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Vilela-Costa, Heloisa H.

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ROLE OF 5-HT$_{1A}$ AND 5-HT$_{2C}$ RECEPTORS OF THE DORSAL PERIAQUEDUCTAL GRAY IN THE ANXIETY- AND PANIC-MODULATING EFFECTS OF ANTIDEPRESSANTS IN RATS

Heloisa H. Vilela-Costa [1], Jhonatan Christian Maraschin [1], Plinio C. Casarotto [2], Ana Beatriz Sant'Ana [1], Valquiria C. de Bortoli [3], Maria Adrielle Vicente [1], Alline Cristina de Campos [1], Francisco S. Guimarães [1], Helio Zangrossi Jr [1]*

[1] Department of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo (USP), Ribeirão Preto, SP, Brazil. [2] Neuroscience Center - HILIFE, University of Helsinki, Finland. [3] Department of Health Sciences, Federal University of Espírito Santo (UFES), São Mateus, ES, Brazil.

*Corresponding author. School of Medicine of Ribeirão Preto, University of São Paulo. Av. Bandeirantes, 3900, Ribeirão Preto, Brazil, CEP: 14049-900.
E-mail address: zangross@fmrp.usp.br

Abstract

Antidepressant drugs are first-line treatment for panic disorder. Facilitation of 5-HT$_{1A}$ receptor-mediated neurotransmission in the dorsal periaqueductal gray (dPAG), a key panic-associated area, has been implicated in the panicolytic effect of the selective serotonin reuptake inhibitor fluoxetine. However, it is still unknown whether this mechanism accounts for the antipanic effect of other classes of antidepressants drugs (ADs) and whether the 5-HT interaction with 5-HT$_{2C}$ receptors in this midbrain area (which increases anxiety) is implicated in the anxiogenic effect caused by short-term treatment with ADs. The results showed that previous injection of the 5-HT$_{1A}$ receptor antagonist WAY-100635 in the dPAG blocked the panicolytic-like effect caused by chronic systemic administration of the tricyclic AD imipramine in male Wistar rats tested in the elevated T-maze. Neither chronic treatment with imipramine nor fluoxetine changed the expression of 5-HT$_{1A}$ receptors in the dPAG. Treatment with these ADs also failed to significantly change ERK1/2 (extracellular-signal regulated kinase) phosphorylation level in this midbrain area. Blockade of 5-HT$_{2C}$ receptors in the dPAG with the 5-HT$_{2C}$ receptor antagonist SB-242084 did not change the anxiogenic effect caused by a single acute injection of fluoxetine or imipramine in the Vogel conflict test. These results reinforce the view that the facilitation of 5-HT$_{1A}$ receptor-mediated neurotransmission in the dPAG is a common mechanism involved in the panicolytic effect caused by chronic administration of ADs. On the other hand, the anxiogenic effect observed after short-term treatment with these drugs does not depend on 5-HT$_{2C}$ receptors located in the dPAG.

Key words: *panic, anxiety, serotonin, dorsal periaqueductal gray, imipramine, fluoxetine*

1. Introduction

In addition to their role in the clinical management of mood disorders, selective serotonin reuptake inhibitors (SSRIs; e.g., fluoxetine and escitalopram) are first-line treatment for several anxiety pathologies, such as generalized anxiety and panic disorder (PD) [1]. Tricyclic antidepressants, such as imipramine, are equally effective [2]. However, important side effects have restricted the use of this class of antidepressants drugs (ADs) to specific situations and conditions [1,3].

Both SSRIs and tricyclic ADs are only effective after chronic treatment. Full beneficial effects are usually observed after 2 to 4 weeks of continuous administration [1,4,5]. At the beginning of treatment, these drugs may worsen anxiety and negatively impact patient adherence to the therapy [1].

Several hypotheses have been formulated to explain the delayed therapeutic action of ADs, which mainly focus on their effects in managing depression. One of the most prominent was that proposed by Pierre Blier and Claude de Montigny [6]. These authors suggest that a net increase in 5-HT neurotransmission in key areas, such as the hippocampus and frontal cortex, is required for ADs' mood-relieving effects. However, after acute treatment ADs raise 5-HT concentrations in raphe nuclei (e.g., dorsal and median raphe nuclei), where it binds to inhibitory somatodendritic 5-HT$_{1A}$ receptors. This leads to a decrease in the firing rate of 5-HT neurons and consequently inhibits 5-HT release. After repeated treatment, these somatodendritic 5-HT$_{1A}$ receptors desensitize, allowing a greater availability of 5-HT in terminal areas [7], which leads to antidepressant effect.

