Defensive freezing evoked by electrical stimulation of the periaqueductal gray: comparison between dorsolateral and ventrolateral regions

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Previous reports indicated that ventrolateral periaqueductal gray (vlPAG) plays a role in the expression of freezing behavior whereas dorsolateral periaqueductal gray (dlPAG) is involved on both freezing and active forms of defensive behaviors. In order to evaluate the role of each of these areas in the occurrence of defensive reactions, rats were electrically stimulated either in the dlPAG or vlPAG with different stimulus frequencies. Stepwise increases in the electrical stimulation of both dlPAG or vlPAG induced initially freezing and then a jumping response. Freezing induced by vlPAG stimulation had a tendency to disappear when the stimulation was turned off whereas freezing induced by dlPAG stimulation remained high in the absence of the stimulation. These results suggest that dlPAG and vlPAG are involved on defensive freezing probably through different neural circuitries. *NeuroReport* 12:4109–4112

Key words: Defensive behavior; Electrical stimulation; Freezing; Periaqueductal gray

INTRODUCTION

The periaqueductal gray (PAG) comprises a well defined area in the mesencephalon surrounding the cerebral aqueduct and is composed by anatomically different subregions probably related to different aspects of defensive reactions [1]. The ventrolateral region of the periaqueductal gray (vlPAG) seems to be involved on defensive freezing behavior: a tense posture that animals display when exposed to innate or learned threatening situations. Chemical [2] or electrical [3] stimulation of the vlPAG produced freezing behavior whereas lesion of the vlPAG reduces freezing to innate [4] or conditioned [5] aversive stimuli. Moreover, innate [6] or conditioned [7] danger stimuli produced an increase in Fos labeling in the vlPAG.

The dorsolateral region of the periaqueductal gray (dlPAG) appears to mediate highly active escape defensive responses as well as freezing behavior (for review see [8]). Chemical stimulation of the dlPAG produces clear-cut active defensive reactions, such as running and jumping, alternated with freezing episodes [9]. Since chemical stimulation of the dlPAG produces a continuous process of cell activation, its is difficult to dissociate these two opposite defensive behaviors. Employing a procedure in which electrical stimulation is stepwisely increased, it has been observed that freezing appears at lower intensities whereas active escape behaviors appears at higher intensities [10]. When the sequence of stimulation follows a random pattern of intensities, the likelihood of observing active escape or freezing behaviors is also random across intensities [11]. Recently, we demonstrated that vlPAG lesions that disrupted conditioned freezing did not affect freezing induced by dlPAG chemical or electrical stimulation [12]. Therefore, it appears that both ventrolateral and dorsolateral PAG regions are involved in freezing behavior probably through different neural circuitries.

The purpose of the present experiment was to compare the pattern of defensive freezing behavior to electrical stimulation of the dorsolateral and ventrolateral regions of the PAG. Although the use of electrical brain stimulation has the disadvantages of activating both cell bodies and fibers of passage, the choice of this technique was made due to necessity for a gradual and discrete stimulation of these regions. Therefore, it was possible to observe the animal's behavior during the presence and the absence of the gradual increase of electrical stimulation. Since the amount of tissue electrically stimulated is a function of the intensity of the stimulus [13], frequency was used in order to vary the stimulation effectiveness

while keeping the stimulated region relatively unchanged [14].

MATERIALS AND METHODS

The subjects were 44 male Wistar rats weighing about 250 g and obtained from local breeding facilities. Animals were housed in individual Plexiglas-walled cages with free access to food and water. Room light:dark cycle was maintained on a 12:12 h cycle (lights on at 07:00 h). The experiment was conducted during the light phase of the cycle.

Rats were anaesthetized with tribromoethanol (250 mg/kg, i.p.) and fixed on a stereotaxic frame. With the skull horizontal between bregma and lambda, bipolar electrodes (Plastics One, USA), insulated except at the cross-section of the tip, were inserted into the dlPAG (angle 16°, 1.9 mm lateral to the lambda to a depth of 5.0 mm below the bony surface, $n = 24$) or vlPAG (angle 16°, 1.0 mm caudal and 2.3 mm lateral to lambda to a depth of 6.2 mm below the bony surface, $n = 20$), and anchored to the skull by two small screws and autopolymerizing resin.

Electrical stimulation of the dlPAG and vlPAG was obtained by means of a square-wave stimulator Grass S44 (Quincy, Mass., USA), connected to an oscilloscope (Tektronix, USA) that indicated the voltage drop through a 100 KΩ resistor in series with the rat. The electrodes were connected to the stimulator by means of a flexible cable and a mercury swivel, which allowed free movement to the animal.

