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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

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Abstract

The defensive responses induced by electrical stimulation of the dorsal periaqueductal gray matter (dPAG) of the rat have been proposed as a model of panic attacks in humans. In the present study we investigated the acute and chronic effects of fluoxetine and diazepam on freezing and escape reactions elicited by electrical stimulation of the dorsolateral (dlPAG) and lateral (lPAG) columns of the periaqueductal gray matter (PAG). The frequencies of crossing, rearing, bouts of micturition and fecal boli were also recorded. Electrodes were unilaterally implanted in the brainstem aimed at the PAG. Drug treatments were given daily for 2 weeks with fluoxetine (5, 10 and 20 mg/kg ip), a selective inhibitor of serotonin reuptake, diazepam (1, 2 and 4 mg/kg ip), or saline. Drug effects were assessed acutely (15 min after the first injection) and chronically (15 min after the 14th injection). Chronic, but not acute, administration of fluoxetine caused a significant increase in the threshold of freezing without affecting the escape response elicited by dlPAG/lPAG stimulation. This characteristic pattern of effects could not be attributed to motor deficit, since this drug did not change the number of crossings and rearings. In contrast, no significant threshold changes were observed following acute and chronic treatment with diazepam. These data give further evidence for (a) an antiaversive effect of chronic treatment with fluoxetine, which caused a selective reduction in freezing behavior and neurovegetative responses associated with fearlike reaction elicited by dlPAG/lPAG electrical stimulation; (b) the involvement of the dlPAG and lPAG in the generation and organization of defensive responses and that freezing may probably be associated with panic attacks; and (c) the lack of effect of diazepam in this model is in line with its inefficacy as a panicolytic drug. The study of the unconditioned freezing behavior evoked by dlPAG/lPAG stimulation may constitute a new and interesting model for the study of panic disorder.

Keywords: Fear; Freezing; Panic attacks; Periaqueductal gray matter; Serotonin; γ-aminobutyric acid/benzodiazepine system

1. Introduction

Gradual increases in a progressive manner, in the intensity of electrical stimulation of the dorsolateral (dlPAG) and lateral (lPAG) aspects of the periaqueductal gray matter (PAG) elicit defensive behaviors characterized by alertness, freezing, and escape accompanied by autonomic responses (Coimbra et al., 1989; Brandão et al., 1990; Coimbra and Brandão, 1993, 1997; Eichenberger et al., 2002). It has been established that the brain and brainstem networks involved with the organization of fear-induced behaviors are composed of the medial hypothalamus, amygdaloid complex, and PAG (as a mesencephalic output). These structures constitute the main neural substrates for the integration of aversive states in the central nervous system (Olds and Olds, 1963; Graeff, 1990; Canteras, 2002; Comoli et al., 2003; Strauss et al., 2003; Sullivan et al., 2003). The midbrain tectum seems to be an important station of this encephalic aversive system (Graeff, 1990; De Oca et al., 1998; Canteras and Goto, 1999; Vianna et al., 2001a,b; Osaki et al., 2003). In fact, brainstem networks, such as neurons situated in the dorsal and lateral aspects of the PAG, in the deep layers of the superior and inferior colliculus, have often been included among the encephalic circuits involved with the generation and elabo-

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ration of defensive behavior (Brandão et al., 1988; Cardoso et al., 1992, 1994; Coimbra and Brandão, 1993; Coimbra et al., 2000; Eichenberger et al., 2002; Osaki et al., 2003).

Several lines of evidence clearly implicate GABAergic, cholinergic, serotoninergic, and opioid-mediated mechanisms in the control of neural substrates commanding defensive behavior in this aversive system (Bandler et al., 1985; Brandão et al., 1982; Graeff, 1990; Cardoso et al., 1992; Coimbra et al., 1992; Coimbra and Brandão, 1993, 1997; Leite-Panissi et al., 2003).

The involvement of GABA and serotonin in anxiety has been established through clinical and basic researches (Zhang et al., 2000; Netto et al., 2002). Several research laboratories have shown that serotonin- and γ-aminobutyric-acid-mediated mechanisms have a different role in the control of neural substrates involved with the organization of aversive responses in the midbrain tectum. Whereas γ-aminobutyric acid exerts a tonic regulation (Leão-Borges et al., 1988; Brandão et al., 1990; Zangrossi et al., 1999; Cruz-Morales et al., 2002), serotoninergic mechanisms phasically control these neural circuits (Castilho and Brandão, 2001; Castilho et al., 2002). However, the components of the defense reaction elicited by stimulation of the midbrain tectum have not been studied systematically. For instance, whereas escape behavior induced by stimulation of the brainstem has been the main focus of studies in this field, freezing behavior has received little attention, although a recent report showed evidence of benzodiazepinic and serotoninergic involvement in conditioned freezing evoked by electrical stimulation of the dorsal aspects of the PAG (Castilho et al., 2002). However, whereas in this article the defensive behavioral responses were studied after conditioned stimuli applied on the dorsal periaqueductal gray matter (dPAG), in the present article, the effect of acute and chronic treatment with a GABA/benzodiazepinic agonist and using a serotonin reuptake inhibitory drug were studied on the nonconditioned fear-induced responses.