In recent years, we have focused on the generality of this proposal in the context of AD effects in anxiety, particularly in generalized anxiety and panic disorders [for extensive reviews, see 8-10]. More specifically, we have

focused on the short- and long-term consequences of AD treatment on 5-HT neurotransmission in the amygdala and the dorsal periaqueductal gray (dPAG).

Our results suggest that the facilitation of 5-HT_{1A} receptor-mediated neurotransmission in the dPAG accounts for the panicolytic effect of ADs. Previous investigations have shown that 5-HT in the dPAG, through its interaction with 5-HT_{1A} receptors, inhibits the expression of panic-related defensive behaviors, specifically escape/flight responses [11-15]. Chronic, but not short-term administration of imipramine, sertraline, or fluoxetine, enhances the anti-escape effect caused by dPAG injections of 5-HT or 5-HT_{1A} receptor agonists, indicating that these ADs increase the responsiveness of this receptor [16-19]. Furthermore, chronic, but not acute, administration of fluoxetine increases 5-HT release in this area, and microinjection of the 5-HT_{1A} receptor antagonist WAY-100635 in the dPAG blocked the anti-escape effect of long-term systemic administration of fluoxetine [20]. Therefore, repeated treatment with fluoxetine can cause a net enhancement of 5-HT_{1A} receptor-mediated neurotransmission in the dPAG and consequently inhibits the expression of panic-associated defensive behaviors.

On the other hand, previous studies have shown that activation of 5-HT_{2C} receptors in the dPAG or the basolateral nucleus of the amygdala (BLA) enhances anxiety in different animal models [21-24]. Vicente and Zangrossi [23] reported that previous microinjection of the 5-HT_{2C} receptor antagonist SB-242084 into the basolateral nucleus of the amygdala counteracted the anxiogenic effect of acute administration of fluoxetine or imipramine in the Vogel conflict test. It is still unknown whether 5-HT_{2C} receptors located in the dPAG may be equally involved in the anxiogenic effect observed after short-term administration of ADs.

In the present study, we first evaluated whether administration of WAY-100635 into the dPAG also blocks the anti-escape effect of chronic treatment with imipramine in rats submitted to the elevated T-maze [for a full description of this test, see 9]. This experiment was performed to verify the generality of the results with fluoxetine. Next, we investigated the effects of chronic treatment with imipramine or fluoxetine on the levels of 5-HT_{1A} receptor protein and ERK1/2 (extracellular-signal regulated kinase) phosphorylation (pERK) in the dPAG. Finally, we evaluated whether the previous injection of SB-242084 in the dPAG interferes with the anxiogenic effect caused by a single systemic administration of fluoxetine or imipramine in the rat Vogel conflict test.

2. Material and Methods

2.1 Animals

Male Wistar rats weighing 290 to 310 g on the day of surgery (n = 152) were group-housed in groups of 4 under a 12-h light/dark cycle (lights on 07:00 hours) at 22±1°C with free access to food throughout the experiment, except during testing. Water was also freely available, except in the experiments with the Vogel conflict test where they were submitted to periods of deprivation (see below). All experiments described in this study were approved by the Experimental Animal Ethical Committee of the University of São Paulo (protocol no. 034/13), which follows the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation (COBEA).

2.2 Elevated T-maze

The elevated T-maze was made of wood and had three arms of equal dimensions (50×12 cm). One arm, enclosed by 40-cm-high walls, was perpendicular to two opposed open arms. To avoid falls, the open arms were surrounded by a 1-cm-high Plexiglas rim. The entire apparatus was elevated 50 cm above the floor.

2.3 Open field

The open-field test was used to assess possible drug effects on locomotion. The test was performed in a wooden square arena (60×60 cm) with 30-cm-high walls. Luminosity at the level of the T-maze arms and open field was 60 lx.

2.4 Vogel Conflict Test

The Vogel conflict test was performed as described by Pelosi et al. [25] in a Plexiglas box (length 42 cm, width 25 cm, height 20 cm) with a stainless-steel grid floor. A metallic spout of a drinking bottle containing water projected into the box. Animal contact with the spout and grid floor closed an electrical circuit controlled by a sensor (Insight Instruments, Brazil), which produced 7 pulses/s whenever the animal was in contact with both components. Each pulse was considered a lick, and at every 20 licks the animal received a 0.5-mA shock for 2 s. The sensor recorded the total number of licks and shocks delivered during the test period. The apparatus was located inside a sound-attenuated cage.

