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Effects of ovine CRF injections into the dorsomedial, dorsolateral and lateral columns of the periaqueductal gray: A functional role for the dorsomedial column

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Abstract

Corticotropin-releasing factor (CRF) and its receptor subtypes have been implicated in the regulation of endocrine, behavioral and autonomic responses to stress, fear and anxiety. Ovine CRF (oCRF) is a nonspecific CRF receptor agonist that produces anxiogenic-like effects when injected locally into the dorsal aspects of the periaqueductal gray (PAG). This structure is subdivided into four distinct longitudinal columns but their exact functional role is not fully understood. The purpose of the present study was to characterize the effects of oCRF (0.25, 0.5 and 1 μg/0.2 μL) injections into the dorsomedial (dmPAG), dorsolateral (dlPAG) and lateral (lPAG) columns of the PAG using an analysis of the exploratory behavior of rats in the elevated plus-maze (EPM) test. The results showed that microinjections of oCRF intra-dmPAG reduced entries and time spent in the open arms and decreased end-arm exploration and head-dipping. In contrast, oCRF intra-dlPAG or lPAG did not affect the exploratory behavior of the animals in the EPM. These findings point to a columnar specificity for the oCRF effects in the PAG, that is, it increased spatial avoidance measures of the EPM test only in the dmPAG. The proaversive effects of oCRF in the dmPAG gain further relevance when combined with previous immunohistochemical studies showing that CRF-containing projections from the periventricular hypothalamic system arch dorsomedially to the PAG, which could function as an important relay station in the midbrain tectum for avoidance behaviors.

Keywords: Anxiety; Fear; oCRF; Elevated plus-maze; Dorsomedial column of the PAG

Introduction

Many reports indicate that the dorsal periaqueductal gray (dPAG) is one of the main structures involved in the integration of defensive behavior in the brain (Blanchard et al., 1981, 1993; Behbehani, 1995; Brandão et al., 1999). Indeed, its electrical or chemical stimulation produces several behavioral and somatic responses characteristic of high fear states, similar to that observed in animals confronted by predators or dangerous environmental cues (for reviews see Brandão et al., 1999, 2005). A brain aversion system essentially composed of the amygdala, medial hypothalamus and dPAG seems to be responsible for the organization of fear and anxiety-like behaviors (Graeff et al., 1997, Graeff, 2004; Blanchard et al., 2005; Brandão et al., 1999, 2005). The amygdala and the medial hypothalamus act downstream, through descending projections to brainstem regions, particularly the PAG (Canteras et al., 1997; Canteras and Goto, 1999; Risold and Swanson, 1997). The superior and inferior colliculi have also been proposed to integrate this system (Brandão et al., 1988, 1999, 2005). Thus, the midbrain tectum has been proposed as part of the neural substrates responsible for the expression of defensive behaviors.

After a long series of studies carried out since the beginning of the 80s, it is well established that a variety of neurotransmitters mediate the defense-related behavior in the brain aversion system, that include γ-amino-butyric-acid (GABA; Brandão et al., 1982, 1988; Melo et al., 1992; Coimbra and Brandão, 1993), serotonin (Brandão et al., 1993; Melo and Brandão, 1995; Castilho and Brandão, 2001; Graeff, 2004; Coimbra et al.,

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2006), excitatory amino acids (Cardoso et al., 1994; Nobre et al., 2004), opioids (Jenck et al., 1986; Bagri et al., 1992; Osaki et al., 2003) and neuropeptides such as cholecystokinin (Ferreira-Netto and Guimarães, 2004) and corticotropin-releasing factor (Martins et al., 1997, 2000; Carvalho-Netto et al., 2007).

Nowadays, great attention has been paid to the corticotropin-releasing factor (CRF) that plays a major role in coordinating the endocrine, autonomic and behavioral responses to stress and anxiety through actions in the periphery and the brain (Dunn and Berridge, 1990; Fisher, 1993; Koob et al., 1993; Arborelius et al., 1999). In the hypothalamic–pituitary–adrenal (HPA) axis, in response to stress, CRF is released from the median eminence of the hypothalamus, which activates hipophyseal corticotrophin (ACTH) secretion. The increase of ACTH into the bloodstream consequently acts at the adrenal cortex to facilitate the release of glucocorticoids such as corticosterone in rodents (Vale et al., 1981; Rivier et al., 2003; Risbrough and Stein, 2006). Studies have shown an increase in the plasma corticosterone triggered either by innate or conditioned fear stimuli (File et al., 1994; Rodgers et al., 1999, Mikics et al., 2005, Albrechet-Souza et al., 2007).

In addition to CRF endocrine effects, there is a widespread distribution of CRF-containing neurons and its receptor subtypes, CRF-1 and CRF-2, in the central nervous system (CNS) and the direct effects of CRF on autonomic function and behavior suggest that endogenous CRF also functions as a neurotransmitter or neurohormone (Swanson et al., 1983; Merchenthaler, 1984; De Souza et al., 1985; De Souza, 1987, 1995; Arborelius et al., 1999). Because CRF was shown to generate neuroendocrine, autonomic and behavioral responses, it was hypothesized that CRF contributes to the development of stress and affective disorders by overactivating its receptors in neocortex, amygdalar complex, hippocampus and brainstem nuclei (Gray and Magnuson, 1992; Hauger et al., 2003). Hence, CRF is well situated to modulate circuits involved in cognition, defensive behavior and emotion.

