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Regulation of conditioned and unconditioned fear in rats by 5-HT$_{1A}$ receptors in the dorsal periaqueductal gray

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Abstract

Studies on the involvement of 5-HT$_1$-mediated mechanisms in the dorsal periaqueductal gray (dPAG) of animals with past stressful experiences have not been conducted so far. We investigated the role of 5-HT$_1$ receptors in the dPAG of rats previously submitted to contextual fear conditioning. Defensive behaviors induced by activation of the dPAG were assessed by measuring the lowest electric current applied to this structure (threshold) able to produce freezing and escape responses during testing sessions of contextual fear conditioning, in which animals were placed in a context previously paired to footshocks. The 5-HT$_{1A}$ function of the dPAG was evaluated by local injections of 8-OH-DPAT (4 and 8 nmol/0.2 μL) and WAY-100635 (10 nmol/0.2 μL), selective agonist and antagonist of 5-HT$_{1A}$ receptors, respectively. In accordance with previous studies, 8-OH-DPAT increased aversive thresholds (antiaversive effects) but injections of WAY 100635 into the dPAG did not produce significant effects on the aversive thresholds in naive rats. However, the aversive thresholds of animals exhibiting contextual fear remained unchanged with both treatments. Moreover, 8-OH-DPAT and WAY 100635 did not change the dPAG post-stimulation freezing. The present results suggest that the stressful experience of being fear conditioned has an effect on the role of the 5-HT$_{1A}$ receptors in mediating unconditioned fear. Also, the reduction in the regulation of the defensive behaviors by 5-HT$_{1A}$-mediated mechanisms in the dPAG of these animals may underlie the stress precipitated psychopathology associated with the neural substrates of aversion of the dPAG.

Keywords: 5-HT1A receptors; Dorsal periaqueductal gray; 8-OH-DPAT; WAY-100635; Unconditioned fear; Contextual fear conditioning

1. Introduction

A brain aversion system made up of the dorsal PAG (dPAG), dorsomedial hypothalamus and amygdala has been associated with unconditioned fear (Graeff et al., 1986; Graeff 1990, 2004). Panic attacks have been related to the deregulation of the dPAG (Graeff et al., 1986; Graeff 1990, 2004), dorsomedial hypothalamus (Johnson and Shekhar 2006) and bilateral temporal poles (Reiman et al., 1989). The electrical or chemical stimulation of the dPAG causes a characteristic pattern of active defense reaction, with alertness, freezing and escape responses, along with autonomic changes that resemble this anxiety disorder (Graeff et al., 1986; Brandão et al., 2003; Borelli et al., 2004; Graeff 2004). It has also been suggested that another system comprised of the hippocampus, amygdala and ventrolateral periaqueductal gray (vPAG) is related to conditioned fear (Gray and McNaughton 2000). Malfunctioning of this system appears to be associated with generalized anxiety disorder (Gray and McNaughton 2000). It seems that these two aversive systems are not entirely independent and some interaction between them may exist. For example, it has been proposed that anxiety states generated at the amygdala level may inhibit panic attacks elicited by activation of the neural substrates of aversion in the dPAG (Graeff 2004). In line with this notion, a recent study has shown that rats exposed to conditioned fear stimuli

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present a reduction in the unconditioned fear when concomitantly stimulated in the dPAG at the escape threshold (Magierek et al., 2004).

Defensive behaviors are hierarchically organized and different behaviors within this class are provoked by aversive stimuli of different intensities or distances from the predators (Blanchard and Blanchard, 1990; Schenberg et al., 2005; Santos et al., 2005). In this context, it has been shown that there are two types of freezing behavior induced by direct stimulation of the PAG; one bound to the stimulus and another one that appears when this stimulation terminates (Vianna et al., 2001). The first freezing appears as a preparatory response for escape (immediate defensive responses) and the post-stimulation freezing is related to the processing of aversive information that is relayed to higher structures (Borelli et al., 2005a; Ruiz-Martinez et al., 2006). Considering the premise that different anxiety disorders might be related to distinct defensive systems, which in turn might involve specific neural mechanism, we have proposed that the dPAG-evoked freezing is related to panic attacks whereas the post-stimulation freezing may be related to agoraphobia-like responses associated with panic disorder (Oliveira et al., 2007). The 5-HT (5-hydoxytryptamine) system is highly involved in the modulatory systems underlying generalized anxiety disorder and panic attacks. Several studies have been conducted to disclose how the multiple 5-HT receptors modulate the aversive states induced by stimulation of the dPAG (Jenck et al., 1989; Brandão et al., 1991; Graeff 2004). The dPAG is rich in 5-HT immunoreactive nerve terminals from serotonin-containing cell bodies located mainly in the dorsal raphe nucleus (Clements et al., 1985; Beitz et al., 1986; Lovick et al., 2000). One prominent function of serotonin is to regulate aversive states induced by electrical or chemical stimulation of the dPAG (Graeff et al., 1986; Graeff 2004). Electrophysiological studies have found that the dPAG is rich in 5-HT_{1A} and 5-HT_2 receptors subtypes (Brandão et al., 1991; Lovick 1993). It has been shown that the activation of these receptors has an inhibitory effect on the neural substrates of aversion in the dPAG (Graeff et al., 1986; Coimbra and Brandão 1997; Castilho and Brandão 2001). Recently, it has been found that animals with previous aversive experience and injected locally with 5-HT_2 antagonists into the dPAG show an enhanced sensitivity to the electrical stimulation of this structure in comparison with naïve animals, in which intra-dPAG injections of 5-HT_2 antagonists do not change the freezing and escape thresholds (Oliveira et al., 2007). Taking into account that 5-HT_{1A} and 5-HT_2-mediated mechanisms play a cooperative role in the regulation of fear in the dPAG (Nogueira and Graeff 1995; Zanoveli et al., 2003), it is of relevance to know whether the fear generated at the level of the dPAG in animals with previous experience with stressful events is also regulated by 5-HT_{1A} receptors. In this study, we evaluated the involvement of the 5-HT_{1A}-mediated mechanisms of the dPAG of rats submitted to the electrical stimulation of the dPAG at the freezing and escape thresholds before or after contextual fear conditioning (CFC). Conditioning was evaluated in a neutral context or in the presence of the contextual cues previously paired with footshock. The 5-HT_{1A} function was assessed by local injections into the dPAG of 8-OH-DPAT (DP) and WAY 100635 (WAY), selective agonist and antagonist of 5-HT_{1A} receptors, respectively (Mundey et al., 1996; Fornal et al., 1996; Fletcher et al., 1996; Ahlenius et al., 1999; Avanzi and Brandão 2001; Borelli et al., 2005b).

