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ORIGINAL ARTICLE

Inactivation of the dorsolateral periaqueductal gray matter impairs the promoting influence of stress on fear memory during retrieval

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Abstract
Exposure to stressful conditions induces long-lasting neurobiological changes in selected brain areas, which could be associated with the emergence of negative emotional responses. Moreover, the interaction of a stressful experience and the retrieval of an established fear memory trace enhance both fear expression and fear retention. Related to this, the stimulation of the dorsolateral part of the mesencephalic periaqueductal gray matter (dlPAG) prior to retrieval potentiates a fear memory trace previously acquired. Therefore, the question that arises is whether the dlPAG mediates the increased fear expression and fear retention after retrieval. Rats were subjected to a contextual fear conditioning paradigm using a single footshock, and 1 day later, rats were subjected to a stressful situation. As previously reported, there was an increase of freezing response only in those rodents that were re-exposed to the associated context at 1 and 5 days after stress exposure. Muscimol intra-dlPAG prior to the restraint event prevented such increase. Conversely, Muscimol intra-dlPAG infusion immediately after the stress experience had no effect on the resulting fear memory. When the neuroendocrine response to stress was explored, intra-dlPAG infusion of muscimol prior to stress decreased Fos expression in the paraventricular nucleus and serum corticosterone levels. Moreover, this treatment prevented the enhancement of the density of hippocampal "mature" spines associated with fear memory. In conclusion, the present results suggest that the dlPAG is a key neural site for the negative valence instruction necessary to modulate the promoting influence of stress on fear memory.

Keywords Fear memory · Stress · Retrieval · Periaqueductal gray matter · Valence instruction

Introduction

There is vast literature supporting the view that emotionally driven events promote the emergence of stronger and long-lasting memories (Roozendaal et al. 2009). Accordingly, diverse-threatening situations prior to learning sensitize specific brain areas potentiating fear memory encoding (Shors 2001; Cordero et al. 2003; Rodriguez Manzanares et al. 2005; Rau and Fanselow 2009). In line with this, a stressful experience prior to retrieval potentiated a fear memory trace previously acquired (Giachero et al. 2013a). Furthermore, such memory enhancement was associated with an increased number of total and mature dendritic spines in the dorsal hippocampus (DH) (Giachero et al. 2013b). This stress influence on retention and on hippocampal structural plasticity was prevented by midazolam (MDZ) intra-basolateral amygdala (BLA) infusion prior to stress (Giachero et al. 2013a, b, 2015). These findings suggest that, under specific conditions, the retrieval of a consolidated memory trace can induce a transient plasticity state during which a memory could be readjusted to contemporary environmental conditions (Dudai 2002), pointing to retrieval as a functionally suitable experience to rewrite emotional memories (Gisquet-Verrier and Riccio 2012).

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The mesencephalic dorsolateral periaqueductal gray matter (dlPAG) is an important area to respond to life-threatening situations (Canteras 2002; Souza and Carobrez 2016) and to modulate aversive emotional states (Carobrez et al. 2001; Canteras et al. 2015; Motta et al. 2017). Defensive responses evoked by dlPAG stimulation require lower electrical current or chemical doses when compared to other PAG columns (Bittencourt et al. 2004). In addition, dlPAG neurons project to all PAG columns, along the rostrocaudal axis (Cameron et al. 1995; Sandkühler and Herdegen 1995). On the other hand, ascending information generated in the dlPAG influences hypothalamic and other prosencephalic targets serving as unconditional stimulus in fear conditioning paradigms (Kincheski et al. 2012). Previous studies have pointed out that the dlPAG also modulates fear associations (Di Scala et al. 1987; Kim et al. 2013) and a recent study suggests the dlPAG as a candidate to modulate positive effect circuits as well (Horovitz et al. 2017). Furthermore, glutamatergic stimulation of the dlPAG was able to support an olfactory fear conditioning, showing that such dlPAG stimulation functions as an unconditioned stimulus (Kincheski et al. 2012). In support of these evidences, electrophysiological studies have suggested that dorsal periaqueductal gray matter relays aversive information to BLA influencing the neural plasticity necessary to cope with stressful situations (Horovitz and Richter-Levin 2015; Horovitz et al. 2017). Finally, it was reported that the interaction between glutamatergic stimulation of the dlPAG and the retrieval of an established trace resulted in a robust and persistent fear memory (Mochny et al. 2012).

Considering the key role for dlPAG in defensive behavior expression and in aversive memory formation, the question that arises is whether dlPAG mediates the increased fear expression and fear retention after retrieval resulting from the combination of fear conditioning followed by a stressor. Therefore, this study was designed to investigate the influence of dlPAG blockade, by muscimol, on a fear conditioning potentiation induced by stress. In addition, we also explored the influence of the dlPAG inactivation on both the neuroendocrine response to stress and the structural plasticity in the DH associated with the enhanced fear memory retention induced by stress.

Materials and methods

Animals

Adult male Wistar rats weighing 280–350 g obtained from the Department of Pharmacology of Federal University of Santa Catarina were used in this study. Animals were housed in polypropylene cages (50 cm × 30 cm × 15 cm) in groups of three or four, under a 12 h light/dark cycle (lights on at 7 am), in a temperature-controlled environment (23 ± 1 °C) with free access to food and water.

Stressor

This procedure was based on previous findings (Giachero et al. 2013a). Briefly, animals were transferred in their own home cages to an experimental room, and located for 30 min inside a plastic cylindrical restrainer fitted close to the body, preventing animal movement except for the tail and the tip of the nose. At the end of the stress session, animals were returned to the colony room. No other subjects were present in the experimental room during stress exposure. Control unstressed animals were transferred to the experimental room, gently handled, and then returned to the colony room.

Conditioning apparatus

The conditioning chamber (23 cm × 20 cm × 26 cm) was placed in an acoustically isolated separated room maintained at a constant temperature of 21 ± 2 °C. It was constructed with stainless-steel walls and a grid floor composed of 1 cm spaced stainless bars connected to a shock generator (Insight, Ribeirão Preto, SP, Brazil) to provide footshock. Illumination was supplied by a 2.5 W white light bulb, and the background noise was made available by ventilation fans and the shock scrambler (55 dB). Conditioning chamber was cleaned with 10% aqueous ethanol solution before and after each session. Experiments were always performed between 2PM and 4PM with the experimenters unaware of the treatment condition.

Behavioral procedures

Contextual fear conditioning

The procedure used was similar to Giachero et al. (2013a). On the day of the experiment, rats were transported from the housing room, individually placed in the conditioning chamber A and left undisturbed for a 3 min acclimation period (pre-shock period), which was followed by a single unsignaled footshock (0.4 mA; 3 s duration). The animals remained in the chamber for an additional 50 s (post-shock period). At the end of this period, rats were removed and subsequently placed in their home cages. A single footshock was given in the conditioning context to elicit a minimal level of freezing, which allowed a potential facilitating influence of the stressful experience on fear memory (Giachero et al. 2013a).

Test sessions

Rats were re-exposed to the associated context without shocks for 3 min, 2 days (test 1) or 7 days (test 2) after training. Freezing behavior was assessed as a measure of fear memory during test 1 and during test 2. The behavior of each rat was continuously videotaped in order to score freezing behavior during the pre-shock and post-shock period, and during the entire 3 min test sessions. The total time spent freezing in each period was quantified (in seconds) using a stopwatch and expressed as the percentage of total time. Freezing, a commonly used index of fear in rats (Blanchard and Blanchard 1969), was defined as a total absence of body and head movement except those associated with breathing.

Surgery and intracranial infusions

Under aseptic conditions, rats were intraperitoneally anesthetized with 1.5 ml/kg of a solution containing ketamine (10%; Cetamin®, Brazil) and xylazine (2%; Xilazin®, Brazil), associated with local anesthetic (3% lidocaine with norepinephrine 1:50,000, Dentsply, Brazil) and placed in a stereotaxic instrument (Stoelting, Wood Dale, IL). The scalp was incised and retracted, and the head position was adjusted to place bregma and lambda in the same horizontal plane with the incisor bar set at − 3.3 mm. For dlPAG experiments, a stainless-steel guide cannula (13 mm length, 22G) was implanted unilaterally into the caudal dlPAG using the following coordinates: anteroposterior − 7.2 mm, midline 1.9 mm, and dorsoventral − 2.0 mm from the skull surface at an angle of 22° (Paxinos and Watson 2007). For BLA experiments, two stainless-steel guide cannulas were bilaterally lowered into the BLA (22-gauge, length 12 mm) using the following coordinates: anteroposterior -2.8 mm, midline 5.0 mm, and dorsoventral 6.1 mm (Paxinos and Watson 2007). The guide cannulas were secured in place using acrylic cement and two stainless-steel screws were anchored to the skull. Stainless "dummy cannulas" protruding 0.5 mm beyond the tips were placed inside the guide cannulas to prevent occlusion. At the end of the surgery, subjects were injected intramuscularly with an antibiotic association of benzylpenicillin and streptomycin (1.0 ml/kg; Pentabiótico®, Brazil) to prevent possible infections. Animals were gently handled every day, replacing missing dummy cannulas when necessary, and were allowed to recover from surgery for 5–7 days before the experimental procedures.

