
Fos-like immunoreactive neurons following electrical stimulation of the dorsal periaqueductal gray at freezing and escape thresholds
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Abstract

Electrical stimulation of the dorsal regions of the periaqueductal gray (PAG) leads to defensive reactions characterized as freezing and escape responses. Until recently it was thought that this freezing behavior could be due to the recruitment of neural circuits in the ventrolateral periaqueductal gray (vlPAG), while escape would be mediated by other pathways. Nowadays, this view has been changing mainly because of evidence that freezing and escape behaviors thus elicited are not altered after lesions of the vlPAG. It has been suggested that there are at least two pathways for periaqueductal gray-mediated defensive responses, one involving the hypothalamus and the cuneiform nucleus (CnF) which mediates responses to immediate danger and another one involving the amygdala and vlPAG which mediates cue-elicited responses, either learned or innate. To examine this issue further we measured Fos protein expression in brain areas activated by electrical stimulation of the dorsolateral PAG (dlPAG) at the freezing and escape thresholds. The data obtained showed that freezing-provoking stimulation caused increases in Fos expression in the dorsomedial PAG (dmPAG), while escape-provoking stimulation led to increases at both dmPAG and dlPAG. Surprisingly, neither escape- nor freezing-provoking stimulations altered Fos expression in the central nucleus of amygdala (CeA). Escape-provoking stimulation caused increased Fos expression in the ventromedial hypothalamus (VMH), dorsal premammillary nucleus (PMd) and in the cuneiform nucleus. Significant increases in Fos labeling were found in the dmPAG and PMd following freezing-provoking stimulation. Therefore, the present data support the notion of a neural segregation for defensive behaviors in the dorsal columns of PAG, with increased Fos expression in the dmPAG following freezing, while dlPAG is affected by both freezing and escape responses. dlPAG, CnF, VMH and PMd are part of a brain aversion network activated by fear unconditioned stimuli. The present data also suggests that the defensive responses generated at the dlPAG level do not recruit the neural circuits of the vlPAG and CeA usually activated by conditioned fear stimuli.

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1. Introduction

It has been established that the longitudinal columns of the periaqueductal gray (PAG), along its rostrocaudal axis, coordinate distinct patterns of behavioral and physiological reactions critical for survival [3,15]. Electrical or chemical stimulation of the dorsal half of the periaqueductal gray of the rat leads to a vigorous defensive response characterized by alertness, freezing and escape behavior [2,7,32,49]. Although escape behavior has been the main focus of attention, interest in freezing behavior induced by electrical stimulation of the dlPAG has grown lately. This freezing behavior is not mediated by ventrolateral periaqueductal gray (vlPAG), as it is not changed by vlPAG electrolytic lesions [49]. It is, thus, logical to assume that other PAG afferents and efferents should mediate this reaction [48]. Indeed, freezing behavior elicited by this procedure has been reported to resemble human panic attacks [49]. The structures with higher chance of being involved in the neural circuits of aversion are also those that when electrically stimulated, rostrally to the PAG, also give rise to defensive responses, such as the amygdala and medial hypothalamus, and the cuneiform nucleus, more caudally [22,28,40]. The medial frontal and cingulate cortices, sources of afferents to PAG, have also been implicated in the mediation of affective responses [1,27,33]. In fact, all these structures show increased Fos expression when rats are submitted to anxiogenic procedures [4,10,12,23,25,31].

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Defensive strategies as distinct as avoidance, freezing and escape are likely to be organized by different networks [5,28,34]. Therefore, it is expected that electrical stimulation of the dIPAG either at freezing or escape thresholds should activate different sets of brain structures. To test this prediction, Fos distribution was presently measured in serial sections of the brain following dIPAG electrical stimulation at freezing and escape thresholds of rats placed in an arena. Sham-stimulated animals were exposed to the same procedure in order to control for novelty of the exposure and locomotor activity. The reason for choosing electrical stimulation in this work is that this procedure makes it possible to clearly dissociate freezing and escape responses with gradual increases in the intensity of the electrical stimulation of the dIPAG. Also, we have chosen to concentrate on those nuclei known for having monosynaptic connections with PAG; that is, cingulate (Cg) and prelimbic (PrL) areas [27], central amygdaloid nucleus (CeA) [39], ventromedial (dorsomedial part, VMHdm) and premammillary (PMd) nuclei of the hypothalamus [11], cuneiform nucleus (CuF) [38], inferior colliculus (IC) [37], the locus coeruleus (LC) [26] and of course the dorsolateral (dIPAG), dorso-lateral (dIPAL), lateral (IPAG) and ventrolateral (vIPAG) subdivisions of periaqueductal gray [3,15].

