Role of amygdala in conditioned and unconditioned fear generated in the periaqueductal gray

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The amygdala and ventral portion of the periaqueductal gray (vPAG) are crucial for the expression of the contextual freezing behavior. However, it is still unclear whether the amygdala also plays a role in defensive behaviors induced by electrical stimulation of the dorsal periaqueductal gray (dPAG). In the present study, rats were implanted with electrodes into dPAG for determination of the thresholds for freezing and escape responses before and after sham or electrolytic lesions in the amygdala. Animals were then submitted to a context fear conditioning procedure. Amygdala lesions disrupted contextual freezing but did not affect defensive behaviors induced by dPAG electrical stimulation. These results indicate that contextual and unconditioned freezing behaviors are mediated by independent neural circuits. *NeuroReport* 15:2281–2285 © 2004 Lippincott Williams & Wilkins.

Key words: Amygdala; Defensive behavior; Fear conditioning; Freezing

INTRODUCTION

When a rat is exposed to an experimental chamber previously associated with footshock the animal freezes [1–3]. This contextual freezing seems to be mediated by the ventral portion of the periaqueductal gray (vPAG). For example, chemical [4] or electrical [5] stimulation of the vPAG produces freezing behavior whereas lesions of this area reduce freezing to innate [6] or learned [7] danger stimuli. Moreover, rats exposed to either innate [8] or conditioned [9] aversive stimuli present an increase in Fos labeling in the vPAG.

Defensive freezing behavior can be also triggered by chemical [10] or electrical [5] stimulation of the dorsal portion of the periaqueductal gray (dPAG). Stepwise increases in the electrical stimulation of the dPAG elicit initially freezing and then escape responses. We have previously shown that vPAG lesions disrupted context conditioned freezing but did not affect freezing induced by chemical or electrical stimulation of the dPAG [10]. Therefore, it seems that there exist at least two different patterns of inhibitory defensive behavior with distinct neural pathways: one, elicited by innate and conditioned stimuli and dependent on the integrity of the vPAG, and another one specific to neural substrates of the dPAG which is not mediated by intra-PAG connections.

It has been suggested that a neural circuitry underlying freezing behavior induced by dPAG stimulation might involve ascending projections to other fear-related brain structures [5,6]. Fibers originating from dPAG innervate forebrain structures, such as amygdala, which is critically involved in the regulation of innate and conditioned reactions to threatening stimuli [11,12]. For example, stimulation of the amygdala can induce several defensive behaviors, including freezing [13]. In the same vein, it has been shown that amygdala lesions block freezing behavior to innate [14] and conditioned [7,15] danger stimuli.

The present study examined the participation of the amygdala on the defensive behavior induced by electrical stimulation of the dPAG. To this end, electrodes were implanted into the dPAG of rats for determination of the freezing and escape thresholds before and after sham or bilateral amygdala electrolytic lesions. Since amygdala lesions disrupt expression of conditioned freezing [7,15], a positive control procedure was employed in order to behaviorally verify the effectiveness of our bilateral amygdala electrolytic lesions. Therefore, after the completion of the dPAG electrical stimulation procedure, conditioned freezing to contextual cues previously associated with footshock was also measured in all sham- and amygdala-lesioned animals.

MATERIALS AND METHODS

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 ($22 \pm 1^{\circ}C$) room and maintained on a 12:12 h light:dark cycle (07.00–19.00 h lights on). Animals were maintained in individual Plexiglas-walled cages and given free access to food and water throughout the experiment. Rats were randomly assigned to sham- or amygdala-lesioned groups.

Surgical procedures: Animals were anaesthetized with tribromoethanol (250 mg/kg, i.p.) and fixed in a stereotaxic frame (David Kopf, Tujunga, CA). Each animal was implanted, with a unilateral bipolar electrode aimed at the dPAG (angle 16°, 1.9 mm lateral to lambda at a depth of 5.1 mm below the skull surface). The electrode was made of stainless steel wire, 160 μm in diameter, insulated except at the cross-section. The electrode wire 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. A bilateral guide-cannula, through which a monopolar stainless-steel electrode could be inserted for electrolytic lesion, was also stereotaxically implanted 3 mm above the amygdala. Taking bregma as reference the coordinates were: AP −3.2 mm, ML 4.2 mm, DV 5.8 mm. The dPAG electrode and the bilateral guide cannula were fixed to the skull by means of acrylic resin and 3 stainless steel screws.

