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A Shift in the Activation of Serotonergic and Non-serotonergic Neurons in the Dorsal Raphe Lateral Wings Subnucleus Underlies the Panicolytic-Like Effect of Fluoxetine in Rats

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Abstract

A wealth of evidence indicates that the lateral wings subnucleus of the dorsal raphe nucleus (lwDR) is implicated in the processing of panic-associated stimuli. Escape expression in the elevated T-maze, considered a panic-related defensive behavior, markedly and selectively recruits non-serotonergic cells within this DR subregion and in the dorsal periaqueductal gray (dPAG), another key panic-associated area. However, whether anti-panic drugs may interfere with this pattern of neuronal activation is still unknown. In the present study, the effects of acute (10 mg/kg) or chronic fluoxetine (10 mg/kg/daily/21 days) treatment on the number of serotonergic and non-serotonergic cells induced by escape expression within the rat DR and PAG subnuclei were investigated by immunochemistry. The results showed that chronic, but not acute, treatment with fluoxetine impaired escape expression, indicating a panicolytic-like effect, and markedly decreased the number of non-serotonergic cells that were recruited in the lwDR and dPAG. The same treatment selectively increased the number of serotonergic neurons within the lwDR. Our immunochemistry analyses also revealed that the non-serotonergic cells recruited in the lwDR and dPAG by the escape expression were not nitrergic. Overall, our findings suggest that the anti-panic effect of chronic treatment with fluoxetine is mediated by stimulation of the lwDR-dPAG pathway that controls the expression of panic-associated escape behaviors.

Keywords Panic · Dorsal raphe · Serotonin · Nitric oxide · Elevated T-maze · Antidepressant

Introduction

Several lines of evidence suggest that the dorsal raphe nucleus (DR), the main source of serotonergic projections to brain areas implicated in anxiety and panic processing [1–4], is not a homogeneous structure, but an aggregate of distinct populations of serotonergic and non-serotonergic neurons that differ anatomically and functionally [5–7]. Although the DR contains the highest concentration of serotonergic neurons in the brain [8, 9], it has been estimated that the percentage of non-serotonergic cells in this structure may vary from 30 to 70% of the total in humans and rats, respectively [3, 10]. Among the non-serotonergic neurons found in this nucleus, there are GABAergic, nitrergic, glutamatergic, and dopaminergic neurons [11–14]. Based on immunohistochemical and behavioral evidence, a division of the DR into five subnuclei has been proposed, namely the dorsal (DRD), ventral (DRV), lateral wings (lwDR), caudal (DRC), and interfascicular (DRI) nuclei [7, 8].

There is now strong evidence to suggest that these DR subnuclei are distinctively activated by anxiety- or panic-inducing agents/situations. For instance, Johnson and co-workers [15, 16] have shown that in rats exposed to panicogenic stimuli, such as lactate infusion [15], or exposure to high concentrations of CO_2 [16], neurons within the lwDR are predominately recruited. On the other hand, upon exposure to anxiogenic drugs, such as caffeine, the CRF1 receptor agonist urocortin 1 [17, 18], or to anxiety-evoking situations, such as exposure to inescapable footshocks or to a bright environment [19, 20], serotonergic neurons within the DRD and DRC are stimulated.

In full agreement with these findings, Spiacci and co-workers [21] reported that whereas inhibitory avoidance acquisition in the elevated T-maze recruits serotonergic cells in the DRD and DRC, escape expression in this test activates non-serotonergic neurons in the lwDR. In the elevated T-maze, a wealth of evidence supports the relationships between

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inhibitory avoidance and anxiety and between escape and panic (for a full description of this test see [22]). For instance, chronic, but not acute, administration of antidepressants drugs such as the selective serotonin (5-HT) reuptake inhibitors (SSRIs) fluoxetine and escitalopram or the tricyclic compound imipramine inhibits escape expression, suggesting an anti-panic effect [23–25], which is in agreement with the clinical effectiveness of these drugs in panic disorder [26–28].

Still, with regard to the role played by the lwDR in the regulation of panic-related defensive behaviors, the main focus of the current study, we recently reported [29] that chemical stimulation of this subnucleus with subtoxic doses of the excitatory amino acid kainic acid evokes a vigorous escape response in rats, which is accompanied by an increase in c-Fos expression within the dorsal periaqueductal gray (dPAG) and medial hypothalamus, two brain areas consistently implicated in the pathophysiology of panic disorder [30–32].

Another set of evidence gathered in our laboratory indicates that the pharmacological manipulation of 5-HT_{1A} receptors located at the lwDR selectively interferes with escape expression, without affecting inhibitory avoidance acquisition in the elevated T-maze. As widely documented, 5-HT_{1A} receptors in raphe nuclei regulate by a negative feedback mechanism the activity of serotonergic neurons, controlling, therefore, serotonin release in terminal areas [33–35]. More specifically, Spiacci and coworkers [36] observed that whereas microinjection of the 5-HT_{1A} receptor antagonist WAY-100635 into the lwDR inhibits escape expression, local infusion of the 5-HT_{1A} receptor agonist 8-OH-DPAT facilitates it. It was hypothesized that WAY-100635, by counteracting the tonic inhibitory influence of 5-HT on the firing rate of lwDR neurons [34, 37], led to an increase of 5-HT release in terminal areas related to panic processing (e.g., the dPAG and medial hypothalamus), where this monoamine is consistently reported to inhibit escape expression [38–41]. Conversely, the pro-escape effect of 8-OH-DPAT was due the opposite effect of this drug on lwDR 5-HT_{1A} autoreceptors and on 5-HT release in the same terminal areas.

Within this framework, in this study, we investigated whether the administration of the widely used anti-panic drug fluoxetine interferes with the pattern of neuronal activation, evaluated by c-Fos and tryptophan hydroxylase (TrpOH) immunostaining, observed in the DR and dPAG of rats submitted to the escape task in the elevated T-maze.

By analyzing the number of immunostained cells for the enzyme neuronal nitric oxide synthase (nNOS) and c-Fos protein in the DR of these animals, we also explored whether nitrergic neurons correspond to the non-serotonergic cells that are recruited in the lwDR by the escape task. We focused our attention on NO, given evidence that nitrergic cells are found in expressive amounts in the lwDR [42] and NO has been shown to exert an important regulatory control on serotonergic neurotransmission [43, 44].

Materials and Methods

Animals

Male Wistar rats weighing 160–180 g on the first day of drug treatment were group-housed ($n = 5$ per cage) under a 12-h light/dark cycle (lights on 07:00 hours) at 22 ± 1 °C, with free access to water and food throughout the experiment, except during testing. The experimental procedures adopted in this study were conducted in conformity with Brazilian Council for the Care and Use of Laboratory Animals (COBEA), which are in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and were approved by our local ethics committee (protocol number: 034/13).

Apparatus

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

The enclosed T-maze had all three arms (50 × 12 cm) surrounded by 40-cm-high wooden walls. Luminosity at the level of both mazes was 60 lx.

