Psychopharmacology
DOI 10.1007/s00213-006-0653-3

ORIGINAL INVESTIGATION

5-HT_2 receptor mechanisms of the dorsal periaqueductal gray in the conditioned and unconditioned fear in rats

Luciana Chrystine Oliveira · Ana Carolina Broiz · Carlos Eduardo de Macedo · J. Landeira-Fernandez · Marcus Lira Brandão

Received: 3 July 2006 / Accepted: 19 November 2006

Abstract
Rationale It is well known that 5-HT_2 mechanisms modulate the defensive behavior produced by the stimulation of the dorsal periaqueductal gray (dPAG). However, in spite of the notion that past stressful experiences play a role in certain types of anxiety, only studies with the stimulation of the dPAG of rats without previous aversive experience have been conducted so far.
Objectives We investigated the mediation of 5-HT_2 receptors of the dPAG in rats previously submitted to contextual fear conditioning (CFC). Defensive behaviors induced by the activation of the dPAG were assessed by measuring the lowest intensity of electric current applied to this structure (threshold) able to produce freezing and escape responses during the testing sessions of CFC in which animals were placed in a context previously paired to footshocks. The 5-HT_2 function of the dPAG in this condition was evaluated by local injections of α-methyl-5-HT (20 nmol/0.2 μl) and ketanserin (5 and 10 nmol/0.2 μl), selective agonist and antagonist of 5-HT_2 receptors, respectively.
Results In accordance with previous studies, α-methyl-5-HT increased the aversive thresholds (antiaversive effects) in naive rats, and injection of ketanserin into the dPAG did not produce significant effects. On the other hand, ketanserin decreased in a dose-dependent manner the freezing threshold (proaversive effect) determined by the dPAG electrical stimulation, whereas α-methyl-5-HT continued to show antiaversive effects in animals under CFC.
Conclusions The present results suggest that past stressful experience can produce changes in the synaptic function of 5-HT_2 receptors within the dPAG with important impact on the expression of defensive behaviors.

Keywords 5-HT receptors · Dorsal periaqueductal gray · Ketanserin · α-methyl-5-HT · Unconditioned fear · Contextual fear conditioning

L. C. Oliveira · A. C. Broiz · C. E. de Macedo · M. L. Brandão (✉)
Instituto de Neurociencias & Comportamento,
Campus USP,
14040-901 Ribeirão Preto, Sao Paulo, Brazil
e-mail: mbrandao@usp.br

L. C. Oliveira · A. C. Broiz · C. E. de Macedo · M. L. Brandão
Laboratório de Neuropsicofarmacologia,
Faculdade Filosofia, Ciências e Letras de Ribeirão Preto,
Universidade de São Paulo (USP),
14040-901 Ribeirão Preto, Sao Paulo, Brazil

J. Landeira-Fernandez
Departamento de Psicologia,
Pontifícia Universidade Católica do Rio de Janeiro,
Rio de Janeiro, Rio de Janeiro, Brazil

Introduction

The hippocampus, amygdala, and ventrolateral periaqueductal gray (vPAG) have been related to conditioned fear. The malfunctioning of this system appears to be associated with generalized anxiety disorder. It has also been suggested that another brain aversion system made up of the dorsal PAG (dPAG), dorsomedial hypothalamus, and amygdala is associated with unconditioned fear (Graeff 1990, 2004). Panic attacks have been related to the deregulation of the dPAG (Graeff 1990, 2004), dorsomedial hypothalamus (Johnson and Shekhar 2006), and bilateral temporal poles (Reiman et al. 1989). As to the dPAG, its electrical or chemical stimulation causes a characteristic pattern of active defense reaction with alertness, freezing, and escape responses, along with autonomic changes that resemble this anxiety disorder (Graeff 1990, 2004). 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 the activation of the neural substrates of aversion in the dPAG (Graeff 1990, 2004). In line with this notion, a recent study has shown that rats exposed to conditioned fear stimuli present a reduction in the unconditioned fear when concomitantly stimulated in the dPAG at the escape threshold (Magierek et al. 2004). However, to reconcile this hypothesis with the fact that panic attacks are frequently preceded by anticipatory anxiety (APA 1994) is still open to investigation.

The serotonin system is highly involved in the modulatory systems underlying generalized anxiety disorder and panic attacks. 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). The dPAG is rich in 5-hydroxytryptamine (5-HT) immunoreactive nerve terminals from serotonin-containing cell bodies located mainly in the dorsal raphe nucleus (Beitz et al. 1986). Because of the multiplicity of 5-HT receptors and their different adaptive properties, several studies have been carried out to disclose how they modulate the aversive states induced by the stimulation of the dPAG (Jenck et al. 1989; Brandão et al. 1991; Graeff 2004). Electrophysiological studies have found that the majority of the serotonergic receptors of the dPAG are of the 5-HT_2 type (Brandão et al. 1991; Lovick 1993). It has been shown that the activation of 5-HT_2 receptors has an inhibitory effect on the neural substrates of aversion in the dPAG (Graeff et al. 1986; Coimbra and Brandão 1997; Castilho et al. 2002). Therefore, the careful characterization of all relevant 5-HT receptors involved in modulating the brain negative reinforcement system is of considerable significance. This should help our understanding of the role of serotonin in this neuronal system that might be an important substrate for drugs proposed to alleviate anxiety disorders.

