Neurobiology of Learning and Memory 121 (2015) 52–58

Contents lists available at ScienceDirect

Neurobiology of Learning and Memory

journal homepage: www.elsevier.com/locate/ynlme

Dorsal periaqueductal gray post-stimulation freezing is counteracted by neurokinin-1 receptor antagonism in the central nucleus of the amygdala in rats

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ARTICLE INFO

Article history:
Received 13 January 2015
Revised 4 April 2015
Accepted 6 April 2015
Available online 13 April 2015

Keywords:
Amygdala
NK1 receptor
Spantide
Dorsal periaqueductal gray
Fear
Anxiety

ABSTRACT

Electrical stimulation of the dorsal periaqueductal gray (dPAG) in rats generates defensive responses that are characterized by freezing and escape behaviors, followed by post-stimulation freezing that resembles symptoms of panic attacks. dPAG post-stimulation freezing involves the processing of ascending aversive information to prosencephalic centers, including the amygdala, which allows the animal to evaluate the consequences of stressful situations. The basolateral nucleus of the amygdala (BLA) is thought to act as a filter for innate and learned aversive information that is transmitted to higher structures. The central (CeA) and medial (MeA) nuclei of the amygdala constitute an output for the expression of fear reactions through projections to limbic and brainstem regions. Neurokinin (NK) receptors are abundant in the CeA, MeA, and BLA, but their role in the expression of defensive responses and processing of aversive information that is evoked by electrical stimulation of the dPAG is still unclear. In the present study, we examined the role of NK1 receptors in these amygdala nuclei in the expression of defensive responses induced by electrical stimulation of the dPAG in rats and fear memory of this aversive stimulation. Rats were implanted with an electrode into the dPAG for electrical stimulation and one cannula in the CeA, MeA, or BLA for injections of vehicle (phosphate-buffered saline) or the NK1 receptor antagonist spantide (SPA; 100 pmol/0.2 μl). Injections of SPA into the CeA but not BLA or MeA reduced the duration of post-stimulation freezing evoked by electrical stimulation of the dPAG, without changing the aversive thresholds of freezing or escape. Twenty-four hours later, exploratory behavior was evaluated in the elevated plus maze test (EPM) in the CeA group of rats. Electrical stimulation of the dPAG rats that received vehicle exhibited higher aversion to the open arms of the EPM than sham rats that did not receive any dPAG stimulation. SPA injections into the CeA prevented the proaversive effects of electrical stimulation of the dPAG assessed in the EPM 24 h later. The present results suggest that neurokininergic modulation via NK1 receptors in the CeA but not BLA or MeA is involved in the processing of aversive information derived from dPAG stimulation. The long-lasting consequences of electrical stimulation of the dPAG may be prevented by NK1 receptor antagonism in the CeA.

1. Introduction

Chemical or electrical stimulation of the dorsal periaqueductal gray (dPAG) in rats generates defensive responses that are characterized by alertness, freezing, and escape behavior (Bandler, Depaulis, & Vergnes, 1985; Brandao, de Aguiar, & Graeff, 1982; Krieger & Graeff, 1985; Schenberg, Costa, Borges, & Castro, 1990; Vianna, Graeff, Landeira-Fernandez, & Brandao, 2001b), responses that resemble those that are displayed by animals when confronted with natural predators (Bandler & Depaulis, 1991; Brandao, Anseloni, Pandossio, De Araujo, & Castilho, 1999; Fernandez de Molina & Hunsperger, 1959; Olds & Olds, 1962). In humans, electrical stimulation of the dPAG has been reported to be extremely unpleasant, with feelings and autonomic changes that are similar to those that occur during a panic

Abbreviations: ANOVA, analysis of variance; SP, Substance P; SPA, spantide; dPAG, dorsal periaqueductal gray matter; BLA, basolateral nucleus of the amygdala; MeA, medial nucleus of the amygdala; CeA, central nucleus of the amygdala; EPM, elevated plus maze; ES, electrical stimulation; PSF, post-stimulation freezing.

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http://dx.doi.org/10.1016/j.nlm.2015.04.001
1074-7427/

attack (Amano et al., 1978; Nashold, Wilson, & Slaughter, 1969). Given the similarities between behavioral responses in rats and symptoms of panic attacks in human, electrical stimulation of the dPAG has been effectively used as a model of panic attacks (Graeff, 1990; Graeff & ZanPlease check whether the given reference 'Graeff, 2002; Jenck, Moreau, & Martin, 1995; Lovick, 2000; Schenberg, Bittencourt, Sudre, & Vargas, 2001).