Microinfusions were made using an infusion pump (Insight, Ribeirão Preto, SP, Brazil) with a thin dental needle (outer diameter = 0.3 mm), sized 16.2 mm, introduced through the guide cannula, extending 2.5 mm below the cannula end, reaching the dlPAG or BLA. A polyethylene catheter (PE10; Clay Adams, USA) was interposed between the upper end of the dental needle and the microsyringe (5 µl; Hamilton), and an air bubble displaced inside the polyethylene was used to monitor the drug flow.

Drugs and dlPAG and BLA infusion procedure

For intra-dlPAG infusion, each rat was infused with 0.4 µl of MUS (0.25 µg/µl) or PBS into dlPAG at a flow rate of 0.4 µl/min over a period of 60 s. This dose of MUS was selected based on previous studies (Johansen et al. 2010). For intra-BLA infusion, each rat was bilaterally infused with 0.5 ml per side of MDZ (1 µg/µl) or SAL into BLA at a flow rate of 0.5 µl/min over a period of 60 s (Giachero et al. 2013a). After completion of the volume injection, the infusion cannulas were kept in place for an additional period of 60 s to allow diffusion of the drug.

Fos immunostaining

Ninety minutes after the behavioral test, animals were deeply anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and perfused transcardially with a solution of 4% (w/v) 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 five series of 40-µm-thick sections were cut with a sliding microtome in the frontal plane. One series was processed for immunohistochemistry with anti-Fos antiserum raised in rabbit (Ab-5; Calbiochem) at a dilution of 1:20,000. The primary antiserum was localized using a variation of the avidin–biotin complex system. In brief, sections were incubated for 90 min at room temperature in a solution of biotinylated goat anti-rabbit IgG (Vector Laboratories) and then placed in the mixed avidin–biotin horseradish peroxidase (HRP) complex solution (ABC Elite Kit; Vector Laboratories) for the same period. The peroxidase complex was visualized by a 5-min exposure to a chromogen solution containing 0.02% 3,3′ diaminobenzidine tetrahydrochloride (DAB; Sigma) with 0.3% nickel ammonium sulfate in 0.05-M Tris buffer (pH 7.6), followed by incubation for 20 min, in chromogen solution with hydrogen peroxide (1:3000) to produce a blue–black product. The reaction was stopped by extensive washing in potassium PBS (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.

Quantification of Fos-labeled cells

Density of Fos-immunoreactive neurons were evaluated by an observer without knowledge of the animal's

experimental treatment and were generated for selected brain regions 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 the density of Fos labeling, we first delineated, as defined in adjoining Nissl-stained sections, the borders of the medial parvicellular part of the paraventricular hypothalamic nucleus, which contains the CRH cells, 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 performed with the aid of a computer program (Image-Pro Plus, version 4.5.1; Media Cybernetics, Silver Spring, MD, USA). Cell densities were obtained on both sides of the brain and averaged for each individual.

Blood sampling and determination of serum corticosterone by ELISA

The procedure of blood sampling from the tail was carried out by negative pressure according to Lee and Goosens (2015) 60 min after restraint session. In brief, each rodent was secured with a clean cloth and its tail immersed in 42 °C water for 40–50 s to dilate blood vessels. After the recognition of the sampling point, a catheter was inserted into the vein for blood collection (minimum of 100 µl/rat). Blood samples were centrifuged at 3000 rpm during 10 min (4 °C) and sera samples were separated immediately afterward and stored at − 20 °C until the assay. Corticosterone concentration was assessed in duplicate by solid-phase enzyme-linked immunosorbent assay kit (Corticosterone ELISA, IBL International®, Germany). At the day of the assay, thawed samples were restored by vortex mixing. Twenty microliters of each standard and blood sera samples were used in the assays. Standard curves were constructed using 0, 5, 15, 30, 60, 120, and 240 nmol/L standard samples provided by the kit manufacturer. The optical density (450 nm) for each microliter plate was measured using an Infinite® 200 Pro reader (TECAN Group Ltd., Switzerland) within 10 min after adding stop solution for ending the enzymatic reaction. Determination of the concentration curve was made by an automated method with four parameter logistics curve fit using MasterPlex® 2010 software (Hitachi Software Engineering Co. Ltd., USA). Calculation of the results was made after constructing a standard curve by plotting the mean absorbance value obtained from each standard against its concentration. Intra- and inter-assay variations were 6.7% and 13.6%, respectively.

Structural plasticity analysis

Dendritic spine visualization and analysis were performed as previously reported by other researchers and by our laboratory (Tyler and Pozzo-Miller 2003; Calfa et al. 2012; Giachero et al. 2013b, 2015; Bender et al. 2018). Concisely, under deeply anesthesia (chloral hydrate, 400 mg/kg i.p.), animals were transcardially perfused first by ice-cold PB (0.1 M, pH 7.4) and then fixed using ice-cold 4% paraformaldehyde (PFA) (in 0.1 M PB, pH 7.4). After brain removed and post-fixed (4% PFA, 24 h, 4 °C), coronal sections (200-µm-thick) containing the DH were obtained with a vibratome and collected in 0.1% PBS. The CA1 DH was stained with small droplets (< 10 µm) of a saturated solution of the lipophilic dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate (DiI, Invitrogen; Carlsbad, CA, USA) in fish oil (Pozzo-Miller et al. 1999) by microinjection with a patch pipette and positive pressure application (Giachero et al. 2013b). Using a Leica DMI6000 B laser-scanning confocal microscope (Laboratório Central de Microscopia Eletrônica, UFSC, Florianópolis, Brasil) with a 100× oil immersion, z-sections from labeled dendritic segments were collected. The images were deconvolved using the LAS AF Lite software (Leica Microsystems, Wetzlar, Germany). A theoretical point spread function was used.

The dendritic spine analysis was achieved manually using ImageJ software. Dendritic protrusions less than 3 µm length and contacting with the parent dendrite were considered for the analysis (Murphy and Segal 1996; Chapleau et al. 2009; Calfa et al. 2012). Special consideration was taken to select a single dendritic segment, presumably from different neurons but from CA1 stratum radiatum, in lights of the high density of labeled dendrites. Thus, from the z-section projection, the total number and also the number of each particular type of dendritic spine normalized to 10 µm of the dendritic segment length were counted and certainly that each spine was counted only once.

Spine types were classified as previously described (Koh et al. 2002; Tyler and Pozzo-Miller 2003): type I or "stubby"-shaped dendritic spines, type II or "mushroom"-shaped dendritic spines, and type III or "thin"-shaped dendritic spines. Different measurements were taken for each dendritic protrusion to classify them, in brief: the length (dimension from the base at the dendrite to the tip of its head, L), the diameter of the neck (measured as the maximum neck diameter, dn), and the diameter of the head (measured as the maximum head diameter, dh) (Koh et al. 2002). Thus, individual spines were included in each category based on the specific ratios L/dn and dh/dn (Koh et al.

2002; Tyler and Pozzo-Miller 2003; Calfa et al. 2012; Giachero et al. 2013b).

As previously reported (Tyler and Pozzo-Miller 2003; Chapleau et al. 2009; Calfa et al. 2012; Giachero et al. 2013b, 2015; Bender et al. 2018), we have included the "stubby"- and "mushroom"-shaped dendritic spines in the category of "mature" spines. This recategorization is in virtue of the widespread Ca^{2+} transients in the parent dendrite and neighboring spines and because of the strength of the excitatory synapses formed on these spines (Harris 1999; Segal et al. 2000; Yuste et al. 2000; Nimchinsky et al. 2002; Kasai et al. 2003).

Histological procedures

After behavioral tests, rats were sacrificed by an overdose of chloral hydrate and their brains were removed and immersed in a 10% formalin fixative solution. Frontal sections were cut in a cryostat (Leica, Nussloch, Germany). An observer blinded to the experimental condition verified cannula placement throughout the dlPAG and BLA under a light microscope. Animals with inaccurate cannula placement or extensive damage were excluded from data analysis.

