



Research article

Therapeutic treatment with fluoxetine using the chronic unpredictable stress model induces changes in neurotransmitters and circulating miRNAs in extracellular vesicles

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ABSTRACT

The most widely prescribed antidepressant, fluoxetine (FLX), is known for its antioxidant and anti-inflammatory effects when administered post-stress. Few studies have evaluated the effects of FLX treatment when chronic stress has induced deleterious effects in patients. Our objective was to evaluate FLX treatment (20 mg/kg/day, i.v.) once these effects are manifested, and the drug's relation to extracellular circulating microRNAs associated with inflammation, a hedonic response (sucrose intake), the forced swim test (FST), and corticosterone levels (CORT) and monoamine concentrations in limbic areas. A group of Wistar rats was divided into groups: Control; FLX; CUMS (for six weeks of exposure to chronic, unpredictable mild stress); and CUMS + FLX, a mixed group. After CUMS, the rats performed the FST, and serum levels of CORT and six microRNAs (miR-16, -21, -144, -155, -146a, -223) were analyzed, as were levels of dopamine, noradrenaline, and serotonin in the prefrontal cortex, hippocampus, and hypothalamus. CUMS reduced body weight, sucrose intake, and hippocampal noradrenaline levels, but increased CORT, immobility behavior on the FST, dopamine concentrations in the prefrontal cortex, and all miRNAs except miR-146a expression. Administering FLX during CUMS reduced CORT levels and immobility behavior on the FST and increased the expression of miR-16, -21, -146a, -223, and dopamine. FLX protects against the deleterious effects of stress by reducing CORT and has an

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antidepressant effect on the FST, with minimally-modified neurotransmitter levels. FLX increased the expression of miRNAs as part of the antidepressant effect. It also regulates both neuro-inflammation and serotonergic neurotransmission through miRNAs, such as the miR-16.

1. Introduction

Depression is a heterogeneous, chronic mental disorder that is among the top five causes of disability in the world and contributes significantly to the global burden of disease by affecting over 264 million people globally, according to the World Health Organization. In severe cases, depression can lead to suicide with an estimated 800,000 people dying from depression annually [1,2]. Depression is etiologically multifactorial, involving genetic and environmental factors, stressful events, and hereditary susceptibility [3,4]. The neurobiology of depression is associated with hypoactivation of the prefrontal cortex and linked limbic structures like the hippocampus and hypothalamus. These factors may have multiple conceptual and physiological overlaps. Stress has been recognized as one of the most important predisposing factors for developing depression but its interaction with, and contribution to, mood disorders are poorly understood [5,6]. Though progress has been made in research and treating depression in recent years, the exact pathogenesis of the disease is still unclear. Stress-based animal models are frequently used to explore the biological mechanisms of depression [7], including CUMS (chronic unpredictable mild stress), a rodent model of depression with high face, theoretical, and predictive validity and great translational potential to reproduce anhedonia, a cardinal symptom of depression [8] that results from a dysfunction of neural reward and motivation circuits [9]. CUMS operates by randomly exposing animals to various stressors in a naturalistic manner to decrease their degree of habituation [10]. It can also reproduce parameters related to clinical depression, such as decreased sucrose consumption, alterations in weight gain, decreased responsiveness to various stimuli, and appropriate responses to antidepressant drugs [8,11,12].

It is now known that miRNAs play a critical role in maintaining normal physiological states [13]. Studies have shown that the dysregulated expression of miRNAs participates in the development of depression [14,15] by affecting neural plasticity [16]. miRNAs regulate microglia activation [17], and some (miR-16, -21, -126, -155, -146a, -223) have been reported as potential biomarkers for diseases related to inflammatory processes [18]. Specifically, miR-146a has been identified as a key regulator of the innate immune response that blocks the expression of several pro-inflammatory factors (TNF- α , IL-6, IFN-gamma) [19–21]. In contrast, miR-21 has been related to the production of cytokines and pro-inflammatory mediators since it modulates NF- κ B activation and promotes c-Jun and AKT activity [21–24]. miR-223 and miR-155 are associated with inflammatory processes [23,25], and the latter is implicated in autoimmune diseases such as multiple sclerosis and asthma [23]. It also induces the activation of microglia [26]. miR-16 is a negative regulator of serotonin transport and is also involved in inflammatory processes [27,28]. Recently miRNAs embedded in extracellular vesicles (EVs) have been implicated in depression and treatment responses [29].