Although escape and freezing behaviors that are evoked by electrical stimulation of the dPAG have been the main research focus, dPAG post-stimulation freezing has also received growing interest. This sort of behavior emerges immediately after the cessation of electrical stimulation of the dPAG (Carvalho, Santos, Bassi, & Brandao, 2013; Martinez, de Oliveira, & Brandão, 2006; Vianna, Graeff, Brandao, & Landeira-Fernandez, 2001a; Vianna et al., 2001b). In contrast to context-conditioned freezing, dPAG post-stimulation freezing is not context-dependent. The context that is paired with electrical stimulation of the dPAG does not evoke dPAG post-stimulation freezing (Vianna, Borelli, Ferreira-Netto, Macedo, & Brandao, 2003; Vianna et al., 2001a,b). This process involves ascending aversive information that is transmitted to prosencephalic centers, including the amygdala, via the medial forebrain bundle, which allows the animal to evaluate the consequences of aversive situation and aids in the recognition of threatening stimuli in fear-experienced animals (Brandao, Zanoveli, Ruiz-Martinez, Oliveira, & Landeira-Fernandez, 2008).

The inter-relationship between the PAG and amygdala in the expression of unconditioned defensive reactions related to anxiety and fear is well established (Canteras, 2002; Comoli, Ribeiro-Barbosa, & Canteras, 2003; Graeff, 1990; Olds & Olds, 1963; Strauss, Maisonnette, Coimbra, & Zangrossi, 2003; Sullivan, Apergis, Gorman, & LeDoux, 2003). The basolateral nucleus of the amygdala (BLA) is predominantly involved in filtering aversive stimuli. The central (CeA) and medial (MeA) nuclei of the amygdala constitute the output for autonomic and somatic components of defensive reactions via major projections to the hypothalamus and brainstem regions (Canteras, Simerly, & Swanson, 1995; Sah, Faber, Lopez De Armentia, & Power, 2003). The excitability of these output neurons is regulated by a tonic inhibitory influence from the BLA (Nitecka & Ben-Ari, 1987). The amygdala synthesizes stimulus inputs from the environment; depending on the type of threat, it acts in concert with the neural substrate of fear in the dPAG (Fanselow, 1991; Gross & Canteras, 2012; Ledoux, 1994; Zhao, Yang, Walker, & Davis, 2009).

Several studies have shown that the amygdala influences affective behaviors related to fear and anxiety, at least partially through actions of substance P (SP; (Bassi, de Carvalho, & Brandao, 2014; Carvalho et al., 2013; Ebner, Rupniak, Saria, & Singewald, 2004; Smith et al., 1999; Zhao et al., 2009). Substance P is involved in the regulation of such behavioral processes as reinforcement, learning, memory, fear, and anxiety and also mediates stress responses (Chahl, 2006; Ebner et al., 2004; Hasenohrl et al., 2000; Huston & Hasenohrl, 1995). Three neurokinin (NK) receptors have been identified to date: NK1, NK2, and NK3. Despite the fact that SP binds to all three receptor subtypes, it has higher affinity for NK1 receptors (Hokfelt, Bartfai, & Bloom, 2003; Mantyh, 2002; Mussap, Geraghty, & Burcher, 1993; Quartara & Maggi, 1998). Many studies have investigated the participation of the SP/NK1 receptor system in the CeA, MeA, and BLA in the expression of defensive responses in rats (Bassi et al., 2014; Boyce, Smith, Carlson, Hewson, Rigby, O'Donnell, Harrison, & Rupniak, 2001; Ebner et al., 2004; Kertes, Laszlo, Berta, & Lenard, 2009a; Smith et al., 1999; Zhao et al., 2009), but it is not known whether or not this amygdale system modulates the expression of defensive behaviors evoked by electrical stimulation of the dPAG and the fear memory of this aversive stimulation. Thus, the present study investigated the effects of the NK1 receptor antagonist SPA injected into the CeA, MeA, and BLA on freezing, escape, and dPAG post-stimulation freezing responses elicited by electrical stimulation of the dPAG in rats and on the exploratory behavior in the elevated plus maze (EPM) 24 h later. With the EPM test we assessed whether the long lasting aversive consequences of the electrical stimulation of the dPAG can be prevented by NK1-receptors antagonism in the amygdala. According to several studies, this time window is enough for memory consolidation process (Colley & Routtenberg, 1993; Izquierdo & Medina, 1997; Izquierdo et al., 2006).

2. Materials and methods

2.1. Animals

The experiments were performed in accordance with the Brazilian Society of Neuroscience and Behavior (SNeC) Guidelines for the Care and Use of Laboratory Animals. The procedures were approved by the Committee on Animal Research and Ethics (CEUA) of the University of Sao Paulo (no. 09.1.84.54.7). All efforts were made to minimize the number of animals used and their suffering. A total of 48 male Wistar rats, weighing 250–270 g, were obtained from the animal house of the Campus of Ribeirão Preto, University of São Paulo, and housed in a temperature-controlled room (22 ± 1 °C) under a 12 h/12 h light/dark cycle (lights on at 7:00 AM). The animals were kept in Plexiglas-walled cages and given free access to food and water throughout the experiment. The rats were randomly assigned to one of three surgery groups: BLA, MeA, and CeA. An additional sham group for the CeA (not exposed to electrical stimulation of the dPAG) served as a control for the EPM test.