Experimental evidence has demonstrated that intracerebroventricular (i.c.v.) administration of CRF increases anxiety related to behaviors in rodents (Momose et al., 1999; Yang et al., 2006). In order to clarify the neural circuitry underlying anxiogenic effects of CRF, several studies have investigated the direct infusion of CRF into discrete brain structures (for review see, Griebel, 1999). Local injections of CRF into the dPAG have also been found to produce an anxiogenic effect in the EPM (Martins et al., 1997). Intra-PAG injections of the CRF antagonist α-helical-CRF blocked the anxiogenic effect of 4 h of restraint on EPM performance (Martins et al., 2000). Recently, intra-PAG injections of oCRF have been shown to enhance avoidance behaviors in the mouse defense test battery (MDTB) and in the rat exposure test (RET) (Carvalho-Netto et al., 2007). These results support a role for CRF-receptor-mediated PAG excitation (Bowers et al., 2003).

Anatomical and histochemical analyses of the PAG have led to its parcellation into four columns: the dorsolateral (dlPAG), dorsomedial (dmPAG), lateral (lPAG) and ventrolateral (vlPAG) (Carrive, 1991, 1993; Bandler and Keay, 1996). Electrical and chemical stimulation of the dlPAG, the superior colliculus and the inferior colliculus of the rat induces aversive behaviors such as freezing, arousal and escape (Bandler and Carrive, 1988; Bandler et al., 1985; Brandão et al., 1990; Brandão et al., 1993), while lPAG stimulation elicits defecation and flight (Bandler and Shipley, 1994; Schenberg et al., 2005). These behaviors are accompanied by changes in autonomic measures such as heart rate, mean arterial blood pressure and respiration (Carrive, 1991; Hayward et al., 2003) and are followed by analgesia (Fanselow, 1991; Coimbra et al., 1992; Coimbra and Brandão, 1997).

The purpose of the present study was to characterize the effects of oCRF (0.25, 0.5 and 1 μg/0.2 μL) injections into the dmPAG, dlPAG and lPAG using a thorough ethological analysis of the exploratory behavior of rats in the elevated plus-maze test (EPM), including standard and novel behavioral categories.

Materials and methods

Animals

Ninety-three male Wistar rats, weighing 230–250 g, from the animal house of the *Campus* of Ribeirão Preto-University of São Paulo, were used. These animals were transported to a room adjacent to the test laboratory 72 h before the test. They were housed in groups of five per cage under a 12:12 dark/light cycle (lights on at 07:00 h) at 23±1 °C, and given free access to food and water. The experiments reported in this article were performed in compliance with the recommendations of the SBNeC (Brazilian Society for Neuroscience and Behavior), which are based on the US National Institutes of Health Guide for Care and Use of Laboratory Animals.

Surgery

The animals were anesthetized with tribromoethanol (250 mg/kg, i.p.) and fixed in a stereotaxic frame (David Kopf, USA). A brain cannula was implanted in the midbrain, aimed at the PAG columns. The stainless steel guide-cannula (11 mm length, o.d. 0.6 mm, i.d. 0.4 mm) was introduced at an angle of 16° using the following coordinates with the lambda serving as the reference for each plane: dmPAG—antero-posterior, 0 mm; medio-lateral, 1.4 mm; and dorso-ventral, 4.1 mm; dlPAG—antero-posterior, 0 mm; medio-lateral, 1.9 mm; and dorso-ventral, 4.1 mm; lPAG—antero-posterior, 0 mm; medio-lateral, 1.9 mm; and dorso-ventral, 4.5 mm (Paxinos and Watson, 2005). The upper incisor bar was set at 2.5 mm below the interaural line so that the skull was horizontal between bregma and lambda. The cannula was fixed to the skull by means of acrylic resin and two stainless steel screws. At the end of the surgery, each guide-cannula was sealed with a stainless steel wire to prevent obstruction. After the surgery, the rats were placed again in their home cages in group of five as before the surgery. The experiments started after a 1-week postoperative delay.

Microinjections

The animals were put in a plastic box and a thin dental needle (o.d. 0.3 mm) could be introduced through the guide-cannula until its lower end was 1 mm below the tip of the cannula. The injection needle was linked to a 5 μL Hamilton syringe by means of polyethylene tubing connected to a microinfusion apparatus (Harvard, USA). A constant volume of 0.2 μL was injected during 30 s. The displacement of an air bubble inside the polyethylene tubing connecting the syringe to the injection needle was used to monitor the microinjections. After the infusions, the microinjection needles were held inside the brain for additional 30 s to allow a complete drug diffusion.

Drugs

Ovine CRF (0.25, 0.5 and 1 μg/0.2 μL, Sigma-Aldrich) was dissolved in saline solution (0.9%). Selection of oCRF dose and the time for testing were based on previous studies (Martins et al., 1997, 2000). Saline injections also

served as control. The injections were done locally into the PAG columns 15 min before the test. The doses used here are in the range of those used in studies reported by other laboratories (Martins et al., 1997, 2000; Litvin et al., 2007; Carvalho-Netto et al., 2007).

Elevated plus-maze testing

The elevated plus-maze (EPM) device was made of wood and consisted of two open arms (50 × 10 cm) and two enclosed arms of the same size, with 50 cm high walls. The maze was configured such that arms of the same type were opposite each other and the apparatus was elevated 50 cm from the floor. A raised edge made of transparent Plexiglas (0.5 cm) on the open arms provided additional grip for the rats (Pellow et al., 1985).

All testing was conducted during the light phase of the LD cycle, between 09:00 and 11:00 h. The apparatus was located inside of a room with a constant noise (50 dB). The animal behaviors was recorded by a video camera (Everfocus, USA) positioned above the maze and the signal was relayed to a monitor in another room via a closed-circuit TV camera. Luminosity at the open arms level was 27 lx. The rats were placed individually in the center of the maze facing a closed arm and allowed 5-min free exploration. Each rat was tested only once. Videotapes were subsequently scored by an observer using ethological analysis software (Observer) developed by Noldus (Amsterdam, The Netherlands).