2. Methods

2.1. Animals

Eighty-six male Wistar rats weighing 250–280 g from the animal house of the Campus of Ribeirão Preto of the University of São Paulo were housed in a temperature-controlled (22± 1 °C) room and maintained on a 12-h light/12-h dark cycle (0700–1900 lights on). These animals were maintained in pairs in Plexiglas-walled cages and given free access to food and water throughout the experiment. The experiments were carried out according to the Brazilian Society of Neuroscience and Behavior Guidelines 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, Tujunga, CA). The rapid induction and recovery, adequate surgical plane of anesthesia, and lack of complications make this anesthetic effective and simple to use in rodents (Papaioannou and Fox, 1993). The upper incisor bar was set at 3.3 mm below the interaural line such that the skull was horizontal between bregma and lambda. A chemitrode made of a stainless steel guide cannula (o.d. 0.6 mm, i.d. 0.4 mm) glued to a brain electrode was aimed at the dPAG. The electrode was made of stainless steel wire, 160 μm in diameter, insulated except at the cross-section, and was introduced with a 16° angle and directed towards midline, with lambda serving as the reference for each plane: antero-posterior (AP)=0.0 mm; medio-lateral (ML)=±1.9 mm; and dorso-ventral (DV)=5.1 mm, according to Paxinos and Watson (1997). For all groups the electrode and cannula were fixed to the skull by means of acrylic resin and two stainless steel screws. The electrode wire was 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. At the end of the surgery each guide cannula was sealed with a stainless steel wire to protect it from obstruction.

2.3. Microinjection procedure

The injection needle was a thin dental needle (0.3 mm, o.d.) connected to a 5 μL Hamilton syringe by means of a polyethylene tube. The injection needle was introduced through the guide cannula until its lower end was 1 mm below the guide cannula. A total volume of 0.2 μL for a 1 min duration was injected into the dPAG driven by an infusion pump (Harvard Apparatus, South Natick, MA, U.S.A.). The displacement of an air bubble inside the polyethylene (PE-10; Becton-Dickinson, Franklin Lakes, NJ, U.S.A.) catheter connecting the syringe needle to the intracerebral needle was used to monitor the microinjection. The needle was held in place for an additional 1 min to maximize diffusion away from the needle tip. We have previously shown that the volume of 0.2 μl has a diameter of diffusion circumscribed to the site of injection (Ferreira-Netto et al., 2007).

2.4. Drugs

The following drugs were used: *N*-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-*N*-2-pyridinyl-cyclohexanecarboxamide maleate (WAY, RBI, USA) and (±)-8-hydroxy-2-(di-*n*-propyl-amino) tetralin hydrobromide (DP; RBI, USA). Both drugs were dissolved in sterile saline. The doses of the drugs were used on the basis of previous studies (Avanzi and Brandão 2001; Borelli et al., 2005b; Nogueira and Graeff, 1995; Beckett and Marsden, 1997).

This study was divided into two parts with independent groups of animals. The first part examined the effects of microinjections of saline, DP or WAY into the dPAG on the defensive reaction induced by electrical stimulation of this region of naïve rats. In the second part, the effects of these drugs on the defensive behavior induced by electrical stimulation of the dPAG were assessed in rats placed in the same context where they received contextual conditioning or in a different context.