Measurement of defensive behaviors induced by dPAG electrical stimulation: One week after surgery, each animal was placed in a circular arena (60 cm diameter and 50 cm high with the floor divided into 12 sections) with the experimental compartment illuminated with a 40 W fluorescent lamp (80 lux at floor level). The animal was allowed a 5 min period of habituation in the arena at the beginning of the session. Next, the brain was electrically stimulated by means of a sine wave stimulator (DelVecchio, Brazil). The stimulation current was monitored by measuring the voltage drop across a 1 KΩ resistor with an oscilloscope (Philips, USA). Brain stimulation (AC, 60 Hz, 15 s) was presented 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 current intensity that produced immobility in two consecutive ascending series of electrical stimulation accompanied by at least two of the following autonomic reactions: urination, defecation, piloerection or exophthalmus. The current intensity that produced running (gallop) or jumping in two successive trials was considered to be the escape threshold. Animals with an escape threshold above 150 μA (peak-to-peak) were discarded from this study. After this baseline session, rats were again anesthetized as described above and monopolar stainless-steel electrodes were lowered 3 mm below the guide-cannula previously implanted. Animals assigned to the amygdala lesioning group received bilateral electrolytic lesions aimed at the amygdala by passing anodal current (2.0 mA, 60 s) through the electrodes (Albarsh, Brazil). Animals assigned to the sham lesion group underwent identical procedure except that no electrical current was delivered. Two days after the sham or amygdala electrolytic lesion, dPAG thresholds for freezing and escape were redetermined.

Contextual fear conditioning: One day after the end of the dPAG electrical stimulation procedure, all animals were exposed to a contextual fear conditioning paradigm in order to behaviorally verify the effectiveness of the amygdala electrolytic lesions. Conditioning took place in an experimental chamber (25 25 15 cm) made of opaque acrylic walls except for the ceiling and the front wall, which were made of translucent acrylic. The floor was made of 18 stainless bars, 2.0 mm diameter spaced 12 mm apart to which scrambled electrical footshock could be delivered through an AC shock source (Albarsh Instruments, Brazil). Each animal was placed in the experimental chamber and after 6 min habituation, five 5 s 1 mA unsignaled footshocks were presented at 60 s intervals. On the next day, animals were placed in the same conditioning chamber and left undisturbed for 8 min. Freezing, defined as the total absence of movement of the body and vibrissa except that required for respiration, was recorded for a 8 min test session through a time sample procedure [10]. Every 2 s the animal was observed and its behavior was scored as freezing or not freezing.

Histology: On completion of the experiment, the animals were overdosed with urethane and perfused intracardially with saline followed by buffered 4% formalin. Serial 60 μm brain sections were cut using a microtome, thaw-mounted on gelatinized slides and stained with neutral red in order to localize the stimulation electrode tips within the dPAG and the lesions within the amygdala according to the Paxinos and Watson [16] atlas.

Statistical analysis: Freezing and escape thresholds were analyzed separately by a two-way ANOVA, with lesions (sham and lesions), and thresholds (before and after) the between and within-group factors, respectively. The percentage of time spent freezing for sham and amygdala lesioned animals was analyzed by Student's *t*-test.

RESULTS

Figure 1 depicts a representative histological section illustrating the location of the electrode tip in the dPAG and an electrolytic lesion of the amygdala. Figure 2 presents a composite of the electrode tip locations within the dPAG as well as representative areas of the smallest and largest lesions in the amygdala. Histological examination of the brain slides in the midbrain area indicated that all electrical stimulation electrodes were located inside the dPAG. Histological analysis of the bilateral electrolytic lesions into the amygdala indicated that they tended to be symmetrical and damaged most of the basolateral, central, lateral portions of the amygdala.