2.2. Surgery

The animals were intraperitoneally anesthetized with 100 mg/kg ketamine/4.5 mg/kg xylazine (Agener União, Embu-Guaçu, SP, Brazil) and fixed in a stereotaxic frame (David Kopf, Tujunga, CA, USA). The upper incisor bar was set 3.3 mm below the interaural line, such that the skull was horizontal between bregma and lambda. A unilateral guide cannula was implanted over the right BLA, MeA, or CeA. The right amygdala was chosen because the right hemisphere is specialized in emotional behavior, particularly negative affect, compared with the left hemisphere (Adamec, Burton, Shallow, & Budgell, 1999; Michelgard et al., 2007). According to the atlas of Paxinos and Watson (2005) and with bregma serving as the reference point, the coordinates were the following: BLA (anterior/posterior [AP], 2.3 mm; medial/lateral [ML], 5.3 mm; dorsal/ventral [DV], 8.6 mm), MeA (AP, 1.9 mm; ML, 3.4 mm; DV, 8.7 mm), and CeA (AP, 1.9 mm; ML, 4.1 mm; DV, 8.0 mm). A bipolar brain electrode was then implanted into the midbrain aimed at the dPAG. The electrodes were made of two twisted stainless-steel wires, each 50 μm in diameter, that were insulated except at the cross-section of the tip. The electrode was introduced at a 22° angle inclined medially, with lambda serving as the reference for each plane (AP, 0 mm; ML, 1.9 mm; DV, 5.3 mm). For all of the groups, the cannulae and electrode were fixed to the skull with acrylic resin and two stainless-steel anchor screws. Each guide cannula was sealed with a stainless-steel wire to protect it from blockage. At the end of surgery, the animals received an injection of a polyvalent veterinary antibiotic (Pentabiótico, 0.2 ml, intramuscular; Fort Dodge, Campinas, SP, Brazil) and an injection of the antiinflammatory and analgesic flunixin meglumine (Banamine, 2.5 mg/kg, subcutaneous; Schering-Plough, Cotia, SP, Brazil). Afterward, the rats were allowed 5 days to recover from the surgical procedure.

2.3. Drug and microinjection procedure

SPA (Sigma–Aldrich, St Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS) solution (0.1 M) shortly before use. The rats received SPA (100 pmol/0.2 μl) into the CeA, MeA, or BLA, and control rats received the same volume of PBS in all of the experiments.

The microinjection procedure was performed in freely moving animals. A thin dental needle (outer diameter, 0.3 mm) was introduced through the guide cannula positioned 7.0 mm below the skull for the CeA, 7.7 mm below the skull for the MeA, and 7.6 mm below the skull for the BLA. The needle protruded 1.0 mm beyond the guide cannula to reach the CeA, MeA, or BLA. The needle was connected to a 5-μl Hamilton syringe by polyethylene-50 tubing (Becton–Dickinson, Franklin Lakes, NJ, USA) that was connected to a microinfusion apparatus (Harvard, Holliston, MA, USA). A constant volume of 0.2 μl was injected over 60 s. The displacement of an air bubble inside the polyethylene tubing that connected the syringe needle to the glass needle was used to monitor the microinjections. Following the end of the injections, the microinjection pipettes remained inside the brain for a further 60 s to allow for diffusion of the drug away from the tip.

2.4. Electrical stimulation of the dPAG

Five days after surgery, the animals were placed in a square Plexiglas box (25 × 20 × 20 cm) in an illuminated room with a 40 W fluorescent lamp (80 lux at the box floor level). The animals were allowed a 10-min period of acclimation in the enclosure at the beginning of each session. Afterward, the brain was electrically stimulated by means of a sine-wave stimulator (DelVecchio, Ribeirão Preto, SP, Brazil). The stimulation current was monitored by measuring the voltage drop across a 1-kΩ resistor with an oscilloscope (Minipa, Houston, TX, USA). Brain stimulation (60 Hz sine wave for 10 s) was presented at pseudorandom intervals (30–120 s), with the current intensity increasing by 5 μA steps to determine freezing and escape response thresholds. The freezing threshold was defined as the lowest intensity that produced the absence of movement, except movements related to respiration, which is believed to reflect the evaluation of distal threats. The intensity of current that produced running (galloping) and/or jumping was considered the escape threshold. Animals with an escape threshold >120 μA (peak-to-peak) were removed from the experiment. To investigate behavior that persisted after escape, the animals remained in the experimental box for another 8 min, without any stimulation, during which the duration of post-stimulation freezing was recorded, which may be related to the memory of aversive stimulation of this structure. At the end of this period, each rat received a microinjection of SPA or PBS into the BLA, MeA, or CeA. Five minutes later, the aversive thresholds for freezing and escape and post-stimulation freezing were again determined (Carvalho et al., 2013).