2.5. Experiment I: effects of DP and WAY on the aversive thresholds determined by electrical stimulation of the dPAG

One week after surgery, the animals were placed in an experimental box, which consisted of a cage (25 × 25 × 15 cm) with lateral walls and ceiling made of black and transparent Plexiglas, respectively, and floor made of 15 stainless bars with 2.0 mm diameter spaced 12 mm apart. The chamber was illuminated with a 40 W fluorescent lamp (54 lx at the floor level). The animals were allowed a 5 min period of habituation in the box at the beginning of each session. Afterwards, the brain was electrically stimulated by means of a sine wave stimulator (Del Vecchio, 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, 10 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 intensity producing interruption of the ongoing behavior longer than 6 s accompanied by, at least, two of the following autonomic reactions: micturition, defecation, arching ears, and pilorerection (Coimbra and Brandão, 1993; Maisonnette et al., 1996). The current intensity producing running (gallop) or jumping in two successive trials was considered to be the escape threshold. These measures were confirmed in another consecutive ascending series of electrical stimulation. A cut-off intensity of 120 μA (peak-to-peak) for the electrical stimulation was used. In order to investigate the behavioral effects of the last electrical stimulation that triggered the escape behavior, the animals remained in the experimental box for another 5 min, without any stimulation, during which period the freezing behavior was recorded. This post-stimulation period is referred to as post-stimulation freezing (Vianna et al., 2001). The animal behaviors was recorded by a video camera (Everfocus, USA) positioned in the ceiling of the experimental box and the signal was relayed to a monitor in another room via a closed-circuit TV camera. The behaviors were measured live by one of us and confirmed later by analysis of the videotapes.

Immediately after the measurements of these baseline values, the rats were randomly assigned to one of the following groups: (a) saline + saline, (b) saline + DP (4 nmol); (c) saline + DP (8 nmol), d) WAY (10 nmol) + saline; (e) WAY (10 nmol) + DP (8 nmol). The injections were separated by an interval period of 5 min. Ten min afterwards the animals were placed in the middle of the experimental box for the redetermination of the aversive thresholds and the time spent in freezing after the dPAG stimulation. The most effective drug doses and waiting time after injections of DP (8 nmol) or WAY (10 nmol) were selected from previous studies of this laboratory (Avanzi and Brandão 2001; Silva et al., 2004; Borelli et al., 2005b). The number of animals per group was equal 8 with the exception of the group 4 nmol DP that was equal 6. Each animal received only one injection.

2.6. Experiment II: effects of DP or WAY on the aversive thresholds determined by dPAG electrical stimulation of rats under contextual conditioned fear

In contrast to Experiment I, in this study all animals were submitted to contextual conditioning sessions and the drug effects on the freezing and escape thresholds of electrical stimulation of the dPAG were determined on the testing day of the CFC.

2.6.1. Training

The control measurements of freezing and escape thresholds were determined through the same dPAG stimulation procedure as described in Experiment I. Immediately afterwards, the animals were submitted to the contextual fear conditioning. Briefly, the animals were placed in the experimental box described above and 6 min later each rat received 10 footshocks (0.6 mA, 1 s) with a variable intertrial interval of 15 to 45 s (training sessions). The shocks were delivered through the cage floor by a constant current generator built with a scrambler (Albarsh Instruments, Brazil). Stimulus presentation was controlled by a microprocessor and an I/O board (Insight Equipment, Brazil). Each animal was removed 2 min after the last shock and returned to its home cage. Each training session lasted for about 15 min.

2.6.2. Testing

Twenty-four hours later the testing sessions were conducted without presentation of footshocks in the chamber described above (same context) or in a different context which consisted of a circular arena (60 cm in diameter and 50 cm high) made of acrylic. Both the experimental box (same context) and the circular arena (different context) were equipped with a system for determining the aversive thresholds and a videocamera for recording the behaviors. Each animal received only one injection. They were injected with saline, DP (8 nmol/0.2 μL) or WAY (10 nmol/0.2 μL) into the dPAG and returned to their home cages. Ten min later they were placed in the same or in a different context (arena) and submitted to the testing sessions, in which the response to the context, the aversive thresholds and the post-stimulation freezing were determined sequentially. The measure used to assess contextual fear was the time rats spent

freezing during the first 3 min of the sessions. Soon after this period the rats were submitted to the dPAG electrical stimulation procedure for the determination of the aversive thresholds and of the time of post-stimulation freezing (5 min). Freezing was operationally defined as the total absence of movement of the body and vibrissa. The total duration of the testing session was about 15 min. As in Experiment I the behaviors were measured live by one of us and confirmed later by analysis of the videotapes. $N=8$ for each treatment group tested in the same or different contexts.

2.7. Histology

On completion of the experiments, the animals were overdosed with urethane and perfused intracardially with saline followed by buffered 4% formalin. After this, Evans Blue (2%) was microinjected into the dPAG at the same volume as drug microinjections in order to mark the drug injection site at the end of each study. The brains were removed and maintained in formalin solution for one day and then were maintained in sucrose 30% for another three days. Serial 60-μm brain sections were cut using a microtome, thaw-mounted on gelatinized slides and Nissl-stained in order to localize the sites of injections according to the Paxinos and Watson atlas (1997).