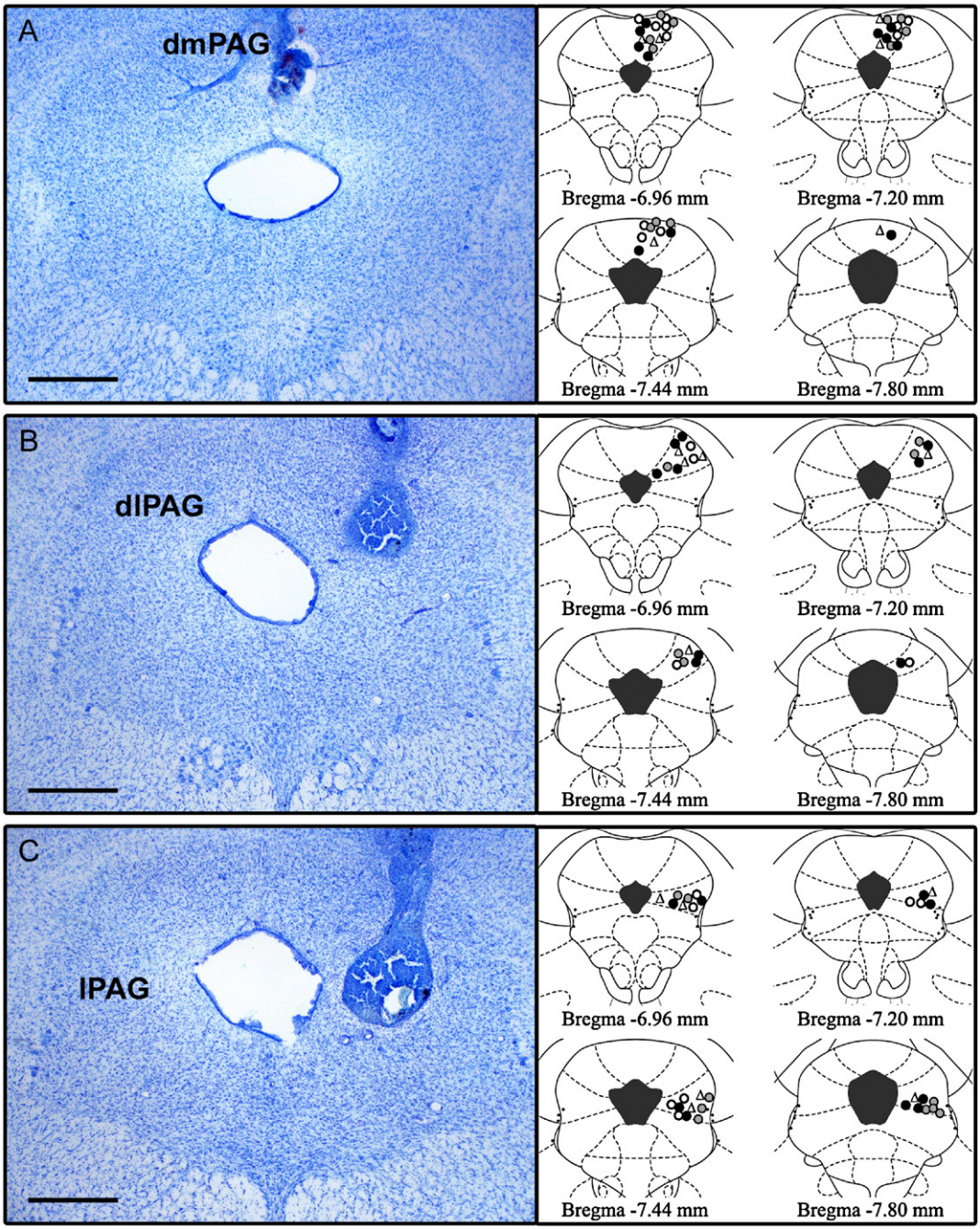

Fig. 1. Representative photomicrographs of microinjections (A) into the dorsomedial PAG, (B) into the dorsolateral PAG and (C) lateral PAG. Scale bar represents 500 μm. The injection sites into each PAG columns are represented beside its respective photomicrographs. The symbol (△) indicates animals injected with saline, (○) oCRF 0.25 μg, (◍) oCRF 0.5 μg and (●) oCRF 1 μg/0.2 μL.

The performance of each animal in the maze was analyzed taking the standard measurements recorded in each section of the maze into account (closed and open arms), which comprised the frequency of open and closed arm entries (an arm entry or exit being defined as all four paws into or out an arm, respectively), total arm entries and the amount of time spent by the animals in each section of the maze. In addition, the frequencies of the following "novel ethological categories" were measured: (1) head-dipping: dipping of the head below the level of the maze floor, (2) end-arm exploration: the number of times the rat reached the end of an open arm, (3) stretched-attend postures: when the animal stretches to its full length with the forepaws (keeping the hind paws in the same place and turns back to the anterior position, (4) flat-back approach: locomotion when the animal stretches to its full length and cautiously moves forward; (5) scanning: horizontal head movements in any direction, including sniffing of maze floor and walls; and (6) peeping out: stretching the head/shoulders from the closed arms to the central platform. Most of these categories were defined following works with rats and mice (Cruz et al., 1994; Blanchard et al., 1993; Rodgers and Johnson, 1995; Anseloni and Brandão, 1997).

Histology

Upon completion of the experiments, the animals were given a lethal dose of chloral hydrate (500 mg/kg, i.p.) and perfused transcardially with 0.9% saline followed by buffered 10% formalin. Brains were removed from the skulls and maintained in formalin solution for 2 h and cryoprotected in sucrose 30% for 3 days. Serial 60 μm brain coronal sections were cut using a cryostat (−19 °C) and mounted on gelatin-coated slides and stained with cresyl violet (5%) (Sigma-Aldrich) in order to localize the positions of the microinjection sites according to the atlas of Paxinos and Watson (2005). The microinjection sites were evaluated by microscopic examination.