Statistical analyses

Behavioral experiments were analyzed by a repeated-measure ANOVA, followed by Bonferroni post hoc test. Data from post-shock freezing and corticosterone levels (experiment 6) were also analyzed by a two-way ANOVA. The significance level used for all statistical analyses was set at $p < 0.05$. For the density of Fos-labeled cells, the data were initially subjected to the Kolmogorov–Smirnov test. Since a normal and homoscedastic profile was identified, a *T* test for independent groups was performed.

For the dendritic spine analysis, dendritic segments that belong to different slices from the same rat and from the same experimental group were considered for the statistical analysis. The distribution of the data does not rely on a normal distribution, and considering that mean values are rather insensitive to subtle changes, we use cumulative frequency plots to measure shifts in the total number of dendritic spines, mature and thin dendritic spines per 10 µm of dendritic segment in the different experimental groups. Cumulative distribution probabilities were compared by Kolmogorov–Smirnov (KS) test. Under this consideration, the total density of dendritic spines as well as mature and thin dendritic spines, no significant differences were observed between rats from each particular experimental group comparing the results from the different dendritic segments ($p > 0.05$, KS test for all the comparisons). Data were also expressed as median (quartile) and compared by Kruskal–Wallis test and multiple comparison of mean rank was used as post hoc. $p < 0.05$ was considered statistically significant. We performed a compromise power analyses to determine the statistical power given the number of observations, sample means, and SD, using G*Power (Faul et al. 2007). All data collection was achieved in a blinded manner.

Results

Experiment 1

The combination of stress and fear conditioning enhances fear memory retention

To evaluate the promoting influence of stress on fear memory during retrieval, the animals were randomly allocated into two groups; one group was subjected to a weak-training procedure (CS-US) and the other one received no shock (CS-noUS). Twenty-four hours later, half of the animals in each group were restrained (S) and another group remained without manipulation (NS). On day 3, the freezing behavior was measured during Test 1 and on day 7 during Test 2 (Fig. 1a).

During the post-shock period, a Student's *t* test analysis revealed a significant effect between CS-US vs. CS-noUS ($p < 0.0001$) where the animals that received the shock showed a significant increased freezing response during the post-shock period [percentage of freezing (mean + SEM) CS-US, 25.06 + 0.98] in comparison to the group that was not foot-shocked (CS-noUS, 6.22 + 1.21).

A repeated-measures ANOVA for the freezing behavior response during Test 1 and Test 2 showed a significant effect of training ($F(1,27) = 45.10$, $p < 0.05$), stress ($F(1,27) = 21.59$, $p < 0.05$), and training × stress ($F(1,27) = 19.47$, $p < 0.05$) (Fig. 1b). Post hoc test revealed that the group receiving a weak training followed by restrained stress showed increased ($p < 0.05$) freezing responses during Test 1 and Test 2 when compared to all remaining groups.

In summary and in line with previous study (Giachero et al. 2013a, b), conditioned animals that were subjected to a weak-training procedure and that later on experienced an unrelated stressful event, showed a robust and persistent fear expression.

Experiment 2

Intra-dlPAG MUS prior to restraint prevent the augmented fear retention following stress in fear conditioned rats

This set of experiments investigated the effect of MUS intra-dlPAG prior to the restraint session on the resulting fear memory after retrieval. Thirty-two dlPAG-cannulated

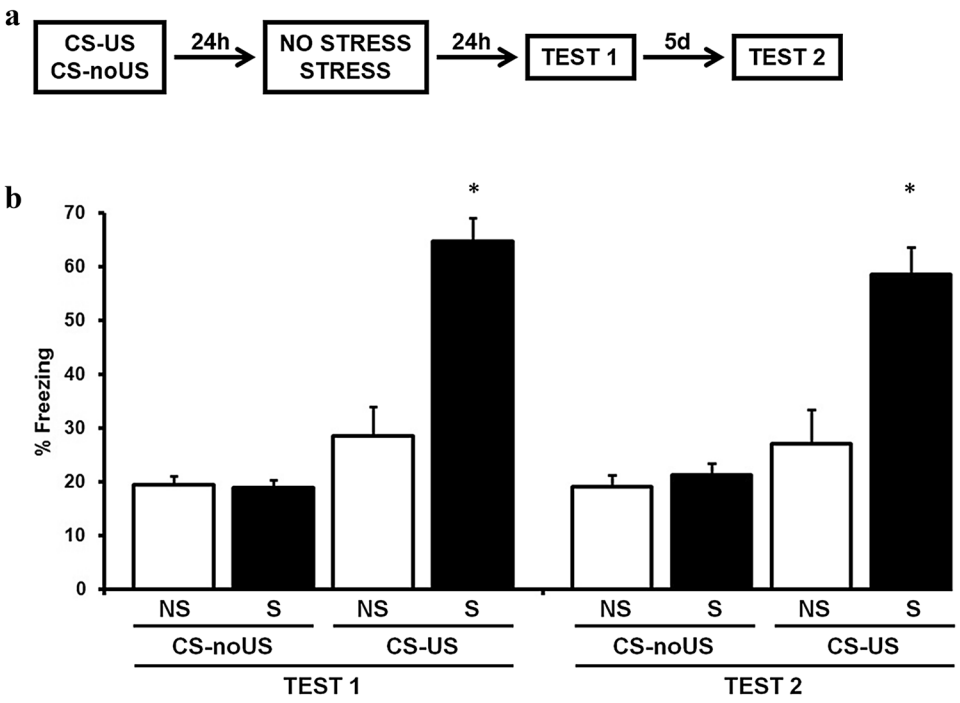

Fig. 1 The combination between stress and the retrieval of a consolidated trace enhanced fear memory retention. Stressed and control non-stressed animals were submitted to a weak-training session or exposed to the context without receiving the electrical footshock. **a** Schematic representation of the experimental design. **b** Bar graph showing the freezing response denoted in animals at 1 (Test 1) and 6 (Test 2) days after the restraint experience. Data are expressed as mean ± SEM percentage of freezing time spent during both test ($n = 8$ rats per group). *$p < 0.05$ compared to the remaining experimental groups (repeated-measure ANOVA, Bonferroni post hoc test)

rats were submitted to the conditioning protocol. One day later, a group of rats was administered with PBS and another group with MUS intra-dlPAG. Ten min after infusion, a group of rats was restrained and another group remained without manipulation. On days 3 and 7, all rats were re-exposed to the associated context and their freezing behavior assessed (Fig. 2a, b).

During the post-shock period, a two-way ANOVA revealed a non significant effect between groups. The percentage of freezing spent during post-shock are as follows: PBS-no stress (mean + SEM), 23.90 + 0.55; PBS-stress, 25.65 + 1.05; MUS-no stress 24.77 + 0.93; MUS-stress, 27.47 + 1.15.

A repeated-measure ANOVA showed a significant effect of drug treatment ($F(1,28) = 24.88$, $p < 0.05$), stress ($F(1,28) = 27.39$, $p < 0.05$), and drug treatment × stress interaction ($F(1,28) = 30.68$, $p < 0.05$) (Fig. 2c). Post hoc analysis revealed ($p < 0.05$) that in the weak-training group, dlPAG MUS infusion before restrained stress blocked the increased freezing response obtained in the PBS group during Test 1 and Test 2. Interestingly, such defensive response was prevented by the inactivation of the dlPAG. Unstressed animals with intra-dlPAG MUS and unstressed animals with intra-dlPAG PBS displayed similar levels of fear.

In conclusion, dlPAG inactivation by local infusion of MUS prior to restraint prevents the promoting influence of stress on fear memory during retrieval.

Experiment 3

MUS intra-dlPAG after stress exposure does not affect the contextual fear memory following retrieval and stress

This experiment investigated whether dlPAG inactivation post-restraint impairs the subsequent conditioned response. Following the same protocol described in Experiment 2, 1 day after conditioning, a group of rats was exposed to the stressful event and another group remained without manipulation. Immediately after the restrained stress, animals received PBS or MUS intra-dlPAG infusions. Freezing was evaluated during Test 1 and Test 2 (Fig. 3a, b).

During the post-shock period, a two-way ANOVA revealed a non significant effect between groups. The percentage of freezing spent during post-shock are as follows: no stress-PBS (mean + SEM), 28.25 + 2.12; stress-PBS, 27.27 + 1.45; no stress-MUS, 26.11 + 1.61; stress-MUS, 26.42 + 0.98.

