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Dorsolateral and ventral regions of the periaqueductal gray matter are involved in distinct types of fear

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Abstract

Stepwise increases in the electrical stimulation of the dorsolateral periaqueductal gray (dlPAG) produces alertness, then freezing and finally escape. This paper examines whether this freezing is (i) caused by Pavlovian fear conditioning to the contextual cues present during stimulation and (ii) the result of the stimulation of neurons located inside the dlPAG or elsewhere. To this end, freezing behavior was assessed in rats exposed either to the same or a different environment (context shift test) following the application of either footshocks or stimulation of the dlPAG at the freezing threshold. Rats submitted to footshocks presented freezing to the context 24 h later whereas rats submitted to the dlPAG stimulation showed freezing only immediately after the stimulation, regardless of the context. In the second experiment, aversive states generated by activation of the dlPAG were assessed either by measuring the thresholds for freezing and escape responses or the duration of these responses following microinjections of semicarbazide inside the dlPAG. The duration of freezing behavior was also measured in rats submitted to a contextual fear-conditioning paradigm using footshocks as unconditioned stimulus. Lesions of the ventral periaqueductal gray (vPAG) disrupted conditioned freezing to contextual cues associated to footshocks but vPAG lesions did not change the threshold of either freezing or escape responses elicited by electrical stimulation of the dlPAG. Lesions of the vPAG did not change the amount of freezing or escape responses produced by microinjections of semicarbazide into the dlPAG. These results indicate that stimulation of dlPAG neurons produce freezing behavior independent of any contextual fear conditioning and add to previously reported evidence showing that the vPAG is a critical structure for the expression of conditioned fear. In contrast, the neural substrate of unconditioned dlPAG stimulation-induced freezing is likely to elaborate unconditioned fear responses to impending danger, which have been implicated in panic disorder.

Keywords: Periaqueductal gray; Defensive behavior; Escape; Freezing; Conditioned fear

1. Introduction

"When the intensity of the stimulating current of the dorsolateral periaqueductal gray (dlPAG) was increased stepwisely, the first change was a behavioral arrest. Next, the animals suddenly stopped, became tense and immobile and often urinated and defecated. With higher intensities, freezing behavior was followed by vigorous running and jumps" [7]. As far as we know this was the first description of freezing behavior induced by electrical stimulation of the dlPAG. Afterwards, many other studies replicated these findings [14,15,41]. It has also been reported that glutamate microinjections or drugs blocking glutamate acid decarboxylase (GAD), an enzyme required for the synthesis of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) produced freezing behavior, alternating with escape, after microinjection into the dlPAG [8,32]. This sequence of defensive behavior replicates the pattern of defensive reactions that animals present when facing threatening situations. Freezing behavior has been the most used measure of conditioned fear in laboratories of psychobiology [3,12,23,24]. In these tests, rats receive electric footshocks inside an experimental chamber. These animals present a significant amount of freezing behavior when later returned to the environment where they previously received footshocks. This kind of Pavlovian learning process has been called contextual fear conditioning [25,29,31].

It is well established that the central nucleus of the amygdala, dorsomedial hypothalamus and ventral and dorsal PAG provide neural substrates for defensive behavioral reactions in the brain (for reviews see Ref. [4,9,10,26,28]). Among these structures, the ventral periaqueductal gray

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(vPAG) seems to be critical for the expression of freezing behavior, as demonstrated by the experiments carried out by Fanselow and coworkers [19,23,24]. According to these authors lesions of the vPAG reduced freezing response to neutral stimuli associated with footshock as well as with the presence of a predator but unconditioned escape responses elicited by footshock were facilitated. In contrast, lesions of the dlPAG abolished unconditioned responses to footshock, but did not affect contextual freezing. Based on this, it has been proposed that the neural circuits of the vPAG are involved in the expression of conditioned freezing, while those in the dlPAG integrate active responses to unconditioned stimuli.

Defensive freezing behavior seems to be modulated by afferent projections that vPAG receives from forebrain structures, especially from the amygdaloid complex [3,25,33,34,42]. On the other hand, the dorsal portion of the PAG (dlPAG) appears to mediate the active behavioral patterns of defensive responses [3,12,23,24]. Until now, little is known about the role of dlPAG in the control of freezing behavior triggered by fear inducing stimuli. It has been suggested that behavior output to dlPAG stimulation closely resembles the reaction of rats to electrical footshock [23]. Therefore, the occurrence of freezing behavior to dlPAG stimulation has also been interpreted as a conditional response to contextual cues associated with this aversive stimulation. To further elucidate this issue the present study examines (1) whether the neural substrates of the freezing behavior induced by electrical stimulation of the dlPAG is context sensitive and (2) whether electrical or chemical stimulation of the dlPAG may be dissociated of the neural substrates of the conditioned fear elaborated at the vPAG level.