The aim of the present article is to investigate the involvement of the γ-aminobutyric acid/benzodiazepine and serotonergic systems on the elaboration of the paniclike behavior elicited by electrical stimulation of the PAG taking into account that (i) freezing behavior induced by electrical stimulation of the dorsolateral aspects of the PAG is not clearly understood; (ii) serotonin and γ-aminobutyric acid have different roles in the regulation of the defense reactions generated at the midbrain tectum level; and (iii) no systematic study had ever been made on the effects of acute and chronic administration of fluoxetine on each component of the so-induced defense reaction.

2. Material and methods

2.1. Animals

Male Wistar rats from the animal house of the Campus of Ribeirão Preto of the University of São Paulo were used. These animals, weighing 220–250 g, were housed in individual Plexiglas-walled cages under a 12:12-h dark/light cycle (lights on at 0700 h) at 20 ± 1 °C, and given free access to food and water throughout the experiment. The experiments reported in this article were performed in compliance with the recommendations of the Brazilian Society of Neuroscience and Behavior (SBNeC), which are based on the U.S. National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

2.2. Surgery

The animals were anaesthetized with tribromoethanol (250 mg/kg ip) and fixed in a stereotaxic frame (David Kopf, USA). A bipolar brain electrode was implanted in the midbrain, aimed at the dlPAG. The electrodes were made of two twisted stainless steel wires, each 50 μm in diameter, insulated except at the cross section of the tip. The upper incisor bar was set at 2.5 mm below the interaural line such that the skull was horizontal between bregma and lambda. The electrode was introduced with a 16° angle using the following coordinates, with the lambda serving as the reference for each plane: anteroposterior, 0.0 mm; mediolateral, 1.9 mm; and dorsoventral, −5.1 mm (tip of the electrode), and fixed to the skull by means of acrylic resin and three stainless steel screws. The electrode wires were connected to male pins; these pins could be plugged into an amphenol socket at the end of a flexible electrical cable used for brain stimulation.

2.3. Apparatus

Four days after surgery, the rats were placed in an arena (circular enclosure, 60 cm in diameter and 50 cm high, with the floor divided into 12 sections) situated in an isolated room illuminated with a 40-W fluorescent lamp (350 lx at the arena floor level). The rats were allowed a 10-min period of habituation in the enclosure. The midbrain was stimulated electrically by means of a sine wave stimulator (Marseillan, 1977). The stimulation current was monitored by measuring the voltage drop across a 1K resistor with an oscilloscope (Philips, USA). The arena was cleaned with a 5% ammonium solution immediately before each animal was placed inside it.

2.4. Procedure

Independent groups of animals were used for evaluating the effects of each dose of the drugs on the aversive thresholds. In the first experiment, the effects of intraperitoneal (ip) injections of fluoxetine and diazepam were assessed in eight groups of animals: saline ($n = 8$), fluoxetine 5 mg/kg ($n = 10$), 10 mg/kg ($n = 13$), and 20 mg/kg ($n = 8$), saline + Tween ($n = 8$), diazepam 1 mg/kg ($n = 12$), 2 mg/kg ($n = 8$), and diazepam 4 mg/kg ($n = 10$). In the second experiment we evaluated the effects of the highest dose of fluoxetine (20 mg/

kg) and diazepam (4 mg/kg) on the exploratory activity and autonomic responses of four groups of rats ($n = 8$): saline, saline + Tween, fluoxetine, and diazepam.

In Experiment I, starting at 4 days after surgery, animals were placed in the experimental apparatus and remained undisturbed during a 10-min period for habituation. Then, the brainstem electrical stimulation procedure was started. The midbrain was stimulated electrically by means of a sine wave stimulator (Marseillan, 1977). Midbrain stimuli (AC, 60 Hz, 15 s) were presented at 1- to 5-min intervals with the current intensity increasing by steps of 5 μA for measurements of the aversive thresholds. Freezing threshold was defined as the lowest intensity, producing a complete immobility except for the respiration movements accompanied by at least two of the following responses: arching back, piloerection, defecation, micturition, exophthalmus, and ear retraction. As soon as the freezing threshold was determined, the electrical stimulation was stopped and started the recording of the freezing behavior during the intertrial period (1–5 min). Escape threshold was defined as the lowest current intensity that produced running or jumping in two successive ascending series of midbrain electrical stimulation. Animals with an escape threshold above 200 μA (peak-to-peak) were discarded from the experiment. After determining the baseline aversive thresholds, the animals received ip administration of fluoxetine, diazepam, or vehicle, and 15 min afterwards the aversive thresholds were redetermined (acute effects). The animals were treated daily during 14 days. Fifteen minutes after the 14th injection the animals were retested (chronic effects).