The interconnected monoaminergic system has led to the development of adaptive capacities that buffer most genetic-environmental impacts through coping mechanisms [30]. Several studies have shown that alterations in serotonergic (5-HT), dopaminergic (DA), and noradrenergic (NA) neurotransmission, and the associated receptors, may contribute to depression-like behaviors, as these neurotransmitters play key roles in motivation and emotional processing in the brain and inherently influence the risk of developing depression [31]. This theory is the cornerstone of existing pharmacological therapies for depression based primarily on tricyclic antidepressants, selective 5-HT or norepinephrine reuptake inhibitors, and monoamine oxidase inhibitors [32], drugs that directly affect the functional tone of these circuits in limbic and frontal cortical areas [33]. The selective 5-HT reuptake inhibitor, fluoxetine (FLX), is the most widely prescribed drug in the world [9,34]. It acts by inhibiting the serotonin transporter (SERT) with antidepressant effects regulating energy metabolism, synthesis of neurotransmitters, and tryptophan metabolism. It also exerts a protective effect on damaging processes, as it has been shown to attenuate neuronal apoptosis, promote motor functions in damaged tissue (e.g., in cases of cerebral ischemia), and improve cognitive functions [35–38]. Finally, FLX has antitumor, anti-inflammatory, antioxidant, and antiplatelet aggregation effects [39,40].

FLX administration at the onset of stress has a potential anti-inflammatory effect and restores sucrose consumption decreased by CUMS [41–43]. Research has shown that miR-22 and -16 respond to FLX administration [44,45]. However, only a few studies have evaluated the drug's effect during stress to protect organisms from the impact of chronic stress when it begins to have deleterious effects on the body. Since the antidepressant effects of FLX apparently involve several mechanisms, our work was designed to evaluate FLX treatment in the stage when chronic stress has begun to show deleterious effects, focusing on its relation to extracellular circulating microRNAs that have been associated with inflammation, such as SERT regulators, and resilience to stress. To address these issues, we evaluated FLX' action on hedonic responses (sucrose intake), behavioral responses on the forced swim test (FST), corticosterone levels (CORT) as a reliable indicator of stress impact, and, finally, monoamine concentrations in limbic areas and inflammation related to miRNA concentrations in circulating EVs.

2. Materials and method

Three-month-old male Wistar rats were obtained from the vivarium of the *Universidad Autónoma Metropolitana-Iztapalapa*. We have carried out all animal experiments in strict accordance with the Official Mexican Standards, NOM-062-ZOO-1999 [46], and the National Institutes of Health Guide for the Care and Use of Laboratory Animals [47]. The experimental protocol was approved by the Ethics Committee of the UAM-I (CBS.310.18). The rats were kept on a 12-h inverted light-dark cycle (lights on 9 p.m.) with food and

water *ad libitum*. They were assigned to the following experimental groups: a) CTRL; b) intact with FLX at a dose of 20 mg/kg/day for three weeks; c) stress group subjected to the CUMS procedure for six weeks; and d) CUMS + FLX with the rats subjected to CUMS for six weeks with FLX (20 mg/kg/day) administered orally for 21 days from the beginning of the fourth week [48,49] (n = 6 per group).

Table 1 describes the stressors applied during the six weeks of CUMS exposure. Body weight and sucrose consumption (SPT) were recorded weekly. The average initial body weights in grams were CTRL = 346, FLX = 345, CUMS = 340, and CUMS + FLX = 341. On the day following the end of CUMS (day 43) or FLX administration, the rats in each group were subjected to the FST for 15 min. After a 24-h period (day 44), the test was repeated for 5 min. Finally, at 72 h after completing CUMS (day 45), the rats were euthanized by decapitation to obtain blood serum and dissect three brain structures: the hypothalamus, hippocampus, and prefrontal cortex, to analyze DA, NA, and 5-HT concentrations. Expression of miR-16, -21, -144, -155, -146a, and -223 and CORT levels were analyzed in plasma and serum, respectively. Fig. 1 describes the experimental design.