It is important to acknowledge that different anxiety disorders might be related to distinct defensive systems, which in turn might involve a specific neural mechanism. For instance, 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 appears when this stimulation terminates (Vianna et al. 2001a,b). 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 et al. 1990; Schenberg et al. 2005; Santos et al. 2005). Going one step further, we have proposed that with gradual increases in the electrical stimulation of the dPAG, freezing first appears as a preparatory response for escape (immediate defensive responses), and after the interruption of this stimulation the animals freeze again when the processing of aversive information is being relayed to higher structures (Ruiz-Martinez et al. 2006; Borelli et al. 2005). However, in spite of the notion that past stressful experiences play a crucial role in the development of mental disorders, the animal models of anxiety using electrical stimulation of the dPAG do not include previous stressful experiences as an important variable. Therefore, it is of relevance to study whether the fear generated at the level of the dPAG can be modulated by previous experience with stressful events (Brandão et al. 2003).

In this study, we evaluated the involvement of the 5-HT_2-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_2 function was assessed by local injections into the dPAG of α-methyl-5-HT and ketanserin, selective agonist and antagonist of 5-HT_2 receptors, respectively (Leysen et al. 1981; Baxter et al. 1995).

Materials and methods

Animals

Seventy-nine male Wistar rats weighing 250–280 g from the animal house of the Campus of Ribeirão Preto of the University of Sao Paulo were housed in a temperature-controlled (22 ± 1°C) room and maintained on a 12-h light/12-h dark cycle (lights on from 0700–1900 hours). 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.

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 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. 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.

Procedure

This study was divided into two parts with independent groups of animals. The first part examined the effects of microinjections of saline, α-methyl-5HT, or ketanserin into the dPAG on the defensive reaction induced by electrical stimulation of this region of naive rats. In the second part, the effects of these drugs on the defensive behavior induced by the electrical stimulation of the dPAG were assessed in rats placed in the same context, where they received contextual conditioning, or in a different context.

Microinjection procedure

A total volume of 0.2 μL was used for drug injections into the dPAG. The control animals received the same volume of vehicle. 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. The solutions were injected into the dPAG (0.2 μL/min) driven by an infusion pump (Harvard Apparatus, South Natick, MA, USA). The displacement of an air bubble inside the polyethylene (PE-10, Becton-Dickinson, Franklin Lakes, NJ, USA.) 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.

Experiment 1: effects of α-methyl-5HT or ketanserin on the aversive thresholds determined by electrical stimulation of the dPAG

One week after surgery, the animals were placed in an experimental box that consisted of a cage (25×25×15 cm) with lateral walls and ceiling made of black and transparent Plexiglas, respectively, and with a floor made of 15 stainless bars with a 2.0-mm diameter spaced 12 mm apart. The chamber was illuminated with a 40-W fluorescent lamp (80 lx at the arena floor level). The animals were allowed a 5-min period of habituation in the box at the beginning of each session. Afterward, 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.

The freezing threshold was operationally defined as the lowest intensity producing an interruption of the ongoing behavior longer than 6 s and attentive postures towards the surroundings (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 180 μA (peak-to-peak) for the electrical stimulation was used. To investigate the behavioral effects of the last electrical stimulation that triggered the escape behavior, the animals remained in the experimental box for another 8 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. 2001a,b).

Soon after the measurements of these baseline values, the rats received saline (N=7), α-methyl-5HT (N=9), or ketanserin (N=6) into the dPAG. Ten minutes afterward, the aversive thresholds were redetermined as well as the time spent in freezing after the dPAG stimulation. The most effective drug doses and waiting time after injections of α-methyl-5HT and ketanserin were selected from the previous studies of this laboratory (Graeff et al. 1986; Coimbra and Brandão 1997; Castilho and Brandão 2001; Castillho et al. 2002).

Experiment 2: effects of α-methyl-5HT or ketanserin on the aversive thresholds determined by dPAG electrical stimulation of rats under contextual conditioned fear

Differently from experiment 1, in this study, the animals were submitted to contextual conditioning sessions, and the drug effects on the freezing and the escape thresholds of the electrical stimulation of the dPAG were determined on the testing day of the CFC.

Training One week after the surgery, the control measurements of freezing and escape thresholds were determined through the same dPAG stimulation procedure as described in Experiment 1. Soon afterwards, the animals were submitted to the contextual fear conditioning. Briefly, the animals were placed in the experimental cage described above (training cage), and 6 min later, each rat received ten

footshocks (0.6 mA, 1 s) with a variable intertrial interval of 15 to 45 s. The shocks were delivered through the training cage floor by a constant current generator built with a scrambler (Albarsh Instruments, Brazil). The 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.

Testing The testing sessions were conducted without the presentation of footshocks in the chamber described above (same context), or in a different context that consisted of a circular arena (60 cm in diameter and 50 cm high) made of acrylic. Ten minutes before the session in the same or different context, each animal received an injection of either saline, α-methyl-5HT, or ketanserin into the dPAG. N=8 for each treatment group tested in the different context, N=8 for saline and α-methyl-5HT, and N=6 for ketanserin groups tested in the same context. The measure used to assess contextual fear was the time rats spent freezing during the test period of 3 min. Freezing was operationally defined as the total absence of movement of the body and vibrissa. Soon after this 3-min period, the rats were submitted to the dPAG electrical stimulation procedure for the determination of the aversive thresholds and the time of post-stimulation freezing in rats under the effects of the drugs injected into the dPAG.

Histology

On completion of the experiments, the animals were overdosed with urethane and perfused intracardially with saline followed by buffered 10% formalin. After this, Evans Blue (2%) was microinjected into the *dPAG* at the same volume as drug microinjections 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 3 days. Serial 60-μm brain sections were cut using a microtome, thaw-mounted on gelatinized slides and Nissl-stained to localize the sites of injections according to the atlas of Paxinos and Watson (1997).