Tests were conducted in a sound-attenuated room, with an air exhaust fan as source of white background noise. The rats' exploratory behavior during test were recorded by a camera positioned above these apparatuses.

After each experimental session, the models were cleaned with a 20% ethanol solution.

2.5 Drugs

The following drugs were used: N-(2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl)-N-2-pyridinyl cyclohexanecarboxamide maleate (WAY-100635, 5-HT_{1A} receptor antagonist; Sigma-Aldrich, St Louis, USA), 6-chloro-2,3-dihydro-5-methyl-N-[6-[(2-methyl-3-pyridinyl)oxy]-3-pyridinyl]-1H-indole-1-carboxyamide dihydrochloride (SB-242084, 5-HT_{2C} receptor antagonist; Tocris, Bristol, UK), imipramine hydrochloride (Sigma- Aldrich, St Louis, USA), and fluoxetine hydrochloride (EMS, Hortolandia, Brazil). All drugs were freshly prepared and administered intraperitoneally (i.p.) at 1 ml/kg. Drugs were dissolved in sterile saline except for fluoxetine, which was dissolved in a solution containing sterile saline with 2% Tween-80.

2.6 Surgery

Animals were deep anaesthetized with 2,2,2-tribromoethanol (250 mg/kg i.p.) followed by local anesthesia (2% lidocaine with vasoconstrictor) and placed in a stereotaxic frame. A stainless-steel guide cannula (12-mm long; outer and inner diameter 0.6 and 0.4 mm, respectively) was implanted 2 mm above the dPAG. The guide cannulae were implanted according to the coordinates of the rat brain atlas by Paxinos & Watson [26] as follows: holding the incisor bar 2.5 mm below the horizontal plane, 2.4 mm anterior to the interaural line, and 1.9 mm lateral to the midline at an angle of 22° with the sagittal plane until the cannula tip was 3.2 mm below the surface of the skull. The guide cannulae were fixed to the skull with acrylic resin and two stainless-steel screws. Stylets the same length as the guide cannulae were introduced inside them to prevent obstruction. At the end of surgery, all animals were injected intramuscularly with 0.3 ml of antibiotic preparation (benzylpenicillin and streptomycin, Pentabiotico Veterinário Pequeno Porte, Brazil) to prevent possible infections. In addition, flunixin meglumine (Schering-Plough, Brazil; 2.5 mg/kg), a drug with analgesic, antipyretic, and anti-inflammatory properties, was administered subcutaneously for post-surgery analgesia. The animals were left undisturbed for 5 to 7 days after the surgery except for normal handling during cage cleaning.

2.7 Procedures

Experiment 1 - Administration of WAY-100635 into the dPAG and the effects of chronic imipramine in the elevated T-maze and open-field tests

The elevated T-maze test was performed as previously described by Zanoveli et al. [20]. Briefly, rats were injected daily (i.p.) with imipramine (15 mg/kg) or saline solution (*n*=8-9) for 21 consecutive days. Stereotaxic surgery was performed on day 14 after the beginning of drug treatment. On day 20 after the beginning of drug or saline treatment, each animal was pre-exposed to one of the open arms of the elevated T-maze for 30 min [for further details see 9]. The next day, 10 min before the last injection of imipramine or saline, animals of each group were injected with WAY 100635 (0.37 nmol) or saline into the dPAG, forming the following groups: Saline dPAG/Saline i.p. (*n*=8), Saline dPAG/Saline i.p. (*n*=9), WAY dPAG/Saline i.p. (*n*=8), and WAY dPAG/Imipramine i.p. (*n*=8). This dose of WAY 100635 in the dPAG counteracts the anti-escape effect caused by chronic systemic injection of fluoxetine in rats tested in the elevated T-maze after [20].

A needle (0.3-mm outer diameter) was introduced through the guide cannula until its tip was 2 mm below the cannula end. A volume of 0.2 µl was injected over a period of 2 min (0.1 µl/min) using a 5 µl microsyringe (Hamilton 701-RN, USA) attached to a microinfusion pump (KD Scientific, USA). The displacement of an air bubble inside the polyethylene catheter connecting the syringe needle to the intracerebral needle was used to monitor the microinjection. The needle was removed 1 min after the end of the injection. The animals were tested in the elevated T-maze 30 min after the last injection of imipramine or saline (i.e. 40 min after injections in dPAG).