As reported previously [17], freezing and escape behaviors occurred in a stepwise fashion as the intensity of electrical stimulation applied to the dPAG increased. Figures 3a and 3b present the mean (±s.e.m.) of the dPAG electrical current threshold required to induce freezing before (56.88±6.74; 58.75±9.11) and after (60.0±5.67; 53.75±5.64), and escape behavior before (67.50±6.12; 68.75±10.52) and after (70.0±6.55; 64.55±5.62) sham (n=8) or amygdala (n=12) electrolytic lesions, respectively. Two-way ANOVA revealed that electrolytic lesion of the amygdala did not affect the dPAG aversive threshold to induce freezing ($F(1.18)=0.05$; $p>0.8$) or escape ($F(1.18)=0.04$; $p>0.8$) behaviors. No interaction between lesioning procedure and freezing ($F(1.18)=1.56$; $p>0.2$) or escape ($F(1.18)=0.49$; $p>0.4$) thresholds was found.

Figure 3c presents the mean (± s.e.m.) percentage of time spent freezing for sham (60.64±8.37) and amygdala (13.8±2.14) lesioned animals during the testing sessions of the contextual fear procedure. Animals with amygdala

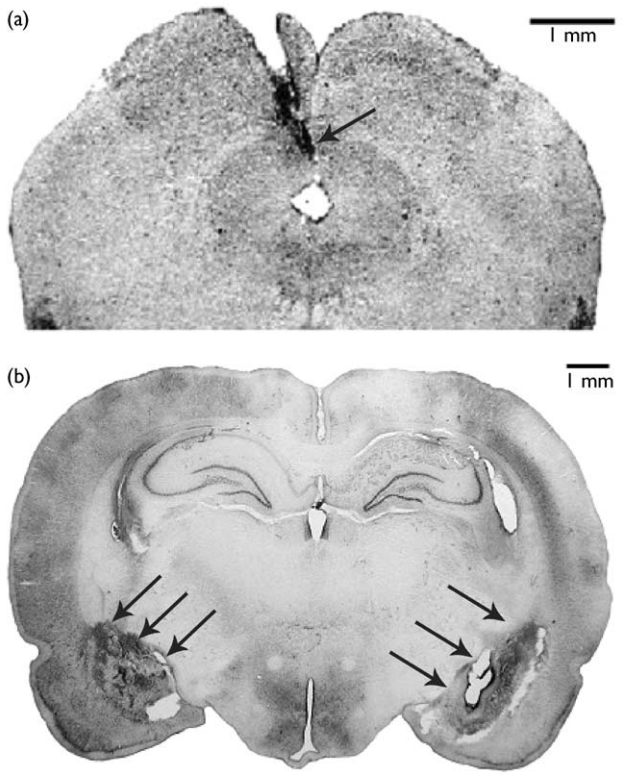

Fig. 1. Representative photomicrographs showing with arrows a typical example of a stimulation electrode tip in the dPAG (**a**) and a bilateral electrolytic lesion in the amygdala (**b**).