In the second experiment the rats were placed into an arena for 10 min for habituation. To record the behavioral responses, the animals were placed in the middle of the arena 15 min after the first (acute treatment), or 14th injection (chronic treatment). The following behavioral responses were scored for each minute during 30 min: number of crossing (number of floor sections traversed), number of "rearing" (standing with the forelegs raised in the middle of the arena or against the walls), and number of autonomic responses (micturition and defecation). These last neurovegetative manifestations were recorded by the determination of the number of events of elimination of excrement and urine. The open field was always cleaned before the introduction of each animal in the experimental protocol. The exploratory activity was recorded at 10, 20, and 30 min after injections, and autonomic responses, such as defecation and micturition, were systematically recorded during the 30 min of the experimental session.

2.5. Drugs

The drugs used in this study were fluoxetine (RBI, USA) at the doses of 5, 10, and 20 mg/kg, and diazepam (Sigma, USA) at the doses of 1, 2, and 4 mg/kg. Diazepam was mixed in Tween 80 and dissolved in physiological saline (0.9%). Fluoxetine was dissolved in physiological saline (0.9%). Systemic injections were given in a volume of 1 ml/kg. The control animals received treatments with saline or saline + Tween 80.

2.6. Histology

Upon completion of the experiments, the animals were deeply anaesthetized with urethane (1.25 g/kg; Sigma, USA) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.4). Brains were removed, immersed (4 °C) in the above fixative for 2 h, and then kept in 30% sucrose in 0.1 M PBS until soaked. Serial 60-μm brain sections were cut using a microtome, and then stained with methylene blue to locate the positions of the electrode tips sites in the midbrain according to Paxinos and Watson's atlas (1997).

2.7. Analysis of results

Data are reported as mean ± S.E.M. The freezing and escape thresholds measured following treatments were subjected to a two-way analysis of variance (ANOVA) with repeated measure. This analysis was performed on the freezing or escape behaviors measured after each drug

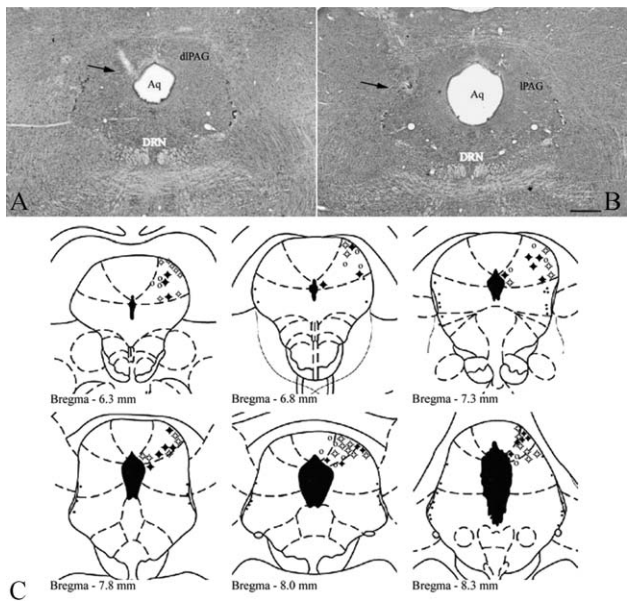

Fig. 1. Photomicrographs (A and B) of transversal sections of the brainstem of *Rattus norvegicus* (Wistar albino rats), at the level of periaqueductal gray matter (PAG). Arrows point to sites of electrical stimulation of the PAG, in dorsolateral (A) or lateral (B) aspects. Aq: sylvius aqueductus; dlPAG: dorsolateral column of PAG; lPAG: lateral column of the PAG; DRN: dorsal raphe nucleus. Bar: 369 μm. (C) Schematic representation of crosswise sections of the midbrain of *R. norvegicus*, illustrating the location of sites of electrical stimulation of the PAG, was performed in animals acutely (24 h) or chronically (14 days) treated with (○) saline (NaCl; 0.9%), (✦) fluoxetine, or (✧) diazepam in different doses.

Table 1
Effect of the acute (24 h) and chronic (14 days) treatments with saline (NaCl 0.9%), fluoxetine, or diazepam on the freezing and escape behaviors elicited by electrical stimulation of the periaqueductal gray matter

	Saline			Fluoxetine 5 mg/kg			Fluoxetine 10 mg/kg			Fluoxetine 20 mg/kg		
	BL	1st day	14th day	BL	1st day	14th day	BL	1st day	14th day	BL	1st day	14th day
Freezing	45.31	45.94	51.25	44.00	45.00	45.50	65.00	70.77	69.23	47.03	48.08	62.41
Escape	54.06	54.38	62.81	50.50	52.50	55.00	77.31	78.85	81.54	39.20	39.31	51.24
	Saline			Diazepam 1 mg/kg			Diazepam 2 mg/kg			Diazepam 4 mg/kg		
	BL	1st day	14th day	BL	1st day	14th day	BL	1st day	14th day	BL	1st day	14th day
Freezing	45.31	45.94	51.25	67.92	69.58	74.58	59.15	62.75	66.41	55.95	59.84	68.06
Escape	54.06	54.38	62.81	79.84	83.04	86.89	60.42	63.28	73.64	68.00	75.00	74.50

Data are presented as absolute values (μA). As there is no statistical difference ($P>.05$) between both control groups (saline and saline plus Tween 80), we decided to form one single control group with the obtained data.

treatment (fluoxetine or diazepam). Doses and days of treatment were the factors. Post hoc differences between treatment means were tested with Newman–Keuls comparisons. $P<.05$ was accepted as significant. The exploratory activity and the neurovegetative responses were also analysed by one-way ANOVA, followed as appropriate for Newman–Keuls post hoc test.