A repeated-measure ANOVA indicated only a significant effect of stress ($F(1,28) = 110.19$, $p < 0.05$). No effect was observed regarding drug treatment ($F(1,28) = 0.11$, $p = 0.74$) or stress × drug treatment ($F(1,28) = 0.28$, $p = 0.60$). As shown in Fig. 3c, stressed animals either infused with PBS or MUS in the dlPAG, after restrained stress, showed an increased ($p < 0.05$) percentage of freezing during Test 1 and Test 2 when compared to unstressed animals. Hence,

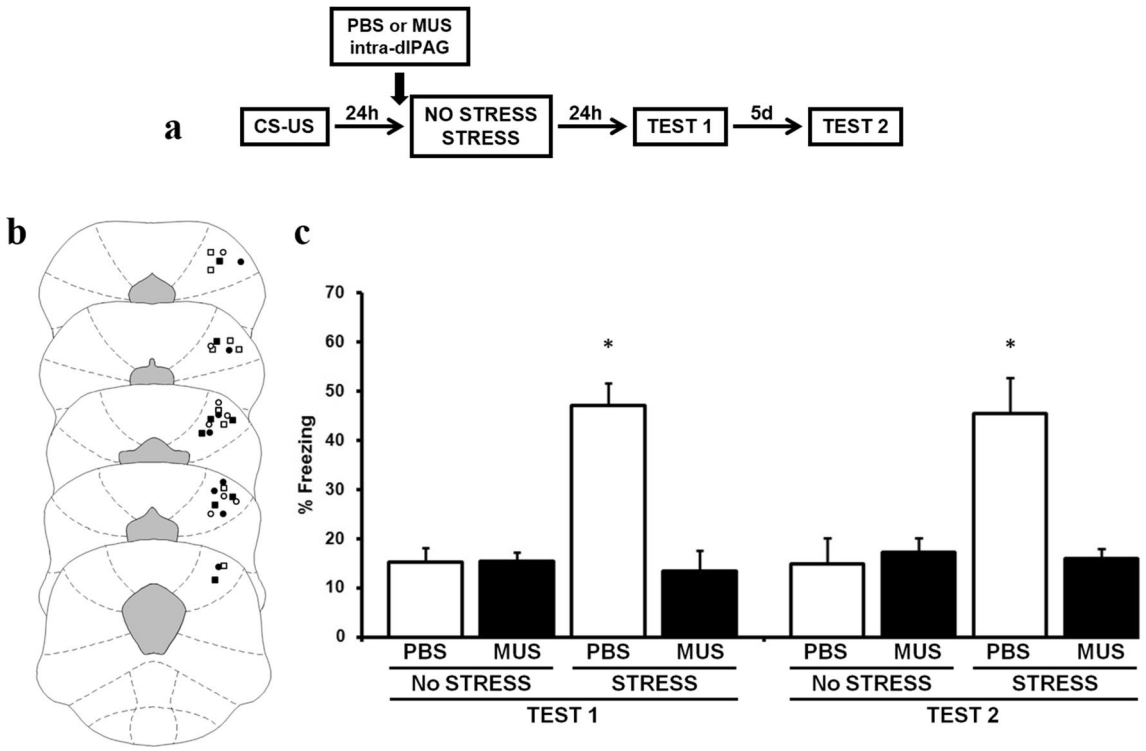

Fig. 2 Intra-dlPAG infusion of MUS prior to restraint prevented the resulting fear memory. **a** Schematic representation of the experimental design. **b** Schematic drawings of coronal sections showing the location of the cannula placement in dlPAG (Adapted from Paxinos and Watson, 2013); black filled circles: PBS/NS; unfilled circles: MUS/NS; black filled squares: PBS/S; unfilled squares: MUS/S. **c** Bar graph showing the freezing exhibited in both tests by rats that either received intra-dlPAG administration of MUS or PBS 10 min prior to restraint. Data are expressed as mean ± SEM percentage of freezing time spent during the tests ($n = 8$ rats for all groups). *$p < 0.05$ compared to the rest of the experimental groups (repeated-measure ANOVA, Bonferroni post hoc test)

MUS intra-dlPAG after restraint did not affect stress-induced memory strengthening.

In an additional experiment, designed to test the same protocol in BLA-cannulated animals, 32 rats received PBS or MDZ intra-BLA immediately after the restrained stress procedure (Fig. 4a, b).

During the post-shock period, a two-way ANOVA revealed a non significant effect between groups. The percentage of freezing spent during post-shock is as follows: no stress-PBS (mean + SEM), 27.40 + 1.30; stress-PBS, 31.52 + 1.77; no stress-MDZ, 28.14 + 1.38; stress-MDZ, 28.27 + 1.16.

A repeated-measure ANOVA indicated a significant effect of stress ($F(1,24) = 45.15$, $p < 0.05$), drug treatment ($F(1,24) = 71.03$, $p < 0.05$), stress × drug treatment ($F(1,24) = 48.31$, $p < 0.05$) and stress × drug treatment × test trial ($F(1,24) = 6.71$, $p < 0.05$). Furthermore, post hoc analysis indicated that stressed rats infused with intra-BLA PBS exhibited higher level of freezing as compared to the remaining experimental groups at both Test 1 and Test 2 ($p < 0.05$). Such increased freezing response was attenuated during Test 1 and Test 2 in stressed animals treated with MDZ (Fig. 4c).

Hence, the lack of effect of post-stress intra-dlPAG MUS suggests that this region is critically involved in the initial stages of experience-related neural plasticity. Conversely, the impairment of the resulting memory by post-stress GABAergic transmission enhancing in BLA is consistent with previous evidence indicating a critical role of this structure on both the processing and storing of aversive information.

Experiment 4

Intra-dlPAG infusion of MUS prior to retrieval does not affect the promoting influence of restraint on contextual fear memory

An attempt to explore the involvement of the dlPAG in influencing fear conditioning retrieval was performed in trained and subsequently stressed rats, as described before. Ten min prior Test 1 context exposure, rats received intra-dlPAG PBS or MUS. Five days later, all rats were relocated in the associated context to assess freezing during Test 2 (Fig. 5a, b).

During the post-shock period, a two-way ANOVA revealed a non significant effect between groups. The percentage of freezing spent during post-shock are as follows:

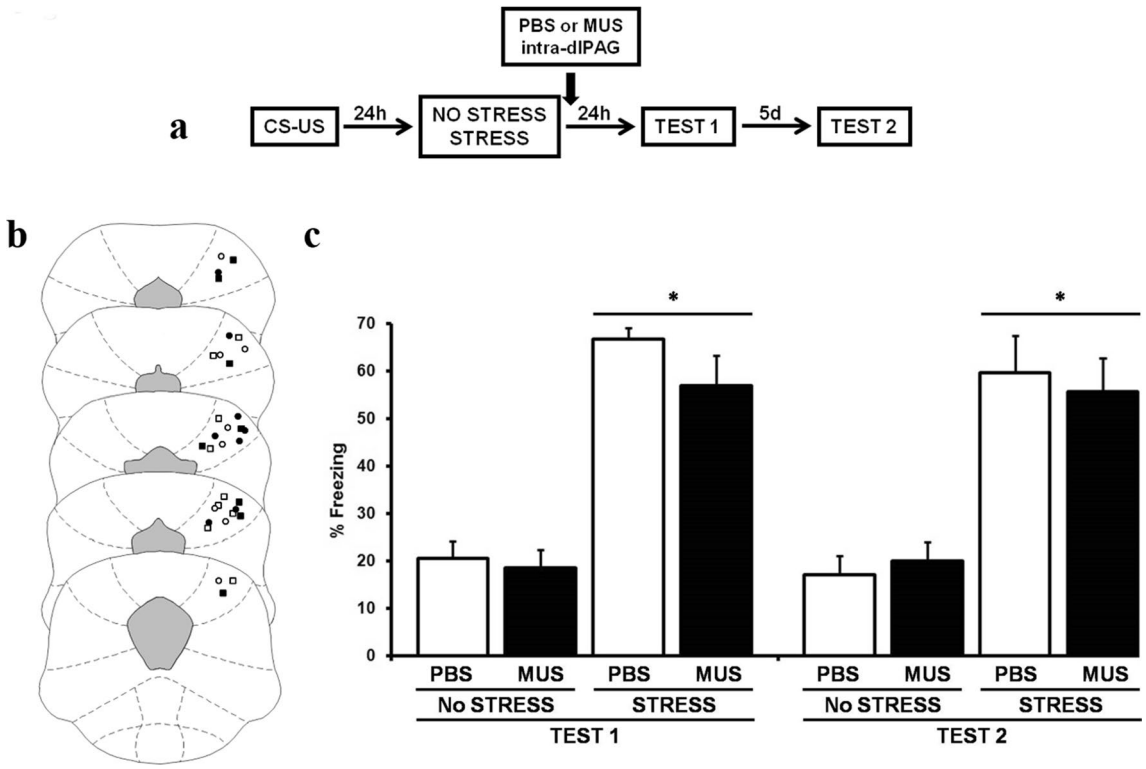

Fig. 3 Intra-dlPAG infusion of MUS immediately after restraint does not affect the contextual fear memory following retrieval and stress. **a** Schematic representation of the experimental design. **b** Schematic drawings of coronal sections showing the location of the cannula placement in dlPAG (Adapted from Paxinos and Watson, 2013); black filled circles: NS/PBS; unfilled circles: NS/MUS; black filled squares: S/PBS; unfilled squares: S/MUS. **c** Freezing behavior observed at Test 1 and Test 2 in animals with intra-dlPAG MUS or PBS infusion after to restraint. Data are expressed as mean ± SEM percentage of freezing spent during both tests ($n = 8$ rats for all groups). *$p < 0.05$ compared to the rest of the experimental groups (repeated-measure ANOVA, Bonferroni post hoc test)