Microinjections of GABA receptor antagonists, such as bicuculline, into the dIPAG cause escape interspersed with freezing behavior. These effects have been related to the release of the tonic inhibition exerted by GABA mechanisms on the efferents responsible for the defense reaction [7,8]. For comparison purposes with the electrical stimulation, which stimulates fibers of passage and does not distinguish between efferents from and afferents to the region under study, chemical stimulation of the dIPAG with bicuculline, which activates only post-synaptic neurons, was also performed in this work.

2. Materials and methods

2.1. Animals

Naive male Wistar rats weighing 240-260 g were used. Animals were kept under controlled temperature (22 ± 2°C) and a 12-h light/12-h dark cycle (lights on at 07:00 h). They were housed in two per cage and had free access to food and water throughout the experiment. The experiments were conducted between 9.00 and 12.00 a.m. The experiments were performed in compliance with the recommendations of SBNC/SC (Brazilian Society of Neuroscience and Behavior), which are based on the US National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2. Surgery

The animals were anaesthetized with tribromoethanol (250 mg/kg, i.p.) and fixed in a stereotaxic frame (David Kopf, USA). A brain electrode was implanted in the midbrain, aimed at the dorsolateral PAG. The electrode was made of stainless steel wire, 160 μm in diameter, insulated except at the cross-section of the tip. The upper incisor bar was set at 3.3 mm below the interaural line so that the skull was horizontal between bregma and lambda. The electrode was introduced at an angle of 16° using the following coordinates with the lambda serving as the reference for each plane: medio-lateral, 1.9 mm; and dorso-ventral, 5.1 mm [36]. For microinjections of bicuculline and saline, a stainless-steel guide-cannula (13 mm length, o.d. 0.6 mm, i.d. 0.4 mm) was similarly implanted, introduced vertically at 2.5 mm at the dorso-ventral plane. The electrode or cannula was fixed to the skull by means of acrylic resin and the three stainless steel screws. At the end of surgery each guide-cannula was sealed with a stainless steel wire to protect it from congestion.

2.3. Microinjections

The animals were gently wrapped in a cloth, hand-held and a thin dental needle (i.e., 0.33 mm) was introduced through the guide-cannula until its lower end was 1 mm below its tip, reaching the same depth as the electrode tip. The injection needle was linked to a 5 μl Hamilton syringe by means of polyethylene tubing connected to a microinfusion apparatus (Harvard, USA). A volume of 0.2 μl was injected during 20 s. Following the end of the injection the microinfusion needle was held inside the brain for 10 s.

2.4. Drugs

Bicuculline hydrochloride (Sigma, USA) was dissolved in physiological saline (0.9%) shortly before use. The dose of bicuculline used was of 40 ng/0.2 μl injected into left dIPAG. Physiological saline served as vehicle control for dIPAG microinjections.

2.5. Apparatus

Six days after surgery, the rats were placed in an open field, which was a circular enclosure 60 cm in diameter and 50 cm high. The rat was placed in the arena and had its brain electrode connected to a flexible wire cable, allowing ample movement inside the box. The cable, in turn, was connected to the stimulator by means of a mercury swivel mounted on the top of the experimental chamber. The rats were allowed a 15 min period of habituation in the enclosure. The brain was stimulated electrically by means of a sine wave stimulator [35]. 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 μA) was presented at 1 min intervals with the current intensity increasing by steps of 5 μA for measurements of the freezing and escape thresholds. Freezing threshold was defined as the lowest intensity producing cessation of the
ongoing behavior in two consecutive series of electrical stimulation, accompanied by at least two of the following autonomic responses: piloerection, defecation, micturition and exophthalmus. Escape threshold was defined as the lowest current intensity that produced running or jumping in two successive ascending series of electrical stimulation. Animals with an escape threshold above 120 μA (peak-to-peak) were discarded from the experiment.