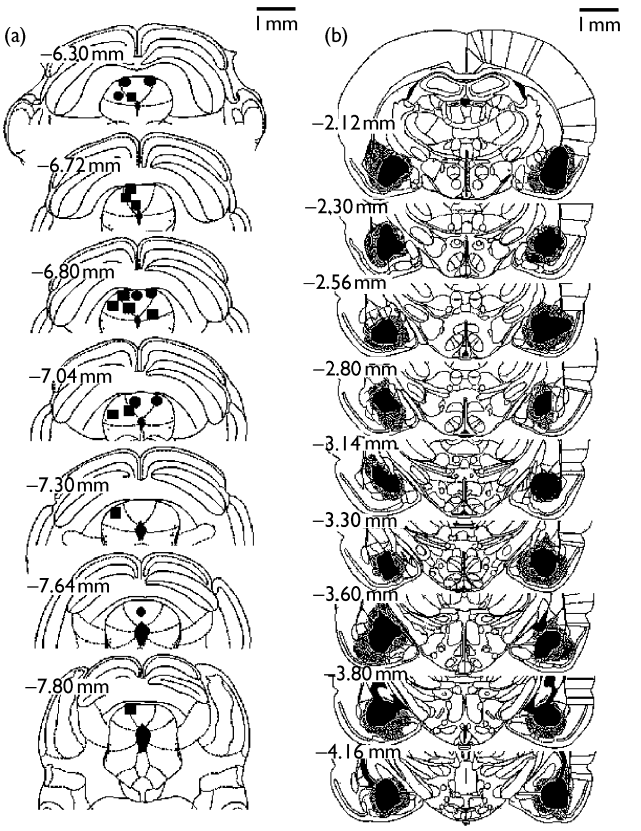

Fig. 2. Composite of coronal sections adapted from Paxinos and Watson atlas. Numbers indicate the distance in millimeters from bregma. The left side of the figure represents the stimulation electrode tips within the dPAG among sham (circles) and amygdala (squares) lesioned animals. The right side of the figure shows the smallest (black) and largest (gray) damaged areas in amygdala lesioned animals.

electrolytic lesions displayed less freezing than sham-lesioned control animals ($t(18)=6.48$; $p<0.001$). Therefore, although amygdala lesions were able to disrupt context fear conditioning, destruction of this structure did not affect the aversive thresholds determined by dPAG electrical stimulation.

DISCUSSION

Contextual cues previously associated with footshock induced freezing behavior, which was disrupted by electrolytic lesions of the amygdala. These results are in line with previous reports showing that the amygdala is crucial for the contextual fear conditioning [7,15]. On the other hand, these same amygdala electrolytic lesions were not able to interfere with freezing and escape behaviors induced by dPAG electrical stimulation. These findings point out that whereas amygdala is important for the occurrence of conditioned freezing it does not seem to play a role in the defensive behaviors induced by dPAG electrical stimulation.

It has been proposed that projections from the dPAG to amygdala might modulate the expression of the freezing response [6]. This suggestion is based on the fact that there are reciprocal anatomical projections between the dPAG and amygdala [11]. However, results from the present experiment do not support this suggestion since amygdala lesions did not interfere with the freezing behavior induced by dPAG electrical stimulation. In fact, context fear conditioning, which depends on the amygdala activity, may inhibit the occurrence of escape responses induced by electrical stimulation of the dPAG [18].

Results from the present study are in agreement with another study showing that electrolytic lesions of the vPAG disrupted contextual fear conditioning but had no effect on freezing and escape behaviors induced by stimulation of the dPAG [10]. Different areas of the periaqueductal gray coordinate different aspects of defensive behaviors [19,20]. The vPAG, which receives direct projections from the amygdala [11], seems to be responsible for the motor performance of this defensive response through descending projections to brainstem regions, which eventually reach the spinal motoneurons [21]. On the other hand, dPAG appears to mediate freezing behavior probably through ascending projections to higher brain structures involved in sensorial processing of aversive stimuli [5]. The fact that neither vPAG [10] nor amygdala lesions (present results) affected freezing or escape behaviors induced by dPAG stimulation but disrupted conditioned freezing to contextual cues indicates that there are at least two independent neural circuitries involved in different forms of defensive reactions: one dependent on the integrity of the amygdala and the vPAG and another one specific to neural mechanism of the dPAG. In agreement with this, dPAG electrical stimulation caused an increase in Fos expression in several brain