Statistical analysis

The behavioral data are expressed as mean ± SEM and were analyzed by a one-way analysis of variance (ANOVA) with drug treatment as the main factor.

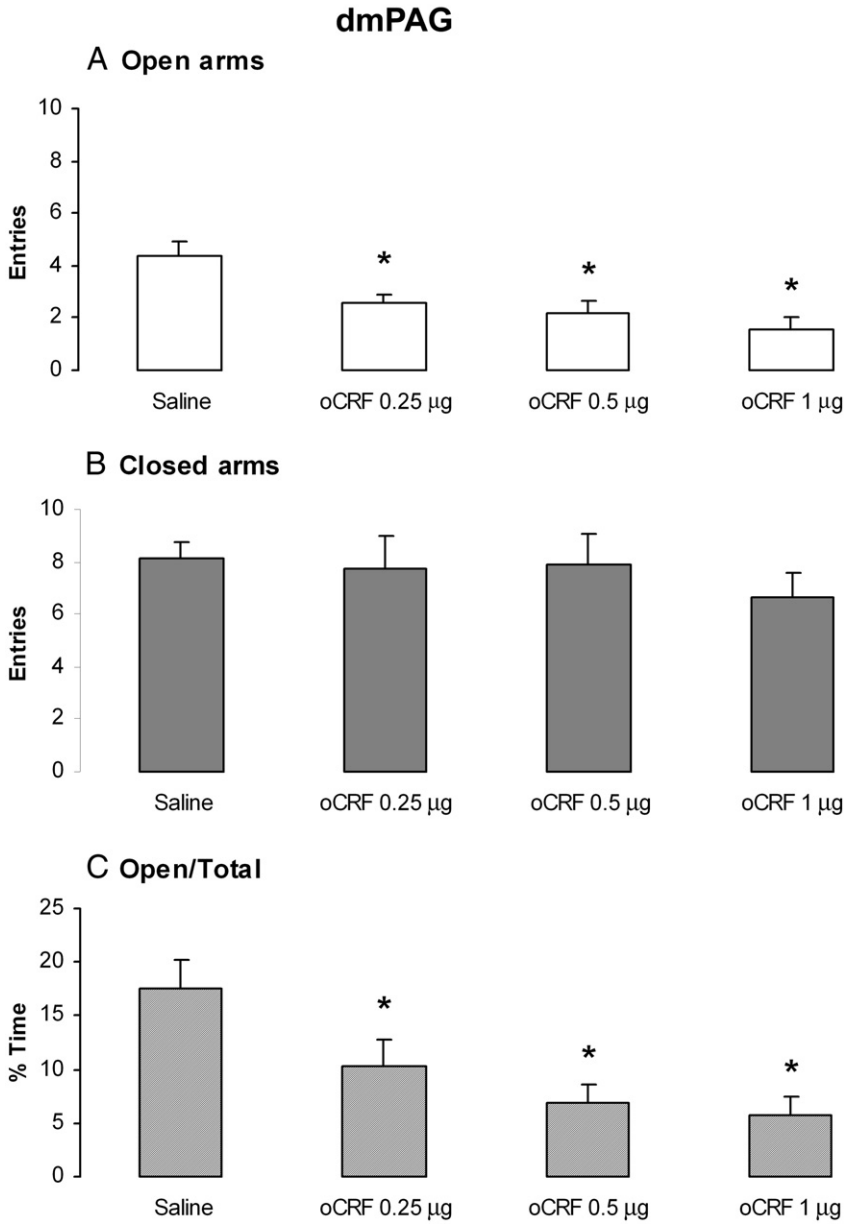

Fig. 2. Effects of oCRF intra-dmPAG on the exploratory behavior of rats submitted to the EPM. Each animal was injected 15 min before test either with saline (n = 17) or oCRF 0.25, 0.5 or 1 μg/0.2 μL (n = 11 in each group). (A) Number of entries in the open arms of the maze. (B) Number of entries in the closed arms of the maze. (C) % of time spent into the open arms in relation to total. The values are mean ± SEM. *Different from the saline group (one-way ANOVA followed by Newman–Keuls post hoc comparisons).

Newman–Keuls post hoc comparisons were carried out if significant overall *F*-values were obtained. *p* values lower than 0.05 were considered significant.

Results

Microinjections sites

All sites of chemical stimulation of the PAG were situated in the dmPAG, dlPAG or lPAG. The great majority of sites of injections were concentrated into the intermediate region of the PAG (−6.96 to −7.44 mm in relation to bregma) and only few injections fell into more caudal regions of the PAG (−7.80 to 8.52 mm). The documented material was first examined by one of us (KGB) and the reported sites of injections were confirmed later (MLB). Representative photomicrographs of microinjections and sites depicted on diagrams modified of the Paxinos and Watson (2005) atlas into each PAG columns are shown in Fig. 1.

Behavioral effects

To examine whether the effects of saline injections were related to the sites of injections into the PAG columns, a one-way ANOVA was performed taking dPAG columns as the main factor. This analysis did not show any significant effects for microinjections sites upon the frequency of open arms entries [$F_{(2,14)}=0.67$; $p>0.05$], closed arms entries [$F_{(2,14)}=1.42$; $p>0.05$] and the percentage of open arm entries [$F_{(2,14)}=0.40$; $p>0.05$] and time spent [$F_{(2,14)}=0.33$; $p>0.05$] on the open arms of the maze. Due to this lack of significant effects, these data were pooled together as constituted the control group for the whole study.