2.6. Experimental procedure

2.6.1. Electrical stimulation

On the seventh day after surgery, animals were randomly allocated to the freezing (N = 8) and escape (N = 10) groups. A control group consisted of rats submitted to the same procedure, except that no current was applied to the electrodes (N = 9). Animals of the three groups were placed in the arena and allowed 15 min for habituation. Next, the aversive thresholds were determined in one series of ascending intensity of electrical stimulation, and then the animals were returned to their home boxes. On the next day, electrical stimulation was applied at the same aversive threshold determined the day before. The animals were returned to their home cages and observed for 2 h. No apparent behavioral changes could be observed during this period. Next, their brains were processed in parallel, as described below.

2.6.2. Chemical stimulation

On the seventh day after surgery, animals were randomly allocated to the bicuculline (N = 8) and saline (N = 8) groups. Animals of these groups were placed in the arena and allowed 15 min for habituation. Afterwards, the animals were returned to their home boxes and observed for 90 min. Next, their brains were processed in parallel, as described below.

2.7. Fos protein immunohistochemistry

About 2 h after the electrical stimulation procedure, the animals were deeply anaesthetized with urethane (1.25 g/kg; Sigma, USA) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.4). Brains were removed, immersed (4°C) in the above fixative for 2 h and then kept in 30% sucrose in 0.1 M PBS until soaked. They were then quickly frozen in isopentane (−40°C) and sliced by the use of a cryostat (−15°C). Two adjacent series of 40-μm thick brain slices were obtained, having as reference the following AP coordinates: bregma +3.7, +1.0, −1.8, −2.7, −4.2, −4.3, −5.8, −6.8, −7.6, −8.7, −9.3 mm. The sections were collected in 0.1 M PBS and subsequently processed free-floating according to the avidine-biotine procedure, using the Vectastain ABC Elite peroxidase rabbit IgG kit (Vector, USA, ref. PK 6101). All reactions were carried out under agitation at room temperature. The sections were first incubated with 1% H2O2 for 10 min, washed four times with 0.1 M PBS (5 min each) and then incubated overnight with the primary Fos polyclonal antibody (Santa Cruz, USA, SC-52) at a concentration of 1/20,000 in 0.1 M PBS enriched with 4% normal goat serum and 0.2% Triton-X. Sections were again washed three times (5 min each) with 0.1 M PBS and incubated for 1 h with biotinylated goat antirabbit antibody (one drop from the kit for 10 ml of 0.1 M PBS enriched with 4% normal goat serum and 0.2% Triton-X). After another series of three 5-min washings in 0.1 M PBS, they were incubated for 1 h with the avidine-peroxidase solution (two drops of each solution labeled A and B of the kit for 25 ml of 0.1 M PBS) and three times washed in 0.1 M PBS (5 min per wash). The sections were finally left for 5 min in a solution of 3,3′-di-aminobenzidine (DAB, 0.02%) to which hydrogen peroxide (0.04%) was added just prior to use and were washed twice with 0.1 M PBS.

2.8. Quantification of Fos-positive cells

Mounted sections of the tissue were photographed with the help of an Olympus BX-50 microscope with a digital camera attached to it (Hamatsu Photonics C2400). Neuronal nuclei expressing levels of DAB reaction product above tissue background were counted by a computerized image analysis system (Image Pro Plus 4.0, Media Cybernetics, USA), as described elsewhere [34,47]. Briefly, counting of Fos-positive cells was performed at a magnification 100×. An area of the same shape and size per brain region was used for each rat. The same light and threshold conditions were employed for all sections. Fos staining could vary from one area to another. However, in order to ensure accuracy of measurement and avoid variations among same areas in different subjects, the background of each area was measured and digitally subtracted from the area to be measured. Accordingly, the threshold conditions were set for each area and maintained for all subjects [34]. All brain regions were bilaterally counted in various sections for each rat depending on the size of the structure. After that, counts for the same section indicated in Fig. 1 according to the Paxinos and Watson atlas were taken. The number of positive nuclei that fell within a 1.0 mm × 0.8 mm viewing field area (0.8 mm²) in each region of interest was counted. In several cases, the designated area was substantially larger than the boundaries of the viewing field. In such cases, the viewing field was placed in a fixed position within the region of interest relative to known anatomical landmarks. In other cases, the designated area to be counted was smaller than the boundaries of the viewing field. In such cases, only the region of interest, and not extraneous areas, was counted. Nuclei were counted individually and expressed as number of Fos-positive nuclei per 0.1 mm² [34]. The nomenclature and nuclear boundaries utilized were based on the atlas of Paxinos and Watson [36] and the planes of analyzed sections were standardized as far as possible. Each region was
bilaterally counted. This procedure has been used in other studies of this laboratory [34].