2.8. Analysis of results

The data are presented as mean+SEM. In Experiment I aversive thresholds differences and post-stimulation freezing duration for the groups injected with saline or drugs were subjected to a one-way ANOVA. In Experiment II, the freezing duration was subjected to a one-way ANOVA and the differences (testing sessions — baseline) in the aversive thresholds and post-stimulation freezing duration were subjected to a two-way ANOVA, using treatment and contexts as factors. The factor treatment refers to injections of saline, DP or WAY into the dPAG. The factor context refers to the same and different contexts. Differences of multiple means were assessed with the Bonferroni's t-test in all experiments ($p<0.05$). Only results of experiments in which the electrode tips and injection sites were positioned in the dPAG were included in the analysis. In general, the electrode fell outside the dPAG in one or two animals per group and four animals were also discarded because the chemitrodes presented problems in the electrical conductivity.

3. Results

The majority of the electrode tips and the injection sites were situated inside the dorsolateral columns of the PAG while few injection sites were located in the dorsomedial division of the PAG. Representative sites of stimulation and microinjections into the dPAG are shown in Fig. 1.

As the intensity of the current applied to the dPAG increased, the animals suddenly stopped, became immobile and often urinated and defecated. With higher intensities, this freezing behavior was followed by vigorous running and jumping.

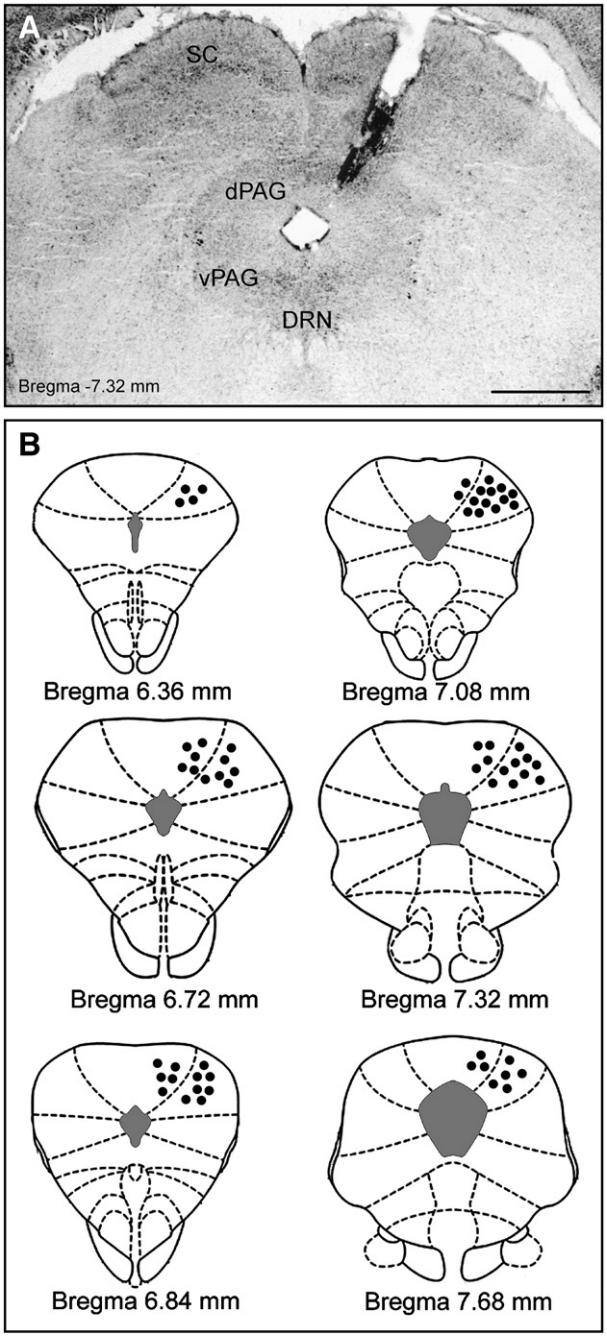

Fig. 1. Representative photomicrograph of electrode tips into the dorsal periaqueductal gray (A) and sites of injections into PAG outline from the atlas of Paxinos and Watson (1997) (B). The number of points in the figure is less than the total number of rats used because of several overlaps. SC = superior colliculus. dPAG = dorsal periaqueductal gray. vPAG = ventral periaqueductal gray. DRN = dorsal raphe nucleus. Scale bar equal to 800 μm in A.

Fig. 2 shows the mean change in the freezing and escape thresholds determined by electrical stimulation of the dPAG across baseline and test phases of the experiment in the groups of animals injected with saline, DP, WAY and WAY+DP into the dPAG of rats non-exposed to contextual conditioning procedure. One-way ANOVA applied on these data showed that treatments caused significant increase in the freezing ($F_{4,33}=6.97$, $p<0.05$) and escape ($F_{4,33}=2.80$, $p<0.05$) thresholds but did not change