2. Experiment 1: dlPAG stimulation vs context shift test

2.1. Animals

Male Wistar rats weighing 250–300 g from the animal house of the Campus of Ribeirão Preto of the University of São Paulo were housed in a temperature-controlled (22 ± 1 °C) room and kept on a 12-h light/12-h dark cycle (0700–1900 lights on). These animals were maintained in individual Plexiglas-walled cages and given free access to food and water throughout the experiment. The rats were randomly assigned to one of four groups: footshock +, footshock − (non-stimulated), dlPAG +, dlPAG − (non-stimulated).

2.2. Surgical procedures

The animals were anaesthetized with tribromoethanol (250 mg/kg, i.p.) and fixed in a David Kopf stereotaxic frame. The upper incisor bar was set at 3.3 mm below the interaural line such that the skull was horizontal between bregma and lambda. Each animal was implanted, with a unilateral bipolar electrode (Plastics One, USA) aimed at the dlPAG. The electrodes were made of stainless steel wire, 160 μm in diameter, insulated except at the cross-section, and were introduced at the dlPAG with an angle of 16°, 1.9 lateral to lambda and 5.1 mm ventral to the skull. The dlPAG electrode was fixed to the skull by means of acrylic resin and three stainless steel screws. This electrode could be connected to a male pin so that it could be plugged into an amphenol socket at the end of a flexible electrical cable and used for brain stimulation.

2.3. Behavioral recordings

Freezing was scored through a time–sample procedure. Every 2 s, an experimenter rated the animal's behavior as freezing or activity. Freezing was operationally defined as the total absence of movement of the body and vibrissae, accompanied by at least two of the following responses: arched back, retraction of the ears, piloerection or exophthalmus. Escape behavior was defined as simultaneous lifting of the four paws from the floor, and was used as criterion to stop the stimulation, but was not coded in a time–sample manner.

2.4. Context shift procedure

In the conditioning experiments, two distinctive boxes served as the same (chamber A) and different (chamber B) contexts. The ceiling, side and back walls of the same context box ($30 \times 25 \times 25$ cm^3) were constructed of stainless steel and the front door was made of transparent Plexiglas. The chamber lay within a sound attenuating chest consisted of a large $50 \times 54 \times 56$ cm^3 plywood container with a hole on the door through which all behavior was observed. The grid floor of this chamber consisted of stainless steel rods spaced 1.2 cm apart. The footshocks (1.0 mA, 1 s with 60 s interval) were delivered through the test cage floor by a constant current generator built with a scrambler (DelVecchio Instruments, Brazil). The different context consisted of a chamber ($30 \times 30 \times 28$ cm^3) with the two facing walls of the chamber made of aluminum and the remaining walls and ceiling made of Plexiglas. In one of the aluminum walls there were two operant levers and a hole with 6 cm diameter and 2 cm deep at floor level. The ground was made of aluminum bars with 3 mm diameter, 1.5 cm far from each other. The box was cleaned with 70% ethanol before each animal was placed inside it. This observational chamber was placed inside the animal colony room. Two 60 W lamps illuminated the room and two air conditioners and exhaust fans made a permanent background noise. Animals were observed by means of a video camera and a monitor placed outside the colony room.

Unoperated animals received 5 min daily handling during 4 days. In the fifth day, each animal was placed in the observational chamber where it remained undisturbed for 6 min (baseline). After that, it was subjected to five

footshocks of 1.0 mA 1 s duration, with 60 s interval between shocks (footshock + group). Controls remained in the chamber for equal time, but did not receive footshock (footshock − group). Immediately after that, half of shocked and half of control rats were removed and placed in the different context. After 6 min of observation all animals returned to their home cages. Twenty-four hours later, all animals were re-exposed to the same chamber where they had previously received footshocks.

For the brain stimulation experiment, the stimulation apparatus was connected to the same experimental chamber described earlier and the same procedure was followed with the exception that one group (dlPAG +) received brain stimulation. The control group was similarly connected to the apparatus but no current was applied (dlPAG −).