The test in the elevated T-maze was started by measuring inhibitory avoidance. For this aim, each animal was placed at the distal end of the enclosed arm of the elevated T-maze facing the intersection of the arms. The time taken by the rat to leave this arm with four paws was recorded (baseline latency). The same measurement was repeated in two subsequent trials (avoidances 1 and 2) at 30-s inter-trial intervals. Following the avoidance task (30 s), each animal was placed at the end of the same previously experienced open arm and the latency to leave this arm with four paws was recorded three consecutive times (escapes 1, 2, and 3), again with 30-s intervals. A cut-off time of 300 s was established for avoidance and escape latencies.

To assess putative drug effects on motor performance, immediately after testing in the elevated T-maze, the total distance traveled by each animal in the open field was evaluated for 5 minutes and analyzed by a video-tracking system (Ethovision, Holland).

Experiment 2 – Western blotting analyses

Rats were injected daily (i.p.) with imipramine (15 mg/kg), fluoxetine (10 mg/kg), or vehicle solution (*n*=9 for each group) for 21 consecutive days. Three hours after the last injection, the

animals were deeply anesthetized with 2,2,2-tribromoethanol (250 mg/kg i.p.) and decapitated by a guillotine. The dPAG was isolated using a punching needle (2.0-mm internal diameter). The tissue was mechanically homogenized in microcentrifuge tubes in RIPA buffer [50mM Tris-HCl pH 8.0; 150mM NaCl; sodium deoxycholate 0.5%; 1.0% triton X-100 and 0.1% sodium dodecyl sulfate] containing a cocktail of protease inhibitors (1 mM SIGMAFAST™, Sigma-Aldrich, St. Louis, USA) and phosphatase inhibitors [1 mM NaF and 1 mM sodium orthovanadate]. Following homogenization, samples were centrifuged at 12000 x g cycles for 20 min at 4ºC. The supernatant containing the proteins was collected and stored at -80ºC until use. The concentration of total proteins was measured by the Bradford method [27].

The levels of 5-HT_{1A} receptors and pERK were determined by western blotting. Briefly, dPAG samples (40 µg total protein) were loaded and separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane (0.45 µm, Amersham™ Protran™, GE Healthcare, Germany). After blocking with 5% bovine serum albumin (Sigma- St. Louis, USA) in TBST buffer (20 mM Tris-HCl; 150 mM NaCl; 0.05% Tween20), membranes were incubated subsequently with primary antibodies (rabbit anti-5-HT_{1A} 1:10000; ThermoFisher, #PA5-28090- Waltham, Massachusetts, USA) overnight at 4°C followed by mouse monoclonal anti-GAPDH (1:5000 Sigma-Aldrich, #G8795- St. Louis, USA) for 2 h at room temperature, rabbit anti-phospho-ERK1/2 (1:1000; Cell Signaling, #9101, Danvers, Massachusetts, USA), and mouse anti-ERK1/2 (1:1000; Cell Signaling #4696, Danvers, Massachusetts, USA) overnight at 4°C. After each incubation with primary antibodies, the membranes were washed and incubated with anti-rabbit IgG HRP-conjugated antibody (1:5000, Cell Signaling, #7074- Danvers, Massachusetts, USA) or anti-mouse IgG (H+L) peroxidase labeled antibody, (1:5000, KPL #04-18-06- Gaithersburg, MD, USA), for 1 h at room temperature. Chemiluminescence was detected by Amersham™ ECL™ Prime (Amersham, #RPN2232, Little Chalfontt, UK) in ChemiDoc™ XRS+, BioRad, Hercules, California, USA) and quantified using ImageJ software (v.1.53a, National Institutes of Health, Bethesda, Maryland, USA). The optical densities relative to 5-HT1A receptors and phospho-ERK1/2 were normalized by GADPH and total ERK1/2, respectively. The data were expressed as percentage of the control group.

Experiment 3 - Administration of SB-242084 into the dPAG and the effects of acute imipramine or fluoxetine in the Vogel conflict test

Animals were water-deprived for 48 h prior to the test. After the first 24 h of deprivation, they were pre-tested in the experimental cage to locate the spout of the bottle containing water. During the pre-test session, rats were allowed to drink water freely for 3 minutes. Animals that failed to locate the spout were not included in the experiment. Twenty-four hours later, animals were injected (see procedure above) with SB-242084 (10 nmol) or saline in the dPAG 10 minutes before systemic injection (i.p.) of imipramine (10 mg/kg) or saline or fluoxetine (15 mg/kg) or vehicle solution. Animals were returned to the test cage 30 min later. The test period lasted for 3 min and the animals received a 0.5-mA shock for 2 s through the bottle spout every 20 licks. The following groups were formed: A) Saline dPAG/Saline i.p. (n=8), Saline dPAG/Imipramine i.p. (n=8), SB dPAG/Saline i.p. (n=8), and SB dPAG/Imipramine i.p. (n=7); B) Saline dPAG/Vehicle i.p. (n=11), Saline dPAG/Fluoxetine i.p. (n=8), SB dPAG/Vehicle i.p. (n=9), and SB dPAG/Fluoxetine i.p. (n=9).