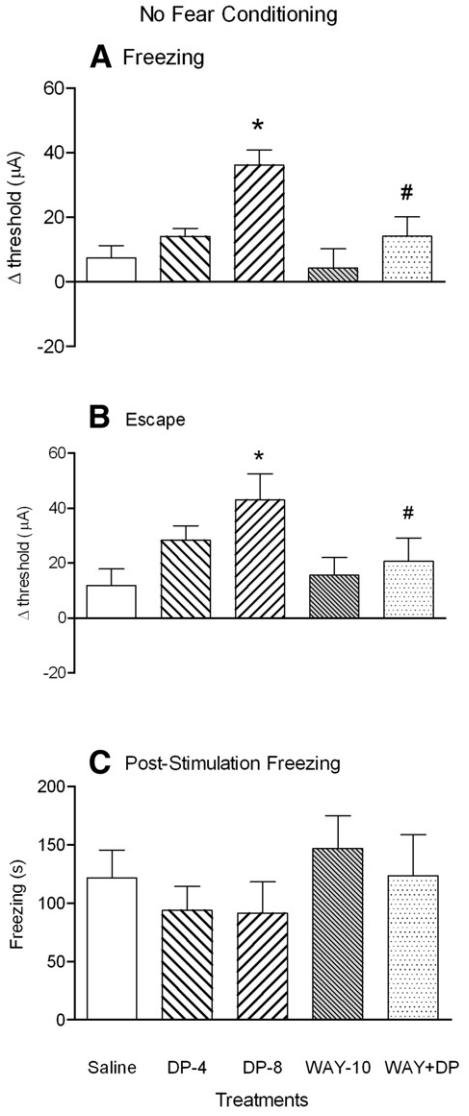

Fig. 2. Mean differences between freezing (A) and escape (B) thresholds determined before and after microinjections of saline, DP (4 and 8 nmol), WAY (10 nmol) and WAY (10 nmol)+DP (8 nmol) into the dPAG of naïve rats, non-exposed to contextual conditioning procedure. The time of post-stimulation freezing is depicted in C. $N=8$ for each group, except the group DP 4 nmol which had 6 animals. *$p<0.05$ in relation to the saline group. # $p<0.05$ in relation to the DP 8 nmol group. ANOVA followed by Bonferroni test.

the dPAG post-stimulation freezing ($F_{4,33}=0.66$, $p>0.05$). *Post-hoc* comparisons revealed that DP 8 nmol, but not WAY, was able to show antiaversive effects on both types of defensive reaction ($p<0.05$). The antiaversive effects of DP 8 nmol on the freezing and escape thresholds were attenuated by the combined treatment with WAY. The baseline values for freezing ($F_{4,33}=1.86, p>0.05$) and escape ($F_{4,33}=2.05, p>0.05$) thresholds were not statistically different in Exp. I (Table 1).

Fig. 3 illustrates the mean time rats spent freezing following contextual conditioning and injection of saline, DP or WAY before the testing sessions. Two-way ANOVA showed that context had a significant effect on freezing duration ($F_{1,42}=75.84$, $p<0.05$) indicating a greater response to the same than to the different context. There were also significant effects of treatments ($F_{2,42}=4.57$, $p<0.05$) and conditions×treatments interaction ($F_{2,42}=5.16$, $p<0.05$), indicating that freezing behavior was highly dependent on the context previously paired with shock. *Post-hoc* analysis revealed that WAY and saline caused similar effects in the conditioned freezing response. However, 8 nmol DP decreased the expression of context-conditioned fear compared to saline-treated rats.

Table 1
Mean±SEM of baseline values (μA) corresponding to the freezing and escape thresholds determined for the groups of animals that received injections of saline, DP — 4 nmol, DP — 8 nmol, WAY — 10 nmol and WAY (10 nmol)+DP (8 nmol) into the dPAG

	Freezing	Escape
Saline ($N=8$)	58.13±4.99	75.63±7.98
DP — 4 nmol ($N=6$)	48.33±3.80	60.00±3.16
DP — 8 nmol ($N=8$)	53.75±5.15	72.50±8.02
WAY — 10 ($N=8$)	60.00±3.54	85.00±5.67
WAY+DP ($N=8$)	46.88±2.98	61.88±4.43

Fig. 4A shows the effects of DP and WAY on the mean change of the freezing threshold determined by the procedure of dPAG electrical stimulation in rats under CFC. Two-way ANOVA revealed a significant effect of treatment ($F_{2,42}=6.71$, $p<0.05$) whereas contexts did not change the freezing threshold ($F_{1,42}=2.26$, $p>0.05$). There was no interaction between treatments vs. contexts ($F_{2,42}=1.17$, $p>0.05$). *Post-hoc* comparisons showed that whereas the freezing threshold was increased by DP in the different context ($p<0.05$) it remained unchanged in the same context ($p>0.05$).