2.9. Statistical analysis

The data are presented as mean ± S.E.M. and were analyzed by means of a three-way analysis of variance (ANOVA), with structures, groups of stimulation (sham, freezing and escape), and side of stimulation (ipsi/contralateral to the electrode placement) as the factors. For each structure this analysis was followed by a two-way ANOVA with groups as between-group factor (sham, freezing and escape) and side of stimulation (ipsi and contralateral) as within-group factor. Newman–Keuls post hoc test was done when appropriate. The same analysis was performed for the bicuculline data with the only difference being the groups (saline and bicuculline). The significance level was set at $P$ equal to 0.05.
Fig. 2. Sites of stimulation electrodes in the dorsolateral periaqueductal gray with reference to the Paxinos and Watson atlas [36]. Animals submitted to electrical stimulation at freezing (○) or escape (●) thresholds. Shams are not depicted.

3. Results

3.1. Behavioral effects

The tips of the electrodes were situated inside or in the immediate vicinity of the dlPAG (see Fig. 2). The intensity of the electric current applied to the dlPAG of the animals to induce freezing and escape responses was 38.13 ± 0.81 and 64.00 ± 2.41 (peak-to-peak), respectively. The freezing response was characterized by immobility for at least 6 s accompanied by piloerection, arching back, lowered ears and autonomic responses such as micturition, defecation or exophthalmus. The escape behavior was characterized as running, galloping or jumpings and the same autonomic responses as in the freezing behavior. Student’s t-test performed on these thresholds data revealed that they were

<table>
<thead>
<tr>
<th>Region</th>
<th>Sham</th>
<th>Freezing</th>
<th>Escape</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ipsi</td>
<td>Contra</td>
<td>Ipsi</td>
</tr>
<tr>
<td>LC</td>
<td>18.38 ± 1.21</td>
<td>18.88 ± 1.30</td>
<td>16.13 ± 1.17</td>
</tr>
<tr>
<td>Ic</td>
<td>67.20 ± 6.09</td>
<td>96.60 ± 9.84</td>
<td>54.71 ± 5.96</td>
</tr>
<tr>
<td>vPAG</td>
<td>70.67 ± 3.82</td>
<td>76.00 ± 3.44</td>
<td>72.25 ± 4.24</td>
</tr>
<tr>
<td>dPAG</td>
<td>30.78 ± 2.07</td>
<td>32.22 ± 1.45</td>
<td>40.75 ± 2.29</td>
</tr>
<tr>
<td>dmPAG</td>
<td>24.33 ± 0.67</td>
<td>23.89 ± 0.71</td>
<td>62.50 ± 6.88*</td>
</tr>
<tr>
<td>VMHdm</td>
<td>15.29 ± 0.99</td>
<td>15.29 ± 1.26</td>
<td>39.29 ± 3.71*</td>
</tr>
<tr>
<td>Cg</td>
<td>10.44 ± 1.19</td>
<td>9.89 ± 0.96</td>
<td>5.57 ± 0.60</td>
</tr>
<tr>
<td>PrL</td>
<td>2.00 ± 0.23</td>
<td>4.89 ± 0.55</td>
<td>4.63 ± 0.60</td>
</tr>
<tr>
<td>Pel</td>
<td>69.56 ± 7.62</td>
<td>59.78 ± 6.95</td>
<td>74.98 ± 7.47</td>
</tr>
<tr>
<td>PMd</td>
<td>84.00 ± 16.54</td>
<td>89.63 ± 12.55</td>
<td>81.88 ± 10.25</td>
</tr>
</tbody>
</table>

Male Wistar rats were placed in an open arena for 15 min and then electrically stimulated in the dlPAG at intensities sufficient to produce freezing or escape behavior, or not stimulated at all (sham). The animals were killed under deep anesthesia 2 h after the behavioral tests and had their brain removed and processed for the detection of Fos-like immunoreactivity. The number of Fos-positive neurons per region in the ipsilateral (ipsi) or contralateral (contra) side in relation to the electrode placement was counted using a computerized image analysis system (Image Pro Plus 4.0, Media Cybernetics). Abbreviations: CeA: central amygdaloid nucleus; IC: inferior colliculus; Cg: cingulate cortex; CnF: cuneiform nucleus; dPAG: dorsolateral periaqueductal gray; dmPAG: dorsomedial periaqueductal gray; LC: locus coeruleus; PMd: dorsolateral prefrontal cortex; PrL: prelimbic cortex; vPAG: ventral lateral periaqueductal gray; VMHdm: ventromedial hypothalamic nucleus, dorsomedial part.