Procedures

Rats were injected once a day (i.p.) with fluoxetine hydrochloride (EMS, Brazil; 10 mg/kg) or vehicle solution (saline with 2% Tween 80) for 20 consecutive days. On the 21st day, half of the animals treated with vehicle were injected again with vehicle solution (control group) and the other half with fluoxetine 10 mg/kg (acute treatment group). The fluoxetine-treated animals received one more injection of fluoxetine 10 mg/kg (chronic treatment group). During the test, half of the animals in the control group were submitted to the escape task of the elevated T-maze and the other half was tested, as described below, in the enclosed T-maze, which served as a behavioral task control. As previously shown [21], exposure to this apparatus controls the effects of handling by the experimenter and the locomotor activity of the animals while exploring the maze. All animals treated with fluoxetine, either acute or chronically, were only submitted to the escape task of the elevated T-maze. The following groups were formed: vehicle/enclosed T-maze ($n = 10$), vehicle/escape ($n = 9$), acute fluoxetine/escape ($n = 8$), and chronic fluoxetine/escape ($n = 10$). Figure 1 shows an overview of the experimental design adopted.

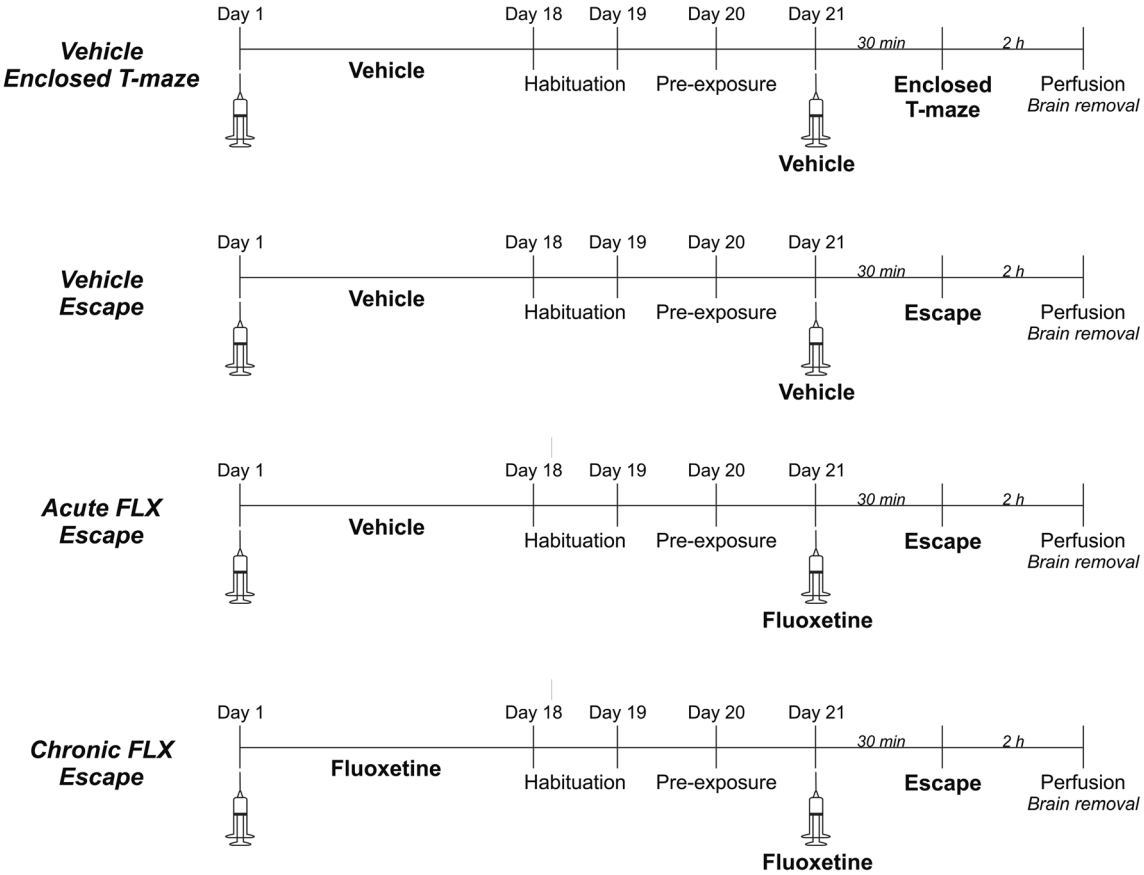

Fig. 1 The experimental design followed in the current study

The dose of fluoxetine was chosen based on its anti-escape effect inthe elevated T-maze as reported by Poltronieri et al. [45] and Zanoveli et al. [24].

Behavioral Test

The animals were handled daily for 5 min for two consecutive days before the experiment in order to familiarize them to the experimental conditions and to reduce non-specific stress responses. Twenty-four hours before the test, all rats were exposed to one of the open arms of the elevated T-maze apparatus for 30 min. A wood barrier mounted on the border of the maze central area and the open arm's proximal end isolated this arm from the rest of the maze. It has been shown that this pre-exposure to the open arm facilitates escape expression in the test. This has been attributed to habituation of behavioral reactions to novelty, which are likely to interfere with the escape response.

On the following day (day 21), escape expression in the elevated T-maze or locomotion in the enclosed T-maze were assessed 30 min after the last systemic drug injection. In the elevated T-maze, the rats were placed at the end of the same previously experienced open arm and the latency to leave this arm with four paws was recorded 6 consecutive times (escape 1 to 6) with 30 s inter-trial intervals. During this time, the animal was left alone in a Plexiglas® box (length 28 cm, width 19 cm, height 34 cm) to which it was previously habituated during the handling sessions. In the enclosed T-maze, the same procedure was followed, except that the previously experienced open arm was surrounded by lateral walls during the test.

Animal behavior was recorded throughout the experiment using a video camera connected to a DVD recorder. Latencies were registered by an experimenter blind to the treatments used.

Tissue Collection and Preparation

Two hours after the end of the test, all animals were deeply anesthetized with urethane (1.25 g/kg; Sigma, USA) and then transcardially perfused with 0.01 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS (pH 7.4) at 4 °C. Brains were immediately removed, post-fixed for 2 h in the same fixative solution, rinsed in PBS, then immersed in cryoprotectant solution (30% sucrose in 0.2 M PB, pH 7.4) at 4 °C for 5 days. Next, brains were then quickly frozen in isopentane in dry-ice, and stored at − 20 °C. Serial coronal sections were cut in three alternate sets of 40 μm using a cryostat (Leica Microsystems, Germany) and collected in PBS.

Immunohistochemistry

One in three series of mesencephalic sections was selected and double-immunostained for c-Fos protein and TrpOH and another set was double-immunostained for c-Fos protein and nNOS, using a free-floating immunohistochemistry methodology conducted on an orbital shaker. The staining for c-Fos was performed first as follows: the slices were washed three times in PBS, and the hydrogen peroxidase was inactivated by immersing in 1% hydrogen peroxide in PBS for 10 min, followed by washing in PBS four times. Tissue sections were then incubated in a blocking solution of 1% bovine serum albumin (BSA) in PBS for 30 min and afterwards incubated overnight with a polyclonal rabbit anti-c-Fos protein antibody (sc-52, 1:900, Santa Cruz, USA). After incubation in the primary antiserum, the slices were washed in PBS and sequentially incubated for 2 h with biotinylated goat anti-rabbit antibody (1:900, Vecstasin, Vector Laboratories). Tissue sections were incubated for 2 h in avidin–biotin complex (Vectastain ABC kit, Vector Laboratories). c-Fos immunoreactivity was revealed after immersing the sections in a solution containing 0.02% chromogen 3,3′-diaminobenzidine tetrahydrochloride, 0.0015% hydrogen peroxide, and 0.05% nickel ammonium sulfate in PBS for 10 min.