3. Results

All sites of electrical stimulation of the PAG were situated in the dlPAG or lPAG and are depicted on diagrams of the Paxinos and Watson atlas (1997) (Fig. 1). Absolute values of the electrical stimulation of the brainstem are provided in Table 1.

3.1. Effect of fluoxetine on fearlike responses

Freezing: Two-way ANOVA with repeated measures showed a statistical significant effect of treatment [$F(3, 43)=9.339$; $P<.001$], days [$F(1,43)=16.040$; $P<.001$], and interaction between treatment and days [$F(3,43)=8.591$; $P<.001$]. Post hoc Newman–Keuls test showed significant differences ($P<.001$) on the freezing threshold between the control group and the group chronically treated

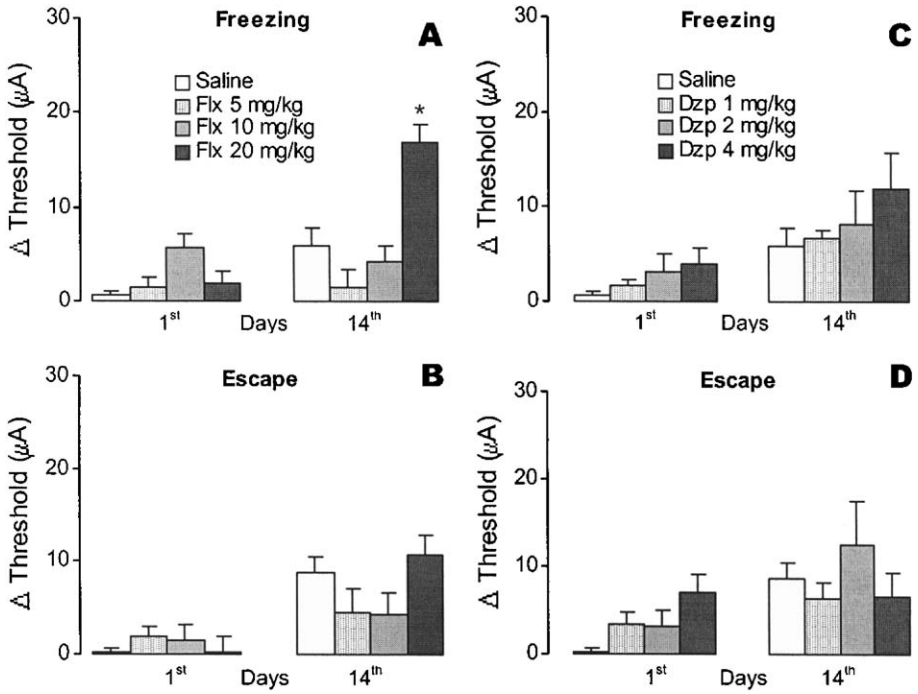

Fig. 2. Effect of peripheral (ip) administration of saline (0.9% NaCl; $n=16$) or fluoxetine at doses of 5 mg/kg ($n=10$), 10 mg/kg ($n=13$), and 20 mg/kg ($n=8$) and lack of effect of peripheral (ip) administration of diazepam at doses of 1 mg/kg ($n=12$), 2 mg/kg ($n=8$), and 4 mg/kg ($n=10$) on freezing (A and C) and escape (B and D) thresholds. The pharmacological drugs were administered acutely (24 h) or chronically (14 days). Defensive behaviors were elicited by electrical stimulation of the PAG. Columns represent means, and bars, the S.E.M. * $P<.001$, as compared to controls, according to Newman–Keuls test.

with fluoxetine 20 mg/kg (Fig. 2A). There was no significant effect of fluoxetine administered at lower doses (5 or 10 mg/kg) on the freezing threshold.

Escape: Two-way ANOVA did not show statistically significant effects of treatment [$F(3,43)=0.839$; $P>.05$], although it showed a significant effect of days [$F(1,43)=18.262$; $P<.001$], but not for the interaction between treatment and days [$F(3,43)=2.002$; $P>.05$]. Post hoc Newman–Keuls test did not reveal statistically significant differences between control and groups treated acutely or chronically with any dose of fluoxetine (Fig. 2B).