The dose of SB-242084 was chosen based on a study showing that in the dPAG it blocks the anxiogenic effect of serotonin in the elevated T maze [24].

2.8 Histology

At the end of the behavioral experiments, animals from experiments 1 and 3 were euthanized with a lethal dose of 2,2,2-tribromoethanol and 0.2 µL of Evans blue was microinjected into dPAG to mark the drug injection site. The brain was then perfused intracardially with saline solution (0.9%) followed by 10% formalin solution before being removed and fixed in 10% formalin. Brain slices of 40 µm were obtained with a cryostat to localize drug injection sites according to the atlas of Paxinos & Watson [26]. Only data from rats with injection sites located within the dPAG (dorsomedial or dorsolateral subnuclei) were included in the statistical analysis.

2.9 Statistical analysis

Repeated-measures analysis of variance (ANOVA) was used to analyze both avoidance and escape data from the elevated T-maze, with systemic and central treatments as independent factors and trials (baseline, avoidance 1 and 2; or escape 1, 2, and 3 latencies) as repeated measures. Locomotion in the open-field and the number of punished licks in the Vogel conflict test were analyzed by two-way ANOVA, with pretreatment and treatment as independent

factors. One-way ANOVA was performed for western blotting data. When appropriate, multiple comparisons were performed by Duncan's *post-hoc* test.

3. Results

Figure 1 depicts the sites of drug injections in the dPAG of animals tested in this study and a representative photomicrograph of an injection site in the dPAG.

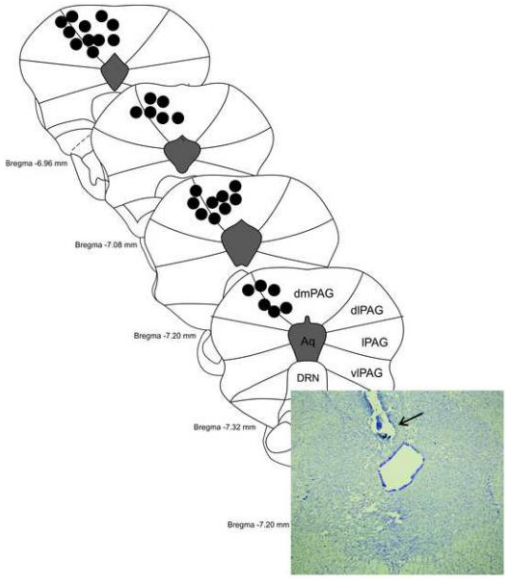

Figure 1. Schematic representation showing injection sites (circles) in the dPAG. Figures represent coordinates from the rat brain atlas Paxinos & Watson (2007) with respect to bregma. The number of points in the figure is fewer than the total number of rats used because of overlapping injection sites. In the bottom panel, a representative photomicrograph of a coronal section of a rat showing an injection site in the dPAG; the arrow represents the injection site. dPAG: dorsal periaqueductal gray; dmPAG: dorsomedial periaqueductal gray; dlPAG: dorsolateral periaqueductal gray; lPAG: lateral periaqueductal gray; vlPAG: ventrolateral periaqueductal gray; Aq: aqueduct; DRN: dorsal raphe nucleus.

Experiment 1 - Intra-dPAG injection of WAY-100635 blocked the panicolytic effect of chronic treatment with imipramine.

Figure 2 shows that a 21-day treatment with imipramine significantly impaired escape performance. This panicolytic effect was blocked by previous administration of WAY-100635 in the dPAG. Repeated-measure ANOVA revealed significant main effects of systemic treatment [$F(1,29)=21.55$, $p<0.05$] but no effect of central injection of WAY-100635 [$F(1,29)=3.54$, $p=0.07$] or trial [$F(2,58)=0.76$, NS]. There was a significant interaction between systemic and central drug treatments [$F(1,29)=14.40$, $p<0.05$]. Repeated-measure ANOVA of inhibitory avoidance indicated a significant trial effect [$F(2,58)=35.96$, $p<0.05$] (Figure 2). However, there was no effect of systemic treatment with imipramine [$F(1,29)=2.97$, NS], central injection of WAY-100635 [$F(1,29)=0.01$, NS], or a significant interaction between systemic and central administration [$F(1,29)=0.47$, NS].