* P < 0.05 in relation to sham group by means of ANOVA followed by Newman–Keuls test.

** P < 0.01 in relation to sham group by means of ANOVA followed by Newman–Keuls test.
statistically different ($t = 3.25; P < 0.01$). Animals injected with bicuculline into the dPAG presented a behavioral activation immediately after the injections. This activation was characterized by escape behavior interspersed with freezing behavior for about 20 min.

3.2. Fos protein expression

Immunoreactive cells exhibited a dark nucleus in neuronal nuclei expressed clearly upon the surrounding background tissue. In the sham-stimulated group, clusters of Fos-like immunoreactive cells could already be identified in some brain regions, namely the frontal cortex, inferior colliculus and ventrolateral periaqueductal gray.

The results obtained with quantitative analyses of Fos-like immunoreactivity in the brain regions studied after induction of freezing and escape responses in rats stimulated in the dPAG are summarized in Table 1. Three-way ANOVA applied on these data revealed that there were significant effects in Fos labeling of the studied structures ($F(11, 537) = 14.63$; 

Fig. 3. Photomicrographs of Fos-immunoreactive cells (dark dots) in coronal sections of the midbrain and diencephalons following electrical stimulation of the dPAG. The left side of the pictures corresponds to the side where the electrode was placed. See Table 1 for abbreviations. Scale bar: 200 μm.
3.2.1. Escape
Two-way ANOVA showed that there was a significant difference between stimulation \( F(7.74; P < 0.05) \) side \( F(10.87; P < 0.05) \) and interaction \( F(4.89; P < 0.05) \) after stimulation of dIPAG at the escape threshold (Fig. 3F). There was a main effect of stimulation \( F(7.14; P < 0.05) \) side \( F(9.81; P < 0.05) \) and interaction between stimulation and side \( F(3.46; P < 0.05) \). However, there was no significant effect that could be detected in the IPAG (Fig. 3F) and vIPAG (Fig. 3C). Stimulation at the escape threshold also caused significant increase in Fos labeling in CnF (Fig. 3C). There was a main effect of stimulation at this nucleus \( F(5.44; P < 0.05) \). Stimulation also produced a higher density of Fos-immunoreactive neurons in PMd \( F(5.81; P < 0.05) \) (Fig. 3I) and VMHdm \( F(6.20; P < 0.05) \) (Fig. 3M). Table 2 presents a complete account of all statistical analysis performed per structure in this work.

3.2.2. Freezing
Stimulation of dorsal regions of PAG at the freezing threshold produced a significant increase in Fos-immunoreactivity in this nucleus itself \( F(5.74; P < 0.05) \) and of the dIPAG \( F(7.14; P < 0.05) \) (Fig. 3E). As observed with stimulation at escape threshold, Fos counts in the LC, IPAG, inferior colliculus, CeA, Cg, and PrL of rats stimulated at the freezing threshold were not different from the sham-control groups (Table 2).

3.2.3. Bicuculline
Three-way ANOVA applied on data revealed that there were significant effects in Fos labeling of the studied structures \( F(11, 240) = 11.28; P < 0.001 \), treatments \( F(11, 240) = 115.32; P < 0.05 \) and significant effect of side of injection \( F(1, 240) = 32.12; P > 0.001 \). Significant interactions occurred between structures and treatments \( F(11, 240) = 6.34; P > 0.001 \) and treatments and side \( F(11, 240) = 13.83; P > 0.001 \). Two-way ANOVA showed that there was a significant difference between treatments \( F(11, 17) = 10.86; P < 0.005 \), side \( F(11, 17) = 7.72; P < 0.05 \) and interaction \( F(11, 17) = 7.58; P < 0.05 \) after bicuculline microinjections into the dIPAG. The same occurred with respect to dIPAG. There was a main effect of stimulation \( F(11, 17) = 12.65; P < 0.005 \), side \( F(11, 17) = 8.35; P < 0.01 \) and interaction between stimulation and side \( F(11, 17) = 4.81; P < 0.05 \). dIPAG stimulation with bicuculline also led to a significant increase in Fos labeling in CnF. There was a significant interaction between stimulation and side of stimulation at this nucleus \( F(11, 17) = 3.74; P < 0.05 \). Stimulation also produced a higher density of Fos-immunoreactive neurons in PMd \( F(11, 15) = 7.84; P < 0.05 \) (Fig. 4F) and VMHdm \( F(11, 17) = 4.67; P < 0.05 \).