3.2. Effect of diazepam on fearlike responses

There was no significant effect of acute or chronic treatment with diazepam on the freezing threshold evoked

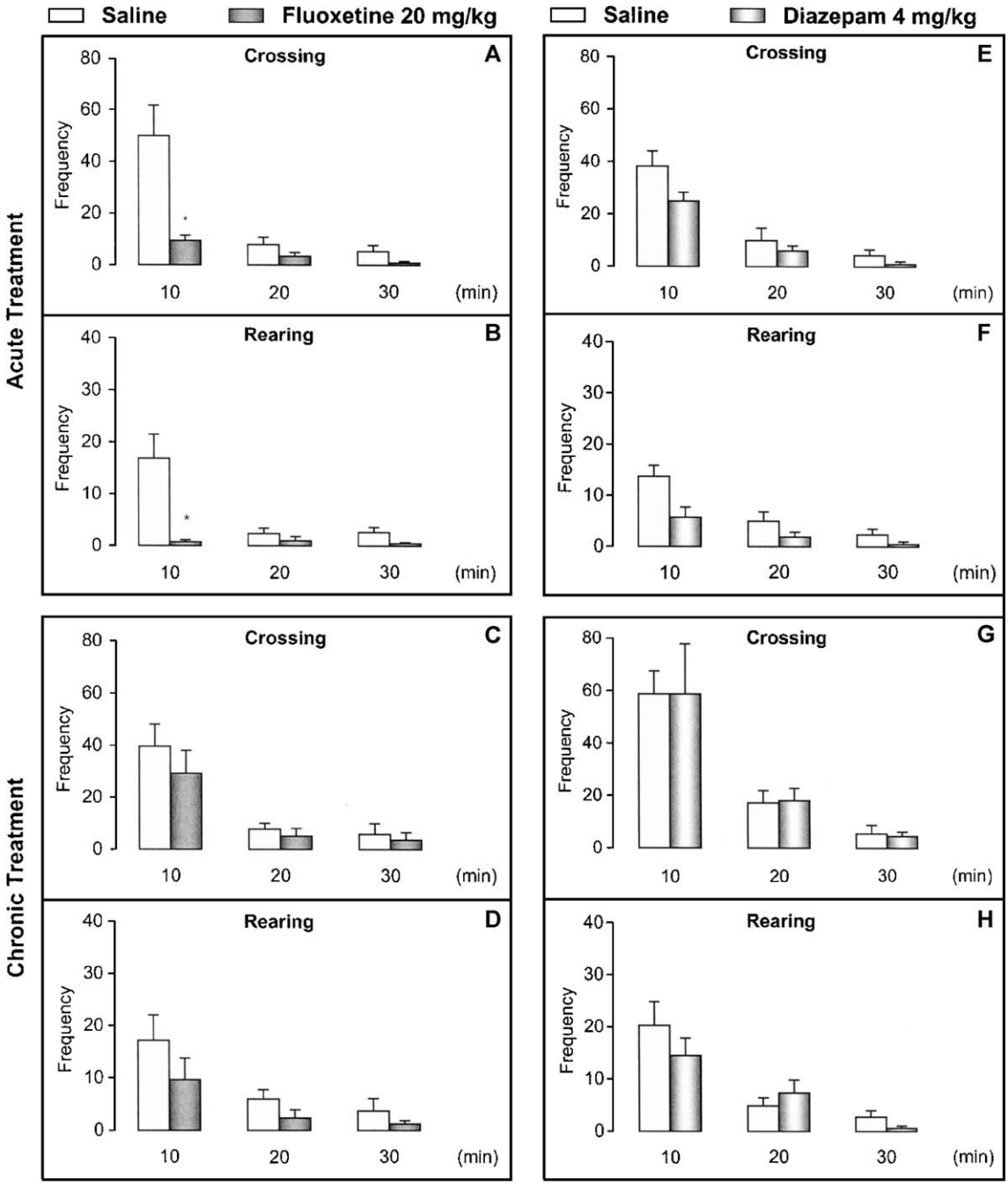

Fig. 3. Effect of peripheral (ip) administration of saline (0.9% NaCl) or fluoxetine at a dose of 20 mg/kg and saline plus Tween 80 or diazepam (at a dose of 4 mg/kg) plus Tween 80 on anticipatory anxiety-like behavioral responses elicited by *R. norvegicus* exposed to open-field test. The mean incidence of crossings (A, C, E, and G) and rearings (B, D, F, and H) was recorded at 10, 20, and 30 min of exposure. The pharmacological drugs and their vehicle were administered acutely (24 h) or chronically (14 days). Columns represent means, and bars, the S.E.M. ($n=8$); * $P<.05$, as compared to controls, according to Newman–Keuls test.

by electrical stimulation of the PAG. Two-way ANOVA with repeated measures did not show statistically a significant effect of treatment [$F(3,42)=2.576$; $P>.05$], although it showed a significant effect of days [$F(1,42)=17,420$; $P<.001$], but not for the interaction between treatment and days [$F(3,42)=0.257$; $P>.05$]. Post hoc comparisons with Newman–Keuls test did not show any significant differences between the control and group acutely or chronically treated with any dose ($P>.05$ in all cases) (Fig. 2C).

Additionally, there was no significant effect of acute or chronic treatment with diazepam for any doses used in the present work (1, 2, or 4 mg/kg) on the escape threshold (Fig. 2D). In fact, two way ANOVA did not show any significant effects of treatment [$F(3,42)=0.913$; $P>.05$] or interaction between treatment and days [$F(3,42)=2.737$; $P>.05$], but showed statistically significant effects of days [$F(1,42)=13.044$; $P<.001$].