None of the treatments used affected locomotion measured in the open field (Table 1).

Table 1. Effect (mean ± S.E.M.) of intra-dPAG injection of saline or WAY-100635 on the distance travelled in the open-field test by rats chronically treated with saline or imipramine

Treatment (i.p.-intra-dPAG)	Dist trav (m)
Saline-Saline	22.51 ± 1.96
Saline-Imipramine	19.64 ± 2.22
WAY-100635-Saline	19.77 ± 2.47
WAY-100635-Imipramine	17.87 ± 2.45

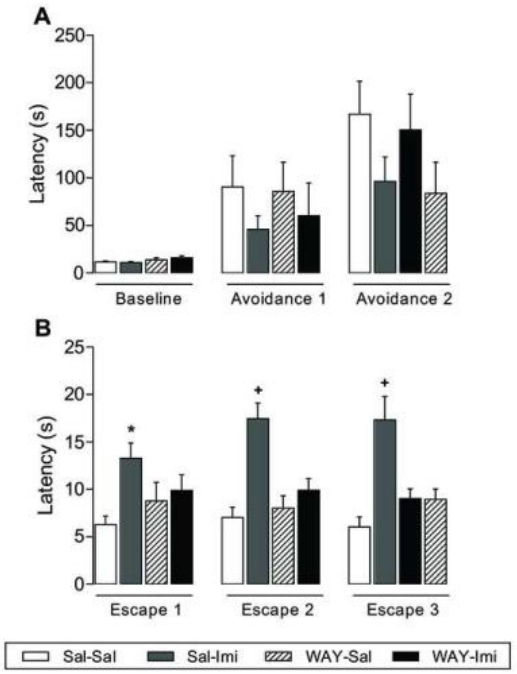

Figure 2. Effect (mean ± S.E.M.) of intra-dPAG injection of saline or WAY-100635 (0.37 nmol) on inhibitory avoidance (A) and escape (B) latencies of rats chronically (21 days) treated with imipramine (15 mg/kg) or saline. n=8-9/group. Sal: saline, Imi: imipramine. *$p<0.05$ compared to Sal-Sal group, $^{+}p<0.05$ compared to all other groups.

Experiment 2 - Chronic treatment with ADs did not significantly change the expression of 5-HT$_{1A}$ receptors or pERK in the dPAG

As shown in Figure 3A, neither chronic treatment with imipramine nor fluoxetine altered 5-HT$_{1A}$ receptor expression in the dPAG [$F(2,24)=0.37$, NS].

Figure 3B shows that the two ADs increased pERK expression, but this effect was only marginal for statistical significance [$F(2,24)=1.23$, $p=0.09$].

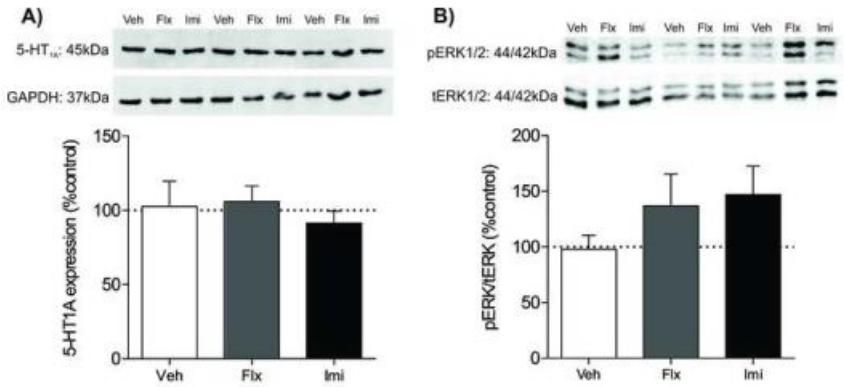

Figure 3. Effect (mean ± S.E.M.) of long-term (21 days) daily i.p. administration of fluoxetine (10 mg/kg) or imipramine (15 mg/kg) on 5-HT_{1A} receptors (panel A) and pERK (panel B) levels in the dPAG. n=9/group.