The experimental setting where brain stimulation was delivered consisted of a box $25 \times 20 \times 20$ cm. Two facing and the rear walls were made of aluminum and the front wall and the ceiling were made of plexiglass. The floor was made of stainless steel bars with 5 mm diameter, 1.5 cm apart. This box was placed inside a sound attenuation chamber made of plywood and illuminated from within by an ordinary 5 W lamp, and 78 dB permanent background noise was delivered by a fan. A 15×25 cm glass window allowed direct observation of the animal during the experiment. The box was cleaned with a 5% ammonium solution immediately before each animal was placed inside it.

Starting on the second day after surgery, animals were handled for 5 min during 4 consecutive days. On the sixth day, each animal was placed in the experimental box, where remained undisturbed for 6 min (baseline period). After that, the brain electrical stimulation procedure started. This procedure, adapted from Sandner *et al*. [15], consisted of holding constant the electrical stimulation intensity at 200 μA, with 1.0 ms pulse duration but varying the electrical stimulation frequency through its interpulse interval (IPI). Starting at an IPI of 80 ms, two groups of animals, one with electrodes implanted in the dlPAG ($n = 9$) and the other with electrodes implanted in the vlPAG ($n = 8$), received a period of 1 min electrical stimulation, alternating with 1 min with no brain stimulation. At each new electrical stimulation period, the IPI was reduced by 5 ms until the rat showed jumping behavior defined as simultaneous lifting of the four paws from the floor. The highest level of brain electrical stimulation was set at IPI equal to 10 ms. Animals in two control groups had electrodes implanted in the dlPAG ($n = 12$) or vlPAG ($n = 11$) and were attached to the apparatus, but remained undisturbed in the experimental box for 35 min. In order to investigate the behavioral effects of the last electrical stimulation that triggered the jumping behavior, a subset of stimulated animals (dlPAG: $n = 6$; vlPAG $n = 5$) was removed and then returned to the experimental box, where they were observed for another 6 min without any stimulation. A subset of control animals that remained in the experimental box for 35 min without any electrical stimulation (dlPAG: $n = 9$; vlPAG: $n = 7$) was also removed and then placed again into the same experimental condition for an extra 6 min. Freezing was scored during the whole experiment through a time-sample procedure. For each 2 s, an experimenter rated the animal's behavior as freezing or activity. Freezing was defined as absence of visible movements, except those due to respiration.

At the end of the experiment, animals were sacrificed with an overdose of sodium thiopental and perfused intracardially with saline followed by 10% formalin containing 1% ferrocianide. A DC current from a 9 V battery (20 s) was applied to the brain electrode to stain the brain tissue around its tip. The brains were then removed and further fixed for a minimum of 3 days with 10% formalin. Serial 60 μm brain slices were sectioned with a cryostat. The stimulation sites, stained by Prussian blue dye, were identified and plotted on diagrams according to the Paxinos and Watson [16] rat brain atlas.

RESULTS

Histological examination of the brain slices indicated that all electrode tips were located inside or at the borders of dlPAG or vlPAG. Figure 1 presents a composite of the electrode tip locations.

A one-way ANOVA of freezing behavior during the baseline period indicated no significant differences among the four groups ($F(3,41) = 2{,}09$; $p > 0.1$). Figure 2 presents the mean (± s.e.m.) percentage of freezing in the presence or in the absence of electrical stimulation of the dlPAG (Fig. 2a) or vlPAG (Fig. 2b) as well during the session in non-stimulated control animals with electrodes implanted in the dlPAG (Fig. 2c) or vlPAG (Fig. 2d) across the seven interpulse intervals. A four-way ANOVA was employed to analyze these results. Two between-group factors, brain

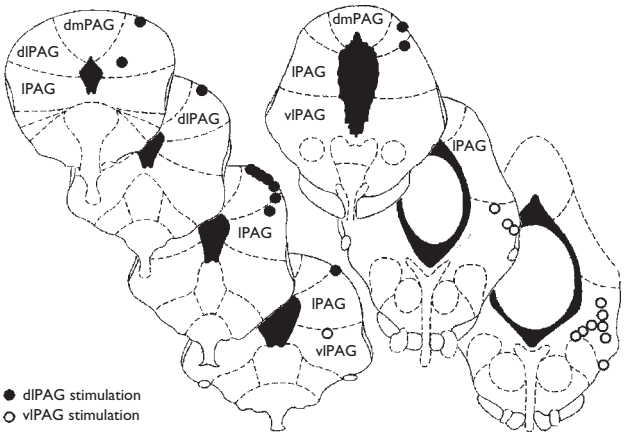

Fig. 1. Composite of stimulation electrodes locations aimed at the dlPAG and vlPAG with the reference to the Paxinos and Watson atlas [16]. Some sites overlapped.