Analysis of results

The data are presented as mean + SEM. In experiment 1, the aversive threshold differences and post-stimulation freezing duration for the groups injected with saline or drugs were subjected to a one-way analysis of variance (ANOVA). In experiment 2, the freezing duration was subjected to a one-way ANOVA, and the differences 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, ketanserin, or α-methyl-5-HT into the dPAG. The factor context refers to the same and different contexts. The differences of multiple means were assessed with the Bonferroni's t test in all experiments (P<0.05).

Results

The electrode tips and the injection sites were situated inside the dPAG. The representative sites of the 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. The baseline values for the freezing and escape thresholds were comparable in the three groups of animals used (Table 1).

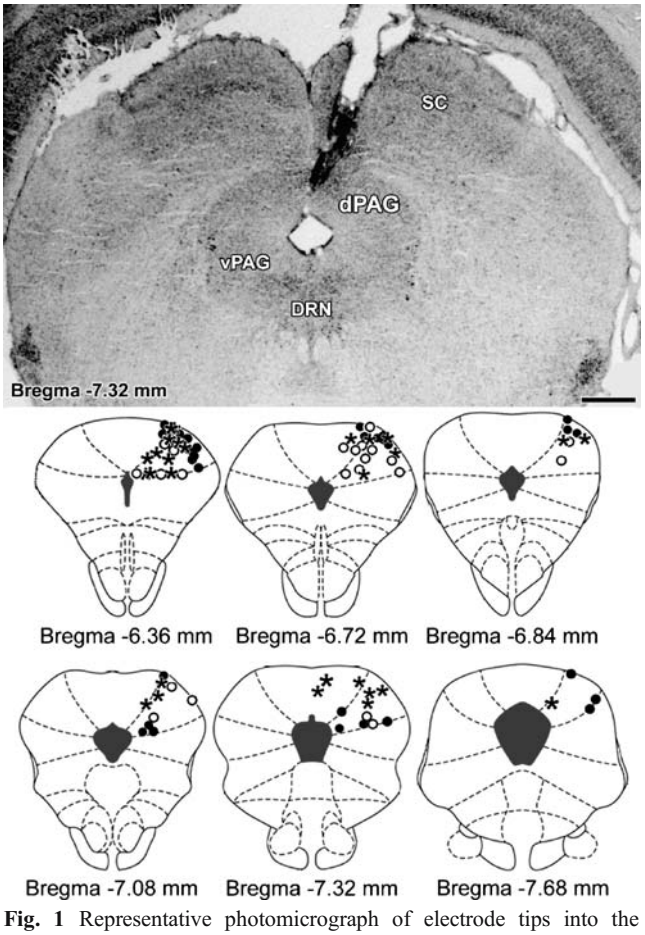

Fig. 1 Representative photomicrograph of electrode tips into the dPAG (**a**) and sites of injections into this region (**b**). *Close circles* represent saline, *open circles* ketanserin, and *asterisks* α-methyl-5-HT. Scale bar is equal to 400 μm in **a**

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, α-methyl-5-HT and ketanserin into the dPAG and were submitted afterwards to contextual fear conditioning, in the same and different contexts

	Same context		Different context	
	Freezing	Escape	Freezing	Escape
Saline	53.18 ± 9.23	74.09 ± 16.09	51.25 ± 8.35	60.00 ± 6.21
α-Methyl-5-HT	55.00 ± 6.87	68.89 ± 10.89	61.25 ± 8.87	81.75 ± 10.06
Ketanserin	45.22 ± 5.47	66.33 ± 6.31	55.63 ± 12.03	66.88 ± 12.88

Figure 2 shows the mean change in the freezing and escape thresholds determined by electrical stimulation of the dPAG across the baseline and test phases of the experiment in the groups of animals injected with saline, α-methyl-serotonin, or ketanserin into the dPAG of rats not exposed to the contextual conditioning procedure. The one-way ANOVA applied on these data showed that treatments caused significant change in the freezing ($F_{2,19}$=3.80, p< 0.05) and escape ($F_{2,19}$=5.27, p<0.05) thresholds. Post-hoc comparisons revealed that α-methyl-serotonin, but not ketanserin, was able to show anti-aversive effects on both types of defensive reaction. Finally, no effect of these drugs was found at the time of dPAG post-stimulation freezing.

Figure 3 illustrates the mean time of the freezing of the animals submitted to the contextual conditioning procedure and treated with saline, α-methyl-serotonin, or ketanserin before the testing sessions. The two-way ANOVA showed that context had a significant effect on freezing duration ($F_{1,40}$=50.52, p<0.05), indicating a greater response to the same than to the different context. There were also significant effects of treatments ($F_{2,40}$=9.64, p<0.05) and conditions × treatments interaction ($F_{2,40}$=10.14, p<0.05), indicating that freezing behavior was highly dependent on the context where shocks were delivered. Post-hoc analysis revealed that ketanserin and saline caused similar effects in the conditioned freezing response. Compared to saline-treated rats, α-methyl-serotonin decreased the expression of context-conditioned fear ($t_{1,14}$=4.09, p<0.05).