Saline-treated groups showed a tendency of increasing effect on the 14th day of the test when compared to the first day, but this was not statistically significant.

3.3. Effect of acute or chronic treatment with fluoxetine or diazepam on the exploratory behavior in the open-field test

The effects of acute and chronic blockade of serotonin uptake with fluoxetine were examined at three time windows of 10 min each. One-way ANOVAs showed a statistically significant effect of treatment [$F(1,14)$ varying from 5.29 to 5.50; $P<.05$ in both cases] only considering the acute ip administration, at the first 10 min, on the mean incidence of crossings and rearing behaviors, which decreased with fluoxetine at a dose of 20 mg/kg (Newman–Keuls test; $P<.001$ in both cases) (Fig. 3A and B). The ethologic performance of the animals acutely or chronically treated with saline or fluoxetine (20 mg/kg) in the other temporal windows did not show any statistical differences (Newman–Keuls test; $P>.05$ in all cases) (Fig. 3C and D).

The same analysis applied on the data collected after the acute or chronic treatments with diazepam at the dose of 4 mg/kg showed that there was no significant effect of the acute and chronic treatments on the mean incidence of

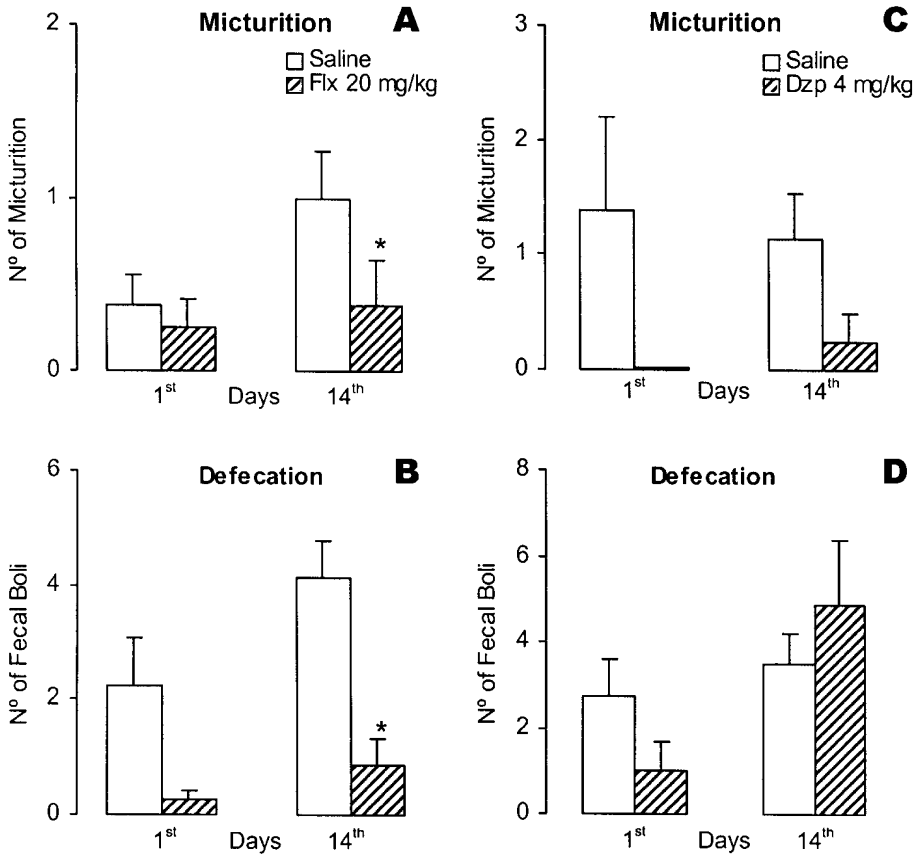

Fig. 4. Effect of peripheral (ip) administration of saline (0.9% NaCl) or fluoxetine at a dose of 20 mg/kg and lack of effect of peripheral (ip) administration of diazepam at a dose of 4 mg/kg on neurovegetative responses elicited by *R. norvegicus* exposed to open-field test. The mean incidence of micturition (A and C) and defecation (B and D) were recorded during 30 min of exposure. The pharmacological drugs and their vehicle were administered acutely (24 h) or chronically (14 days). Columns represent means, and bars, the S.E.M. ($n=8$); $*P<.05$, as compared to controls, according to Newman–Keuls test.

crossings or rearings (Newman–Keuls test; $P>.05$ in all cases). These data are shown in Fig. 3E–H. There was a progressive decrease in the behavioral exploratory activity elicited from the first minute to 30 min of exposition on the open field in all experimental groups.

3.4. Effect of fluoxetine or diazepam on the autonomic responses recorded in the open-field test

The chronic treatment with fluoxetine (20 mg/kg) decreased the mean incidence of micturition in the open-field test (Fig. 4A). Two-way ANOVA, repeated measures, showed a statistically significant effect of treatment [$F(1,14)=21.816$; $P<.001$] and days [$F(1,14)=4.682$; $P<.05$], but no interaction between treatment and days [$F(1,14)=1.170$; $P>.05$]. Post hoc Newman–Keuls test showed significant differences of chronic treatment with fluoxetine ($P<.01$) compared to the control.