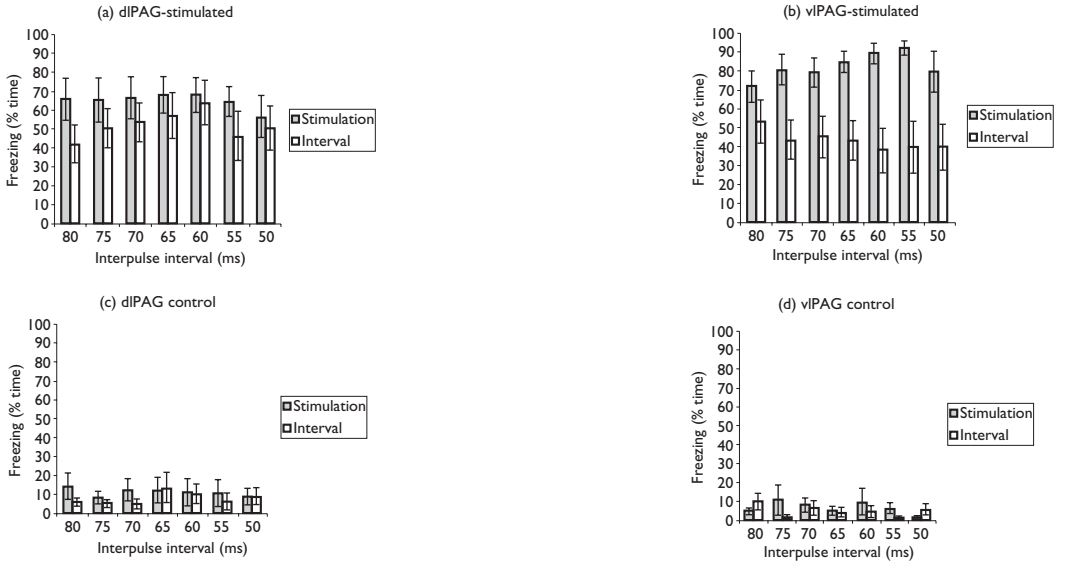

Fig. 2. Mean (± s.e.m.) freezing behavior during the presence or absence of electrical stimulation of the dlPAG or vlPAG (stimulated animals) or 35 min comparison period in non-stimulated control animals with electrodes implanted in the dlPAG or vlPAG across the 7 interpulse intervals. (**a**) located at the upper left, presents the dlPAG-stimulated group; (**b**) located at upper right, presents the vlPAG-stimulated group; (**c**) located at lower left, presents the dlPAG control group; (**d**) located at lower right, presents the vlPAG control group.

electrical stimulation (stimulated × non-stimulated animals) and electrodes placed in different PAG regions (dlPAG × vlPAG), and two within-group facators, freezing in the presence or absence of brain stimulation (2 levels) and freezing across the seven interpulse intervals (7 IPI levels), made up the $2 \times 2 \times 2 \times 7$ factorial design. Animals that jumped before IPI 45 ms (dlPAG: $n = 3$; vlPAG: $n = 1$) were not included in these analyses, to allow more IPI levels to be compared. For the same reason, an IPI cut-off time of 50 ms was set for freezing.

As can be observed in Fig. 2, animals electrically stimulated in either the dlPAG or vlPAG clearly displayed more freezing than control non-stimulated animals. Moreover, it can be observed that dlPAG-stimulated animals presented a different pattern of freezing when compared to vlPAG stimulated animals during the interval between electrical stimulation across the seven interpulse intervals levels. This impression was confirmed by the four-way interaction ($F(1,36) = 6.69$; $p < 0.05$). *Post hoc* comparison indicated that only the vlPAG-stimulated group presented differences in the amount of freezing between stimulation and interval periods across the seven levels of the interpulse intervals (all $p < 0.05$).

Figure 3 shows the mean (± s.e.m.) freezing behavior during the 6 min following jumping (stimulated animals) or after the 35 min session for the two non-stimulated control groups. A one-way ANOVA indicated a difference in the amount of freezing among the four groups ($F(3,25) = 4.93$; $p < 0.01$]. A *post hoc* comparison revealed that animals in the dlPAG-stimulated group presented more freezing than the other three groups (all $p < 0.05$).