Figure 4a shows the drug effects on the mean change of the freezing threshold determined by the procedure of dPAG electrical stimulation. Treatments had context-dependent effects on freezing thresholds ($F_{2,40}$=7.29, p<0.05). Post-hoc comparisons showed that, although these thresholds were increased by α-methyl-5HT in the fear conditioning context ($t_{1,14}$=3.43, p<0.05), they were decreased by ketanserin ($t_{1,12}$=2.93, p<0.05). The treatments had a significant effect on the freezing threshold ($F_{2,40}$=12.95, p<0.05), and the contexts did not cause significant effects ($F_{1,40}$=0.26, p>0.05). Figure 4b shows the drug effects on the mean change of the escape threshold determined by the

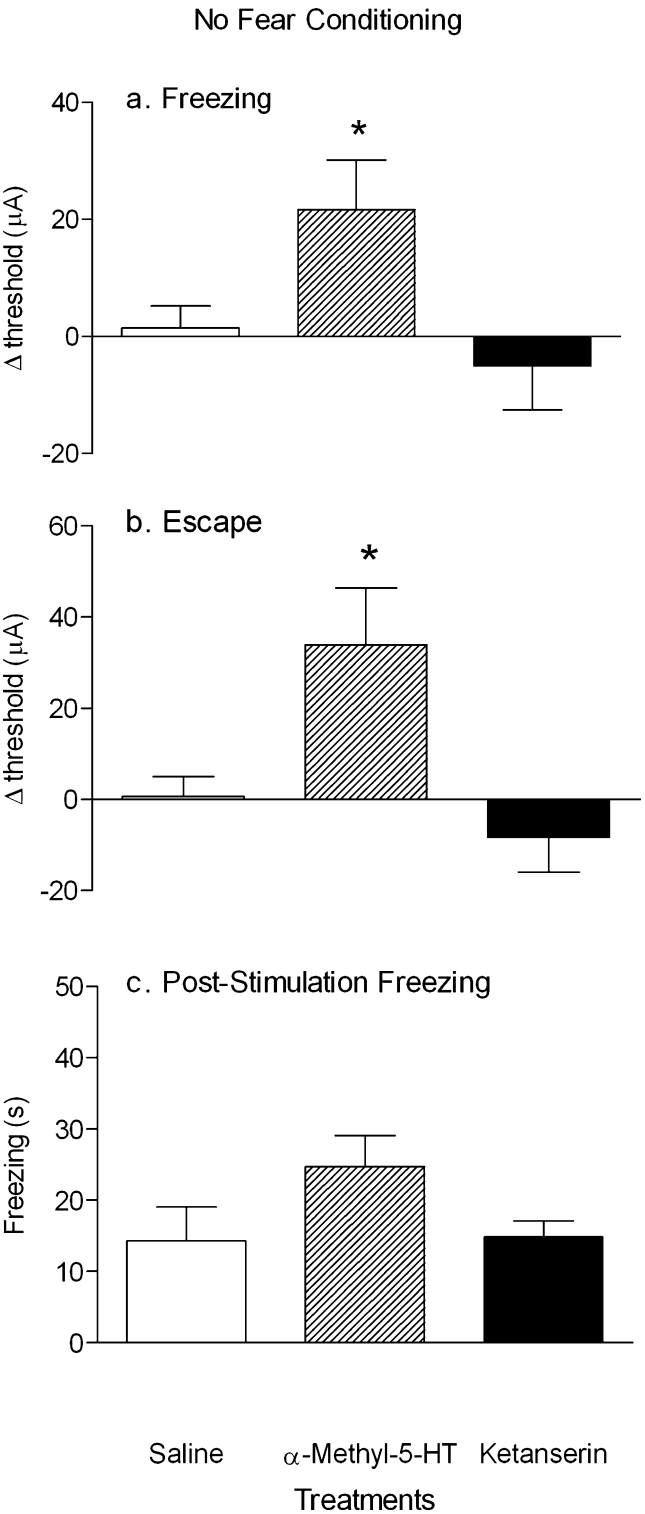

Fig. 2 Mean differences between freezing (*above*) and escape (*below*) thresholds determined before and after microinjections of saline, α-methyl-5-HT, or ketanserin into the dPAG of naive rats nonexposed to contextual conditioning procedure. N=7, 6, and 9 for the saline, α-methyl-5-HT, and ketanserin groups, respectively. *P<0.05 in relation to the saline group (ANOVA followed by Bonferroni test)

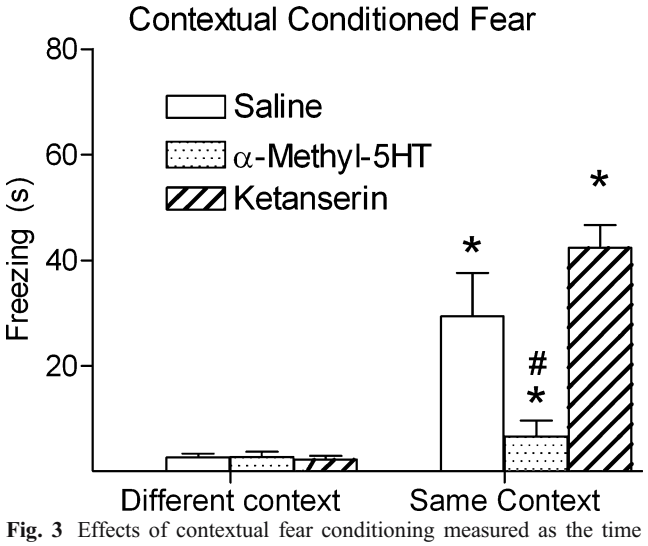

Fig. 3 Effects of contextual fear conditioning measured as the time (s) spent per minute of freezing rats when placed into the same or different context chamber where they received footshock (10× 0.6 mA, 1 s). Mean + SEM. N=8 for each treatment group tested in the different context, N=8 for saline and α-methyl-5HT, and N=6 for the ketanserin treatment group tested in the same context. *Asterisk*, different from the corresponding group tested in the different context. *Sharp*, different from the saline group tested in the same context (ANOVA followed by Bonferroni test)