After c-Fos immunostaining, the tissue sections were washed four times in PBS and processed for either TrpOH or nNOS immunostaining. For this, one set of sections was incubated overnight with polyclonal sheep anti-TrpOH antibody (AB1541, 1:1200, Millipore, USA), and another set with rabbit anti-nNOS (sc-648, 1:1000, Santa Cruz, USA). After incubation in the primary antiserum, the sections were washed in PBS, sequentially incubated for 2 h with biotinylated anti-sheep (PK-6106, 1:1200, Vecstasin, Vector Laboratories) or anti-rabbit antibody (PK-6101, 1:1000, Vecstasin, Vector Laboratories), respectively, and washed three times with PBS. Sections were then incubated with avidin–biotin complex (Vectastain ABC kit, Vector Laboratories), and washed three times in PBS. The set of sections for TrpOH or nNOS immunoreactivity was revealed after immersing the sections in a solution containing 0.02% chromogen, 3,3′-diaminobenzidine tetrahydrochloride and 0.0015% hydrogen peroxide in PBS for 10 min. Sections were then washed four times in PBS to stop the reaction.

The tissue sections were then mounted on gelatin-coated glass slides, dehydrated in a graded alcohol series, cleared in xylol, and coverslipped using Permount histological mounting medium (Fisher Scientific, UK).

Analysis and Quantification

The immunostaining was visualized using a microscope (Olympus DX50), and representative photomicrographs (Fig. 2) were taken using a digital camera linked to a computer with Image Pro Plus 6.0 software (Media Cybernetics, USA). Cell counts were conducted at 200× magnification by an investigator blinded to the assignment of treatment groups. The color reaction of the c-Fos immunostaining was blue-black and localized to the nucleus while TrpOH or nNOS immunostaining was orange-brown and located in the cytoplasm (Fig. 2).

The total number of c-Fos-immunoreactive cells (c-Fos-IR cells), TrpOH-immunoreactive neurons (TrpOH-IR neurons), nNOS-immunoreactive neurons (nNOS-IR neurons), c-Fos + TrpOH-IR double-immunostained neurons (c-Fos + TrpOH-IR double-immunostained neurons), and c-Fos + nNOS-immunoreactive neurons (c-Fos + nNOS-IR double-immunostained neurons) were counted in different regions of the DR at four rostrocaudal levels (from − 6.84 to − 9.00 mm bregma), as described in previous studies [21, 46]. The subdivisions of the DR analyzed were the dorsal subnucleus (DRD) at the rostral level (− 6.84 to − 7.08 mm bregma), the DRD, the ventral (DRV), and the lwDR subnuclei at the mid-rostral (− 7.20 to − 7.80 mm bregma) and mid-caudal levels (− 7.92 to − 8.28 mm bregma), and the caudal (DRC) and interfascicular (DRI) subnuclei at the caudal level (− 8.52 to − 9.00 mm bregma). The number of cells sampled within the lwDR was the sum of cells counted in the right and left sides of this subnucleus. The number of animals analyzed per group was the same as in the behavioral test: vehicle/enclosed T-maze ($n = 10$), vehicle/escape ($n = 9$), acute fluoxetine/escape ($n = 8$), and chronic fluoxetine/escape ($n = 10$).

In the PAG, the number of c-Fos-IR cells were analyzed as follows: at the rostral (− 6.60 to − 6.84 mm bregma) and mid-rostrocaudal levels (− 7.32 to −7.80 mm bregma), the columns sampled were the dorsomedial (dmPAG), dorsolateral (dlPAG), lateral (lPAG), and ventrolateral (vlPAG). At the caudal level (− 8.28 to −8.52 mm bregma), the columns sampled were dmPAG, lPAG, and vlPAG. We also counted the number of c-Fos + nNOS-IR neurons in the dlPAG at rostral and middle levels, given the preponderant presence of nNOS-IR neurons in this subregion when compared to all other PAG columns [47]. The number of cells sampled within the dlPAG, lPAG, or vlPAG were the sum of cells counted in the left and right sides of these columns. The number of animals analyzed per group for the PAG was smaller compared to the DR: vehicle/enclosed T-maze ($n = 9$), vehicle/escape ($n = 9$), acute fluoxetine/escape ($n = 5$), and chronic fluoxetine/escape ($n = 10$), since four animals were lost due to damage in the tissue portion comprising the PAG.

The number of all cells counted was expressed by the area of the corresponding subregion of the DR or the PAG.

Statistical Analysis

The effects caused by escape performance (vehicle-treated animals exposed to the enclosed T-maze × to the elevated T-maze open arm) were analyzed separately from those caused