The electrical stimulation of the dlPAG was obtained by means of a square-wave stimulator Grass S44 (Quincy, MA, USA), connected to an oscilloscope (Tektronix, USA) that indicated the voltage drop through a 100 KΩ resistor in series with the rat. The electrode was connected to the stimulator by means of a flexible cable and a mercury swivel, which allowed free movement to the animal. The procedure, adapted from Sandner et al. [39], consisted in holding constant the electrical stimulation intensity at 70 μA, with 1.0 ms pulse duration but varying the electrical stimulation frequency through its interpulse intertrial (IPI). Starting at an IPI of 80 ms the animals received electrical stimulation of the dlPAG (60 s) at 1 min intervals until the rat presented escape behavior. In order to investigate the effects of the last stimulation electrical stimulation that triggered escape behavior the animal was observed for another 6 min inside the same or in a different experimental box as in the paradigm using footshocks. Rats that did not escape from the dlPAG electrical stimulation were discarded from the experiment. The control group was connected to the apparatus but no current was applied.

2.5. Analysis of results

Data are reported as mean + SEM. Two-way ANOVA was used to analyze scores of freezing behavior recorded in the 6-min before (baseline), immediately after and 24 h after footshock or dlPAG stimulation. Animals exposed to footshocks or dlPAG stimulation was the group factor (footshock + and footshock − or dlPAG + and dlPAG −) and exposure to the context (same × different) was the second factor.

2.6. Histology

At the end of the experiment, animals were deeply anaesthetized with urethane and perfused intracardially with saline followed by formalin solution (10%). Three days later the brains were removed and frozen. Serial 50 μm brain sections were cut using a microtome (Cryocut 1800-Leica, Germany) in order to localize the stimulation electrode tips within the dlPAG and the electrolytic lesions within the vPAG according to Paxinos and Watson's atlas [37].

2.7. Results

The results of the validation of the context shift procedure in the present study with the use of footshocks are shown in Fig. 1. As can be seen there were no significant differences among groups at baseline; no main effects for groups [$F(1,16) = 1.03$; $p > 0.05$], context [$F(1,16) = 0.00$; $p > 0.05$] or interaction between factors [$F(1,16) = 2.32$; $p > 0.05$]. Immediately after the footshocks, when half of the animals were placed in the different context, there were main effects for groups [$F(1,16) = 39.53$; $p < 0.001$] and context [$F(1,16) = 23.55$; $p < 0.001$], as well as interaction between groups and context [$F(1,16) = 13.60$; $p < 0.005$]. This suggested that footshock was able to produce freezing immediately after shock presentation only if the rat was at the place where it received footshock (the shock context). Twenty-four hours after the footshock, when all animals were re-exposed to the shock context, only footshock had a main effect [$F(1,16) = 17.77$; $p = 0.001$], with no effect for context [$F(1,16) = 0.59$; $p > 0.05$]. There was no interaction between factors [$F(1,16) = 0.62$; $p > 0.05$]. Our data with electric footshock replicated previous findings showing that in absence of an explicit to-be-conditioned stimulus (CS), it is the context cues that function as CS [5,23,24]. Thus, removal of these cues by changing the context caused the conditioned response (freezing) to disappear.

Results of the experiments with rats stimulated in the dlPAG and submitted to the shift context procedure are shown in Fig. 2. As can be seen in Fig. 2, similar analyses performed on freezing behavior in the dlPAG stimulation experiment revealed that there were no significant differences among groups at baseline; no main effects for groups [$F(1,16) = 0.00$; $p > 0.05$], context [$F(1,16) = 1.15$; $p > 0.05$] or interaction [$F(1,16) = 1.10$; $p > 0.05$]. Immediately after stimulation, when half of the animals were placed in the alternative context, only the main effect for stimulation reached significance [$F(1,28) = 31.33$; $p < 0.001$], suggesting that stimulated animals showed an increase in freezing behavior regardless of the context where they were placed. There were no context [$F(1,16) = 2.10$; $p > 0.05$] or interaction [$F(1,16) = 3.63$; $p > 0.05$] effects. Twenty-four hours after stimulation, when all animals were re-exposed to the stimulation context, there were no differences among groups. Neither stimulation [$F(1,16) = 1.53$; $p > 0.05$], context [$F(1,16) = 1.23$; $p > 0.05$] or interaction [$F(1,16) = 1.62$; $p > 0.05$] reached significance.