ANOVA indicated a significant effect of oCRF injections into the dmPAG upon the frequency of open arms entries [$F_{(3,46)}=6.24$; $p<0.01$], percentage of open arm entries [$F_{(3,46)}=4.49$; $p<0.01$] and time spent [$F_{(3,46)}=5.50$; $p<0.01$] on the open arms of the maze (Fig. 2). Post hoc analysis showed that all doses of oCRF (0.25, 0.5 and 1 μg/0.2 μL) reduced these parameters.

The novel ethological categories confirmed the anxiogenic effect of oCRF into de dmPAG. There was a decrease of the end-arm exploration [$F_{(3,45)}=4.50$; $p<0.01$] and "'head-dipping [$F_{(3,45)}=7.95$; $p<0.01$] after microinjections of all doses of oCRF. Besides, there was an increase of stretched attend postures [$F_{(3,45)}=11.68$; $p<0.01$] and flat-back approach [$F_{(3,45)}=5.51$; $p<0.01$]. Newman–Keuls post hoc analysis ($p<0.05$) revealed that these effects were due to all doses of oCRF, with the exception of flat-back approach, which was increased only at the dose 0.25 and 0.5 μg/0.2 μL of oCRF. The remaining behaviors were not affected by oCRF; peeping-out [$F_{(3,45)}=0.83$; $p>0.05$] and scanning /[$F_{(3,45)}=1.65$; $p>0.05$]. All these effects are depicted in Fig. 3.

One-way ANOVA did not reveal any significant effects of the oCRF injections into the dlPAG upon the frequency of open arms entries [$F_{(3,32)}=1.09$; $p>0.05$], percentage of open arm entries [$F_{(3,32)}=0.60$; $p>0.05$] and time spent [$F_{(3,32)}=1.81$; $p>0.05$] on the open arms of the maze (Fig. 4).

One-way ANOVA did not reveal any significant effects of the oCRF injections into the lPAG upon the percentage of open arm entries [$F_{(3,38)}=2.84$; $p>0.05$] and time spent [$F_{(3,38)}=2.65$; $p>0.05$] on the open arms of the maze. There was a trend towards significance for the treatments with oCRF injections into the lPAG upon the frequency of open arms entries [$F_{(3,38)}=2.94$; $p=0.05$] but the post hoc analysis did not reveal any significant effects (Fig. 5). The novel ethological measures were not significantly changed by injections of oCRF into the dlPAG or lPAG (Table 1).

The lack of effects of oCRF injections into the dlPAG or lPAG makes it unlikely that the volume of the injection into one of these columns had significantly diffused to the dmPAG, where the anxiogenic effects were demonstrated. Besides, we have previously demonstrated that a volume of 0.2 μL has a

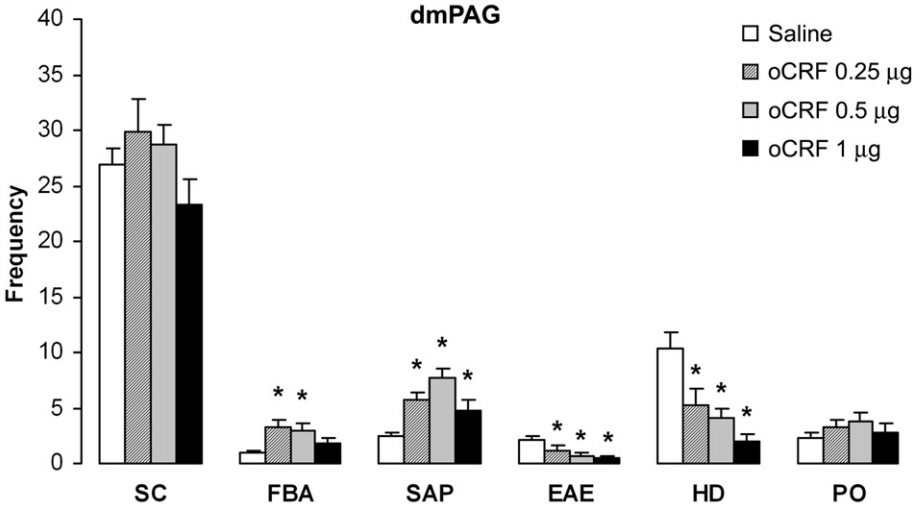

Fig. 3. Effects of oCRF intra-dmPAG on the novel ethological categories measured in rats submitted to the test on the EPM. Each animal was injected 15 min before test either with saline ($n=17$) or oCRF 0.25, 0.5 or 1 ± μg/0.2 μL ($n=11$ in each group). SC: scanning; FBA: flat-back approach; SAP: stretched-attend postures; EAE: end-arm exploration; HD: head-dipping; PO: peeping-out. The values are mean±SEM. *Different from the saline group (one-way ANOVA followed by Newman–Keuls post hoc comparisons).