2.5. Elevated plus maze

In order to assess the time course of the consequences of the electrical stimulation of the dPAG and its modulation by NK-1 receptors of the amygdala, the same rats were also submitted 24 h later to the height and open spaces of the EPM. The EPM apparatus was described in detail elsewhere (Pellow, Chopin, File, & Briley, 1985), which consisted of two open arms (50 cm × 10 cm) crossed at right angles with two closed arms of the same size. The two closed arms were enclosed by 50 cm high walls, with the exception of the central part of the maze (10 cm × 10 cm) where the open and closed arms intersected. The entire apparatus was elevated 50 cm above the floor. To prevent the rats from falling, a Plexiglas rim (0.5 cm high) surrounded the perimeter of the open arms. The experimental sessions were recorded by a video camera interfaced with a monitor and DVD recorder in an adjacent room.

Twenty-four hours after the assessment of the SPA effects on the aversive thresholds and post-stimulation freezing determined by the procedure of electrical stimulation of the dPAG, the rats were gently placed in the central area of the EPM with their nose facing one of the closed arms. An additional group of rats that were sham-operated and not exposed to electrical stimulation of the dPAG was used as a control for this test. The rats were then allowed to freely explore the maze for 5 min. Before the next rat was tested, the maze was cleaned with a 20% ethanol solution. The number of entries into and time spent on the arms were subsequent analyzed (Anseloni & Brandao, 1997; Carvalho, Moreira, Zanoveli, & Brandao, 2012; Carvalho et al., 2013). Fig. 1 shows the timeline of the procedures for testing the involvement of NK1 receptors in the consolidation of the aversive consequences of electrical stimulation of the dPAG.

2.6. Histology

Upon completion of the experiments, the animals were overdosed with urethane (Sigma Aldrich, St Louis, MO, USA) and intracardially perfused with saline followed by buffered 4% formalin. To mark the drug injection sites at the end of each study, Neutral Red dye (2%) was microinjected into the CeA, MeA, or BLA (0.2 μl/min). The brains were removed and maintained in formalin solution for 24 h and then kept in 30% sucrose solution for another 3 days. Serial 60 μm brain sections were cut using a cryostat (Leica, Wetzlar, Germany), thaw-mounted on gelatinized slides, and stained with Cresyl violet to visualize the injection sites with reference to Paxinos and Watson (2005).

2.7. Statistical analysis

The data are expressed as mean ± SEM. To assess the effects of SPA injections into the distinct nuclei of the amygdala on the defensive responses induced by electrical stimulation of the dPAG, differences in aversive thresholds between groups were subjected to two-way repeated-measures analysis of variance (ANOVA), with treatment (PBS and SPA) and defensive response (freezing and escape) as factors. The duration of post-stimulation freezing was analyzed using two-way repeated-measures ANOVA, with treatment (SPA and PBS) and condition (pre- and post-injections) as factors. For the EPM test, a one-way ANOVA was used to assess the effects of SPA in the CeA in rats exposed to electrical stimulation of the dPAG 24 h before the test. Newman–Keuls *post hoc* comparisons were performed when significant overall F values were obtained in the ANOVA ($p < 0.05$).

3. Results

The tips of the electrodes were located within the dorsal part of the PAG, and the injection sites in the amygdala were located inside the CeA, MeA, or BLA (Fig. 2).

The actual values (means ± SEM) and the difference (Δ) in the freezing and escape thresholds in response to electrical stimulation of the dPAG before and after treatment with PBS or SPA (100 pmol/0.2 μl) in the CeA, MeA, and BLA are shown in Table 1 and Fig. 3A, respectively. Two-way repeated-measures ANOVA revealed no significant differences between treatments for the CeA ($F_{1,13} = 0.05$, $p > 0.05$), MeA ($F_{1,11} = 0.51$, $p > 0.05$), or BLA ($F_{1,11} = 0.0006$, $p > 0.05$). A lack of significant effects on freezing and escape responses was also observed for the CeA ($F_{1,13} = 0.25$, $p > 0.05$),

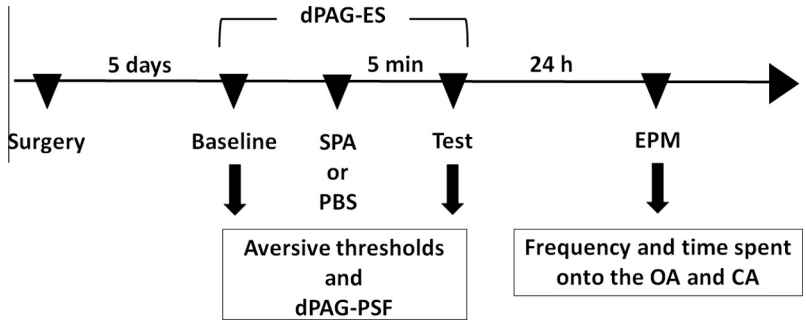

Fig. 1. Timeline of the procedures for testing the involvement of NK1 receptors in the central, medial, and basolateral nuclei of the amygdala in the expression of defensive responses evoked by electrical stimulation (ES) of the dorsal periaqueductal gray (dPAG) and exploratory behavior in the elevated plus maze (EPM) in rats. dPAG-PSF, post-stimulation freezing evoked by ES of the dPAG; CA, closed arms; OA, open arms; PBS, 0.1 M phosphate-buffered saline solution; SPA, spantide.