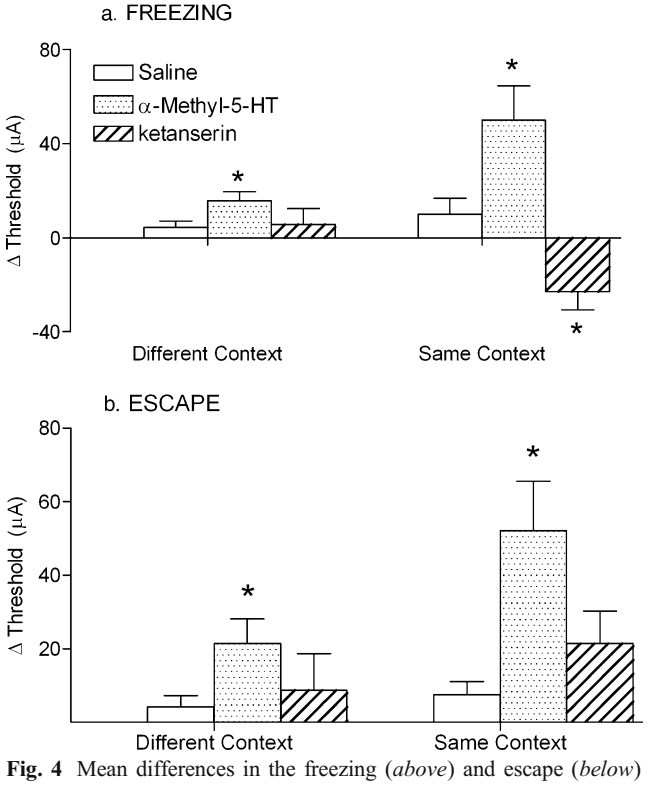

Fig. 4 Mean differences in the freezing (*above*) and escape (*below*) thresholds determined before and after microinjections of saline, α-methyl-5-HT, or ketanserin into the dPAG of rats under a contextual conditioning procedure. Mean + SEM. N=8 for each treatment group in the different context, N=8 for saline and α-methyl-5HT, and N=6 for the ketanserin treatment group tested in the same context. *Asterisk*, different from the saline group of the corresponding condition (two-way ANOVA followed by Bonferroni test)

dPAG electrical stimulation procedure. There was a main effect of the treatments ($F_{2,40}$=7.01, p<0.05) and conditions ($F_{1,40}$=5.13, p<0.05), but there was no significant interaction between conditions×treatments. Post-hoc comparisons showed that only α-methyl-serotonin increased the escape threshold in both contexts ($t_{1,14}$=3.80, p<0.05).

Figure 5 presents the mean time of freezing the post-dPAG stimulation in rats that passed by the CFC procedure. ANOVA indicated that the factor condition had a significant influence on the results ($F_{1,40}$=42.22, p<0.05). There were no significant effects of the treatment or the conditions×treatments interaction, indicating that the freezing behavior was highly dependent on the context where shocks were previously delivered and that treatment did not change the freezing behavior in both conditions.

Taking into account that the effects of the dose of 10 nmol of ketanserin on the freezing threshold depended on whether the animals were naive or had prior experience with foot shocks, we examined the effect of a second dose of this 5-HT$_{-2}$ receptor antagonist (5 nmol) to determine the effective dose that causes this shift in the dose-effect curve for freezing in rats with prior experience with footshock. In this additional experiment, the rats were randomly allocated to two groups of six subjects each: (a) control group injected with saline (N=5) and (b) group injected with 5 nmol ketanserin into the dPAG (N=6). The procedure was similar to that described above for experiment 2. The effects obtained with 5 nmol of ketanserin were less pronounced but qualitatively similar to the dose of 10 nmol. There was a statistically significant reduction ($t_{1,9}$=2.99, p<0.05) in the freezing threshold (−11.67± 4.22 μA for the ketanserin group against the 6.00±4.00 μA for the saline-injected animals). Similar to the dose of 10 nmol, a lower dose of ketanserin (5 nmol) did not cause

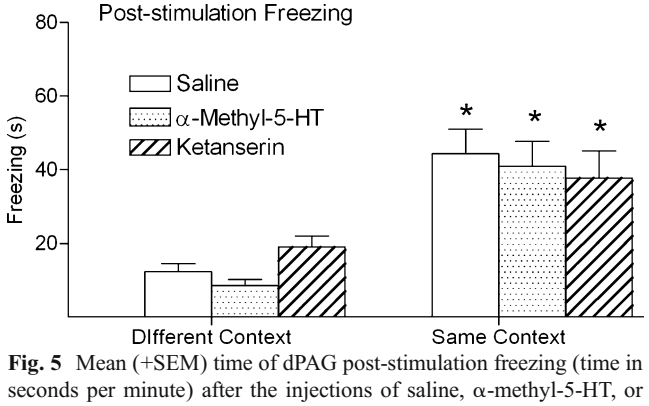

Fig. 5 Mean (+SEM) time of dPAG post-stimulation freezing (time in seconds per minute) after the injections of saline, α-methyl-5-HT, or ketanserin into the dPAG of naive, nonconditioned rats (different context) or in animals previously submitted to a contextual conditioning procedure (same context). N=8 for each treatment group in the different context, N=8 for saline and α-methyl-5HT, and N=6 for the ketanserin treatment group tested in the same context. *p<0.05 in relation to the corresponding group in the different context (two-way ANOVA followed by Bonferroni test)

significant changes in the dPAG-evoked escape and dPAG post-stimulation freezing.