Fig. 4B shows the effects of DP and WAY on the mean change of the escape threshold determined by the dPAG electrical stimulation procedure. There was a main effect of treatments ($F_{2,42}=5.48$, $p<0.05$). There was no significant effect of contexts ($F_{1,42}=0.44$, $p>0.05$) or interaction between contexts×treatments ($F_{2,42}=1.64$, $p>0.05$). *Post-hoc* comparisons showed that only DP increased the escape threshold in the different context whereas WAY did not produce any effect

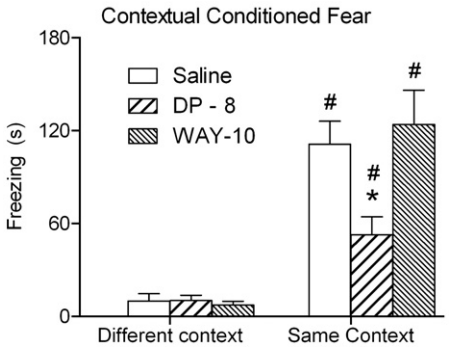

Fig. 3. Effects of contextual fear conditioning measured as time (s) per min rats spent freezing when placed into the same or different context chamber where they had received footshock (10×0.6 mA, 1 s). Mean+S.E.M. ($n=8$). *$p<0.05$, different from the saline group tested in the same context. # $p<0.05$, different from the corresponding group tested in the different context (ANOVA followed by Bonferroni test).

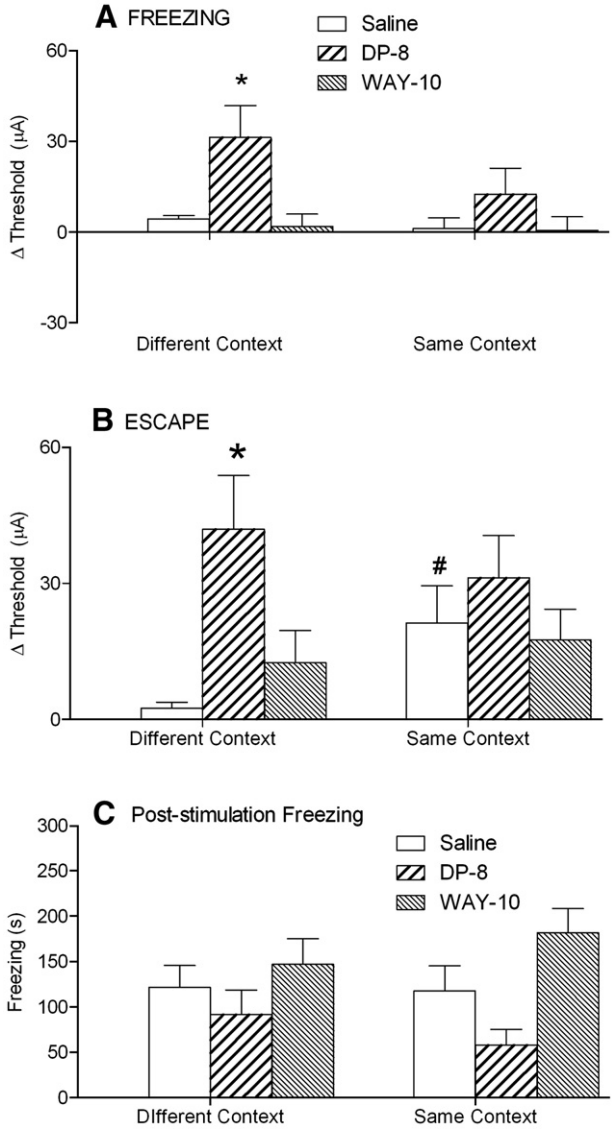

Fig. 4. Mean difference in the freezing (A) and escape (B) thresholds determined before and after microinjections of saline, DP (8 nmol) and WAY (10 nmol) into the dPAG of rats non-conditioned (different context) or contextual fear conditioned (same context). C: Post-stimulation freezing. Mean+S.E.M. (n=8). *p<0.05, different from the saline group of the same condition. # p<0.05 in relation to the saline group of the different condition (two-way ANOVA followed by Bonferroni test).

regardless of the contexts. The baseline values for freezing ($F_{2,42}$=1,64, p>0.05) and escape ($F_{2,42}$=0.72, p>0.05) thresholds were not statistically different in Exp II (Table 2).

Fig. 4C presents the mean time of post-dPAG stimulation freezing in rats exposed to the CFC procedure. ANOVA indicated that the factor context did not have a significant influence on the results ($F_{1,42}$=0.02, p>0.05). There was a significant effect of treatment ($F_{2,42}$=6.22, p<0.05) and no significant contexts×treatments interaction ($F_{2,42}$=0.91, p<0.05). *Post-hoc* comparisons indicated that the significant effects were due to differences between the DP and WAY groups but not between saline and the other groups.