no stress-PBS (mean + SEM), 30.01 + 1.89; stress-PBS, 29.11 + 1.05; no stress-MUS, 27.66 + 1.33; stress-MUS, 29.74 + 1.61.

A repeated-measure ANOVA indicated a significant effect of stress ($F(1,29) = 155.19$, $p < 0.05$), drug treatment ($F(1,29) = 17.75$, $p < 0.05$), stress × drug treatment ($F(1,29) = 12.43$, $p < 0.05$), test trial as repeated measures ($F(1,29) = 49.48$, $p < 0.05$), test trial × stress ($F(1,29) = 48.03$, $p < 0.05$), test trial × drug treatment ($F(1,29) = 21.61$, $p < 0.05$), and stress × drug treatment × test trial ($F(1,29) = 18.10$, $p < 0.05$). Furthermore, post hoc test indicated that stressed animals with PBS intra-dlPAG infusion exhibited a higher freezing at Test 1 compared to those with MUS intra-dlPAG infusion and to unstressed rats either administered with PBS or MUS ($p < 0.05$). More important, stressed animals infused intra-dlPAG with PBS or MUS exhibited a higher freezing score at Test 2 compared to those unstressed rats either administered with PBS or MUS ($p < 0.05$) (Fig. 5c).

These results showed that PAG inactivation prior retrieval, despite reducing freezing expression, did not affect the fear memory trace, since the promoting influence of stress on fear memory was fully exhibited in Test 2.

Experiment 5

Intra-dlPAG infusion of MUS prior to restraint decreases both Fos levels in the hypothalamic paraventricular nucleus and corticosterone secretion

This set of experiments tested the hypothesis whether, in addition to preventing fear memory increase, MUS intra-dlPAG could also affect the neuroendocrine response to stress. dlPAG-cannulated rats were trained as in Experiment 1. One day later, a group of rats was administered with PBS and another group with MUS intra-dlPAG. Ten minutes after infusion, all rats were restrained and 90 min after stress sacrificed for the quantification of Fos-labeled cells in the paraventricular nucleus of the hypothalamus (PVH) (Fig. 6a, d).

A Student's t test for the density of Fos-labeled cells yielded significant group effects for both dorsal and ventral zones of the medial parvicellular part of the paraventricular

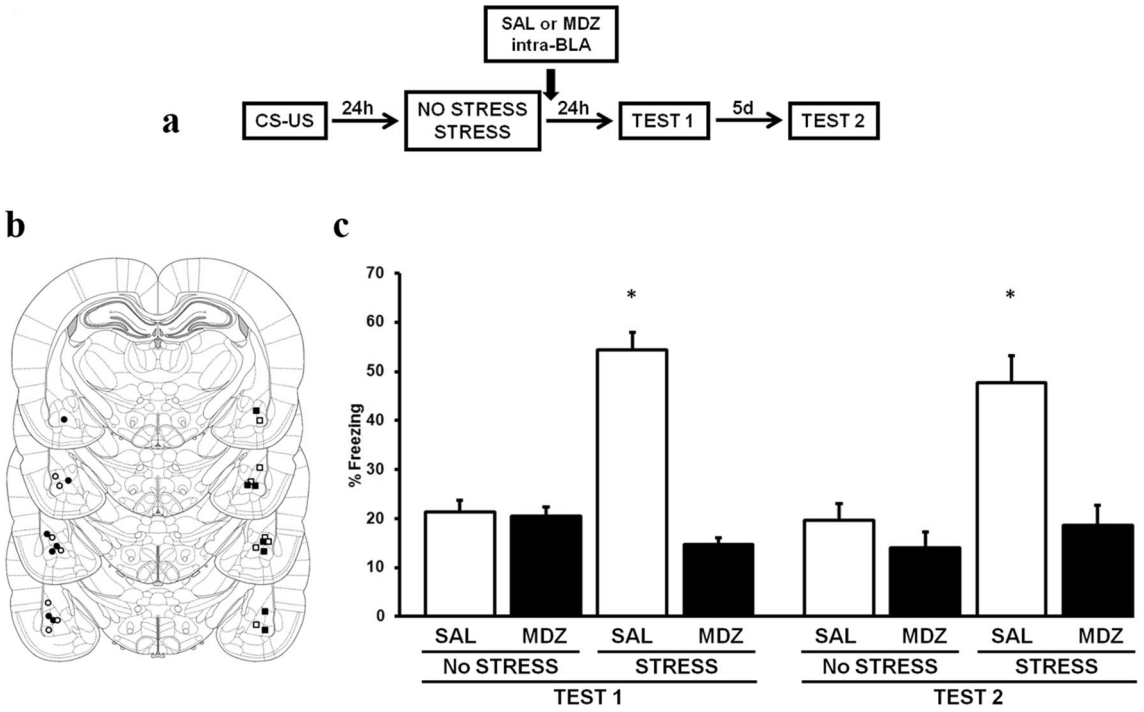

Fig. 4 Intra-BLA infusion of MDZ immediately after restraint impairs the contextual fear memory following retrieval and stress. **a** Schematic representation of the experimental design. **b** Graphic drawings of coronal sections showing the location of the cannula placement in BLA (Adapted from Paxinos and Watson, 2013); black filled circles: NS/PBS; unfilled circles: NS/MUS; black filled squares: S/PBS; unfilled squares: S/MUS. **c** The freezing exhibited in both tests by rats that either received intra-BLA infusion of MDZ or SAL immediately after restraint. Data are expressed as mean ± SEM percentage of freezing spent during the tests ($n = 7$ rats for all groups). *$p < 0.05$ compared to the rest of the experimental groups (repeated-measure ANOVA, Bonferroni post hoc test)

nucleus (PVH-mpd and mpv, respectively), where, compared to the PBS stressed group, restrained animals with intra-dlPAG MUS showed a significant decrease in the density of Fos-labeled cells in both the PVH-mpd ($t = 4.58127$; $df = 8$; $p = 0.00179$) and the PVH-mpv ($t = 3.439$; $df = 8$; $p = 0.00883$) (Fig. 6c). Notably, the PVH-mpd corresponds to the part of the nucleus that contains CRH cells, whereas the PVH-mpv is one of the autonomic parts of the nucleus providing projections to autonomic cell groups in the brainstem and spinal cord (see Swanson 1987).

In an additional procedure, dlPAG-cannulated rats were subjected to the weak-training procedure. One day later, a group of rodents was administered with PBS and another group with MUS intra-dlPAG. Ten minutes after infusion, a group of rats was restrained and another group remained without manipulation. Sixty minutes after stress, collection of blood sample was realized for measure of serum corticosterone concentrations (Fig. 6a, d).

During the post-shock period, a two-way ANOVA revealed a non significant effect between groups. The percentage of freezing spent during post-shock are as follows: PBS-no stress (mean + SEM), 24.38 + 1.02; PBS-stress, 28.91 + 1.73; MUS-no stress 23.29 + 1.22; MUS-stress, 26.88 + 1.47.

A two-way ANOVA analysis reflected a significant effect of treatment ($F(1,19) = 35.97$, $p < 0.05$), stress ($F(1,19) = 38.04$, $p < 0.05$) and treatment × stress interaction ($F(1,19) = 16.61$, $p < 0.05$). Post hoc test revealed a higher CORT level only in stressed animals previously infused with PBS when compared to all other groups ($p < 0.05$) (Fig. 6e).

This finding showed that both PVH activation and CORT levels elevation, typically observed following stressful experiences, were blocked after dlPAG inhibition with local infusion of MUS prior to restraint.