It is noteworthy to mention that there appeared to be more baseline freezing in the rats in the dlPAG stimulation experiment than in the footshock experiment perhaps due to surgical and post-operative stress in the former.

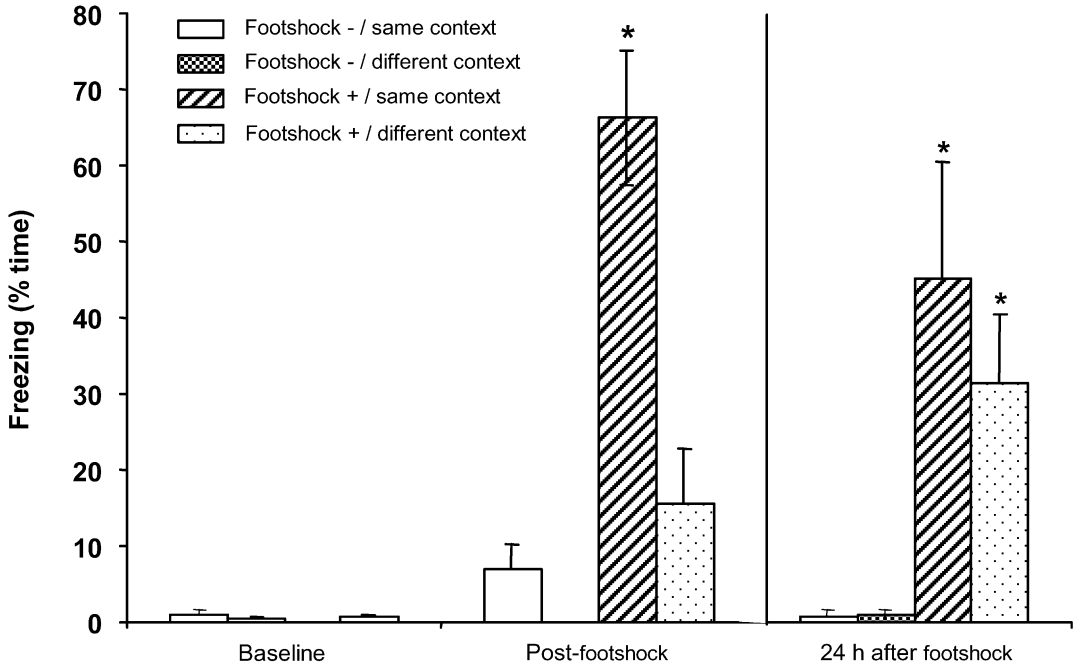

Fig. 1. Mean (+SEM) percent freezing in animals exposed to electrical footshock (+) or no shock (−) conditions. Baseline refers to the period before footshock presentation. After footshock, half of the animals were tested in the same context where footshock was delivered (same context) and the other half was tested in a different context. Twenty-four hours after shock presentation, all animals returned to the same context. *$p < 0.01$ different from the respective control condition.