DISCUSSION

The present results suggest that dlPAG and vlPAG are differently involved in the occurrence of on defensive freezing behavior. Electrical stimulation of the dlPAG triggered a freezing response that persisted when the stimulation was switched off. Conversely, electrical stimulation of the vlPAG induced a transient freezing response that had a tendency to disappear as soon as the stimulation was ceased. The freezing behavior during the 6 min after the last electrical stimulation illustrates this effect even more clearly, as over 6 min the vlPAG-provoked freezing reached control values, whereas dlPAG-stimulated animals still presented an enhancement of this response. Therefore, freezing behavior seems to be regulated differently by dlPAG and vlPAG: freezing induced by dlPAG electrical stimulation remained after stimulation while freezing trig-

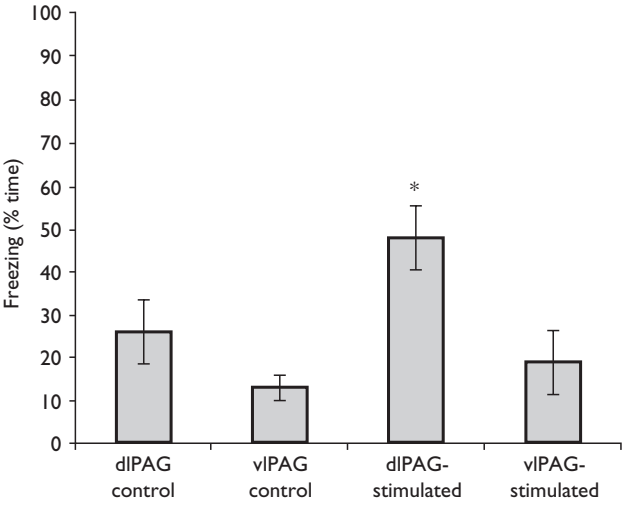

Fig. 3. Mean (± s.e.m.) freezing behavior 6 min after jump (stimulated animals: dlPAG $n = 6$; vlPAG $n = 5$) or after 35 min (non-stimulated control animals: dlPAG $n = 9$; vlPAG $n = 7$). * $p < 0.05$ *vs* all other groups.

gered by vlPAG electrical stimulation did not endure with the termination of the stimulation.

The long lasting freezing induced by dlPAG that persisted after the termination of the stimulation can not be attributed to some sort of associative learning to contextual cues present during the stimulation procedure. Recently, we reported that a context shift procedure that was able to block conditioned freezing to contextual cues did not affect freezing induced by electrical stimulation of the dlPAG [17]. Moreover, we also found that lesions of the vlPAG that disrupted conditioned freezing did not affect freezing behavior induced by dlPAG electrical or chemical stimulation [12]. Therefore, it seems clear that dlPAG neurons are actively involved in the occurrence of defensive freezing behavior.

A possible neural circuitry responsible for the occurrence of the freezing behavior elicited by the dlPAG might be through ascending projections to forebrain structures involved in the sensorial recognition of aversive stimuli. Fibers originating from dlPAG innervate various forebrain regions including the amygdaloid complex through the medial forebrain bundle [18]. Accordingly, it has been suggested that connections between the dPAG and the amygdaloid complex might in fact modulate the occurrence of freezing behavior [19]. Alternatively, the dlPAG also send projections to the intralaminar thalamic nuclei, which in turn reach the anterior cingulate cortex, another forebrain structure involved in the identification of threatening stimuli [20].

On the other hand, the fact that freezing induced by vlPAG disappears with the termination of the stimulation is in agreement with the notion that this area is related to the motor performance of this defensive response probably through descending projections to brainstem regions that in turn projects to spinal motor neurons [19,21]. There are direct connections between the vlPAG and motorneuronal cell groups in the spinal cord which control the neck, back and hind limbs [22]. Neurons from the vlPAG also send projections to medullary regions, including the raphe nuclei and both rostral and caudal ventromedial and ventrolateral medulla from which inibitory defense posture can be evoked by chemical stimulation [23]. Direct connections between vlPAG and the median raphe nucleus might be also a relevant pathway in the inhibitory defense output since electrical stimulation of this raphe nucleus induced a transient but effective inhibitory reaction similar to the freezing response pattern observed in the vlPAG-stimulated group [24].

Finally, the participation of dlPAG on active forms of defensive reactions is well established [8]. However, the occurrence of jumping behavior observed after vlPAG electrical stimulation is not in agreement with a previous report [3]. A possible explanation for the occurrence of jumping behavior induced by electrical stimulation of the vlPAG is the fact that a fixed and a rather high current intensity was employed to allow variation of frequency so that the stimulated area could be maintained unchanged. In accordance with this possibility, Sandner *et al.* [25] reported that vlPAG stimulation induced jumping behavior when using a similar electrical stimulation procedure employed in the present study.

CONCLUSION

Stepwise increase in the electrical stimulation of the dlPAG and vlPAG produced initially freezing and then active escape behaviors. Freezing induced by electrical stimulation of the vlPAG decreased with the termination of the stimulation. Electrical stimulation of the dlPAG induced a long-lasting freezing behavior that remained at high levels after the stimulation. It is suggested that vlPAG is involved on the motor aspect of freezing response through descending projections to the spinal cord whereas the dlPAG activates this defensive posture through ascending projections to forebrain structures related to the sensorial processing of aversive stimuli.

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