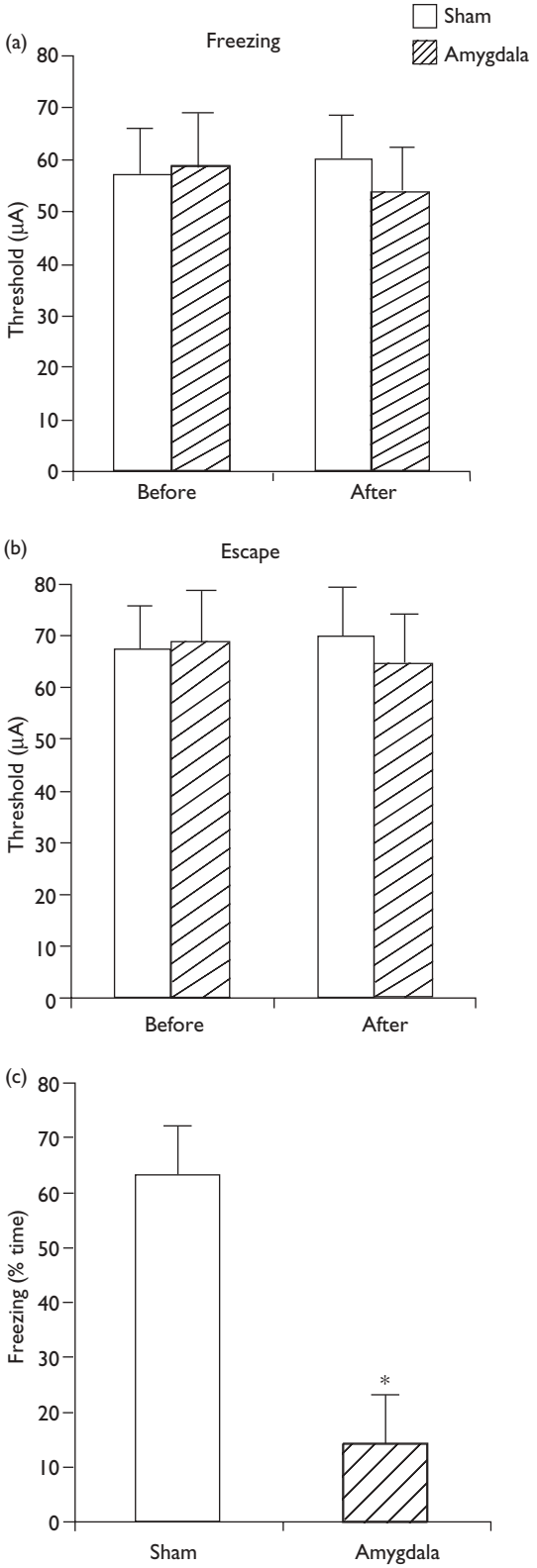

Fig. 3. Mean percentage (±s.e.m.) of the aversive dPAG electrical stimulation threshold to induce freezing (**a**) and escape (**b**) defensive behaviors before and after sham (open columns) or amygdala electrolytic lesions (hatched columns), and the mean percentage (±s.e.m.) of conditioned freezing (**c**) during an 8 min session between sham and amygdala lesioned animals exposed to contextual cues previously associated with footshock. *$p < 0.001$.

structures, such as the dorsal portion of the ventromedial hypothalamus, the dorsal premammilary and cuneiform nuclei, but not in the basolateral amygdala or vPAG [22].

It is possible that the neural pathways from amygdala to vPAG or dPAG may subserve distinct defensive behaviors. The amygdala is involved in the processing of innate or conditioned fear-provoking stimuli [12]. Descending projections from amygdala can reach the vPAG, which in turn organizes the expression of freezing response to distal aversive stimuli [21]. On the other hand, the neural substrates of aversion in the dPAG seem to belong to a more primitive system activated by proximal threatening stimuli of tactile or nociceptive nature, which sends impulses to higher levels of the neuroaxis [23]. Moreover, these stimuli can also trigger a defense reaction similar to that observed after dPAG stimulation [24]. Therefore, it appears that the neural substrates of aversion in the dPAG could be activated by immediate danger and are independent from the amygdala -vPAG axis, which is activated by conditioned aversive situations.

CONCLUSION

Electrical stimulation of the dPAG produces freezing and escape behaviors. Amygdala electrolytic lesions did not affect the production of these defensive reactions but disrupted the conditioned contextual freezing. Therefore, the organization of defensive behaviors in the dPAG does not seem to depend on the integrity of the amygdala, whose projections to the vPAG, are crucial for the contextual fear conditioning.

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