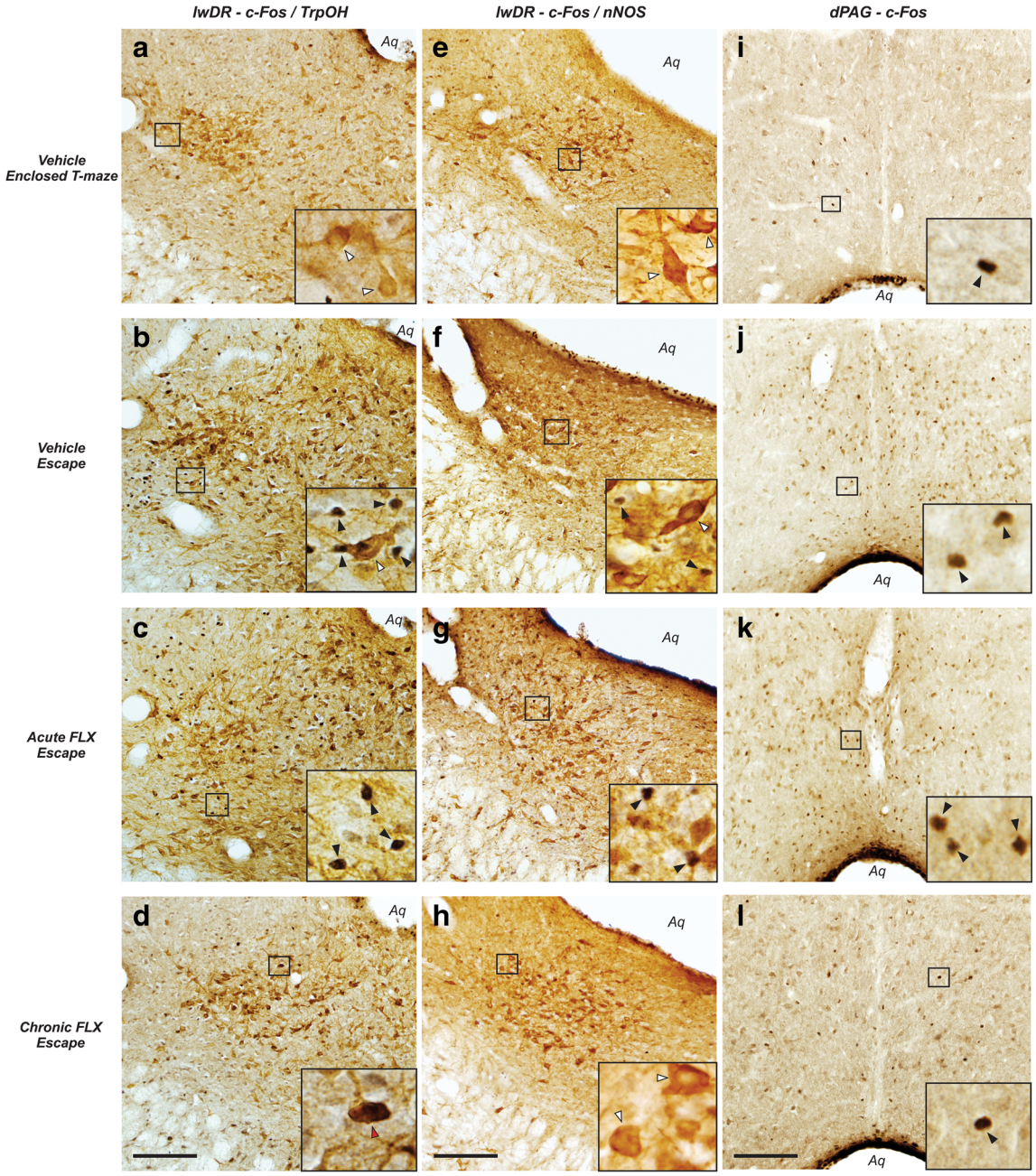

Fig. 2 Photomicrographs depicting c-Fos, TrpOH, and nNOS immunolabeled cells in the lwDR (**a**–**h**) or c-Fos-stained cells in the dPAG (**i**–**l**) of representative animals of the different groups tested in the current study. Black boxes indicate regions shown at higher magnification in insets in the lower right corner of each panel. White arrowheads indicate TrpOH- or nNOS-immunoreactive cells; black arrowheads indicate c-Fos-immunoreactive cells; red arrowhead indicate c-Fos + TrpOH-IR double-immunostained neurons. Aq, cerebral aqueduct. Scale bar = 100 μm (**a**–**h**) and 50 μm (**i**–**l**)

by fluoxetine treatment (vehicle × acute × chronic administration in animals submitted to the escape task).

Behavioral data in both cases was analyzed by repeated measures ANOVA with test condition or drug treatment as independent factors and trials as the repeated measure, except for the merged escape index which was analyzed by Student's *t* test (escape task effect) or one-way ANOVA (fluoxetine effect). The merged escape index was calculated for each rat as the mean ± standard error of the mean (S.E.M.) of the six trials performed.

The number of c-Fos-IR, TrpOH-IR, nNOS-IR, c-Fos + TrpOH-IR, or c-Fos + nNOS-IR cells counted in each subregion of the DR or the number of c-Fos-IR cells or c-Fos + nNOS-IR cells counted in the PAG were analyzed using Student's *t* test (escape task effect) or one-way ANOVA (fluoxetine effect).

When appropriate, post hoc comparisons were performed using Duncan's test. Values of $p < 0.05$ were considered significant. All statistical analyses were carried out using IBM SPSS Statistics 20.0 for Windows.

Results

Behavioral Analysis

Figure 3 shows the effect of fluoxetine treatment on the escape response measured in the elevated T-maze test.

The analysis of the vehicle-treated groups showed that the latencies to leave the enclosed T-maze were longer than those obtained in the open arm of the elevated T-maze (task: $F(1,17) = 22.2$, $p < 0.001$; trial: $F(5,85) = 2.5$, $p < 0.05$; trial × task interaction: $F(5,85) = 1.2$, $p = 0.3$), and this effect reached statistical significance on trials 4–6.

In the groups tested for escape in the elevated T-maze, repeated measures ANOVA revealed significant effects of drug treatment ($F(2,24) = 8.0$, $p < 0.01$), trials ($F(5,120) = 8.5$, $p<0.001$) and a treatment × trial interaction ($F(10,120) = 3.4$, $p < 0.01$). Duncan's post hoc test showed that chronic treatment with fluoxetine significantly increased escape latency in trials 4–6, when compared to the other escape-tested groups. Acute fluoxetine injection prolonged escape 1 latency when compared to the control group.

As show in Fig. 3b, chronic fluoxetine treatment increased the merged escape latency ($F(2,24) = 8.0$, $p < 0.01$) compared to the other escape-tested groups.

Immunohistochemistry

Number of TrpOH-IR and nNOS-IR Neurons in the DR

As shown in Table 1, neither escape expression nor treatment with fluoxetine changed the number of TrpOH-IR or nNOS-IR cells within the different DR subnuclei.

Number of c-Fos-IR Cells in the DR

The statistical analyses of the total number of c-Fos-IR cells in the vehicle-treated groups revealed that animals submitted to the escape task showed an increased c-Fos

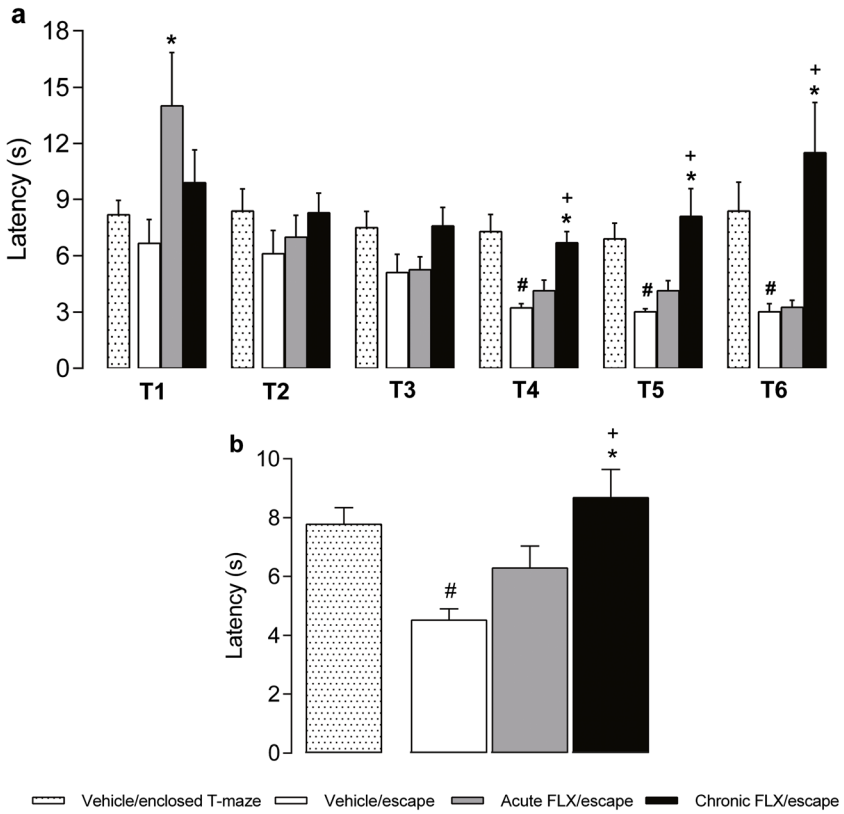

Fig. 3 Effect (mean + S.E.M.) of acute or chronic (21 days) systemic treatment (i.p.) with fluoxetine (FLX, 10 mg/kg) on the elevated T-maze escape task measured in 6 consecutive trials (T1-T6; panel **a**) or as a merged latency index (T1 + T2…+ T6/6; panel **b**). The first column in the graphs represents the values obtained in animals that were treated with vehicle solution and tested in the enclosed T-maze (see the text for more details). $n = 8$–10. $^{\#}p < 0.05$ compared with the vehicle/enclosed T-maze group (Student's t test). $^{*}p < 0.05$ compared with the vehicle/escape group. $^{+}p < 0.05$ compared with acute FLX/escape group (Duncan's test)