Experiment 6

Intra-dlPAG MUS administration prevents the hippocampal structural plasticity associated with the resulting contextual fear memory following stress and retrieval

In this group of experiment, the influence of intra-dlPAG MUS infusion prior to stress on hippocampal dendritic remodeling generated by the combination of fear training and restraint was evaluated.

Sixteen dlPAG-cannulated animals were submitted to the fear conditioning protocol. One day later, a group of rats was administered with PBS and another group with MUS

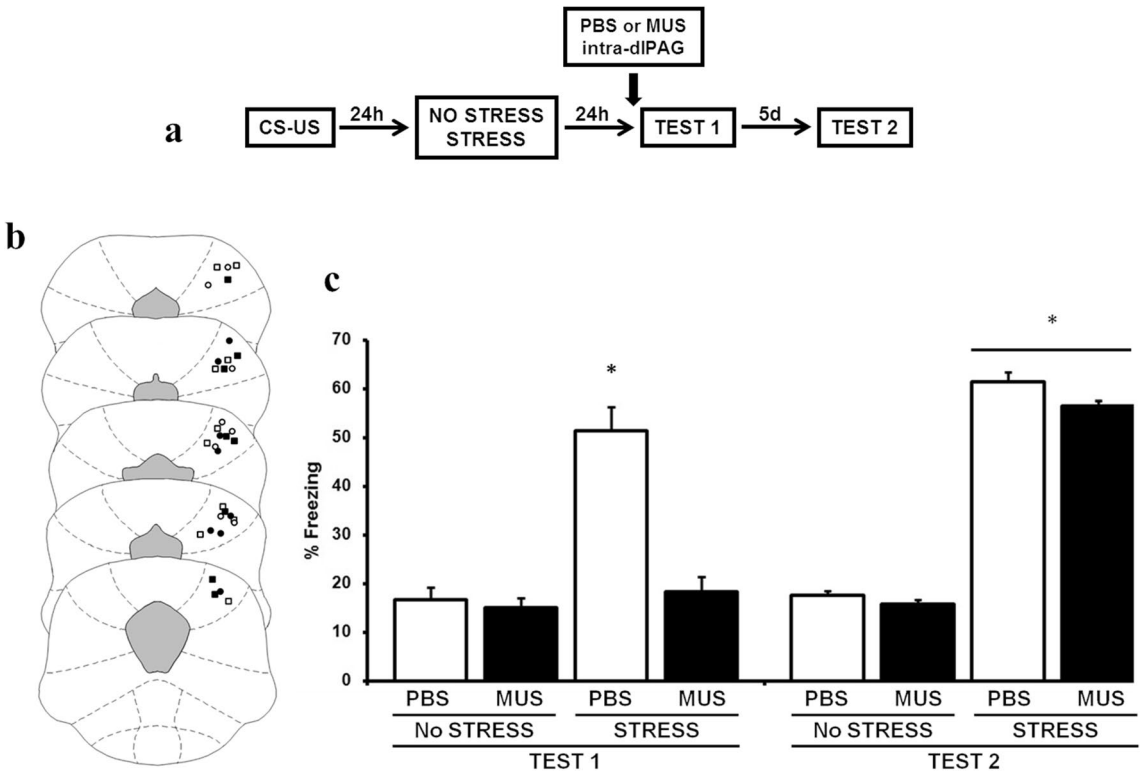

Fig. 5 Intra-dlPAG infusion of MUS prior to Test 1 does not prevent the promoting influence of stress on fear memory. **a** Schematic representation of the experimental design. **b** Schematic drawings of coronal sections showing the location of the cannula placement in dlPAG (Adapted from Paxinos and Watson, 2013); black filled circles: NS/PBS; unfilled circles: NS/MUS; black filled squares: S/PBS; unfilled squares: S/MUS. **c** Freezing behavior observed at Test 1 and Test 2 in animals with intra-dlPAG MUS or PBS infusion prior to Test 1. Data are expressed as mean ± SEM percentage of freezing spent during both tests ($n = 8$–9 rats for all groups). *$p < 0.05$ compared to the rest of the experimental groups (repeated-measure ANOVA, Bonferroni post hoc test)

intra-dlPAG. Ten minutes after the infusion, a group of rats was restrained and another group remained without manipulation. Twenty-four hours later, the animals were sacrificed and the brains removed for spine analysis in the dorsal hippocampus (Fig. 7a).

During the post-shock period, a two-way ANOVA revealed a non significant effect between groups. The percentage of freezing spent during post-shock are as follows: PBS-no stress (mean + SEM), 30.66 + 1.59; PBS-stress, 29.17 + 1.27; MUS-no stress 31.01 + 1.32; MUS-stress, 28.83 + 1.20.

Spine counts were performed on a total of 153 dendritic segments as follows: PBS/NS ($n = 36$ segments, 1260.08 µm of total dendritic length analyzed, four rats), MUS/NS (40 segments, 1136.74 µm, four rats), PBS/S (42 segments, 1188.07 µm, four rats), and MUS/S (35 segments, 1141.55 µm, four rats). Figure 6b shows representative examples of the different dendritic segments in the DH stratum radiatum CA1 for each particular experimental group.

For the total density of dendritic spines as well as for mature and thin dendritic spines, no significant differences were observed between rats from each particular experimental group comparing the results from the different dendritic segments ($p > 0.05$, KS test for all the comparisons).

The dendritic spine analysis demonstrates that dlPAG inactivation by local infusion of MUS prevented the structural changes in DH induced by both fear conditioning and restraint. The analysis of the cumulative probability distributions for the total density of dendritic spines reflected a significant rightward shift, toward higher numbers of dendritic spines in PBS/S rats in comparison to the rest of the experimental groups ($p < 0.05$ for each individual comparison, KS test) (Fig. 7c). Interestingly, the MUS/S group presented a cumulative probability distribution that is comparable to non-stressed animals infused with PBS or MUS ($p > 0.05$ for each individual comparison, KS test) (Fig. 7c). The shift toward higher numbers of dendritic spines in PBS/S also resulted in a higher median (quartiles, total density/10 µm), 16.2 [15.9–17.1], with respect to the rest of the groups, PBS/NS 12.1 [11.2–12.5], MUS/NS 12.2 [11.7–12.6], and MUS/S 12.5 [11.9–12.9] (Kruskal–Wallis test = 65.642, $p < 0.001$).