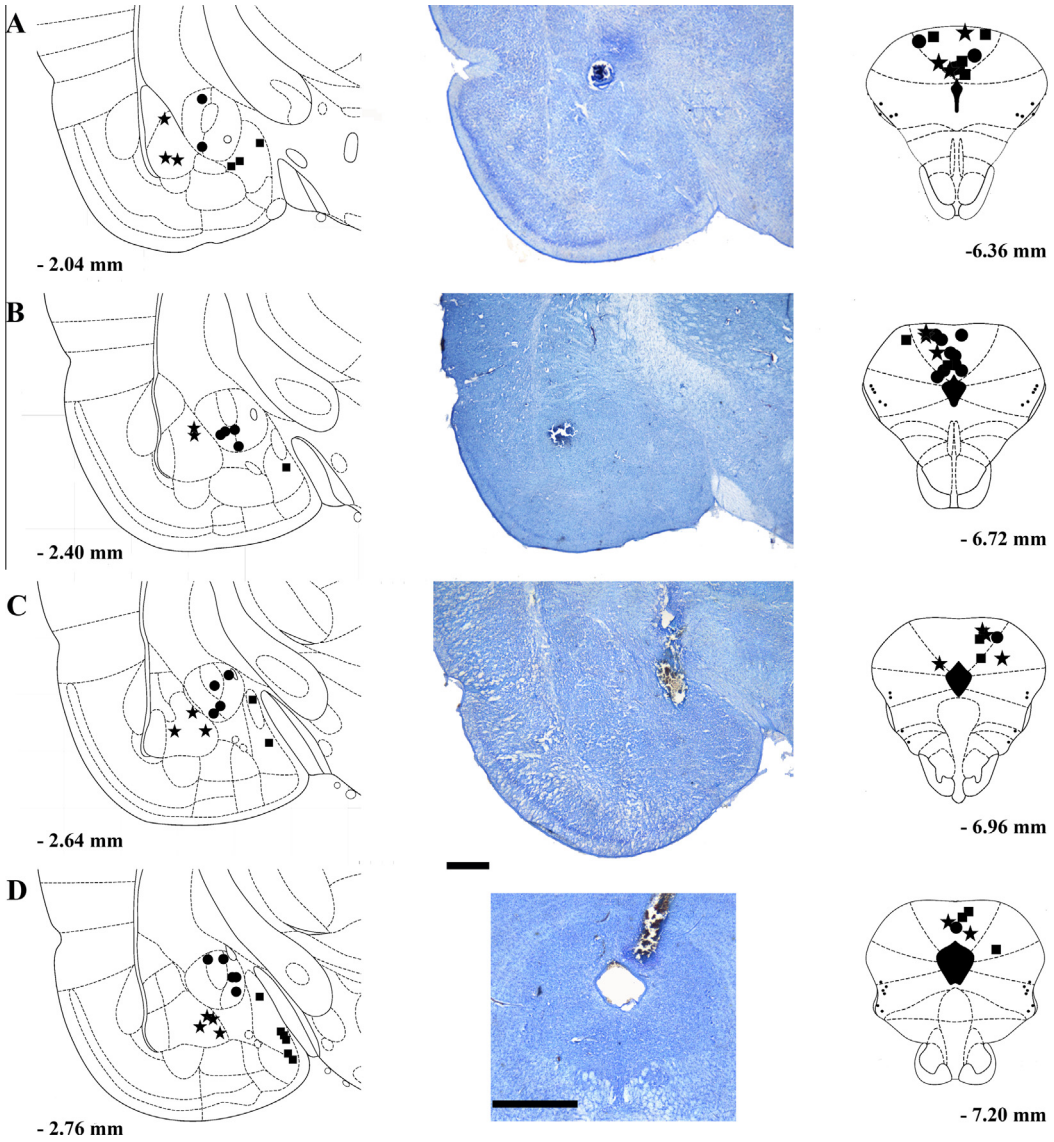

Fig. 2. Representative photomicrographs of the injections sites in the (A) central nucleus of the amygdala (CeA), (B) basolateral nucleus of the amygdala (BLA), and (C) medial nucleus of the amygdala (MeA) and (D) electrode tips in the dorsal periaqueductal gray (dPAG). The points represent the injection sites in the amygdalar nuclei and electrode tips in the dPAG (circles for CeA, squares for MeA, and stars for BLA). Scale bars = 500 μm. The number of sites indicated in the figures is less than the actual number of injected animals because of overlap.