Evidence of a decrease in the autonomic responses after the chronic blockade of the serotonin uptake with fluoxetine was corroborated by a similar decrease in the mean incidence of defecation (Fig. 4B) in the open-field test (Newman–Keuls; $P<.05$). Two-way ANOVA showed a significant effect of days [$F(1,14)=4.67$; $P<.05$], but not treatment [$F(1,14)=2.0$; $P>.05$], or Treatment × Days interaction [$F(1,14)=2.07$; $P>.05$]. There was a lack of effects of acute treatment with fluoxetine (20 mg/kg) on the neurovegetative responses elicited in the open-field test (Newman–Keuls; $P>.05$ in all cases) (Fig. 4A and B).

The acute or chronic treatment with diazepam, however, did not cause similar antiaversive effects (Newman–Keuls; $P>.05$ in all cases) (Fig. 4C and D). There was a tendency in increasing (not statistically significant) the autonomic reactions on the 14th day test when compared to the first-day test.

4. Discussion

The present results confirm the involvement of dlPAG and lPAG in the generation and elaboration of fear-evoking defensive responses. The neural substrate involved with the aversive behavior organization in these regions was susceptible to the chronic treatment with fluoxetine, but not diazepam. Considering that the freezing threshold was increased by chronic blockade of serotonin reuptake, it is possible that the paniclike responses are mediated by a dysfunction of serotonergic system in the dorsolateral and lateral aspects of the PAG. In fact, the dorsal and lateral columns of the PAG are pointed out in the literature as important sites from which the defensive behavior may be organized and modulated. Behavioral defensive reactions are elicited in dangerous situations or through electrical and chemical stimulation of the mesencephalic tectum in experimental approaches (Graeff, 1990; Canteras and Goto, 1999; Vianna et al., 2001a,b; Eichenberger et al., 2002). This PAG stimulation procedure has been suggested as an experimental model of panic attack in humans (Deakin and Graeff, 1991; Jacob et al., 2002; Zanoveli et al., 2003).

In the present work, the electrical stimulation of the dPAG clearly induced aversive responses, which were characterized by alertness, followed by freezing and escape behavior. In humans, the spontaneous and recurrent panic attacks consist of the basic condition of the panic syndrome. This neuropsychiatric disease is a chronic anxiety disorder characterized by intense fear accompanied by symptoms of cognitive and autonomic arousals that peak within 10 min. Phobic avoidance of situations in which attacks have occurred or any situation in which help might be unavailable or ease escape difficult in the event of an attack is a frequent complication (Pollack and Marzol, 2000).

Interestingly, although patients commonly report sensations of imminent danger and fear, they elicit a behavioral inhibition characterized by defensive immobility (a "blockade while walking," according to some patient reports). This behavioral response is commonly associated with neurovegetative manifestations and phobic avoidance. In addition, some patients report fear of losing control of anal and vesical sphincters (Cassano and Savino, 1993). Despite recent evidence of the involvement of the nucleus raphe magnus in hypoxia-induced hyperventilation (Gargaglioni et al., 2003), autonomic responses, such as increases in breathing and in mean blood pressure and heart rate, are reported following behavioral reactions evoked by stimulation of the dorsal aspects of PAG and also of the deep layers of the superior colliculus (Leão-Borges et al., 1988; Brandão et al., 1994; Vargas et al., 2000). In humans, the electrical stimulation of the dPAG induces intense anxiety, panic, and a sensation of imminent death (Nashold et al., 1969). These findings are in accordance with our present results that pointed out the involvement of the dorsomedial and mainly dlPAG and deep layers of the superior colliculus in the control of behavioral manifestations of fear. However, our results showed that acute treatment with fluoxetine did not cause a significant effect on freezing or escape thresholds. As a matter of fact, it is known that in clinical treatment for panic disorder, the antipanic effect does not occur immediately after the use of typical medicines, but only after 2 weeks of chronic treatment (Guimarães et al., 1987; Londborg et al., 1998; Pohl et al., 1998; Pollack et al., 1998). In fact, in the present work, the chronic administration of fluoxetine (20 mg/kg) caused an attenuation of the freezing threshold and decreased the neurovegetative responses that follow the elicitation of fear, such as micturition and defecation. These results are corroborated by the findings of Zhang et al. (2000), showing that inhibitors of reuptake of serotonin reduced either the acquisition or the expression of freezing behavior during exposure to the conditioned stress. In addition, there is evidence that conditioned freezing is also under control of both benzodiazepine and serotoninergic mechanisms (Castilho et al., 2002). Curiously, during panic attack, patients report sensations of imminent death,

palpitations, and intense fear, but although they often report the intention of escape and phobic avoidance of places and situations in which the panic attack occurred, the usual behavior evoked by them is characterized by defensive immobility, in a behavioral expression very similar to freezing responses displayed by experimental animals (Cassano and Savino, 1993; Vargas and Schenberg, 2001).