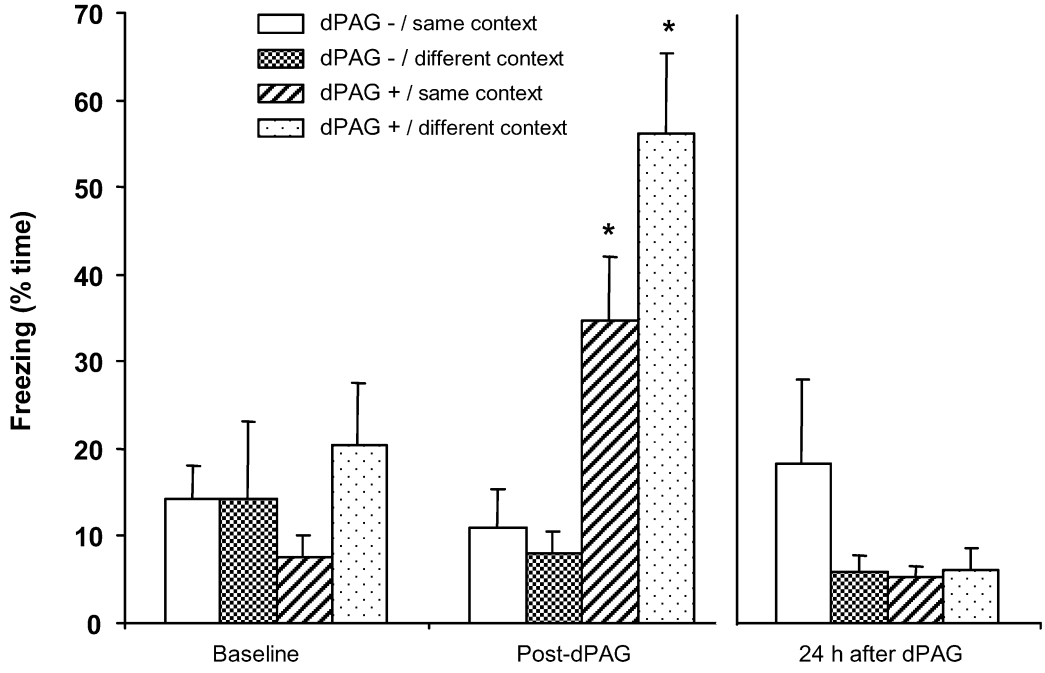

Fig. 2. Mean (+SEM) percent freezing in animals stimulated in the dlPAG (+) or no stimulation (−) conditions. Baseline refers to the period before brain stimulation. After dlPAG stimulation, half of the animals were tested in the context where stimulation was delivered (same context) and the other half was tested in a different context. Twenty-four hours after brain stimulation, all animals returned to the same context. *$p < 0.01$ different from the respective control condition.

3. Experiment 2: vPAG lesions

3.1. Animals and surgery

The animals used in this experiment were similar to those of the first experiment. They were implanted either with a bipolar electrode or a cannula (0.6 mm o.d., 0.4 mm i.d.) aimed at the dlPAG as described in Section 2. Electrolytic lesions in the vPAG were made bilaterally with a monopolar stainless steel electrode (insect pin insulated till the exposed blunt tip) lowered at 0.8 mm posterior to lambda, 1.0 mm lateral to each side of midline and 6.0 mm ventral to the skull. Lesions were made by passing anodal DC current (ESF-108, DelVecchio, Brazil) for 60 s (2 mA) through the electrode. The sham lesion group had identical surgery procedures except that no current was delivered through the electrode. For the experiment with microinjections of semicarbazide into the dlPAG each rat was implanted with a stainless-steel guide cannula directed to the dlPAG as described earlier. Each cannula was fixed with polyacrylic cement anchored to the skull with three stainless-steel screws, and was plugged with stainless-steel stylets. The experiments started after a 1-week postoperative delay. Even so, eight animals with vPAG lesions were discarded from the present study for reasons of poor health.

3.2. Intracranial injection procedure

The animals were gently wrapped in a cloth, and a thin dental needle (o.d. 0.3 mm) was introduced through the guide-cannula until its lower end was 2.5 mm below the guide-cannula. The injection needle was linked to a 5 μl Hamilton syringe by means of polyethylene tubing. A volume of 0.2 μl was injected over 20 s with the aid of an infusion pump (Harvard Apparatus, USA) and the needle was held in place for an additional 10 s. The displacement of an air bubble inside the polyethylene (PE-10) catheter connecting the syringe needle to the intracerebral needle was used to monitor the injection. Each rat received only one injection of either saline or semicarbazide (Sigma, 8 μg/0.2 μl).

3.3. Procedure

The animals of this experiment bearing sham- or vPAG lesions were allocated to two groups: (a) dlPAG electrical stimulation (also submitted to the context fear conditioning procedure), and (b) dlPAG chemical stimulation.

3.3.1. Electrical stimulation

These tests took place in a circular arena 60 cm in diameter and 50 cm high. The arena was in a quiet experimental room illuminated with three 40 W fluorescent lamps (120 lx at the arena floor level). One week after the surgery, the animals were placed in the circular arena and allowed a 6 min period of habituation. At the end of this period brain stimulation (AC, 60 Hz, 15 s) was presented for 15 s at 1 min intervals with the current intensity increasing by steps of 5 μA for measurements of the aversive thresholds. Freezing threshold was operationally defined as the lowest intensity producing a complete immobility except the movements for respiration, defecation or micturition, for at least 6 s during the period of stimulation. The current intensity producing running (gallop) or jumping was considered to be the escape threshold. Animals with an escape threshold above 70 μA (peak-to-peak) were discarded from the experiment. After the determination of the aversive thresholds the animals were taken to their home cages. After approximately 24 h, all animals were submitted to the context fear conditioning procedure described earlier in the Section 2. Each animal was placed in the conditioning chamber and allowed a 6 min of acclimatization and afterwards received five 1 s, 1.0 mA footshocks spaced for 60 s. On the next day, the rat was replaced into the conditioning chamber and remained undisturbed for the 6 min context conditioning test. No shock was presented during this period. Freezing, defined as the absence of movement of the body and vibrissa except that required for respiration was scored according to a time–sample procedure. Every 2 s the animal was observed and its behavior was scored as freezing or not freezing.