Table 1
Means ± SEM of freezing and escape thresholds before and after microinjections of phosphate-buffered saline 0.1 M (PBS) or spantide (SPA) into the central (CeA), medial (MeA) or basolateral (BLA) nuclei of the amygdala of rats submitted to the electrical stimulation of the dorsal periaqueductal gray.

		Freezing	Escape
CeA	Pre-PBS	28.38 ± 5.51	35.50 ± 5.97
	PBS	29.88 ± 5.93	37.38 ± 6.78
	Pre-SPA	22.14 ± 6.44	27.86 ± 6.16
	SPA	22.14 ± 6.44	28.57 ± 7.21
MeA	Pre-PBS	40.71 ± 7.11	51.43 ± 8.00
	PBS	40.71 ± 7.11	50.00 ± 7.24
	Pre-SPA	47.50 ± 11.67	64.17 ± 17.48
	SPA	49.17 ± 12.94	65.00 ± 18.71
BLA	Pre-PBS	30.50 ± 4.82	36.33 ± 5.52
	PBS	31.33 ± 5.04	38.00 ± 5.97
	Pre-SPA	21.43 ± 3.03	27.57 ± 3.44
	SPA	20.71 ± 2.97	28.57 ± 3.89

MeA ($F_{1,11} = 1.71$, $p > 0.05$) and BLA ($F_{1,11} = 0.004$, $p > 0.05$). Likewise, the interaction between treatments and defensive responses was not statistically significant for the CeA ($F_{1,13} = 0.74$, $p > 0.05$), MeA ($F_{1,11} = 0.12$, $p > 0.05$), or BLA ($F_{1,11} = 0.38$, $p > 0.05$).

Two-way repeated-measures ANOVA was also performed for the duration of post-stimulation freezing behavior when electrical stimulation of the dPAG at the escape threshold had ceased (Fig. 3B). For the CeA, the analysis showed a significant effect of condition ($F_{1,13} = 10.77$, $p < 0.05$) but not treatment ($F_{1,13} = 1.53$, $p > 0.05$) and a significant condition × treatment interaction ($F_{1,13} = 13.37$, $p < 0.05$). *Post hoc* comparisons indicated that intra-CeA SPA injections significantly reduced the duration of post-stimulation freezing compared with the pre-injection condition and the PBS group. However, for the MeA and BLA, the analysis revealed no significant effects of condition ($F_{1,11} = 2.49$ and 1.09, $p > 0.05$), treatment ($F_{1,11} = 0.11$ and 0.02, $p > 0.05$) and no condition × treatment interaction ($F_{1,11} = 2.02$ and 0.009, $p > 0.05$), respectively.

The same groups of rats that were injected with SPA (100 pmol/0.2 μl) or PBS into the CeA and exposed to electrical stimulation of the dPAG were subjected to the EPM test 24 h later. A sham-operated group of rats that was not exposed to electrical stimulation of the dPAG was used as a control for this test. One-way ANOVA revealed significant differences in the number of open-arm entries ($F_{2,20} = 4.29$, $p < 0.05$) and percentage of time spent on the open arms relative to total time ($F_{2,20} = 3.90$, $p < 0.05$). *Post hoc* comparisons revealed that the group of rats that was treated with PBS and exposed to electrical stimulation of the dPAG 24 h previously exhibited reductions of the number of openarms entries and percentage of time spent on the open arms compared with the control group (sham-operated group; Fig. 4A and B, respectively). This analysis also revealed no significant differences in the number of closedarms entries ($F_{2,20} = 3.25$, $p > 0.05$; Fig. 4C).

4. Discussion

The present findings showed that SPA injections into the CeA but not MeA or BLA reduced the duration of post-stimulation freezing evoked by electrical stimulation of the dPAG, without changing the aversive thresholds of freezing and escape responses. SPA injections into the CeA also prevented the aversive consequences of electrical stimulation of the dPAG on exploratory behavior of rats in the EPM 24 h later.

The participation of the neurokininergic system via NK1 receptors in some nuclei of the amygdala in the expression of behavioral responses related to fear and anxiety has been suggested in several studies (Bassi et al., 2014; Boyce et al., 2001; Carvalho et al., 2013; Ebner et al., 2004; Smith et al., 1999; Zhao et al., 2009). The present findings showed that NK1 receptors in the CeA, an output of the