Table 1 Number (mean ± S.E.M.) of TrpOH- and nNOS-immunoreactive neurons sampled in the different DR subnuclei

Rostrocaudal level (mm from bregma)	Subnucleus	Number of TrpOH-IR neurons/mm^2				Number of nNOS-IR neurons/mm^2			
		Vehicle/ enclosed T-maze	Vehicle/ escape	Acute FLX/ escape	Chronic FLX/escape	Vehicle/ enclosed T-maze	Vehicle/ escape	Acute FLX/ escape	Chronic FLX/escape
− 6.84 to − 7.08	DRD	360.8 ± 7.0	339.5 ± 10.1	307.1 ± 21.6	317.7 ± 11.6	217.7 ± 39.4	229.4 ± 15.9	178.5 ± 22.5	198.8 ± 16.3
− 7.20 to − 7.80	DRD	501.8 ± 37.5	486.2 ± 26.2	458.3 ± 29.1	447.2 ± 18.2	217.7 ± 14.3	216.9 ± 10.5	202.0 ± 17.9	249.8 ± 16.8
	DRV	434.3 ± 27.3	413.9 ± 14.9	378.7 ± 15.1	401.5 ± 9.3	302.8 ± 20.5	274.7 ± 23.2	284.9 ± 31.9	271.4 ± 34.7
	lwDR	212.9 ± 12.3	214.9 ± 7.1	201.0 ± 5.1	198.8 ± 5.0	110.5 ± 5.8	121.5 ± 7.7	115.6 ± 7.3	116.7 ± 4.2
− 7.92 to − 8.28	DRD	474.8 ± 47.3	435.2 ± 15.9	416.4 ± 14.4	424.8 ± 16.8	198.8 ± 31.7	206.2 ± 15.5	157.8 ± 19.0	172.9 ± 19.0
	DRV	354.8 ± 38.0	332.1 ± 16.4	333.3 ± 18.9	313.0 ± 10.2	212.0 ± 27.0	213.1 ± 7.9	178.5 ± 21.7	179.5 ± 19.0
	lwDR	201.3 ± 15.3	181.2 ± 10.6	181.2 ± 18.5	188.8 ± 8.7	247.7 ± 30.3	251.5 ± 26.7	319.7 ± 16.3	311.8 ± 18.9
− 8.52 to − 9.00	DRC	420.8 ± 7.7	427.6 ± 17.2	376.2 ± 16.9	403.1 ± 22.8	202.4 ± 30.8	190.9 ± 13.2	176.3 ± 18.0	202.0 ± 14.2
	DRI	557.8 ± 18.2	553.8 ± 16.7	507.1 ± 18.4	504.7 ± 21.7	371.8 ± 37.6	359.3 ± 38.7	371.8 ± 45.9	342.6 ± 64.2

DRC caudal, *DRD* dorsal, *DRI* interfascicular, *DRV* ventral, *lwDR* lateral wings, *TrpOH* tryptophan hydroxylase, *nNOS* neuronal nitric oxide synthase

immunostaining only within the lwDR, at both the mid-rostral ($t(17) = 2.8$, $p < 0.05$) and mid-caudal ($t(17) = 3.4$, $p < 0.01$) levels. The difference in the DRD at both the mid-rostral ($t(17) = 1.8$, $p = 0.07$) and mid-caudal ($t(17) = 1.8$, $p = 0.07$) levels was marginal to statistical significance (Fig. 4).

Regarding the effect of fluoxetine, one-way ANOVA revealed significant differences among the escape-tested groups in the lwDR, at both mid-rostral ($F(2,24) = 6.5$, $p < 0.01$) and mid-caudal ($F(2,24) = 6.0$, $p < 0.01$) levels, and within the DRD ($F(2,24) = 4.8$, $p < 0.05$), at the mid-rostral level. Duncan's post hoc test revealed that chronic, but not acute, treatment with fluoxetine significantly decreased the total number of c-Fos-IR cells in the lwDR compared to all other escape-tested groups, and in the DRD compared to the acute-treated group (Fig. 4).

Number of Double-Labeled Neurons (c-Fos + TrpOH-IR) in the DR

No double-labeled neurons were found at the DR rostral level, or in the DRV and DRI.

As shown in Fig. 5, the statistical analysis revealed that the number of c-Fos + TrpOH-IR cells did not differ between the vehicle-treated groups in any of the DR subnuclei analyzed.

One-way ANOVA revealed that in animals submitted to the escape task, treatment with fluoxetine changed the number of c-Fos + TrpOH-IR neurons only in the lwDR, at both mid-rostral ($F(2,24) = 6.6$, $p < 0.01$) and mid-caudal ($F(2,24) = 4.0$, $p < 0.05$) levels (Fig. 5). Duncan's post hoc test showed that chronic treatment with fluoxetine significantly increased the number of double-labeled cells within the lwDR at both levels when compared to all other escape-tested groups.

Number of Double-Labeled Neurons (c-Fos + nNOS-IR) in the DR

As shown in Table 2, neither escape performance nor treatment with fluoxetine changed the number of c-Fos + nNOS-IR cells within the different DR subnuclei. No double-labeled neurons were found at the DR rostral level.

Number of c-Fos-IR Cells in the PAG

The statistical analyses of the total number of c-Fos-IR cells in the vehicle-treated groups revealed that the group submitted to the escape task showed increased c-Fos immunostaining within the dmPAG, at both rostral ($t(16) = 2.1$, $p < 0.05$) and middle ($t(16) = 7.8$, $p < 0.001$) levels, and in the dlPAG at the middle level ($t(16) = 2.4$, $p < 0.05$) (Fig. 6).

Regarding the effect of fluoxetine, one-way ANOVA revealed significant differences among the escape-tested groups in the dmPAG, at both rostral ($F(2,21) = 4.7$, $p < 0.05$) and middle ($F(2,21) = 4.7$, $p < 0.05$) levels. At the dlPAG rostral level, the treatment effect was only marginal to statistical significance ($F(2,21) = 2.9$, $p = 0.07$). Duncan's post hoc test revealed that chronic treatment with fluoxetine significantly decreased the total number of c-Fos-IR cells in the dmPAG compared to all other escape-tested groups at the rostral level and compared to the acute-treated group at rostral, middle, and caudal levels (Fig. 6).