4. Discussion
The present results show that the stepwise increase in the electrical stimulation of the dorsal half of PAG elicits the characteristic pattern of behavioral activation with alertness, freezing and escape accompanied by autonomic responses, such as piloerection, exophthalmus, micturition and defecation. This stimulation led to increased Fos expression in the dIPAG and dPMd, confirming previous reports that show Fos immunoreactivity after chemical and electrical stimulation of these regions [41,42]. The increase of Fos expression...
in the dmPAG and dIPAG, but not in the IPAG or vIPAG, is in harmony with the current view of parcellation of PAG into distinct subdivisions with different functional roles [3,15]. In agreement with this, the dIPAG of rats placed into semi-natural settings is preferentially activated when the animal encounters its predator [13], while the IPAG is activated when the animal is engaged in predatory behavior [18]. On the other hand, the vIPAG is preferentially activated when the animal is under a classical fear-conditioning paradigm [17,50]. Therefore, our results are in line with the studies that indicate a role of dIPAG and dmPAG in the production of defensive behaviors during threatening situations related to immediate dangers, as when the rat faces the predator.
Electrical stimulation of the dIPAG at the escape but not at freezing threshold led to a significant increase in Fos expression in the VMHdm. This result is in line with behavioral studies that show that electrical or chemical stimulation of this hypothalamic area elicits escape, but not freezing, behavior [8, 28, 44]. Indeed, it has been proposed that when the animals encounter predators, dIPAG, medial hypothalamus and amygdala act in concert in the production of escape behavior or flight, when flight is not possible [6, 28].

The CnF is thought to be the main relay station for dIPAG- and SC-mediated defensive responses and route to the medulla oblongata and spinal cord [20, 30]. These defense-specific projections course through the midbrain tegmentum, without crossing the midline, to the ipsilateral CnF. A second pathway from the SC to the CnF, crossing the midline, also exists, but it is related to orientation and not to defensive responses [20]. Accordingly, our results showed that only the ipsilateral CnF presented significant increases in Fos immunoreactivity after escape-producing stimulation.

Freezing and escape elicited by exposure to a predator leads to a rise in the expression of Fos in the PMd, and is abolished after its lesion [12]. The PMd is one of the main sources of afferent to the dIPAG [14], and is thought to be a key structure in the mediation of innate fear responses [11]. Our results showed that escape-eliciting stimulation enhanced Fos expression in the PMd, bilaterally. It has been suggested that the route from dIPAG to PMd could involve the anterior hypothalamus and VMHdm [11].

Another important source of information to the PAG is the CeA [39]. But unlike the PMd, the CeA targets primarily the vIPAG, and seems to be involved in the production of conditioned freezing [16]. In the present study there was no significant change in Fos expression in these nuclei following dIPAG electrical stimulation at freezing and escape thresholds. Therefore, the present findings support the notion that this CeA-vIPAG circuit is not involved in the expression of dIPAG-mediated defensive responses. In contrast with this, using the same technique employed in the present work, it was found that Fos expression increases in the CeA after stimulation of the IC [34]. Therefore, the fact that in the present study Fos increases in neither CeA nor IC were found can mean that different neural networks are activated by electrical stimulation of dIPAG and IC. Indeed, it has been noted that the neural organization of fear-like behaviors in mesencephalic structures is dependent on multiple factors, including the kind of aversive sensory stimuli that trigger the defensive behavioral repertoire, whether visual, acoustic or tactile [9, 48].

