EW* Edinger–Westphal nucleus, *III* oculomotor nucleus, *MRN* mesencephalic reticular nucleus, *ND* nucleus of Darkchewitsch, *PAG dl, dm, l* periaqueductal gray, dorsolateral, dorsomedial, lateral parts, *SC* superior colliculus. Scale bar = 500 µm

and Keay 1996; Bittencourt et al. 2004; Assareh et al. 2016; Deng et al. 2016). Interestingly, rostrodorsal PAG stimulation may evoke either freezing or escape and jumps, where low-magnitude stimuli produce freezing, and slightly higher intensities evoke circa strike responses (Vianna et al. 2001; Bittencourt et al. 2004; Assareh et al. 2016). At this point, how the rostrodorsal PAG mediates freezing remains to be investigated. One possible explanation is that the rostrodorsal PAG influences freezing through the ventral PAG, an important projection target, particularly for the dorsolateral PAG (N.S. Canteras and S.R. Mota-Ortiz, personal observation).

Both rostrodorsal and caudal ventral PAG-lesioned animals, apart from a reduction in freezing, presented significant increases in risk assessment during cat exposure. Similar results have been obtained with lesions in the dorsal premammillary nucleus (Blanchard et al. 2003; Cezário et al. 2008) and in the lateral, posterior basomedial, and anterior basolateral amygdalar nuclei (Martinez et al. 2011; Bindi et al. 2018), as well as in the PAG–cerebellar pathway that mediates the freezing response (Koutsikou et al. 2014). Fear responses to the actual predator include mostly freezing, whereas risk assessment is thought to be an anxiety-like behavior through which a potentially aversive stimulus can be cautiously explored, allowing the gathering of information (Blanchard et al. 2011). Thus, during cat exposure, this lesion-induced shift from high intensity fear or freezing to anxiety-related risk assessment is compatible with a pattern of decreased defensiveness to predator stimuli (Blanchard et al. 2003). Findings from our laboratory suggest that risk assessment responses during cat exposure depend on the integrity of the PAG, where combined cytotoxic lesions, including lesions of rostrodorsal and caudal ventral parts, extending to the adjacent cuneiform nucleus, completely abolished defensive responses (Rufino 2015). The elaborated display of risk assessment responses that involves information gathering and decision making can be reasonably believed to be organized by prosencephalic circuits. The rostrodorsal PAG is in a position to influence prosencephalic circuits mediating risk assessment responses. Notably, the rostrodorsal PAG may influence the septo-hippocampal system through projections to the anterior hypothalamic nucleus and adjacent parts of the subfornical region of the lateral hypothalamic area (Kincheski et al. 2012). Of particular relevance, the septo-hippocampal system is known to play a pivotal role in anxiety in response to conflict situations, such as those observed in animals presenting risk assessment (Gray and McNaughton 2000; for review see; Motta et al. 2017).

Apart from influencing innate fear responses, PAG lesions impaired learned contextual fear to predator threat. Thus, rostrodorsal PAG lesions but not caudal ventral lesions disrupted learned contextual fear responses to cat exposure. In support of the present findings, a number of studies using classical fear conditioning to sound-, light- or odor-conditioned stimuli (CSs) have shown that electrical, chemical or optogenetic stimulation of the dorsal PAG may be used as a useful US to support associative learning (Di Scala et al. 1987; Di Scala and Sandner 1989; Kincheski et al. 2012; Kim et al. 2013; Deng et al. 2016). In sharp contrast to contextual fear learning to predatory threat, fear conditioning to a footshock US does not seem to involve the dorsal PAG (LeDoux et al. 1988; Kim et al. 2013). In fact, the ventral, and not the dorsal, PAG is known to influence fear conditioning to footshocks by responding to painful stimuli and relaying expectancy-modulated information to instruct