4. Discussion

Infusion of the 5-HT$_{1A}$ agonist DP into the dPAG increased in a dose dependent manner the aversive thresholds determined by dPAG electrical stimulation. The present results agree with reported evidence showing that 5-HT$_{1A}$ receptors are likely to inhibit aversion generated in the dPAG (Nogueira and Graeff 1995; Graeff et al., 1986). Moreover, these receptors may be located postsynaptically because the antiaversive effect of DP was antagonized by local injections of the 5-HT$_{1A}$ antagonist WAY, at a dose that was ineffective when given alone. The same pattern of effects was observed in a similar study using α-methyl-5-HT and ketanserin, agonist and antagonist of 5-HT$_{2A}$ receptors, respectively (Oliveira et al., 2007). It has been proposed that 5-HT$_{1A}$- and 5-HT$_{2A}$-mediated mechanisms act in a cooperative way to regulate the neural substrates of fear in the midbrain tectum (Nogueira and Graeff 1995; Castilho et al., 2002; Zanoveli et al., 2003; Soares and Zangrossi 2004). The lack of effect of the 5-HT antagonists administered alone contrasts with the major aversive effects caused by injections of GABA-A blockers into the dPAG (Brandão et al., 1982; Graeff et al., 1986; Coimbra and Brandão 1997; Castilho et al., 2002; Graeff 2004). For this reason, it has been suggested that GABAergic terminals tonically inhibit dPAG neurons involved in defensive behavior whereas 5-HT systems might exert a phasic inhibition within this area (Brandão et al., 1986, 2005; Graeff 2004). That is, while GABAergic mechanisms exert a persistent control on the neural substrates of aversion in the dPAG, 5-HT mechanisms do so only during acute threatening situations.

A similar pattern of effects was also observed in rats exhibiting conditioned freezing triggered by contextual cues previously paired with footshocks. Intra-dPAG injection of DP but not of WAY impaired the conditioned freezing elicited by context cues previously paired with footshocks. Thus, the contextual conditioned freezing behavior also appears to be mediated by 5-HT$_{1A}$ synapses within the dPAG. In line with this finding it has been shown that the inhibitory avoidance acquisition of rats submitted to the T-maze test was also impaired by injections of 5-HT$_{1A}$ agonists into the dPAG (Soares and Zangrossi 2004). However, the antiaversive effects of this 5-HT$_{1A}$ agonist on freezing and escape responses induced by electrical stimulation of the dPAG were suppressed when these unconditioned fear responses were determined in the same context where the animals had previously received footshocks. These results indicate that the stressful

Table 2
Mean±SEM of baseline values (μA) corresponding to the freezing and escape thresholds determined for the groups of animals that received injections of saline, DP — 8 nmol and WAY — 10 nmol into the dPAG and were submitted afterwards to the contextual fear conditioning in the same and different contexts

	Same context		Different context	
	Freezing	Escape	Freezing	Escape
Saline (N=8)	49.36±3.19	66.25±6.32	45.63±3.71	63.13±6.54
DP — 8 (N=8)	48.75±3.24	72.50±8.02	44.38±3.05	70.00±5.04
WAY — 10 (N=8)	53.75±2.63	73.13±6.74	55.75±5.15	78.13±4.32

experience of being fear conditioned had an impact on the role of dPAG 5-HT_{1A} receptors in mediating unconditioned fear. Indeed, when dPAG stimulation takes place in a non-fear conditioned context animals respond the same as naïve rats to local infusions of the 5-HT_{1A} agonist into the dPAG. It is important to mention that, in the same way as in naïve animals, dPAG treatment with WAY did not cause significant effects on the freezing and escape responses induced by dPAG electrical stimulation in rats that were exposed to the CFC procedure. It is worth mentioning that taking together the data obtained in Experiments I and II our results show that prior experiences with footshock shift the effect of the most effective dose (8 nmol) of the DP on the aversive thresholds to the right of the curve. This supports further our contention that the neural substrates of fear of the dPAG become resistant to the antiaversive effect of intra-dPAG infusion of DP in animals under conditioned fear.

There was an increase in the escape threshold induced by electrical stimulation of the dPAG in rats exposed to conditioned fear stimuli. Thus, the freezing behavior induced by the exposure to the contextual conditioned stimuli led the animals to become less able to exert further physical activity in response to the dPAG electrical stimulation at the escape threshold. The interaction between the context-conditioned freezing and the dPAG-evoked unconditioned freezing finds a parallelism with what has been discussed in terms of the behavioral and affective consequences of the interaction between two different types of aversive stimulation. Studies using footshocks associated to foreground (light or tone) or background (context) stimuli as conditioned stimulus have shown that the amplitude of the acoustic startle response is markedly enhanced by moderate fear, and may be depressed by higher fear levels (Davis and Astrachan 1978; Walker et al., 1987; Santos et al., 2005). In the latter case, the freezing response increases but the animals startle less due to a performance deficit. Thus, making a parallel with these studies it is likely that the animals under the conjoint influence of CFC and dPAG electrical stimulation are even more fearful rendering them more resistant to the antiaversive effects of local injections of DP into the dPAG.