Discussion

The stimulation of the dPAG has been reported to produce strong unpleasant fearful sensations in human subjects (Nashold et al. 1969). The rats under dPAG stimulation show rapid acquisition and maintenance of operant responses (e.g., lever pressing) that interrupt the stimulation and allow them to escape from it (Brandão et al. 1982). In the present study, stepwise increases of the current of the electric stimulus applied to this region caused the whole pattern of defense reaction with freezing followed by escape responses. Confirming previous studies, dPAG infusion of the 5-HT_2 agonist α-methyl-5-HT increased, whereas ketanserin did not change the aversive thresholds determined by the dPAG electrical stimulation (Graeff et al. 1986; Nogueira and Graeff 1995). This finding contrasts with the major aversive effects caused by injections of gamma-aminobutyric acid (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, whereas 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. In the present study, the 5-HT_2 antagonist ketanserin was effective in increasing the aversiveness of the dPAG electrical stimulation only in rats placed in the same context in which they had previously received footshocks, but not in the rats tested in a context different from where they received footshocks.

The most prominent effect of injections of ketanserin into the dPAG was a pro-aversive effect on freezing response induced by electrical stimulation of the dPAG, but no effect was obtained on conditioned freezing triggered by contextual cues previously paired with footshock. Differently from ketanserin, the treatment with α-methyl-5-HT caused the expected antiaversive effects on the freezing response induced by dPAG electrical stimulation in rats that passed by the CFC procedure. These results indicate that the regulation of the unconditioned defensive responses by 5-HT_2 mechanisms at the level of the dPAG depend on past stressful experiences.

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 the two different types of aversive stimulation. Studies using footshocks associated to foreground (light or tone) or background (context) stimuli as CS 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. 1997; Silva et al. 2004; Santos et al. 2005). Other studies looking at the influences of the intensity of the fearful stimuli on the defensive responses have also shown that association of different stressful events may cause increased fear along with hypoactivity (Maisonnette et al. 1993; Maritjena et al. 1997). In this study, the effects of ketanserin on the dPAG-evoked freezing of previously shocked rats differ from those of naive rats in the absence of footshock-conditioning clues. Thus, the influence of the conditioned aversive stimuli (context cues) on the dPAG-evoked freezing behavior appears to be mediated by 5-HT_2 synapses within the dPAG.

It is important to mention that the fact that ketanserin caused an increase in the escape threshold induced by electrical stimulation of the dPAG in rats exposed to conditioned fear stimuli cannot be interpreted as a general depressor effect on motor activity. Instead, rats under the conjoint presentation of these aversive stimuli are even more afraid, as indicated by their higher freezing responses in relation to dPAG-evoked unconditioned fear of rats placed in a context different from where they received footshocks. This is substantiated by the fact that the dPAG post-stimulation freezing is much higher in CFC animals than in nonconditioned rats. Consequently, this association would make the animals more able to receive all kinds of sensory information that could have led the animals to become less able to exert further physical activity in response to the dPAG electrical stimulation at the escape threshold. This possibility establishes a parallel between the nonmonotonic function of fear reaction and the increasing threatening conditions (Davis and Astrachan 1978; Walker et al. 1997; Santos et al. 2005, 2006). Consistent with this hypothesis, a recent study from this laboratory demonstrated that aversive stimulation of the midbrain tectum caused a significant reduction in the startle response of rats (Nobre et al. 2003). Other studies looking at the influence of the intensity of the fearful stimuli on the defensive responses have also shown similar deficit in performance after intense threatening conditions or combination of aversive stimuli. This emotional shift may be related to fear states different from generalized anxiety. Indeed, midazolam did not produce any effect al all on the startle of rats at high fear conditioning but caused an anxiolytic-like effect on the contextual fear-potentiated startle (Santos et al. 2005). Differently from ketanserin, α-methyl-5-HT increased the escape response induced by dPAG electrical stimulation in both groups of rats that passed or did not pass by the CFC procedure.

The conditioned freezing was reduced by injections of (α-methyl-5-HT into the dPAG. A claim that can be made is that 5-HT_2-mediated mechanisms of the dPAG indirectly inhibit the neural substrates of the conditional fear in other regions such as the ventral PAG. In this context, electrophysiological studies have found that the majority of the serotonergic receptors of the dPAG are of the 5-HT2 type and that serotonin regulates aversive states in this region through the activation of GABAergic inhibitory transmission (Brandão et al. 1991; Lovick 1993). The enhancement of this inhibitory input by α-methyl-5-HT would allow greater activity in the GABAergic efferents of the dPAG to the vPAG, leading, as a result, to an inhibition of the conditioned fear. In support of this view, it has been shown that dPAG lesions enhance the conditional freezing organized in the vPAG (De Oca et al. 1998).

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. 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. 2001a,b). They did not have any previous experience with foot shocks. In the present study, the post-stimulation freezing of saline-injected rats was potentiated in the footshock-conditioning context. These findings indicate that post-stimulation freezing, whether conditioned or not, persists sometime in a new neutral context.

The participation of 5-HT_2 receptors of the dPAG does not seem to be involved in the modulation of the dPAG post-stimulation freezing, as no effect at all on this variable could be obtained after the injections of α-methyl-5-HT and ketanserin into the dPAG. The post-stimulation freezing undergoes a regulation different from the dPAG-evoked fear because the electrolytic lesions or inactivation of the 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). The post-dPAG stimulation freezing mediates the aversive ascending information that is, probably, relayed through the amygdala. Thus, ascending dPAG efferents seem to be activated during this condition, and the lack of change in the thresholds for freezing and escape observed in amygdala-lesioned or -inactivated animals may be linked to the fact that the dPAG itself contains neural substrates for the production of unconditioned fear. Thus, stimulating a structure closer to the motor output, as is the case for the dPAG, overrides influences from upstream structures. On the other hand, other reports have already shown that $5HT_2$ agonists enhance the regulation of the neural substrates responsible for the production of fear in the dPAG (Nogueira and Graeff 1995; Castilho et al. 2002). 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. 2005).