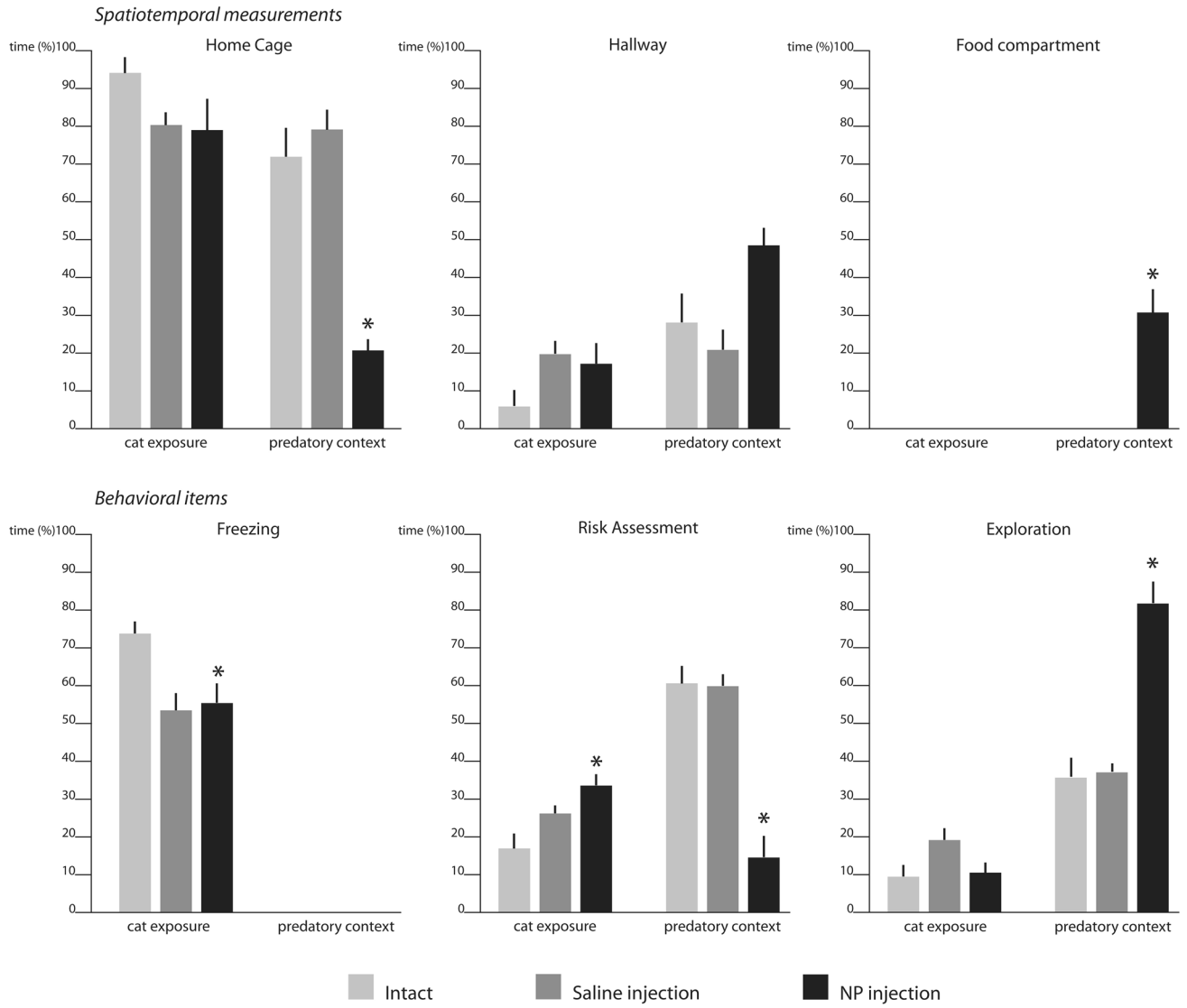

Fig. 9 Experiment 3: Behavioral analysis. Bar graphs representing the spatiotemporal and behavioral measurements during cat exposure and predatory context; for the intact ($n=5$), saline-injected ($n=5$) and *N*-propyl-L-arginine-injected (NP; $n=5$) groups. Values are mean ± SEM of the percentage of the time during a 10 min observation period. *$p<0.05$ vs. intact group (Tukey's HSD post hoc test)

associative plasticity in the amygdala during fear learning (Johansen et al. 2010).

In Experiment 2, muscimol inactivation of the rostrodorsal PAG during cat exposure resulted in similar effects to those found for rostrodorsal PAG cytotoxic lesions, influencing innate responses by decreasing freezing, increasing risk assessment responses and impairing contextual fear learning. Moreover, muscimol inactivation of the rostrodorsal PAG immediately after cat exposure but not 20 min later also impaired contextual fear learning. Taken together, the present results suggest that the rostrodorsal PAG is involved in the acquisition but not the consolidation of contextual fear memory to predatory threat. Similarly, blockade of NMDA receptors within the rostral dorsolateral PAG suppressed defensive responses to cat odor and impaired the acquisition but not the consolidation of contextual fear (Souza and Carobrez 2016). Experiments using the olfactory fear conditioning paradigm associating a neutral odor as the CS and *N*-Methyl-D-Aspartate (NMDA) stimulation of the rostral dorsolateral part of the PAG (dlPAG) as the US revealed that higher doses of NMDA, which provoked strong defensive reactions, failed to induce contextual learning and that there is an optimal level of dorsal PAG activation that could predispose the animal to fear learning (Kincheski et al. 2012; Back and Carobrez 2018). In the present case, our results suggest that the optimum level of predator-induced rostrodorsal PAG activation leading to contextual learning extends from during to shortly after predator exposure; however, at

the moment, the exact time line of this window is difficult to determine. The neurotransmission in the dorsal PAG that influences learning processes has been found to depend on glutamatergic transmission, and an ideal condition for learning to occur includes the well-adjusted activation of NMDA, AMPA/kainate and group I metabotropic glutamate receptors (Back and Carobrez 2018).