3.3.2. Chemical stimulation

One week after the surgery, the animals of this group were placed in the circular arena and allowed a 6 min period of habituation. At the end of this period semicarbazide was injected into the dlPAG of the rats and they were run in a 60-min session in the open field. The behavior of the animals was recorded by a video camera positioned beside the open field and monitored via a closed circuit TV camera. Freezing and escape defined as described earlier were subsequently scored from videotape by an observer with the aid of an appropriate software (The observer, Noldus, Netherlands). The open field was thoroughly cleaned after each test with a 20% ethanol solution and dried. Each rat was tested only once.

3.4. Analysis of results

Time of freezing or thresholds for freezing and escape responses are presented as means ± SEM before and after lesions. Data were analyzed using Student's *t*-test. Eight animals were studied in each lesion and control group, except for the freezing threshold comparison, where one animal of each group had to be excluded for having not shown freezing before the escape threshold was reached.

3.5. Histology

The same as described earlier in Section 2.

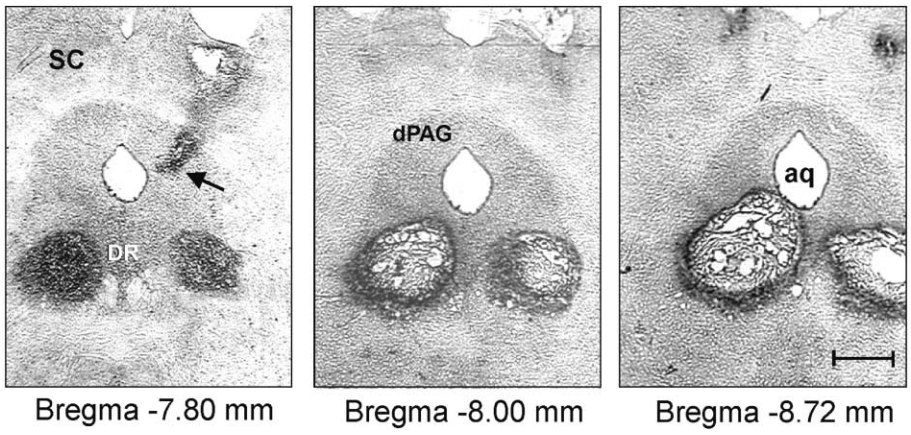

Fig. 3. Photomicrograph showing a typical example of a microinjection site (arrow) into the dorsal periaqueductal gray (dlPAG) and of lesions in the ventral periaqueductal gray in one of the animals used in Section 3. Aq: aqueduct of sylvius; SC: superior colliculus; DR = dorsal raphe nucleus. Bar = 500 μm.