In summary, the present results are indicative that the usual defensive reaction with freezing and escape responses generated by the simple stimulation of the dPAG of naive animals is shifted to a distinct defense response mode when rats are placed in a context where they had experienced past stressful experience. Nowadays, there is good agreement that proximal aversive stimulus, such as the presence of a predator, stimulates the dPAG. 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 the electrical stimulation of the dPAG of rats under CFC was enhanced by ketanserin, but inhibited by α-methyl-5-HT when microinjected into the dPAG. Knowing that the administration of selective serotonin reuptake inhibitors 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 selective serotonin receptor actions that may mediate the therapeutic actions of 5-HT agonists in stress-precipitated psychopathology associated to the dPAG activation, summing up to the evidence that the chronic administration of antidepressants attenuates both the PAG-evoked defensive behaviors (Borelli et al. 2004) and, in clinical doses, the escape behavior (Schenberg et al. 2001). In the light of the present study, it would be reasonable to think that the enhancement of the 5-HT_2 function in the dPAG would be useful in these conditions.

Acknowledgment This research is supported by FAPESP (proc. no. 02/03705-0). L. C. Oliveira and A. C. Broiz are recipients of scholarships from CAPES. M. L. Brandão and J. Landeira-Fernandez are research fellows from CNPq.

References

APA (1994) American Psychiatric Association, Diagnostic and statistical manual of mental disorders, 4th edn. American Psychiatric Association, Washington, DC

Baxter G, Kennett G, Blaney F, Blackburn T (1995) 5-HT2 receptor subtypes: a family re-united? Trends Pharmacol Sci 16:105–110

Beitz AJ, Clements JR, Mullet M, Ecklund L (1986) Differential origin of brainstem serotonergic projections to the midbrain periaqueductal gray and superior colliculus of the rat. J Comp Neurol 250:498–509

Blanchard DC, Blanchard RJ, Rodgers RJ (1990) Pharmacological and neural control of antipredator defense in the rat. Aggress Behav 16:165–175

Borelli KG, Nobre MJ, Brandão ML (2004) Effects of acute and chronic fluoxetine and diazepam on freezing behavior induced by electrical stimulation of dorsolateral and lateral columns of the periaqueductal gray matter. Pharmacol Biochem Behav 77:557–566

Borelli KG, Ferreira-Netto C, Coimbra NC, Brandão ML (2005) Fos-like immunoreactivity in the brain associated with freezing or escape induced by inhibition of either glutamic acid decarboxylase or GABAA receptors in the dorsal periaqueductal gray. Brain Res 105:100–111

Brandão ML, Aguiar JC, Graeff FG (1982) GABA mediation of the anti-aversive action of minor tranquilizers. Pharmacol Biochem Behav 16:397–402

Brandão ML, DiScala G, Bouchet MK, Schmitt P (1986) Escape behavior produced by the blockade of glutamic acid decarboxylase (GAD) in mesencephalic central gray or medial hypothalamus. Pharmacol Biochem Behav 24:497–502

Brandão ML, Lopez-Garcia JA, Roberts MHT (1991) Electrophysiological evidence for excitatory 5-HT_2 and depressant 5-HT_{1A} receptors on neurones of the rat midbrain tectum. Bran Res 556:259–266

Brandão ML, Troncoso AC, Silva MAS, Huston JP (2003) The relevance of neuronal substrates of defense in the midbrain tectum to anxiety and stress: empirical and conceptual considerations. Eur J Pharmacol 463:225–233

Brandão ML, Borelli KG, Nobre MJ, Santos JM, Albrechet-Souza L, Oliveira AR, Martinez RC (2005) Gabaergic regulation of the neural organization of fear in the midbrain tectum. Neurosci Biobehav Rev 29:1299–1311

Castilho VM, Brandão ML (2001) Conditioned antinociception and freezing using electrical stimulation of the dorsal periaqueductal gray or inferior colliculus as unconditioned stimulus are differentially regulated by 5-HT2A receptors in rats. Psychopharmacology 155:154–162

Castilho VM, Macedo CE, Brandão ML (2002) Role of benzodiazepine and serotonergic mechanisms in conditioned freezing and antinociception using electrical stimulation of the dorsal periaqueductal gray as unconditioned stimulus in rats. Psychopharmacology 165:77–85

Coimbra NC, Brandão ML (1993) GABAergic nigro-collicular pathways modulate the defensive behavior elicited by midbrain tectum stimulation. Behav Brain Res 59:131–139

Coimbra NC, Brandão ML (1997) Effects of 5-HT_2 receptors blockade on fear-induced analgesia elicited by electrical stimulation of the deep layers of the superior colliculus and dorsal periaqueductal gray. Behav Brain Res 87:97–103

Davis M, Astrachan DI (1978) Conditioned fear and startle magnitude: effects of different footshock and backshock intensities used in training. J Exp Psychol Anim Behav Process 4:95–103

De Oca BN, DeCola JP, Maren S, Fanselow MS (1998) Distinct regions of the periaqueductal gray are involved in the acquisition and expression of defensive responses. J Neurosci 18:3426–3432

Ferreira-Netto C, Borelli KG, Brandão ML (2005) Neural segregation of Fos-protein distribution in the brain following freezing and escape behaviors induced by injections of either glutamate or NMDA into the dorsal periaqueductal gray of rats. Brain Res 1031:151–163