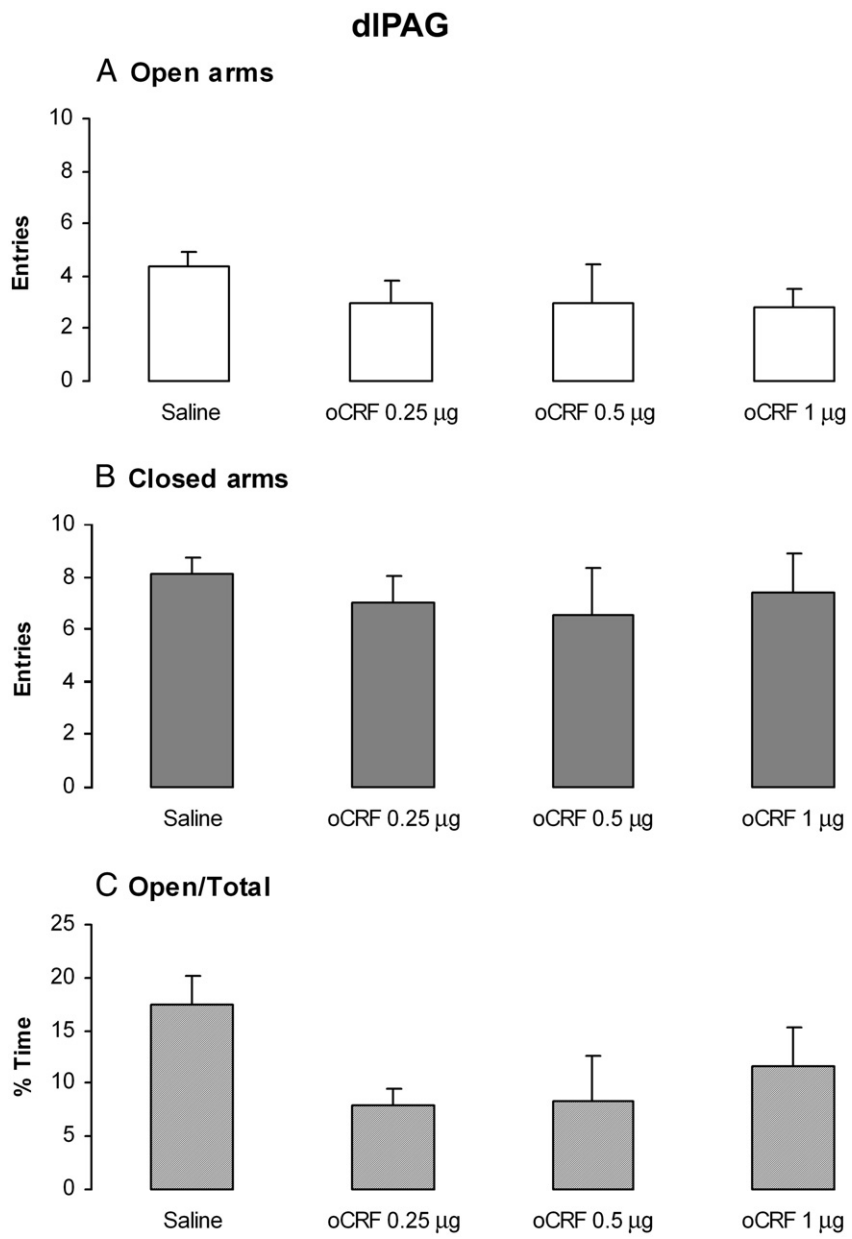

Fig. 4. Effects of oCRF intra-dlPAG on the exploratory behavior of rats submitted to the EPM. Each animal was injected 15 min before test either with saline ($n=17$) or oCRF 0.25, 0.5 or 1 µg/0.2 µL ($n=5$, 5 and 9, respectively). (A) Number of entries in the open arms of the maze. (B) Number of entries in the closed arms of the maze. (C) % of time spent into the open arms in relation to total. The values are mean ± SEM.

diameter of diffusion circumscribed to the site of injection (Ferreira-Netto et al., 2007).

Discussion

Morphologic studies of the PAG have led to its parcellation into four columns: dlPAG, dmPAG, lPAG and vlPAG (Carrive and Bandler, 1991; Carrive, 1993; Bandler and Keay, 1996). Electrical and chemical stimulation of the dlPAG induces defensive behaviors such as arousal, freezing and escape (Bandler et al., 1985; Bandler and Carrive, 1988; Brandão, et al., 1985, 1990), of the lPAG elicits defecation and flight (Bandler and Shipley, 1994; Schenberg et al., 2005) and of the vlPAG causes quiescence (Morgan and Carrive, 2001). It has been suggested that conditioned fear stimuli activate the neural substrates in the vlPAG responsible for the production of freezing behavior and antinociception (De Luca et al., 2006). However, the functional role of the dmPAG has not been clearly dissociated from the dlPAG. For this reason, this study specifically evaluates the behavioral effects of the oCRF injections into the dmPAG, dlPAG and lPAG of rats. It is worthwhile highlighting that in contrast with precedent studies focusing on the standard measures of the EPM only the present experiment provides an entire ethological analysis including the so-called novel categories of the exploratory behavior of rats in the EPM thus providing detailed analysis of the subsets of the