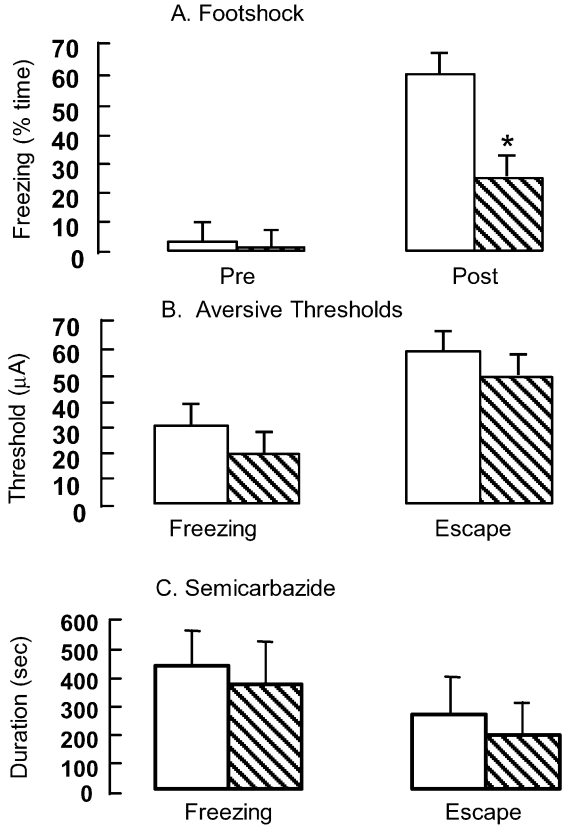

Fig. 4. (A) Percentage of time spent freezing by rats with sham (open columns) or vPAG lesions (hatched columns) during 6 min. PRE refers to the baseline period and POST to the testing session 24 h after conditioning. $N = 8$ for all groups, except for the freezing threshold (lesion = 8, control = 7). (B) Aversive thresholds determined in rats bearing or not (sham) electrolytic lesion of the vPAG. (C) Freezing and escape behaviors induced by microinjections of semicarbazide (8.0 μg/0.2 μl) into the dlPAG in rats bearing (open columns) or not (sham-hatched columns) electrolytic lesion of the vPAG. $N = 8$ for sham- and $N = 9$ for lesion-groups. Columns represent the mean and bars the SEM. *$p < 0.01$ different from the respective control condition.

4. Results

A representative electrolytic lesion of the vPAG and of the cannula placements for microinjections of semicarbazide into the dlPAG can be seen in Fig. 3. As expected, vPAG lesions attenuated freezing induced by the context previously paired with footshock ($t = 2.65$; $p < 0.05$; df = 14), as shown by the right side pair of columns in Fig. 4A. The left side pair of columns of the same figure shows that the amount of freezing behavior before conditioning was very small and similar in both experimental groups ($t = 1.45$; $p > 0.05$; df = 14).

As previously demonstrated in many works from this laboratory [8,9,13,14] freezing and escape behaviors occurred in a stepwise fashion as the intensity of electric current applied to dlPAG was gradually increased. These aversive responses were always accompanied by at least two of the following autonomic reactions: urination, defecation, piloerection or exophthalmus. As shown in Fig. 4B, lesions of the vPAG did not change the aversive threshold of electrical stimulation of the dlPAG either for freezing ($t = 0.95$; $p > 0.05$; df = 13) or for escape ($t = 1.25$; $p > 0.05$; df = 14) responses.

With a latency of around 7 min microinjections of semicarbazide into the dlPAG produced a prolonged behavioral activation interspersed with freezing which lasted for about 10 min. As shown in Fig. 4C lesions of the vPAG did not change the defensive behavior elicited by chemical stimulation of the dlPAG either for freezing ($t = 0.38$; $p > 0.05$; df = 16) or for escape ($t = 0.10$; $p > 0.05$; df = 16) responses.

5. Discussion

The role of the dlPAG in the mediation of the rat's defensive behavior and in the fear motivational system is well established [4,9,10,12,28]. Several studies have shown that

stepwise increases in the electrical stimulation of the dlPAG elicit, at first, a freezing response and then vigorous escape reactions [7,15,41]. Also, it has been reported that glutamate microinjections in the same area produced freezing behavior [32]. Finally, freezing, alternating with escape, has been described after microinjection into the dlPAG of drugs blocking the GAD, an enzyme required for the synthesis of the inhibitory neurotransmitter GABA [8]. This work is an attempt to go one step further at elucidating the distinctive characteristics of the freezing behavior generated at the dlPAG level. The present data show that the amount of freezing displayed by animals stimulated in the dlPAG was similar regardless whether they were placed in the same or in the different context immediately after the stimulation. However, no freezing occurred in dlPAG-stimulated or control animals when the rats were re-exposed to the same environment 24 h later. These results suggest that context fear conditioning was not responsible for the freezing behavior observed after dlPAG electrical stimulation. Therefore, the persistence of freezing immediately after dlPAG stimulation could not be attributed to the contextual cues associated with this midbrain stimulation.

The reported freezing behavior induced by electrical stimulation of the dlPAG may raise the possibility that this behavior may originate from other structures with which this midbrain structure is interconnected. In fact, direct projections from dlPAG to the paraventricular nucleus of the hypothalamus and vPAG, or indirect projections by way of the bed nucleus of the stria terminalis and preoptic area, may mediate the defense reaction brought about by fearful or stressful stimuli [11,19,38,40]. At least regarding freezing behavior our data do not support this prediction. These data point to generation of freezing behavior within the dlPAG. It has been shown that dlPAG has significant numbers of GABAergic interneurons on which GAD blocking agents act reducing the tonic inhibitory control exerted by GABA on the neural circuits of defense therein to produce behavioral activation of an aversive nature [1,8,22]. Indeed, this defensive behavior induced by the blockade of GAD with semicarbazide locally injected into the dlPAG was not affected by vPAG lesions. Overall, the present paper presents evidence for the important role played by local GABAergic interneurons of the dlPAG in the regulation of the neural circuits responsible for the expression of the defensive behavior.