Number of c-Fos + nNOS-IR Neurons in the dlPAG

As shown in Table 2, neither escape expression nor treatment with fluoxetine changed the number of c-Fos + nNOS-IR cells within the dlPAG at the rostral and middle levels.

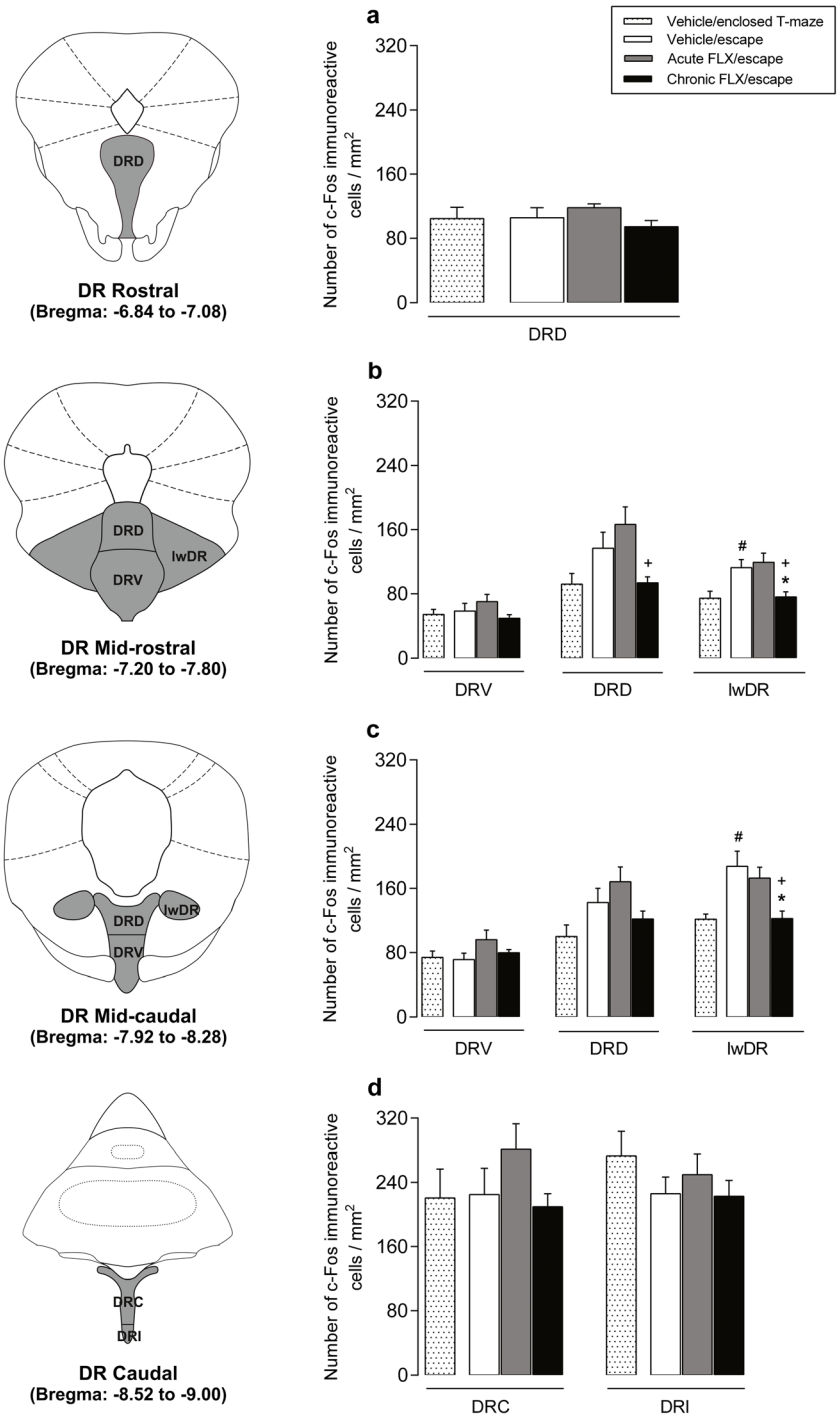

Fig. 4 Number (mean ± S.E.M.) of c-Fos-immunoreactive cells counted in the different DR subnuclei of animals treated acute or chronically with fluoxetine (FLX, 10 mg/kg; i.p., 21 days) and submitted to the elevated T-maze escape task. For further specifications, see the legend of Fig. 3. $n = 8–10$. $^{\#}p < 0.05$ compared with the vehicle/enclosed T-maze group. $^{*}p < 0.05$ compared with the vehicle/escape group. $^{+}p < 0.05$ compared with Acute FLX/escape group

Discussion

We here investigated whether treatment with fluoxetine interferes with the pattern of neuronal activation induced by escape expression within the DR and PAG subnuclei.

As previously reported [23, 24, 45], we observed that chronic systemic administration of fluoxetine impaired escape expression, suggesting a panicolytic-like effect. Although the acute injection of the drug also impaired escape 1 latency, this effect was neither detected on the subsequent escape trials nor significantly interfered with the merged escape index as it was observed after the prolonged treatment.

Also in agreement with the results of a previous investigation [21], we observed that escape expression in the elevated T-maze, recruited non-serotonergic neurons within the lwDR and in the dPAG. This latter finding adds to a series of evidence that associates these two brain areas with the genesis/regulation of panic-related defensive responses [30, 31, 48].

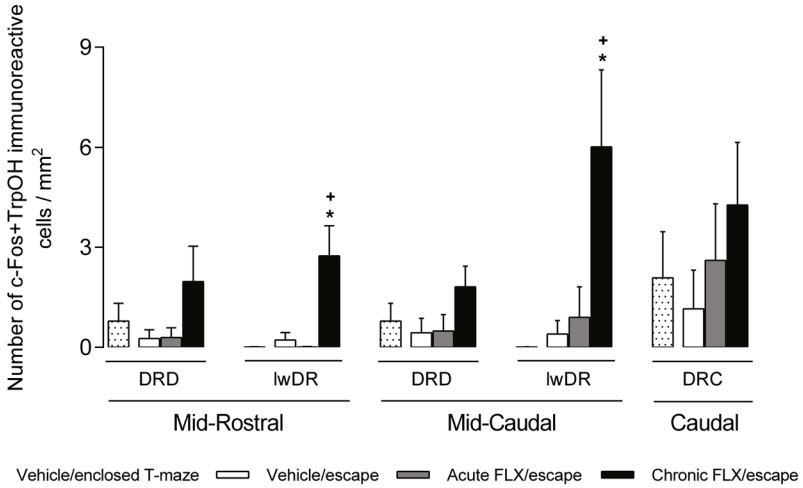

Fig. 5 Number (mean ± S.E.M.) of double-labeled (c-Fos + TrpOH-immunoreactive) neurons counted in the different DR subnuclei of animals treated acute or chronically with fluoxetine (FLX, 10 mg/kg; i.p., 21 days) and submitted to the elevated T-maze escape task. For further specifications, see the legend of Fig. 3. $n = 8–10$. $^{\#}p < 0.05$ compared with the vehicle/enclosed T-maze group (Student's t test). $^{*}p < 0.05$ compared with the vehicle/escape group. $^{+}p < 0.05$ compared with acute FLX/escape group

The most novel of the present findings is that long-term, but not acute, administration of a SSRI reduced the number of c-Fos-IR cells recruited by escape expression within both the lwDR and dPAG. Concomitantly, chronic treatment with fluoxetine also increased the number of serotonergic neurons activated in the DR, and this effect reached statistical significance only in the lwDR subnucleus. Therefore, chronic fluoxetine treatment caused a shift in the balance between activation of non-serotonergic and serotonergic cells recruited by escape expression in the DR, and this was primarily observed in the lwDR.