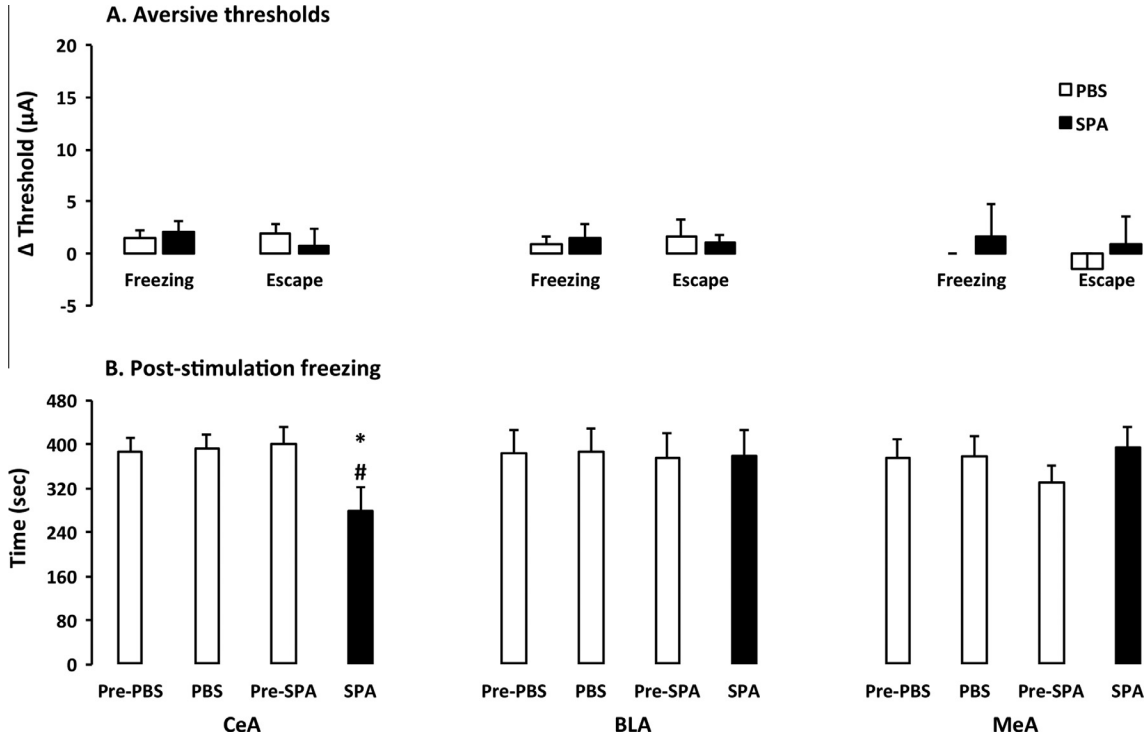

Fig. 3. Effect of spantide (SPA; 100 pmol/0.2 μl) injected into the central nucleus of the amygdala (CeA), medial nucleus of the amygdala (MeA), and basolateral nucleus of the amygdala (BLA) in rats on defensive behaviors elicited by electrical stimulation of the dorsal periaqueductal gray. Differences (Δ) in the thresholds of freezing and escape responses determined after PBS or SPA injections into the CeA, MeA, and BLA (A) and duration (in seconds) of post-stimulation freezing before and after of PBS or SPA injections into the CeA, MeA, and BLA (B). $^{*}p < 0.05$, compared with pre-injection; $^{\#}p < 0.05$, compared with PBS. Number of animals: PBS ($n = 8$, 7, and 6 for CeA, MeA, and BLA, respectively) and SPA ($n = 7$, 6, and 7 for CeA, MeA, and BLA, respectively).

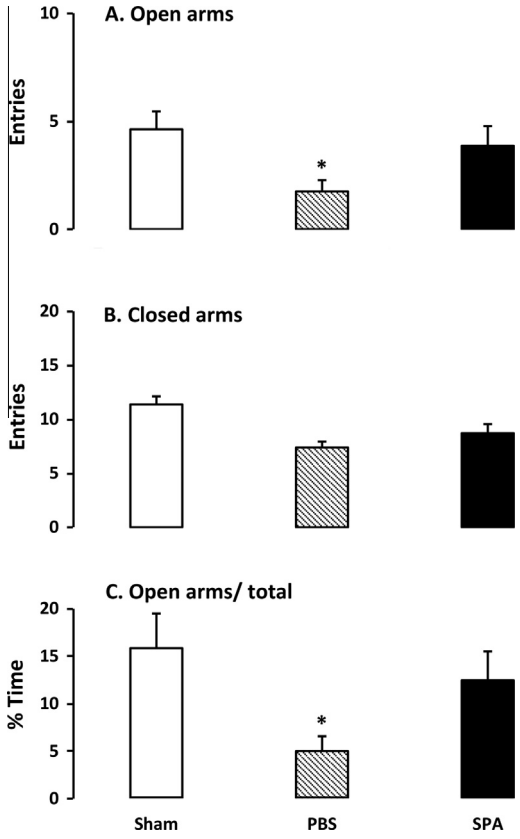

Fig. 4. Exploratory behavior of rats in the elevated plus maze 24 h after PBS or SPA (100 pmol/0.2 μl) injection into the central nucleus of the amygdala and electrical stimulation of the dorsal periaqueductal gray. Each bar represents the mean + SEM of the number of entries into the open and closed arms (A and B) and percent of time spent on the open arms relative to total time (C). $^{*}p < 0.05$, compared with control (sham-operated) group ($n = 8$ for PBS; $n = 7$ for SPA).