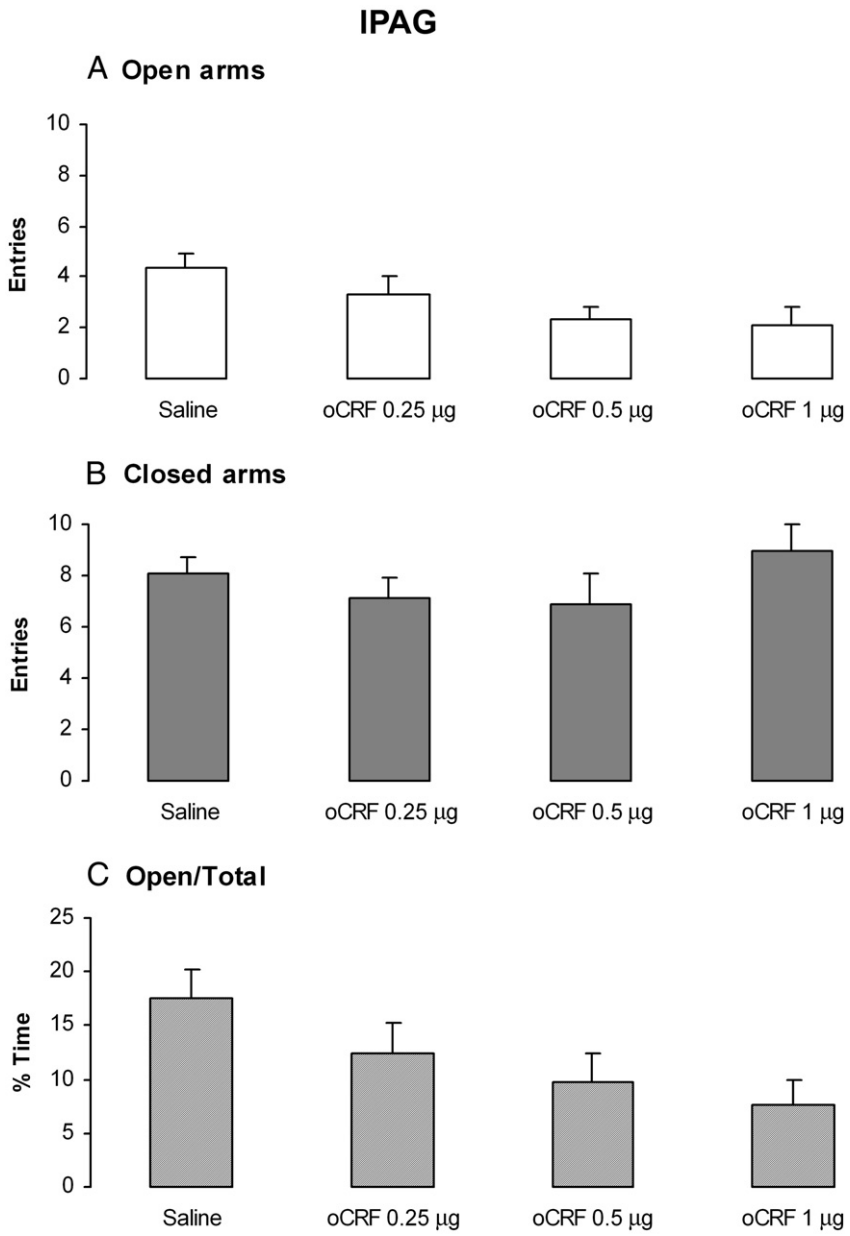

Fig. 5. Effects of oCRF intra-lPAG on the exploratory behavior of rats submitted to the EPM. Each animal was injected 15 min before test either with saline ($n=17$) or oCRF 0.25, 0.5 or 1 µg/0.2 µL ($n=7$, 9 and 9, respectively). (A) Number of entries in the open arms of the maze. (B) Number of entries in the closed arms of the maze. (C) % of time spent into the open arms in relation to total. The values are mean±SEM.

test, evaluating defensive coping strategies, such as direct exploration, risk assessment and decision making.

The measures of behavioral categories in the EPM reflect the conflict resulting from the natural tendency of the animals to explore new environments and avoid dangerous situations. The strong association between activation of the HPA axis and risk assessment in the EPM may be related to information-processing in novel and potentially dangerous environments (Anseloni and Brandão, 1997; Rodgers et al., 1999). Recent evidence has shown that in hormonal terms the exposure of rats to the EPM caused a clear increase in the plasma corticosterone level which was reversed by pretreatment with midazolam (Albrechet-Souza et al., 2007). High level of this hormone in the plasma is a physiological and adaptive response of the animal facing with stressful events (File et al., 1994; Rodgers et al., 1999, Albrechet-Souza et al., 2007). The CRF system also exerts direct action on structures of the limbic system regulating the emotional behavior. Indeed, several studies with rodents have reported that CRF agonist and antagonist injections into the dPAG cause anxiogenic and anxiolytic effects, respectively (Martins et al., 1997, 2000; Carvalho-Netto et al., 2007). Furthermore, it has also been shown that a prolonged depolarization of dPAG neurons could underlie the CRF effects in this region (Bowers et al., 2003). In this line, the present findings showed that oCRF injections into the dmPAG, but not into the dlPAG or lPAG, promote a clear anxiogenic-like effect. Indeed, injections of oCRF causing a decrease on number of open arm entries and percent time spent in the open arms as well as

Table 1
Effects of oCRF intra-dl-PAG or into the lPAG on the novel ethological categories measured in rats submitted to the EPM

Behavior	Saline	dlPAG				
		oCRF 0.25 μg	oCRF 0.5 μg	oCRF 1 μg	$F_{(3,32)}$	p
SC	23.94±1.57	20.80±1.24	26.20±1.25	24.56±2.60	0.69	0.57
FBA	1.77±0.33	3.00±0.84	2.60±0.60	2.67±0.41	1.54	0.23
SAP	5.06±0.98	6.60±0.93	6.20±1.16	5.11±1.01	0.37	0.78
EAE	2.12±0.46	0.80±0.37	1.00±0.55	0.78±0.28	2.18	0.11
HD	11.82±2.06	9.00±0.84	8.60±1.08	7.22±0.68	1.23	0.32
PO	2.18±0.48	3.60±0.68	2.60±0.67	2.70±0.88	0.74	0.54
Behavior	Saline	lPAG				
		oCRF 0.25 μg	oCRF 0.5 μg	oCRF 1 μg	$F_{(3,36)}$	p
SC	23.94±1.57	26.57±2.98	25.33±2.44	22.67±2.65	0.46	0.71
FBA	1.77±0.33	3.15±0.40	2.78±0.54	2.21±0.49	2.02	0.13
SAP	5.06±0.98	6.00±0.69	6.78±0.55	5.33±0.930.67	0.58	
EAE	2.12±0.46	1.14±0.41	1.22±0.32	1.00±0.241.71	0.18	
HD	11.82±2.06	13.13±1.89	11.56±1.18	8.89±1.30	0.68	0.57
PO	2.18±0.48	2.15±0.59	2.90±0.68	2.67±0.530.47	0.71	

The values are mean±SEM. SC: scanning; FBA: flat-back approach; SAP: stretched-attend postures; EAE: end-arm exploration; HD: head-dipping; PO: peeping-out. One-way ANOVA did not show significant effects on any behavioral item analyzed in this study.

decreased head-dipping and end-arm exploration, behavioral categories associated with direct exploration, indicating a decreased tendency to actively explore the potentially dangerous areas.