The hypothesis that freezing and escape responses activate different sets of brain structures was confirmed by the present results. Escape-provoking stimulation caused increased Fos expression in the VMHdm and in the CnF, as well as in the dmPAG and dIPAG. Conversely, freezing-provoking stimulation led to a significant increase of Fos expression only in the dmPAG, while marginal increases were observed in some other structures (PMd, dIPAG and vIPAG). It was proposed [48] that there are at least two paths for PAG-mediated defensive responses, one involving VMHdm–PMd–dIPAG–CnF and mediating responses to immediate danger, and a second one comprising CeA–vIPAG, more related to cue-elicited responses, either learned or innate. In this context, freezing and escape behaviors were produced by dIPAG stimulation in rats bearing vIPAG lesions [49]. Thus, vIPAG is not critical for the generation and elaboration of the defensive responses induced by dIPAG stimulation. This result supports the notion that dissociated neural circuits subserve different kinds of defensive behaviors. In agreement with this, increases in Fos expression in vIPAG are much more pronounced after conditioned fear [17] than after exposure to a predator [13]. On the contrary, exposure to a predator led to a clear predominance of dIPAG activation [13]. In line with these reports in the present study c-fos expression more in the dorsal than in the ventral portions of the PAG, suggesting a predominant role of the former regions in the production of defensive responses to immediate or proximal danger. Overall, our results suggest that vIPAG could have a limited role in the mediation of dIPAG-provoked freezing, but not escape, while VMHdm and CnF have a predominant role in the mediation of escape, but not of freezing behavior. From the present results it can be suggested that PMd is implicated in the generation and elaboration of escape responses and also in the freezing induced by dIPAG stimulation.

It is well established that cortical inputs to the PAG exert a control on the neural operations of this midbrain structure [3, 27, 45, 46]. In the present study no significant change in Fos expression was detected in the cortical structures studied herein (Cg, PrL) in the mediation of freezing and escape responses elicited by dIPAG stimulation. However, a significant increase in Fos expression was reported at the Cg level after microinjection of nitric oxide donors into the dIPAG, which also causes defensive responses [21]. These apparently conflicting results could be due to differences in the techniques employed for stimulating the dIPAG, namely electrical versus chemical stimulation. Anyway, a possibility that emerges from the present results is that cortical structures have a one-way control over the neural substrates of aversion in the dorsal PAG. Indeed, to our knowledge, ascending projections to the cortical regions from the dorsal columns of the PAG have not been clearly characterized so far [3, 27, 48]. In agreement with this view, it has been reported that the onset of an unexpected panic attack is related to an increase of the regional cerebral blood flow in the hypothalamus, but not in the medial prefrontal cortex [29], which suggests that the activation of the hypothalamic-PAG axis is not necessarily related to prefrontal functioning. The activation of this axis, without the participation of cortical structures, could possibly be a source for panic disorder [45]. Interestingly, increased extracellular levels of dopamine in the prefrontal cortex, which has been considered as a biological marker for stressful
conditions, has been observed following aversive stimulation of the IC, but not following similar stimulation at dorsal PAG [19].

It cannot ruled out that the lack of significant change in Fos expression in certain regions could be due to limitations of the technique employed, as negative results of Fos immunoactivity cannot guarantee that a given brain region is not involved in the generation of a response under study [24]. Moreover, a weak effect could have been masked by the fact that even our sham-stimulated animals exhibited an appreciable number of Fos-immunoreactive cells in some regions. Besides, electrical stimulation has the disadvantage of exciting not only perikarya but also axons and fibers of passage. Rats injected with bicuculline exhibited freezing interspersed with escape behavior. Bicuculline microinjections into the dIPAG confirm the overall pattern of results obtained with electrical stimulation. Additionally, this combined behavioral reaction causes some distinctive labeling such as the vPAG, locus coeruleus and CeA. This pattern of effects, which is different from that obtained with dIPAG electrical stimulation, is probably related to the type of responses obtained with each procedure. Whereas electrical stimulation procedure can be switched off as soon as the response is obtained, the defense reaction elicited by microinjections of bicuculline into the dIPAG lasts as long as the drug acts so that freezing or escape alone can not be elicited by this procedure. Therefore, while dIPAG injections of bicuculline cause a full-blown reaction associated with a widespread activation of the neural substrates of aversion, the ease of eliciting distinctive responses of freezing and escape by electrical stimulation of the dIPAG allows a more specific pattern of c-fos expression associated with freezing or escape responses. This assumption is corroborated by the widespread activation of brainstem structures involved in the organization of fear following microinjections of bicuculline into the dIPAG.

In view of these results we have now undertaken the task to examine the effects of excitatory amino acid compounds including compounds that stimulate only AMPA/kainate receptors and produce freezing and others that stimulate NMDA receptors and produce escape behavior. Even considering the present methodological limitations, taking into account our results we suggest that VMHdm, PMd and the CeA are part of a network activated during dIPAG-mediated defensive responses and that dIPAG and PMd mediates a particular kind of freezing that seems not to be shared by the neural substrates of the vPAG and central nucleus of amygdala.

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