The 5-HT_{1A} receptors of the dPAG do not seem to be involved in the modulation of the dPAG post-stimulation freezing since no significant effect could be obtained after the injections of DP or WAY into the dPAG. It is reasonable to assume that the dPAG post-stimulation freezing is a conditional response to the context where the dPAG electrical stimulation was previously presented. However, in previous studies from this laboratory using the context shift procedure it was shown that this freezing behavior persists when the animal is placed in a different context soon after the dPAG stimulation (Vianna et al., 2001). Moreover, the post-stimulation freezing undergoes a regulation different from the dPAG-evoked freezing since electrolytic lesions or inactivation with muscimol of the amygdaloid complex reduce the dPAG post-stimulation freezing but do not affect the dPAG-evoked freezing and escape (Oliveira et al., 2004; Ruiz-Martinez et al., 2006). This finding is consistent with the notion that the dPAG is functionally downstream from the amygdala for the production of the sequential freezing and escape behaviors (Oliveira et al., 2004). On the other hand, the post-dPAG stimulation freezing mediates the aversive ascending information which is probably relayed through the thalamus. Thus, stimulating a structure closer to the motor output, as is the case for the dPAG, overrides influences from upstream structures. Thus, although the dPAG-evoked freezing and dPAG-post-stimulation freezing are interrelated they seem to have different neural substrates in the same way as dPAG-evoked freezing and escape behaviors appear to have (Ferreira-Netto et al., 2005; Borelli et al., 2005a).

Although the activation of 5-HT_{1A} and 5-HT_2 receptors of the dPAG causes antiaversive effects they do so through distinct local pathways or circuits as can be suggested from data obtained with electrophysiological studies on the dPAG cells (Brandão et al., 1991; Griffiths and Lovick 2002). Indeed, at least three differences could be noted between these two mechanisms. They distribute unevenly in the dPAG, the cells where they were found have a distinct firing pattern — 5-HT_2-sensitive cells are silent and 5-HT_{1A}-sensitive cells discharge continuously. Moreover, as activation of 5-HT_2 mechanisms produces increased firing rate of these cells they are supposed to act through activation of inhibitory processes, probably GABAergic interneurons. On the other hand, 5-HT_{1A} mechanisms are supposed to reduce the activity of facilitatory processes since injections of 5-HT_{1A} agonists into the dPAG cause a reduction of the firing of the cells containing these receptors. It is suggested that in contrast with the regulatory role of 5-HT_2 mechanisms through GABAergic mechanisms on the output neurons of defense in the midbrain tectum, 5-HT_{1A} mechanisms appear to act reducing the response of dPAG neurons excited by the incoming aversive stimuli. In the same way as the on- and off-cells regulate nociception in the ventral PAG a similar process may be operant in the dPAG in the regulation of defense (Fields and Basbaum, 2000). While on-cells are excited by aversive stimuli, off-cells are inhibited by these stimuli. Thus, in terms of descending influence of the output pathways for the defensive responses, the activity of off-cells may be related to suppression of aversive transmission whereas on-cells facilitate aversive transmission. It is likely that 5-HT_2 receptors activate an excitatory input to off-cells whereas 5-HT_{1A} receptors activate an inhibitory input to the on-cells. GABAergic neurons could represent the pool of off-cells of the dPAG. The injection of the GABA receptor antagonist bicuculline into this region causes fear while injection of an agonist of the GABA-benzodiazepine receptors produces the opposite effects, i.e. antiaversive effects (Brandão et al., 1982). The excitation of the latter cells by a process of enhanced inhibition, leads to reduction of fear. On the other hand, excitatory amino acids neurons would constitute the pool of on-cells in the dPAG and 5HT-$_{1A}$ mechanisms would inhibit them counteracting the fear-related pathways. Indeed, this hypothesis agrees with reported evidence showing that intra-dPAG administration of the 5-HT_{1A} agonist DP attenuated the escape behavior induced by local microinjection of the excitatory amino acid D,L-homocisteic acid (Beckett et al., 1992). Similar regulatory mechanisms also appear to be present in the vPAG and are highly influential in the sensitivity of the organism to nociceptive stimulation but instead of being regulated by 5-HT mechanisms they are mainly

controlled by opioid ones (De Luca-Vinhas et al., 2006). It is worth mentioning that whereas GABA mechanisms modulate tonically these descending pathways, the control exerted by 5-HT fibers is phasic, that is, only the presence of the aversive stimulus calls them into action.

In summary, the present results are indicative that the usual defensive reaction with freezing and escape responses generated by simple stimulation of the dPAG of naïve animals is shifted to a distinct defense response mode when rats are placed in a context where they had experienced past stressful experience. The electrical stimulation of the dPAG mimics the natural unconditioned fear stimuli and the resultant unconditioned fear reaction has been considered a model of panic attack (for a review see Graeff, 2004). Unconditioned freezing elicited by electrical stimulation of the dPAG of rats under CFC was a little affected by DP and not at all by WAY when microinjected into the dPAG. It is suggested that in contrast with earlier evidence for a regulatory role of 5-HT_2-mechanisms on the output neurons of defense in the midbrain tectum, 5-HT_{1A} mechanisms exert a direct inhibitory action on the neurons responsible for receiving the incoming aversive stimuli on the dPAG. Knowing that administration of selective serotonin reuptake inhibitors (SSRI's) is effective in ameliorating many of the anxiety disorder symptoms, such as anticipatory anxiety and panic attacks, the present findings encourage further search for compounds with serotonin receptor actions that may mediate the therapeutic actions of 5-HT agonists in stress precipitated psychopathology associated to the dPAG activation. In the light of the present and previous studies from this laboratory, it would be reasonable to think that the concomitant enhancement of the 5-HT_{1A} and 5-HT_2 function in the dPAG would be useful in these conditions.

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