Notably, the dorsolateral PAG contains a distinct population of neurons containing the enzyme neuronal nitric oxide synthase (nNOS) (Onstott et al. 1993; Vincent and Kimura 1992), which is involved in the synthesis of nitric oxide (NO). Conversely, NO released from nNOS neurons modulates the release of several neurotransmitters, including glutamate (see Guimarães et al. 2005). Cat exposure increased nNOS activity in the dorsal PAG (Chiavegatto et al. 1998), and injections of *N*-propyl-L-arginine, a selective nNOS inhibitor, into the dorsolateral PAG attenuated defensive responses during exposure to a live cat (Aguiar and Guimarães 2009). In Experiment 3, injection of *N*-propyl-L-arginine into the rostrodorsal PAG immediately after cat exposure, using the same doses effective in reducing innate defensive responses (Aguiar and Guimarães 2009), was able to impair contextual fear learning. The results suggest that NO release induced by cat exposure seems to be critical in providing an optimum level of glutamatergic activation in the dorsal PAG to influence contextual fear learning. Importantly, the dorsolateral PAG, one of the sites most responsive during predator exposure (Comoli et al. 2003; Cezário et al. 2008), contains a characteristic population of nNOS neurons, which are involved in influencing learned fear responses to predatory threats.

The dorsal PAG is in a position to influence a complex cortical-hippocampal-amygdalar circuit related to fear learning (Motta et al. 2017). Supporting this view, the fear conditioning effect induced by dorsal PAG stimulation has been shown to be abolished by inactivation of the BLA (Kim et al. 2013). As previously discussed, the dorsolateral PAG sends massive projections to the anterior hypothalamic nucleus and adjacent parts of the subfornical region of the lateral hypothalamic area, both of which are known to provide strong inputs to the dorsal premammillary nucleus (PMd; Comoli et al. 2000; Goto et al. 2005; Risold et al. 1994). The PMd, in turn, works as a critical hub to transfer information of predatory threats to a cortico-hippocampal-amygdalar circuit involved in the processing of fear memories (see de Lima et al. 2018) and is thought to influence associative learning through its main thalamic target, i.e., the ventral part of the anteromedial thalamic nucleus, where muscimol inactivation blocks the acquisition of contextual fear memories (de Lima et al. 2017). Moreover, the PMd has been shown to be a critical element in the ascending pathway involved in fear learning using dorsal PAG activation as the US, and PMd beta-adrenergic blockade impaired fear conditioning in a paradigm that associates neutral odor cues as the CS and chemical stimulation of the dorsal PAG as the US (Kincheski et al. 2012). In addition to the hypothalamic projection, the dorsal PAG provides a number of parallel thalamic paths likely to influence fear learning, including projections to the nucleus reuniens, the lateral dorsal nucleus, the suprageniculate nucleus, and the parvicellular subparafascicular nucleus (Kincheski et al. 2012). The nucleus reuniens represents the main thalamic source of projections to the hippocampal formation (Vertes et al. 2006); the lateral dorsal nucleus projects to cortical areas that influence fear learning (i.e., the anterior cingulate and retrosplenial areas) (van Groen and Wyss 1992; Furlong et al. 2010; de Lima et al. 2018); and the suprageniculate and the parvicellular subparafascicular nuclei project to the lateral amygdalar nucleus (Linke et al. 2000). At this point, a great deal needs to be learned regarding how the ascending dorsal PAG pathways influence fear learning.

In conclusion, we have examined how the PAG influences both innate and learned contextual fear responses and found that the integrity of both the rostrodorsal and caudal ventral PAG is critical for the expression of freezing during exposure to a predator and that the rostrodorsal but not the caudal ventral PAG seems critical for the acquisition of contextual-learned responses to predatory threat. Accordingly, the acquisition of contextual fear learning is influenced by an optimum level of dorsal PAG activation, which extends from during to shortly after predator exposure and depends on local NO release. This study offers new perspectives for the understanding of fear learning and opens an interesting path of investigation for the dorsal PAG and its NO-producing neurons as conceivably involved in pathologic fear learning conditions, such as the posttraumatic stress disorder.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest.

Research involving human participants and/or animals Experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, 1996). All experimental procedures had been previously approved by the Committee on The Care and Use of Laboratory Animals of the Institute of Biomedical Sciences, University of São Paulo, Brazil (Protocol number 085/2012). In the present study, the experiments were planned to minimize the number of animals used and their suffering. In addition, all surgical procedures were performed under deep anesthesia, and analgesic and antibiotic medications were given postoperatively.

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