Experiment 3 - Intra-dPAG injection of SB-242084 did not interfere with the anxiogenic effect of acute imipramine or fluoxetine

Figure 4A shows that acute treatment with imipramine significantly decreased the number of punished licks in the Vogel conflict test. This anxiogenic effect was not counteracted by the previous microinjection of SB-242084. Two-way ANOVA showed a significant effect of systemic [$F(1,27)=12.7$, $p<0.05$] but not central treatment [$F(1,27)=0.03$, NS]. There was no significant interaction between the two factors [systemic x central injection: $F(1,27)=3.17$, NS]. As observed with imipramine, fluoxetine also decreased the number of punished licks in the Vogel conflict test. The previous microinjection of SB-242084 in the dPAG did not affect this anxiogenic effect (see Figure 4B). Two-way ANOVA showed a significant effect of systemic [$F(1,33)=10.46$, $p<0.05$] but not central treatment [$F(1,33)=0.02$, NS]. No significant interaction between these two factors was found [$F(1,33)=0.03$, NS].

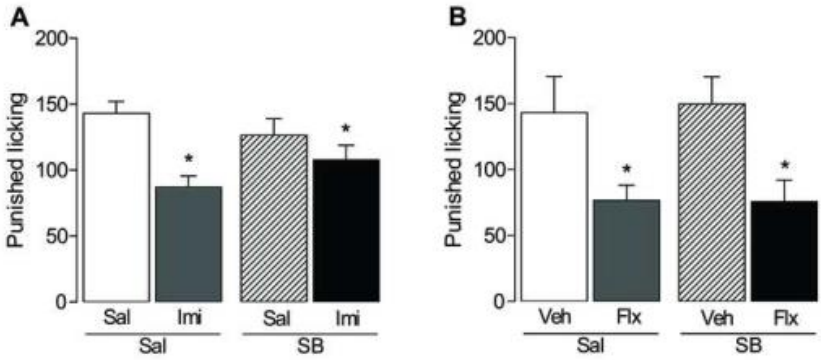

Figure 4. Effect (mean ± S.E.M.) of intra-dPAG injection of saline or SB-242084 (10 nmol) in rats acutely treated with imipramine (15 mg/kg, panel A) or fluoxetine (15 mg/kg, panel B) and tested in the Vogel conflict test. n=7-11/group. *$p<0.05$ compared to Sal-Sal (A) or to Veh-Sal (B) group.

4. Discussion

The present study sought to further investigate the role of dPAG 5-HT_{1A} and 5-HT_{2C} receptors in the anxiety- and panic-modulating effects of fluoxetine and imipramine in rats. The results showed that previous injection of the 5-HT_{1A} receptor antagonist WAY-100635 in the dPAG counteracted the anti-escape effect caused by repeated systemic treatment with imipramine in the elevated T-maze. None of the treatments used significantly affected the total distance travelled by animals in the open field, indicating that this result was not due to a nonspecific effect of the drugs on locomotion. These findings are consistent with previous evidence obtained in the same test showing that the microinjection of WAY-100635 into the dPAG also blocked the panicolytic-like effect caused by chronic fluoxetine administration [20], indicating that activation of 5-HT_{1A} receptors in this midbrain area is a common mechanism for the antipanic action of different classes of ADs.

The results of experiment 2 revealed that chronic treatment with imipramine or fluoxetine did not significantly alter 5-HT_{1A} receptors or pERK levels in the dPAG. Previous pharmacological studies showed that chronic, but not subchronic, treatment with imipramine, sertraline, or

fluoxetine facilitates the anti-escape effect caused by administration of 5-HT_{1A} receptor agonists in this midbrain area [16,18,19]. As such, ADs seem to enhance the functional responsiveness of 5-HT1A receptors in the dPAG [8,9]. Based on our western blotting findings, it is unlikely that upregulation of 5-HT_{1A} receptors in the dPAG drives this phenomenon. This is also consistent with previous results that suggest that fluoxetine fails to increase 5-HT_{1A} mRNA expression in the dPAG after chronic treatment [28].

However, it is worth noting that the absence of 5-HT_{1A} receptor expression changes observed here contrast with the effects observed after chronic fluoxetine in the mouse PAG. Baptista-de-Souza and coworkers [29] recently reported that a 21-day subcutaneous treatment with fluoxetine (5 and 20 mg/kg) increased the density of these receptors. It is unknown if this discrepancy reflects differences between the species used (seemingly the most evident variable), although other methodological factors should also be considered (e.g., tissue harvest, in the mouse brain isolating the dPAG from surrounding areas may be difficult).