It is noteworthy that only a small proportion of all TrpOH-IR neurons immunostained within the lwDR (Table 1) were activated by the SSRI. Previous studies have similarly found that exposure of rats to different aversive stimuli or pharmacological manipulations such as forced swim, GABAergic disinhibition of the prelimbic cortex, systemic injection of saline, or WAY-100635 recruited very few double-labeled neurons for TrpOH and c-Fos within the DR [34, 46, 49]. Importantly, in the first two cases, the behavioral consequences evoked by these stimuli have been associated with changes detected in the lwDR.

Our study also indicates that the non-serotonergic cells recruited by escape expression both in the lwDR and dPAG are not nitrergic. These two areas are rich in NO-producing neurons [42, 47, 50–52], and there is evidence to suggest that nitrergic cells may be implicated in mediation of panic-related defensive responses. For instance, facilitation of NO signaling in both DR and dlPAG evokes escape reactions [53–59]. Exposure of rats to a cat, which also induces escape responses, recruits NO-containing neurons in these brain regions [60–62]. Notwithstanding these findings, our results show that the number of c-Fos + nNOS-IR in the lwDR and in the dlPAG was not significantly altered by escape performance or fluoxetine treatment, ruling out a role of nitrergic neurons. It remains to be elucidated whether the magnitude/salience of the threatening stimuli that motivate escape accounts for the discrepancy found between these studies.

As with nitrergic neurons, the lwDR is rich with GABAergic interneurons [6, 12, 63]. Roche and co-workers

Table 2 Number (mean ± S.E.M.) of c-Fos + nNOS-immunoreactive neurons sampled in the different DR subnuclei and within the dorsolateral PAG

Rostrocaudal level	Subnucleus	Number of c-Fos + nNOS-IR neurons/mm^2			
		Vehicle/enclosed T-maze	Vehicle/escape	Acute FLX/escape	Chronic FLX/escape
Mid-rostral DR	DRV	0.4 ± 0.4	0.0	0.9 ± 0.9	0.2 ± 0.2
	lwDR	0.0	0.0	0.8 ± 0.8	0.0
Mid-caudal DR	DRD	0.0	0.0	0.0	0.7 ± 0.7
	lwDR	0.4 ± 0.4	0.0	0.0	0.0
Caudal DR	DRC	0.0	1.6 ± 1.0	0.0	0.0
Rostral PAG	dlPAG	1.6 ± 0.6	1.2 ± 0.3	1.9 ± 1.1	1.5 ± 0.6
Middle PAG	dlPAG	1.3 ± 0.6	1.2 ± 0.5	0.3 ± 0.2	0.5 ± 0.3

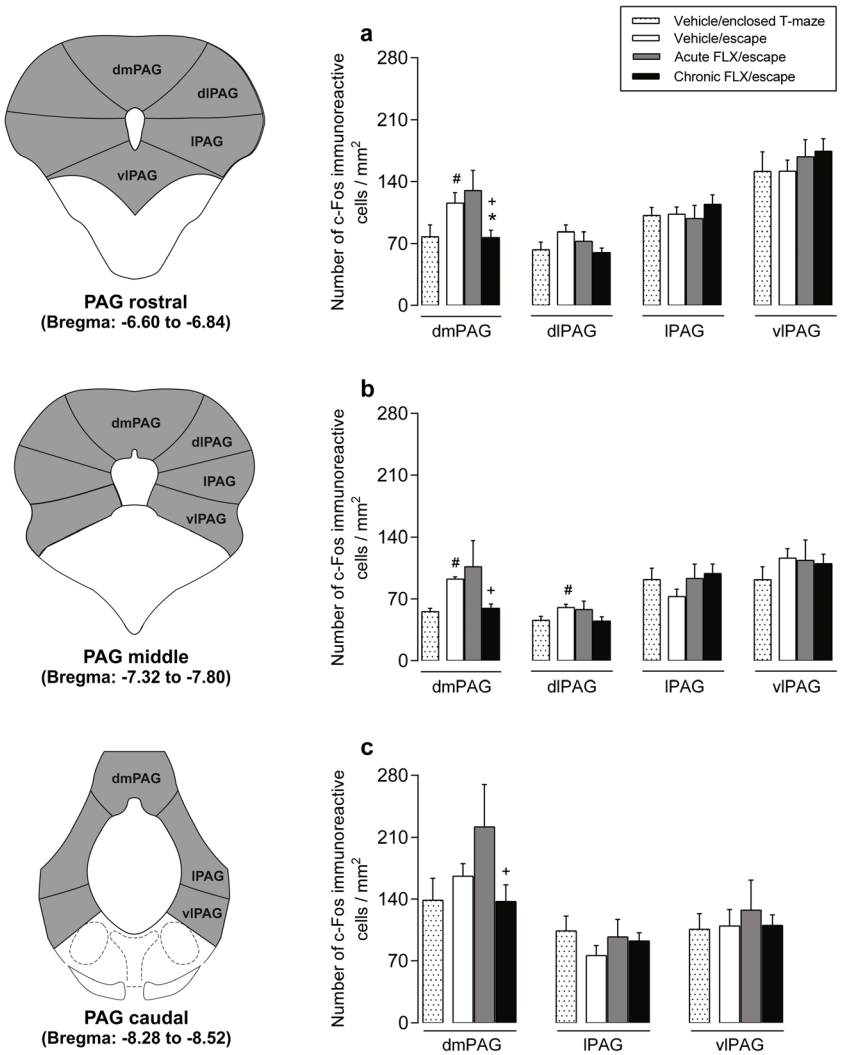

Fig. 6 Number (mean ± S.E.M) of c-Fos-immunoreactive cells counted in the different columns of the PAG of animals treated acute or chronically with fluoxetine (FLX, 10 mg/kg; i.p., 21 days) and submitted to the elevated T-maze escape task. For further specifications, see the legend of Fig. 3. $n = 5–10$. $^{\#}p < 0.05$ compared with the vehicle/enclosed T-maze group. $^{*}p < 0.05$ compared with the vehicle/escape group. $^{+}p < 0.05$ compared with acute FLX/escape group

[49] reported that rats submitted to a forced swimming task, which evokes active escape-like coping behaviors (e.g., swimming, climbing), recruit GABAergic cells within the lwDR. A wealth of evidence indicates that GABA exerts tonic inhibitory control over serotonergic cells with the DR [64–68] and that acute swim stress inhibits 5-HT release in terminal regions of the DR [69, 70]. As such, it is conceivable that facilitation of GABAergic-mediated neurotransmission within the lwDR decreases serotonin release in projections areas, such as the dPAG, which ultimately favor escape expression. In support, inhibition of 5-HT neuron firing in the lwDR by local injection of 8-OH-DPAT facilitates escape expression in the elevated T-maze [36].