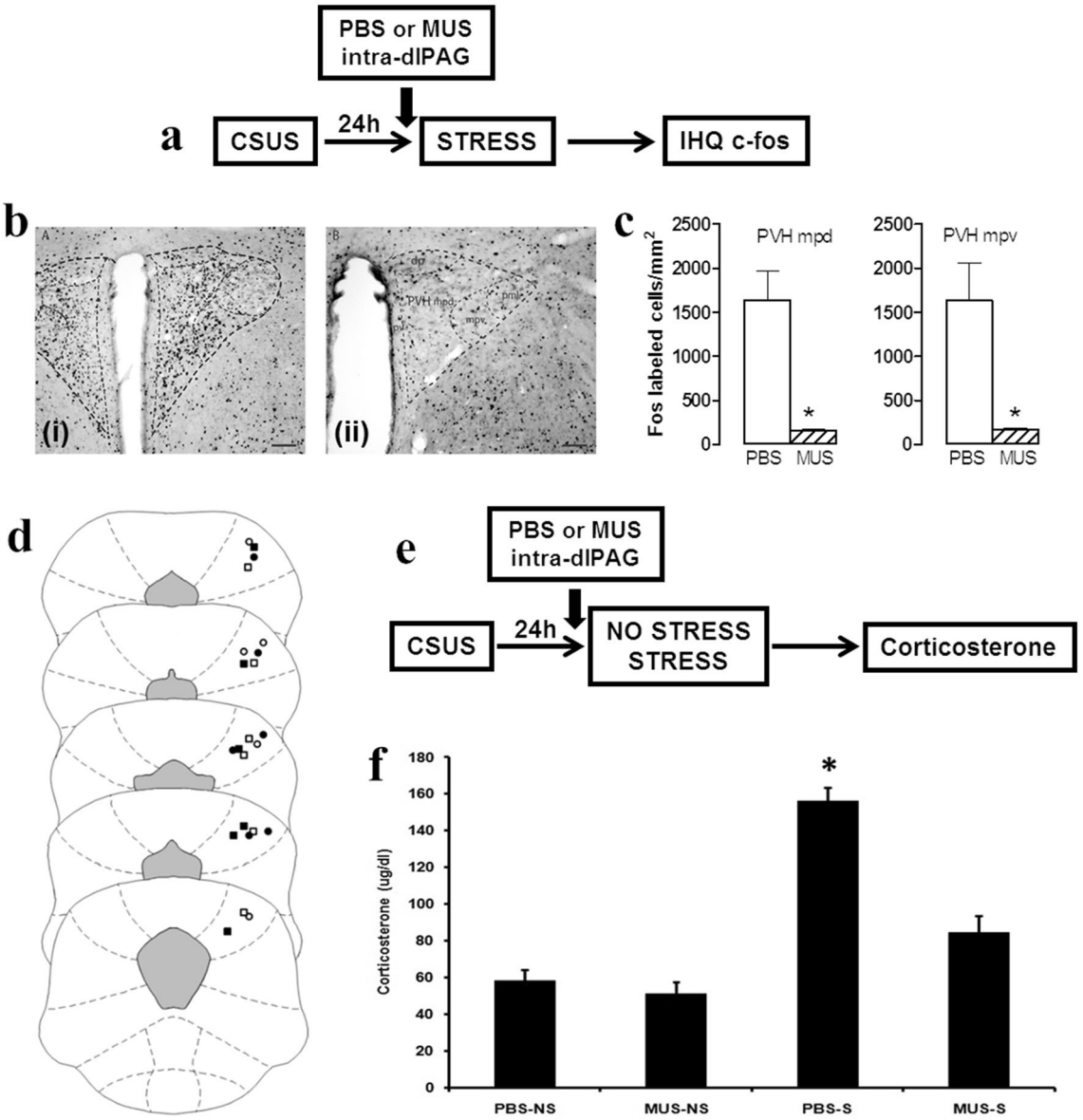

Fig. 6 Intra-dlPAG MUS infusion affects both PVH activation and increased CORT level induced by the restraint stress. **a** Schematic representation of the experimental design. **b** Photomicrographs of transverse Fos-stained sections at the levels of PVH of representative cases of the restrained groups pre-infused intra-dlPAG with PBS (i) or MUS (ii). *PVHdp* paraventricular hypothalamic nucleus, dorsal parvicellular part, *mpd* medial parvicellular part, dorsal zone, *mpv* medial parvicellular part, ventral zone, *pml* posterior magnocellular part, lateral zone, *pv* periventricular part. Scale bars, 100 µm. **c** Reduced density of Fos-labeled cells in the PVH-mpd ($N=5$, t [8] = 4.58, $^*p=0.001$) and PVH-mpv ($N=5$, t [8] = 3.44, $^*p=0.008$) of restrained groups pre-infused intra-dlPAG with MUS when compared to PBS treated rats. **d** Schematic drawings of coronal sections showing the location of the cannula placement in dlPAG (Adapted from Paxinos and Watson, 2013); black filled circles: PBS/NS; unfilled circles: MUS/NS; black filled squares: PBS/S; unfilled squares: MUS/S. **e** Schematic representation of the experimental design. **f** Bar graph showing the serum corticosterone in animals with intra-dlPAG MUS or PBS infusion before to stress. Data are expressed as mean ± SEM of the CORT level measured 60 min after the restraint session ($n=5$–6 rats for all groups) $^*p<0.05$ compared to the rest of the experimental groups (two-way ANOVA, Bonferroni post hoc test)

Similar to total dendritic spines, a significant rightward shift toward higher numbers of mature dendritic spines in PBS/S compared to the rest of the experimental groups was noticeable ($p<0.05$ for each individual comparison, KS test) (Fig. 7d). MUS/S animals presented cumulative probability distributions comparable to the PBS- or MUS-infused nonstressed animals ($p>0.05$ for each particular comparison, KS test; Fig. 7d). In parallel, a higher median (quartiles, mature spines/10 µm) in PBS/S 13.5 [13.1–14.0] was observed in comparison to PBS/NS

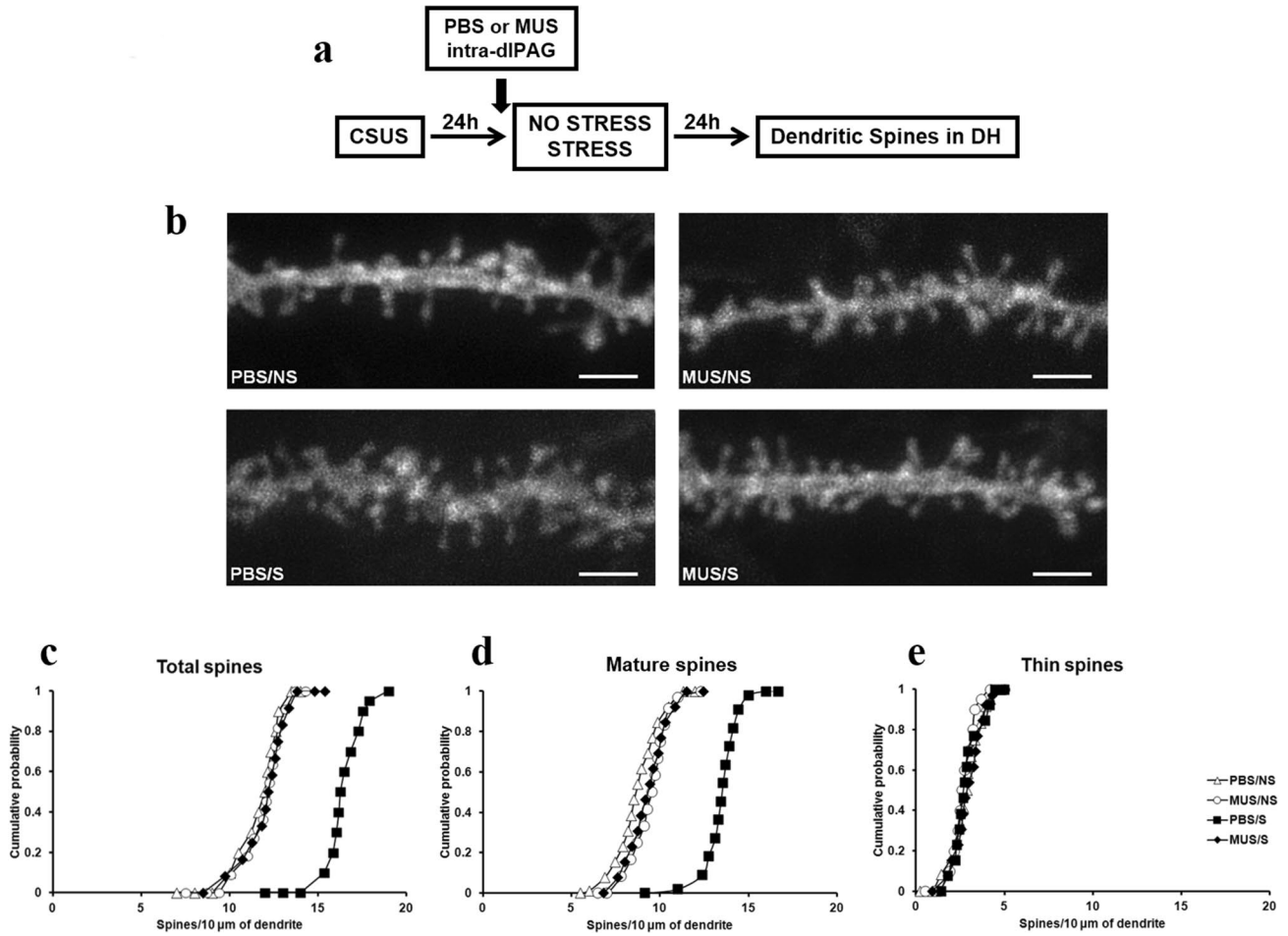

Fig. 7 Intra-dlPAG infusion of MUS prior to restraint prevented the promoting influence of stress DH structural remodeling. **a** Schematic representation of the experimental design. **b** Confocal microscopic photos of representative examples of apical dendritic segments of DH CA1 pyramidal neurons which were selected for quantitative analysis of dendritic spines from animals: PBS/NS ($n=4$ rats), MUS/NS ($n=4$ rats), PBS/S ($n=4$ rats), and MUS/S ($n=4$ rats). Bar, 2 mm. **c**–**e** Cumulative frequency of total (**c**), mature (**d**), and thin (**e**) spine density on apical dendrites of hippocampal pyramidal cells CA1 stratum radiatum ($p<0.05$, by Kolmogorov–Smirnov test)

9.0 [8.3–9.7], MUS/NS 9.4 [8.7–10.0], and MUS/S 9.4 [8.9–10.0] (Kruskal–Wallis test = 64.864, $p<0.001$).