amygdalar complex, did not play a modulatory role in freezing and escape responses evoked by electrical stimulation of the dPAG but appeared to mediate the processing of aversive information generated by electrical stimulation of the dPAG (Carvalho et al., 2013). The freezing produced by electrical stimulation of the dPAG is a preparatory response for the escape reaction to imminent danger and apparently depends on other brainstem structures, such as the cuneiform nucleus (Vianna et al., 2003). dPAG post-stimulation freezing, which is elicited once the flight reaction ceases, is thought to reflect the processing and transfer of information coupled to this type of aversive experience to prosencephalic structures, including the amygdala. Electrolytic lesions or inactivation of the CeA with the γ-aminobutyric acid-A agonist muscimol reduced dPAG post-stimulation freezing but did not change the aversive thresholds determined by electrical stimulation of the dPAG (Martinez et al., 2006; Oliveira, Nobre, Brandao, & Landeira-Fernandez, 2004). The present findings suggest that SP may be released in the CeA during the course of the expression of dPAG post-stimulation freezing, and inhibition of the actions of SP at NK1 receptors by SPA treatment caused a reduction of dPAG post-stimulation freezing.

Considering that the effects of SPA injections into the CeA occurred after the interruption of electrical stimulation of the dPAG, one issue is the significance of dPAG post-stimulation freezing to animal behavior. The present study provides additional information on the importance of this type of freezing for memories of the aversiveness of the activation of the neural substrates of fear. The reduction of post-stimulation freezing by SPA injection into the CeA prevented the proaversive effects of electrical stimulation of the dPAG 24 h later in the EPM. Some evidence indicates that SP via NK1 receptors can have memory-promoting effects when administered into the CeA or systemically (Costa & Tomaz, 1998; Hasenohrl et al., 2000; Kertes, Laszlo, Berta, & Lenard, 2009b; Kertes et al., 2009a). Moreover, of all the areas in the central nervous system, the amygdala is most clearly implicated in evaluating the emotional meaning of incoming stimuli (LeDoux, 1986). Several researchers have suggested that the amygdala assigns free-floating feelings of significance to sensory input, which the neocortex then further elaborates and imbues with personal meaning (Adamec, 1991; LeDoux, 1986; MacLean, 1985; O'Keefe and Bouma, 1969; Van Der Kolk, 2001). The neurokininergic system via SP/NK1 receptors in the CeA may contribute to the attribution of significance to the memory of an aversive stimulus associated with electrical stimulation of the dPAG, thereby strengthening emotional reactions to novel and stressful stimuli that are present in the EPM. SPA injections into the CeA in rats that did not receive prior electrical stimulation of the dPAG did not cause any effects on exploratory behavior in the EPM (Carvalho et al., 2013).

Although the MeA is an output for autonomic and somatic components of emotional reactions via their projections to the hypothalamus and brainstem regions (Canteras et al., 1995; Sah et al., 2003), SPA treatment did not change defensive behaviors evoked by electrical stimulation of the dPAG. This lack of effect was unexpected because some evidence suggests the involvement of SP/NK1 receptors in the MeA in defensive reactions evoked by other aversive stimuli that are different from electrical stimulation of the dPAG (Bassi et al., 2014; Ebner et al., 2004; Singewald et al., 2008). SPA injections into the BLA also did not produce any effect on defensive behaviors evoked by electrical stimulation of the dPAG. However, some studies have demonstrated the participation of the neurokininergic system via SP/NK1 receptors in the BLA (Boyce et al., 2001; Smith et al., 1999; Zhao et al., 2009). Thus, the present data emphasize that the aversiveness of electrical stimulation of the dPAG has properties that are particular to recruitment of the CeA as the output pathway during its consolidation process (Bassi et al., 2014; Carvalho et al., 2013).

In summary, the present results suggest that neurokininergic modulation via NK1 receptors in the CeA, but not in the BLA or MeA, is involved in the processing of aversive information related to dPAG stimulation. The long-lasting consequences of electrical stimulation of the dPAG, assessed in the EPM, could be prevented by NK1 receptor antagonism in the CeA. The SP/NK1 receptor system modulates the defense mechanisms of the CeA only when they are recruited by aversive situations that trigger the consolidation of fear memories, such as those that are derived from the activation of neural substrates related to fear in the dPAG. The present study suggests a possible pharmacotherapy for some anxiety disorders, such as panic attacks, that are triggered by traumatic and stressful events.

Acknowledgments

This work was supported by CAPES Project Grant 23038.027765/2009-10; Aux-PE-PNPD 2193/2009 to MCC. MCC was funded by a CAPES postdoctoral award. JMS contributed to the discussion of the results and drafting of this article. The authors would like to thank Michael Alan Arends for editing this manuscript.

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