Alternatively, our results suggest that ADs may enhance 5-HT_{1A} receptor reactivity by facilitating intracellular signaling pathways. For example, it has been shown that fluoxetine or imipramine increase expression of ERK, pERK, or both in brain areas such as the hippocampus, cortex, amygdala, and striatum [30-32]. There is also evidence to suggest that fluoxetine prevents stress-induced decreases in pERK levels in the hippocampus and prefrontal cortex of rats exposed to stressors but did not change these levels in non-stressed animals [31,33]. In the present study, imipramine and fluoxetine tended to increase pERK, which suggests that different intracellular signaling pathways are concomitantly affected by these ADs; this accounts for the reported increased responsiveness of 5-HT_{1A} receptors.

Accordingly, a wealth of evidence indicates that ADs also recruit signaling pathways such as Akt [32, 34-37], glycogen synthase kinase 3 beta (GSK3β) [34,36-38], and the transcription factor cyclic-AMP response element binding protein (CREB) [31,35,39]. It is worth noting that stimulation of 5-HT_{1A} receptors may engage any of these downstream pathways [for a review, see 40] and may conceivably mediate changes in the reactivity of these receptors after AD treatment.

Finally, the results of experiment 3 revealed that previous administration of the 5-HT_{2C} receptor antagonist SB-242084 in the dPAG did not interfere with the anxiogenic effect mediated by a single systemic injection of imipramine or fluoxetine in rats submitted to the Vogel conflict test. It is important to note that at the dose tested, neither fluoxetine nor imipramine interfered with unpunished water consumption in this test, as reported in a previous study from our laboratory [23].

In contrast with the panicolytic-like role of 5-HT_{1A} receptors in the dPAG [12,15], activation of 5-HT_{2C} receptors in this midbrain structure with the full agonist MK-212 enhanced anxiety without interfering with expression of panic-associated behaviors. This anxiogenic effect is significantly counteracted by previous intra-dPAG administration of SB-242084 at the same dose (10 nmol) used here [24]. Therefore, it seems unlikely that the lack of SB-242084 effect observed here is due to the use of a low dose (see below). Taken together, these findings suggest that 5-HT_{2C} receptors located in the dPAG are not involved in the anxiogenic effect caused by acute administration of imipramine or fluoxetine.

In contrast with these results in the dPAG, administration of an even lower dose of SB-242084 (0.01 nmol) in the BLA blocked the anxiogenic effect of acute fluoxetine or imipramine in the Vogel conflict test [23]. Supporting a prominent role of this amygdaloid subnucleus in the anxiety-modulating effects of ADs, Vicente and Zangrossi [41] reported that BLA 5-HT_{2C} receptors are desensitized after chronic treatment with these two drugs, which therefore limits the negative impact caused by the initial recruitment of these receptors. Given the evidence that activation of 5-HT_{1A} receptors within the BLA causes consistent anxiolytic effects [for a review see 42], we proposed that a shift from anxiogenesis to anxiolysis caused by ADs over time involves both the desensitization of 5-HT_{2C} and the recruitment of 5-HT_{1A} receptors located in this subnucleus [9,10].

As a cautionary note, it should be emphasized that long-term treatment with imipramine in the present study (experiment 1) did not cause a significant anxiolytic effect on inhibitory avoidance acquisition, as reported by Vicente and Zangrossi [41] in their analyses with the BLA. However, a trend toward anxiolysis was observed which did not seem to be affected by the previous intra-dPAG injection of WAY-100635. It is worth noting that as in the BLA, stimulation of 5-HT_{1A} receptors in the dPAG decreases anxiety [15,18]. Therefore, while our current study offers sound evidence that 5-HT_{2C} receptors of the dPAG are not involved in the anxiogenic effect of short-term administration of ADs, other analyses are required to better assess the role of 5-HT_{1A} in this midbrain area for the anxiolytic effect caused by chronic AD treatment.

In conclusion, our results indicate that different mechanisms and neural substrates mediate the

anxiety- and panic-modulating effects promoted by ADs. These results reinforce the view that the facilitation of 5-HT_{1A} receptor-mediated neurotransmission in the dPAG is a common mechanism involved in the panicolytic effect caused by chronic administration of ADs. At a molecular level, this facilitatory effect seems not to depend on 5-HT_{1A} receptor expression changes, but could involve modifications in intracellular signaling pathways, with ERK phosphorylation having only a modest influence. 5-HT_{2C} receptors in the dPAG are not recruited for the anxiogenic effect observed after a single injection of ADs, in contrast to what has been reported in the BLA.

5. Conflicts of interest

The authors declare no conflict of interest.

6. Acknowledgements

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