Graeff FG (1990) Brain defense systems and anxiety. In: Roth M, Burrow GD, Noyes R (eds) Handbook of anxiety, vol 3. Elsevier, New York, pp 307–354

Graeff FG (2004) Serotonin, the periaqueductal gray and panic. Neurosci Biobehav Rev 28:239–259

Graeff FG, Brandão ML, Audi EA, Schultz MTB (1986) Modulation of the brain aversive system by GABAergic and serotonergic mechanisms. Behav Brain Res 21:65–72

Jenck F, Broekkamp CLE, Van Delft ML (1989) Effects of serotonin receptor antagonists on PAG stimulation induced aversion: different contributions of 5-HT1, 5HT2 and 5HT3 receptors. Psychopharmacology 97:489–495

Johnson PL, Shekhar A (2006) Panic-prone state induced in rats with GABA dysfunction in the dorsomedial hypothalamus is mediated by NMDA receptors. J Neurosci 28:1015–1022

Leysen JE, Awonters F, Kennis L, Laduron PH, Vandenberk J, Jansem PAJ (1981) Receptor binding profile of R41468, a novel antagonist of 5-HT2 receptors. Life Sci 28:1015–1022

Lovick TA (1993) Serotonergic influence from raphe nucleus obscurus on neurons in the periaqueductal grey matter in the rat. Brain Res 606:92–98

Magierek V, Ramos PL, Silveira-Filho NG, Nogueira RL, Landeira-Fernandez J (2004) Context fear conditioning inhibits panic-like behavior elicited by electrical stimulation of dorsal periaqueductal gray. NeuroReport 14:1641–1644

Maisonnette SS, Morato S, Brandão ML (1993) Role of resocialization and of 5-HT1A receptor activation on the anxiogenic effects induced by isolation in the elevated plus maze test. Physiol Behav 54:753–758

Maisonnette SS, Kawasaki MC, Coimbra NC, Brandão ML (1996) Effects of lesions of amygdaloid nuclei and substantia nigra on aversive responses induced by electrical stimulation of the inferior colliculus. Brain Res Bull 40:93–98

Maritjena ID, Calvo N, Volosin M, Molina VA (1997) Prior exposure to a brief restraint session facilitates the occurrence of fear in response to a conflict situation: behavioral and neurochemical correlates. Brain Res 752:136–142

Nashold BS, Wilson WP, Slaughter DG (1969) Sensation evoked by stimulation in the midbrain of man. J Neurosurg 30:14–24

Nobre MJ, Sandner G, Brandão ML (2003) Enhancement of acoustic evoked potentials and impairment of startle reflex induced by reduction of GABAergic control of the neural substrates of aversion in the inferior colliculus. Hear Res 184:82–90

Nogueira RL, Graeff FG (1995) Role of 5-HT receptor subtypes in the modulation of aversion generated in the dorsal periaqueductal gray. Pharmacol Biochem Behav 52:1–6

Oliveira LC, Nobre MJ, Brandão ML, Landeira-Fernandez J (2004) Role of amygdala in conditioned and unconditioned fear generated in the periaqueductal gray. NeuroReport 15:2281–2285

Papaioannou VE, Fox JG (1993) The efficacy of tribromoethanol anesthesia in mice. Lab Anim Sci 43:189–192

Paxinos G, Watson C (1997) The rat brain in stereotaxic coordinates. Academic, San Diego, CA

Reiman EM, Raichle ME, Robins E (1989) Neuroanatomical correlates of a lactate-induced anxiety attack. Arch Gen Psychiatry 46:493–500

Ruiz-Martinez RC, Oliveira AR, Brandão ML (2006) Conditioned and unconditioned fear organized in the periaqueductal gray are differentially sensitive to injections of muscimol into amygdaloid nuclei. Neurobiol Learn Mem 85:58–65

Santos JM, Gárgaro AC, Oliveira AR, Masson S, Brandão ML (2005) Pharmacological dissociation of moderate and high contextual fear as assessed by freezing behavior and fear-potentiated startle. Eur Neuropsychopharmacol 15:239–246

Santos JM, Martinez RC, Brandão ML (2006) Effects of acute and subchronic treatments with fluoxetine and desipramine on the memory of fear in moderate and high-intensity contextual conditioning. Eur J Pharmacol 542:121–128

Schenberg LC, Bittencourt AS, Sudre EC, Vargas LC (2001) Modeling panic attacks. Neurosci Biobehav Rev 25:647–659

Schenberg LC, Póvoa RMF, Costa ALP, Caldellas AV, Tufik S, Bittencourt AS (2005) Functional specializations within the tectum defense systems of the rat. Neurosci Biobehav Rev 29:1279–1298

Silva RCB, Gárgaro AC, Brandão ML (2004) Differential regulation of the expression of contextual freezing and fear-potentiated

startle by 5-HT mechanisms of the median raphe nucleus. Behav Brain Res 151: 93–101

Vianna DML, Landeira-Fernandez J, Graeff FG, Brandão ML (2001a) Lesion of the ventral periaqueductal gray reduces conditioned fear but does not change freezing induced by stimulation of the dorsal. periaqueductal gray. Learn Mem 8:164–169

Vianna DML, Landeira-Fernandez J, Brandão ML (2001b) Dorsolateral and ventral regions of the periaqueductal gray matter are involved in distinct types of fear. Neurosci Biobehav Rev 25:711–719

Walker DL, Cassella JV, Lee Y, De Lima TCM, Davis M (1997) Opposing roles of the amygdala and dorsolateral periaqueductal gray in fear-potentiated startle. Neurosci Biobehav Rev 21:743–753