With regard to the dPAG, there is evidence to suggest that the c-Fos-immunostained cells detected in the current study may be glutamatergic. Evans and coworkers [71] reported, using optogenetic and calcium imaging, that these neurons in the dPAG are responsible for encoding the choice to escape and control escape vigor, in contrast with their function in the deep layers of the medial superior colliculus, which is to encode the saliency of the threat stimulus. However, given scant information in the literature, a fuller understanding of the role played by lwDR GABA and dPAG glutamatergic neurons in escape mediation awaits further investigations.

One important question raised by our results is what mechanisms drive the shift caused by chronic fluoxetine treatment in the activation of non-serotonergic and serotonergic neurons in the lwDR. As widely reported, long-term treatment with fluoxetine and other SSRIs by desensitizing 5-HT_{1A} autoreceptors located at the cell membrane of serotonergic neurons, allows a higher accumulation of serotonin in projection areas, such as the hippocampus and frontal cortex [72–75]. This desensitization has been attributed not to a change in the number of 5-HT_{1A} receptor binding sites, but rather a reduction in the capacity to activate G protein [76–78]. The occurrence of these changes in the functioning of 5-HT_{1A} autoreceptors in the raphe, which is only seen after continuous administration of SSRIs, has been associated with the delayed therapeutic onset of these drugs in the treatment of depression (for a more detailed account of this proposal see [79]).

With regard to panic, previous studies conducted in our laboratory revealed that pharmacological manipulation of 5-HT_{1A} receptors in the lwDR selectively interferes with escape expression [36] and that chronic, but not acute, fluoxetine treatment increases 5-HT release in the dPAG [24]. In this latter area, whereas facilitation of 5-HT_{1A}- and 5-HT_{2A}-mediated neurotransmission consistently inhibits escape expression [39, 80–82], blockade of 5-HT_{1A} receptors counteracts the anti-escape effect caused by chronic systemic administration of fluoxetine [24]. Results such as these support the hypothesis that stimulation of the serotonergic pathway that connects the lwDR to the dPAG [48, 83, 84], presumably due to desensitization of 5-HT_{1A} receptors in the former region, is critically implicated in the anti-panic effect of fluoxetine.

One important question that derives from this idea is why this pathway is selectively recruited in face of data showing that 5-HT_{1A} autoreceptors are found throughout the DR [6, 85]. There is evidence to suggest that the sensitivity of 5-HT_{1A} receptors differs across the DR subnuclei, being greater in the DRV and lwDR when compared with the DRD or median raphe nucleus [6]. Moreover, the systemic administration of WAY-100635 recruits a greater number of 5-HT neurons in the lwDR and to a lesser extent in the DRC, but not in other DR subareas. Besides these differences in responses to agonists and antagonists, distinct 5-HT cell membrane properties across subregions were also found. Whereas these neurons in the DRD have more hyperpolarized resting potential compared to the DRV, in the lwDR, they have greater action potential amplitude [6]. In mice, 5-HT neurons within the lwDR have active and passive intrinsic membrane properties that make them more excitable than DRV 5-HT neurons [86]. Therefore, it is conceivable that some or all of these particularities, or even still unknown characteristics, may have contributed to the presently observed distinctive effect of chronic fluoxetine in activating 5-HT neurons, likely by desensitizing 5-HT_{1A} receptors, in the lwDR and not in other DR subregions.

It is important to highlight that the functioning of 5-HT_{1A} autoreceptors in the DR is not only determined by regionally specific characteristics, as suggested by the above evidence but is also dependent on the behavioral state. For instance, in mice exposed to a sociability test, which has been used to measure social approach behavior in mouse models of autism spectrum disorder, double-labeled cells for c-Fos and TrpOH were detected in all DR subnuclei. Chronic treatment with fluoxetine, which increases social approach behavior, decreased the activation of these serotonergic cells in only some subpopulations, being without effect in the rostral and mid-rostrocaudal DRD and DRC [87], two subnuclei thought to implicated in the regulation of anxiety-related defensive behaviors [21, 88, 89].

Another important question raised by our findings concerns the effect of chronic fluoxetine in decreasing the number of non-serotonergic cells activated by escape exposure. Kirby and co-workers [90] observed that in the DR, differently from the median raphe nucleus, 5-HT_{1A} receptors are also found in non-serotonergic neurons. They also reported that the distribution of these 5-HT_{1A} heteroreceptors varies across the different DR subnuclei, being detected in the DRV and lwDR, but not in the DRD [6, 90, 91]. Importantly, whereas in the DRD agonist-induced stimulation of 5-HT_{1A} receptors in non-serotonergic cells has no significant hyperpolarizing effect, in the DRV and lwDR, it causes a hyperpolarization of the membrane potential, but with approximately half the magnitude found in serotonergic cells [6]. Therefore, the dynamics of 5-HT_{1A}-receptor mediated inhibition on cell firing is different in these two types of cells. One intriguing possibility arising from these findings is that 5-HT_{1A} receptors in serotonergic and non-serotonergic neurons may also be differently affected by chronic administration of fluoxetine and other SSRIs, being desensitized in the former, but not in the latter cells. If this is the case, chronic fluoxetine, by increasing 5-HT release in the DR, would decrease the number of c-Fos-immunolabeled cells by acting on these 5-HT_{1A} heteroreceptors. Although this remains a speculative hypothesis, it certainly deserves further investigation.

A final remark concerns the effect of fluoxetine in decreasing the number of c-Fos-immunostained cells in the dPAG. In this brain area, it has been consistently shown that chronic, but not acute treatment with fluoxetine and other antidepressants (e.g., sertraline and imipramine) potentiated the anti-escape effect of 5-HT or 8-OH-DPAT [80, 81, 92, 93], suggesting a sensitization and not desensitization of the 5-HT_{1A}-signaling pathway. As such, after chronic fluoxetine, 5-HT is able to more efficiently hyperpolarize the cell membrane of dPAG output neurons, presumably excitatory neurons, leading to escape inhibition.

It may sound apparently contradictory that, as proposed here, chronic fluoxetine desensitizes 5-HT_{1A} autoreceptors in the lwDR, while sensitizing these ligand sites located in the dPAG. However, 5-HT_{1A} autoreceptors and heteroreceptors have diverse intracellular signaling capabilities, which may rely on recruitment of canonic (Gi/Go proteins, GIRKs) and non-canonic (e.g., MAPK and Akt) signaling pathways (for a review see [94, 95]. This diversity clearly contributes to the complex regulatory function exerted by serotonin on its own system as well as on other neuronal substrates.

Altogether, our findings suggest that the anti-panic effect of chronic treatment with fluoxetine is mediated by stimulation of the lwDR-dPAG pathway that controls the expression of panic-associated escape behaviors. This is achieved by complementary, and apparently concomitant mechanisms that result in adaptive changes in the functioning of 5-HT_{1A} auto- and heteroreceptors located in these two brain areas.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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