The present results are in good agreement with those reported by others showing that lesions of the vPAG reduce freezing to contextual cues paired with footshock whereas dlPAG lesions do not cause this effect [19,23,24]. Also, neurotoxic lesion studies describe similar results with reduction of conditioned freezing after lesions of the vPAG [30]. Altogether, there is general agreement that the vPAG is necessary for the expression of conditioned freezing, while the dlPAG integrates active responses to aversive stimuli. As further support of this assumption, it has also been reported that vPAG lesions did not affect freezing response induced by electrical stimulation of the inferior colliculus, another brain structure involved in the organization of defensive reactions [35].

Several reports indicate that the periaqueductal gray is critically involved in the regulation of innate reactions to impending danger stimuli [6,10,16,17]. The exact nature of the freezing induced by dlPAG electrical stimulation is not well established yet. This defensive response seems to be distinct from the unlearned freezing to a cat or the fear-potentiated startle. In fact, it has been shown that dlPAG lesions enhanced unconditional freezing to a cat [19]. Moreover, dlPAG lesions blocked the attenuation of fear-potentiated startle observed when a conditional stimulus is trained with high shock intensities while stimulation of the dlPAG with non-toxic doses of kainic acid attenuated the fear-potentiated startle reflex [43].

The signal inputs for the occurrence of freezing behavior generated by dlPAG stimulation might involve projections from other structures related to the processing of aversive information. It is likely that the dlPAG integrates sensory information relayed from the amygdala that allows recognition of threatening stimuli. On the other hand, the neural circuits responsible for escape responses may involve other structures. This assumption is supported by previous studies that show that substantia nigra pars reticulata (SNpr) lesions did not change freezing induced by electrical stimulation of the midbrain tectum [35] but greatly enhances the aversiveness of the dlPAG stimulation [14,15]. As a consequence, it has been suggested that defense motor patterning mechanisms recruit GABAergic fibers from the SNpr, which project to the midbrain tectum [20], suggesting that while nigrocollicular pathways are involved in the expression of escape responses the same does not hold true for freezing responses.

The present paper shows that freezing can be obtained without conditioning, and it is not a side-effect of the procedure, but a direct effect of dlPAG stimulation. The failure of these animals to acquire contextual cue conditioning cannot be attributed to a weaker conditioning than that used for footshocks. Animals remained for similar amount of time in the chamber following either footshocks or dlPAG stimulation. When applied in only one session it did not cause context fear conditioning indicating distinct neural substrates for fear in the periaqueductal gray. It is not to say that associative learning cannot occur using stimulation of the aversive substrates of the dlPAG as already clearly demonstrated by other authors [21]. In this context, recently we have shown the necessity of at least two sessions of pairings of dlPAG electrical stimulation and light in order to produce conditioned fear [13]. Therefore, we do not exclude that freezing could be a conditioned response to this conditioning.

It has been demonstrated that vPAG lesions do not affect circa-strike defensive responses elicited by footshock, namely running, jumping and vocalization [19], which are evoked by stimulation of the dlPAG [10,14,15].

Accordingly, in the present study vPAG lesions did not influence the escape response elicited by dlPAG stimulation. The resistance of dlPAG-induced freezing to the vPAG lesions evidences the first and major difference between the neural substrates of the two types of freezing: one mediated through the vPAG and another vPAG independent. Recently, it has been reported that electrolytic lesions of the vPAG produced a deficit in conditioned freezing but had no effect on the suppression of an operant response in a conditioned emotional response paradigm [2]. These results together with the present report indicate that there might exist different patterns of inhibitory defensive behavior with distinct neural pathways.

In summary, the present results show that freezing induced by electrical stimulation of the dlPAG is not context sensitive and that neither freezing nor escape so-induced are changed by vPAG lesions. These data indicate that defense reactions induced by activation of the dlPAG are independent of the neural mechanisms in the vPAG involved in fear conditioning. These behaviors are likely to represent unconditioned fear responses to impending danger, which have been implicated in panic disorder [18,27]. In support of this view, there are human reports showing that electrical stimulation of the dlPAG produced feelings of imminent death accompanied by autonomic reactions similar to panic attacks [36].

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