The increase in the freezing threshold induced by chronic treatment with fluoxetine observed in the present study cannot be due to a motor deficit induced by nonexpected action of the antagonist on the motor system because the animals chronically treated with fluoxetine did not show a significant decrease in the mean incidence of crossings and rearing. Therefore, the antipanic effects of the chronic blockade of the uptake of serotonin are the result of improvement of the serotonergic neurotransmission on the neural substrates involved in generating and organizing the defensive behavior. However, reduction in crossing and rearing with the acute administration of fluoxetine was observed, possibly due to the sedative effect of this drug, already reported by patients in the beginning of the treatment (Nutt, 1990). In clinical settings, panic disorder does not respond to the acute treatment, and hence commonly used medicine, such as clomipramine, paroxetine, and fluoxetine must be chronically administered to achieve their therapeutic efficacy on the control of panic crises (Caetano, 1985). The progressive decrease in the behavioral exploratory activity elicited from the first to the last of the 30 min of exposition on the open field observed in all experimental groups, either in acute or in the chronic pharmacological treatment, was probably due to the habituation in the arena. However, the motivational effect of the contact of rodents in the experimental enclosure was similar for both acute (first day) and chronic (14th day) experimental groups. The apparent increasing effect of control groups in the 14th day test, when compared to the first electrical stimulation of the brainstem, was not statistically significant. The small but consistent increase in escape thresholds across days is usually observed in rats chronically implanted with brain electrodes.

Humans and other animals show an increase of autonomic responses in dangerous or stressful situations (Miyata et al., 1992). Anxiolytic drugs, such as diazepam, inhibit the increase of peristaltic waves of the intestinal straps during exposition to stressful situation in man (Narducci et al., 1985). Encephalic substrates involved in stress causing defecation recruit many neurotransmitters, such as catecholamines, β-endorphin, and serotonin (Sullivan and Gratton, 1999). Fluoxetine may exert its inhibitory effect on defecation possibly through pharmacological action on the neurovegetative network involved in the regulation of gastrointestinal motility or influencing the neural circuits that elaborate anxiety. In agreement with this possibility, Zhang et al. (2000) showed evidence that the chronic use of fluoxetine induces behavioral anxiolytic effects and inhibits defecation in 60% of the cases studied. Our results suggest that the effects of chronic treatment with fluoxetine on these neurovegetative responses may be related to the increase of thresholds of fear in the midbrain tectum. Considering the saline-treated groups, the tendency of increasing in the autonomic reactions in the 14th-day test when compared to the first-day test may be due to the reexposition of the animals in the open field, an experimental model that may represent an aversive stimulus to albino rodents.

On the other hand, the acute or chronic treatment with diazepam did not change either freezing or escape thresholds elicited by nonconditioned stimulation of the dorsal and dorsolateral aspects of the periaqueductal gray matter. Despite evidence in the literature suggesting the involvement of benzodiazepine mechanisms in conditioned freezing induced by electrical stimulation of the dorsal column of the PAG (Castilho et al., 2002), the present results, showing the lack of effects of diazepam on the freezing and escape thresholds organized in the similar network, may suggest that unconditioned freezing is more appropriately related to panic syndrome than conditioned defensive behavior. As a matter of fact, we might consider the possibility of existing fine neuroanatomical differences in the functional neural circuitry situated inside the dorsal and dorsolateral aspects of the periaqueductal gray matter, responding to conditioned and/or unconditioned stimulus, whose activity may organize behavioral responses in dangerous situations, or in case of chronic anxiety disorder, such as the panic syndrome. In fact, a recent report showed differences in the genic expression in the neural network of the PAG and in other limbic structures after acute and chronic treatment with fluoxetine in stressed and nonstressed animals (Lino-de-Oliveira et al., 2001).

There was also a lack of effect of diazepam on the mean incidence of crossing and rearing in the open-field test after both acute and chronic treatments. It is known that the use of high doses of benzodiazepines for panic treatment consists in the main disadvantage of the use of these compounds. The advantage of using benzodiazepines for panic disorder is their rapid therapeutic effect on the control of anticipatory anxiety that can remain even after the pharmacological effect of the chronic blockade of 5-hydroxytryptamine reuptake. It is interesting to note that neither the freezing or escape thresholds nor the neurovegetative responses studied in the present work were significantly modified by the acute or chronic treatments with diazepam. In fact, benzodiazepines are not the first-choice drug in the treatment of panic disorder, being used in low doses for generalized anxiety (Nutt, 1990). Our data are in agreement with these findings, showing an increase of the freezing threshold after chronic treatment with fluoxetine, but not with diazepam. As fluoxetine exerts its pharmacological effect inhibiting the reuptake of serotonin in the presynaptic terminals, serotonergic circuits may be crucially involved in the elaboration of panic syndrome in the PAG.

The present results are in agreement with the hypothesis that electrical stimulation of the dorsal and lateral aspects of

the periaqueductal gray may be used as an unconditional stimulus for the elaboration of defensive reactions, and may become an interesting model of panic, contributing to the comprehension of the functional neuroanatomy of the elaboration of fear and anxiety disorder. dlPAG and lPAG are strong candidates as loci of neuromorphological and neurochemical mechanisms mediating panic syndrome.

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