The analysis of thin dendritic spines resulted in no significant differences between the experimental groups ($p>0.05$ for each individual comparison, KS test) (Fig. 7e). This also resulted in comparable median (quartiles, thin spines/10 µm) between the experimental groups, PBS/NS 3.0 [2.4–3.4], MUS/NS 2.7 [2.3–3.2], PBS/S 2.8 [2.4–3.2], MUS/S 2.8 [2.4–3.3] (Kruskal–Wallis test = 1.480, $p=0.687$).

Overall, these findings suggest that MUS intra-dlPAG prior to restraint prevented the enhancement of the density of hippocampal "mature" spines elicited by the combination of fear conditioning and stress.

Discussion

The present study shows that conditioned rats with a weak conditioning protocol that were later submitted to an unrelated stressful experience exhibited a robust and persistent fear memory. These results suggest that, a certain time after stress, the retrieval experience of a weak memory trace previously established becomes a powerful mnemonic enhancer. In addition, previous data showed that this stress-promoting influence on the fear memory following retrieval was dependent on the previous context–shock association and unrelated to an unspecific generalization of

fear or to a sensitized response to restraint resulting from a prior-shock experience (Giachero et al. 2013a).

It has been proposed that the facilitating influence of the environmental challenge on fear memory is a consequence of BLA hyperexcitability, due to the decrease of recurrent GABA inhibition in BLA (Rodriguez Manzanares et al. 2005; Isoardi et al. 2007). Related to this, activating GABAa sites with MDZ in BLA prior to the emotional arousing stimulus prevented the enhancement of fear retention (Maldonado et al. 2011; Giachero et al. 2013a, 2015). On the other hand, it has been reported that a negative emotional state caused by the chemical stimulation of the dlPAG prior to retrieval potentiates a consolidated fear memory (Mochny et al. 2012). Here, dlPAG inactivation by local infusion of MUS prior to restraint attenuated the stress-induced enhancement of both expression and retention of fear memory. It is possible to ascertain that such influence might have been due to an impairment of the instructive function given by the dlPAG on high-level processing brain regions such as the BLA, and hippocampus (Maren and Fanselow 1995; Kim et al. 2013) preventing the generation of neurobiological changes (i.e., BLA hyperexcitability or hippocampus discriminative properties) necessary for memory enhancement. Moreover, ventral subiculum (vSub) stimulation induced LTP in the BLA and electrical priming of the dorsal periaqueductal gray (dPAG) influenced this vSub HFS induced plasticity (Kim et al. 2013; Horovitz and Richter-Levin 2015), suggesting a functional link between dPAG and BLA.

The present data also demonstrated that MUS intra-dlPAG infusion immediately following the restraint stress has no effect on the resulting fear memory, indicating that the involvement of this region is relevant in the initial stages of the experience-related neural plasticity. dlPAG inactivation pre- but not post-training impairs the subsequent conditioned response (Kim et al. 1993; De Oca et al. 1998; Souza and Carobrez 2016). Altogether, this evidence suggests a temporally graded instructive role for the dlPAG. Conversely, an impairment of this enhancement was observed following BLA inactivation by MDZ post-stress, consistent with previous evidence indicating a critical role of this structure in both process and storage of aversive information (LeDoux 2007). Overall, it is relevant to highlight that dPAG stimulation supported fear learning in life-threatening situations (Kincheski et al. 2012; Kim et al. 2013) and this modulation of fear associations was impaired by BLA inactivation (Kim et al. 2013) or hypothalamic defensive circuit inhibition (Pavesi et al. 2011). It is possible to argue that the difference observed between the post-stress inactivation of BLA and dlPAG could be due to the fact that for BLA MDZ was used, whereas MUS was applied in the dlPAG. On the other hand, although the specific target for each drug in the GABA-A receptor is diverse, both treatments produce an inactivating net effect on the structure in question, leading to a deterioration of its function during the current phenomenon (Rodriguez Manzanares et al. 2005; de Menezes et al. 2008).

As previously determined, the promoting effect of stress depends critically on the recall of the associative fear memory (Giachero et al. 2013a). Here, the infusion of MUS intra-dlPAG immediately prior to retrieval prevented fear expression (Test 1), but did not affect the resulting contextual memory (Test 2). This result dissociates the dlPAG mediating effect during fear expression from its minor relevance in previous established fear memory association, mediated by prosencephalic structures including, the BLA and the DH. It has been proposed that retrieval can be subdivided into two separate processes: reactivation and expression (Tulving 1983). The reactivation corresponds to the activation of neural systems that encode the memory trace. Operationally, this stage results from the re-exposure to information related to the initial experience. Thus, this passage from an inactive to an active state may or may not manifest itself behaviorally (Lewis 1979); that is, the non-behavioral expression is not an absolute indicative of retrieval absence. Therefore, the reactivation of memory is both necessary and sufficient for the influence of stress on contextual memory. Finally, the lack of fear expression following the inactivation of dlPAG is consistent with the argument that this is a region of integration known to respond to threatening stimuli directing motor outputs toward the appropriate defensive behavior (Carobrez et al. 2001; Cezario et al. 2008).

One of the classic responses to stress is the release of glucocorticoids following the activation of the hypothalamic pituitary adrenocortical (HPA) axis (De Kloet 2004). The PVH is crucial for the activation of the HPA system and, recently, it has been reported that restrained rats presented a substantial increase in Fos expression in this nucleus, mostly in the region that contains CRH neurons (Motta and Canteras 2015). In addition, restrained stress increases c-fos mRNA in several areas related to fear memory processing, such as BLA, CA1 area of hippocampal formation, and PAG (Cullinan et al. 1995). In the present study, we found that dlPAG inactivation prevented both the increase of c-Fos expression in the PVH-mpd, which contains CRH cells, and the glucocorticoids release induced by stress, suggesting an excitatory influence of the dlPAG on the HPA-axis activation. Moreover, dlPAG inactivation decreased the activation of the PVH-mpv, which contain cell groups that control autonomic centers in the brainstem and spinal cord, and is likely to be mobilized during stress responses (Swanson 1987). At the moment, it is not clear how the dlPAG may influence the HPA axis and the autonomic part of the PVH.

It is well known that hippocampal synaptic rearrangement, in the form of an increase in spine density, is associated with the formation of long-term memories, including

contextual fear memories (Kandel 2001; Leuner et al. 2003; Restivo et al. 2009; Vetere et al. 2011). Likewise, a previous report has shown that the robust contextual fear memory resulting from the interaction of stress and retrieval is associated with an elevated number of "mature" spines in the DH (Giachero et al. 2013b). In addition, intra-BLA infusion with MDZ prior to restraint prevents hippocampal CA1 structural remodeling following stress and fear conditioning (Giachero et al. 2013a, 2015). Similarly, we showed that MUS intra-dlPAG prior to the environmental challenge prevents the enhancement of the density of "mature" spines in DH elicited by the combination of fear conditioning and stress, suggesting that the stressful influence on hippocampal structural plasticity is, at least in part, under the modulation of the dlPAG activity.

A functional interaction between the hippocampus, the amygdala complex, and the dPAG has been previously reported (Johansen et al. 2010; Kim et al. 2013; Horovitz and Richter-Levin 2015; Watson et al. 2016). Also, it was demonstrated that the attenuation of GABAergic transmission within BLA prior to contextual fear conditioning induced hippocampal dendritic spine rearrangement associated with long-term fear memory facilitation (Giachero et al. 2015). Pharmacological inactivation of the dlPAG during acute stress is likely to impair both BLA instruction by PAG and the subsequent hippocampal dendritic spine remodeling induced by BLA hyperexcitability via the entorhinal cortex. Additional experiments, such as assessment of hippocampal spine density after chemical stimulation of dlPAG, are necessary for the elucidation of this dlPAG's influence on plasticity in other brain regions, such as the BLA and the DH.

Considering that the reduced dlPAG activation produced by MUS was able to impair the potentiated contextual fear expression, the HPA-axis activation, and the DH neuroplasticity only when applied before the restraint stress, the present data suggest that the dlPAG is a key neural site for the negative valence instruction necessary to modulate the promoting influence of stress on fear memory.

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Compliance with ethical standards

Conflict of interest We have no commercial associations which impact on this work. Funding for this study was provided by Brazilian public agencies CAPES, CNPq, and FAPESP; they had no further role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Ethical approval The current research was approved by the Federal University of Santa Catarina, Animal Ethics Committee (23080.0055752/2006-64/UFSC), and was performed in accordance with the Brazilian Society of Neuroscience and Behavior Guidelines for the Care and Use of Laboratory Animals.

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