We also found an increase of flat-back approach and stretched-attend postures, behavioral categories related to risk assessment (Blanchard et al., 1993; Rodgers et al., 1999; Albrechet-Souza et al., 2007). The present data are in line with recent report showing that i.c.v. infusion of oCRF robustly increased risk assessment probably through the activation of structures of the brain defensive system such as the PAG of the animals submitted to MDTB (Yang et al., 2006). On the other hand, the effects observed in this work are partially in contrast with similar study in mice in which oCRF injected intra-dPAG selectively increased measures of avoidance, decreasing risk assessment (as measured by stretch attend postures) in the MDTB and the RET (Carvalho-Netto et al., 2007). The latter study further implicated CRF-1 as a primary receptor mediating the avoidance behaviors (Litvin et al., 2007). These effects were reported to be due to the excitatory effect of CRF in the dorsal aspects of the PAG. It is worth noting that in these studies the great majority of the injection sites were inside the dmPAG.

The observed specificity of the oCRF in producing open arm avoidance when injected in the dmPAG indicates that this neuropeptide has a selective action in this particular column of the dPAG in which it produces as main effect an increased avoidance response from proximal dangerous stimuli. In the latter case, the EPM test is probably one of the best animal models of anxiety to detect this effect given that the animals rapidly avoid the height and open spaces of the open arms. This proposed functional role of the dmPAG in the organization of avoidance behavior finds support in a recent study from this laboratory, in which the dmPAG was the only mesencephalic region with significant Fos immunoreactivity in rats submitted to a place avoidance paradigm (Zanoveli et al., 2007). Obviously, an involvement of CRF in other components of the defensive behaviors associated with the other PAG columns, such as freezing, escape and tonic immobility, which are not amenable to be assessed by the EPM test, is still open to investigation. In this line of research, other immunohistochemistry studies using electrical stimulation of the dorsal aspects of the PAG at freezing and escape thresholds raised the hypothesis that freezing and escape responses activate different sets of brain structures (Vianna et al., 2003). Besides, reduction of GABA transmission in the inferior colliculus or the dPAG with injections of the GABA synthesis blocker semicarbazide or the GABA receptor antagonist bicuculline caused freezing and escape, respectively, which were also accompanied by a distinct pattern of Fos distribution in the brain. Interestingly, these latter studies also reported that escape-provoking stimulation caused an increase in Fos expression in a large number of limbic structures whereas freezing elicited by semicarbazide caused a significant labeling of the dmPAG and laterodorsal nucleus of the thalamus (Borelli et al., 2005, 2006). It is reasonable to assume that the animal is also engaged in the acquisition of aversive information during the freezing behavior induced by semicarbazide, through a pathway comprised of the dmPAG, laterodorsal nucleus of the thalamus and higher brain structures. In harmony with this, immunohistochemical mapping of the brain of rats that passed by a conditioned place aversion paradigm showed significant Fos labeling in the dmPAG, laterodorsal nucleus of the thalamus and basolateral amygdala implicating this structures in place aversion behavior (Zanoveli et al., 2007).

Corroborating the proposal discussed above, recent study showed that rats exposed to cats displayed a robust freezing response along with a high density of Fos immunoreactive cells in the dorsomedial column of the PAG, whereas other PAG columns contained relatively sparse Fos staining (Comoli et al., 2003). In the same way, the contextual fear conditioning also significantly increased the Fos expression in the dmPAG (Zienowicz et al., 2007).

A question that arises from the present findings is where could be the origin of these CRF neurons in the dmPAG. It has been found that CRF-stained cells centered in the parvocellular division of the paraventricular nucleus of the hypothalamus projects massively to the median eminence and to other discrete fiber systems that are not directly related to neuroendocrine mechanisms, e.g. the periventricular system that innervates the central gray, *locus coeruleus* and parabrachial nucleus (Swanson et al., 1983). In the same way, it has been found that CRF immunoreactive terminals in the dPAG are originated from the amygdala, bed nucleus of the stria terminalis and hypothalamus (Gray and Magnuson, 1992). All these regions are somehow related functionally or anatomically with the brain aversion system. In view of this evidence, CRF-containing neurons may well link the hypothalamic regions involved with the generation and elaboration of defensive behavior with the dmPAG, the output center in the brainstem responsible for the expression of freezing and avoidance. The dorsal Schultz bundle linking the periventricular hypothalamic regions to the dmPAG could serve as a bridge for an integrated action of the brain aversion system (Gomita et al., 1988). Thus, it makes sense that a given region of the brain aversion system, like the dmPAG, could intermediate the avoidance component of the defense reaction and contribute to the overall pattern of somatomotor and autonomic responses expressed by animals facing natural threats. Recent studies have implicated CRF-1 as a primary receptor mediating avoidance behavior in the MDBT (Carvalho-Netto et al., 2007; Litvin et al., 2007). To go one step further in this line of research, we plan to assess the effects of injections of antagonists of CRF-1 receptors into the dmPAG of rats submitted to the EPM test.

In summary, the present results are in agreement with previous findings indicating that the emotional responses caused by CRF in dmPAG are the result of an excitatory effect of this neurotransmitter in this region (Bowers et al., 2003). Moreover, these findings indicate that the columnar specificity in the modulation of aversive states in the PAG points to the dmPAG as an important relay station in the midbrain tectum to higher brain centers for avoidance-related information.

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