2.1. FST

The FST procedure has been adapted from previous studies [2,50,51,53]. Rats were placed individually in glass cylinders (40 cm high × 20 cm in diameter) containing 30 cm of water (maintained at 24 ± 1 °C), not allowing to support themselves by touching the bottom. Two swimming sessions were conducted between 10:00 a.m. and 13:00 p.m.: an initial 15-min test followed 24 h later by a 5-min test. After the two sessions, the rats were dried with towels and returned to their cages. All testing took place during the dark phase of the cycle. All recorded sessions were used for scoring. Fresh water was used for each evaluation to prevent confounding results from interference from urine or feces.

This is a valid reliable method used to assess the effects of various antidepressant drugs [50–52]. During the 5-min test at each 5-s interval, we rated the rats behavior into the following categories: (1) immobility, when rats remained passively floating in the water by only making the movements necessary to keep the head above the water; (2) swimming, when the movements of the four extremities allowed the animals to move around or across the cylinder; and (3) climbing, as active attempts with the forepaws movements in and out of the water, mostly toward the cylinder walls. A previously trained observer blind-sighted to the treatment conditions scored and conducted all behavioral scoring. The total number of counts per 5-min session was scored for each individual behavior. Where the number of times that specific behavior (immobility, swimming, or climbing) represented each count observed during the 5-min test.

2.2. Sucrose preference test (SPT) and body weight

We used the SPT to assess anhedonia by measuring the intake of a 1% sucrose solution once a week on different days (days 0, 7, 14, 21, 28, 35, 42) in a 1-h window after 24 h of water and food deprivation. Two bottles, one with tap water and the other with the sucrose solution were randomly placed (right or left) each week to avoid side preference. Consumption was measured by comparing the volume in ml before and after the 1-h window. The baseline was measured one week before the onset of chronic stress, as described in Ref. [53]. The period of water deprivation preceding the measurement of sucrose intake can be considered a stressor applied in addition to the chronic stress protocol; however, the control rats were also exposed to this period of deprivation as part of the test [7, 30,54]. Consumption of water or the sucrose solution was determined by subtracting the residual volume at the end of the test from the initial volume. Sucrose preference (SP) was determined as the percentage of sucrose solution consumed over the total amount of liquid ingested (*i.e.*, water intake plus sucrose intake) as follows: $SP = [(sugar\ solution\ intake/total\ liquid\ intake) \times 100]$. Body weights were

Table 1

Scheme of the types and sequence of the stressors applied to the rats during 6 weeks of subjection to stress. CUMS procedure.

Day	Stressor	Day	Stressor
1	Foreign object in cage 24 h	22	Movement restriction 1.5–3 h
2	Overcrowding [15 rats per cage] 24 h	23	Social isolation + Persistent light 24 h
3	Inclined cage of 45° 24 h	24	Immersion in cold water [8–10 °C] 15 min
4	Movement restriction 1.5–3 h	25	Cold exposure [4 °C] 1.5–3 h
5	Persistent light 24 h	26	Wet bedding [100 ml water/individual cage] 24 h
6	Food and water deprivation 24 h	27	Food and water deprivation 24 h
7	Cold exposure [4 °C] 1.5–3 h	28	Overcrowding [15 rats per cage] + Cage tilt of 45° 24 h
8	Immersion in cold water [8–10 °C] 15 min	29	Social isolation 24 h
9	Wet bedding [100 ml water/individual cage] 24 h	30	Movement restriction 1.5–3 h
10	Social isolation	31	Persistent light 24 h
11	Movement restriction + Cold exposure [4 °C] 1.5–3 h	32	Immersion in cold water [8–10 °C] 15 min
12	Inclined cage of 45° + Persistent light 24 h	33	Wet bedding + Foreign object in cage 24 h
13	Food and water deprivation 24 h	34	Food and water deprivation 24 h
14	Immersion in cold water [8–10 °C] 15 min	35	Inclined cage of 45° 24 h
15	Social isolation + Foreign object in cage 24 h	36	Overcrowding [15 rats per cage] 24 h
16	Movement restriction 1.5–3 h	37	Cold exposure [4 °C] 1.5–3 h
17	Wet bedding [100 ml water/individual cage] 24 h	38	Movement restriction 1.5–3 h
18	Overcrowding [15 rats per cage] 24 h	39	Social isolation + Foreign object in cage 24 h
19	Cold exposure [4 °C] 1.5–3 h	40	Immersion in cold water [8–10 °C] 15 min + Wet bedding 24 h
20	Food and water deprivation 24 h	41	Food and water deprivation 24 h
21	Inclined cage of 45° + Foreign object in cage 24 h	42	Overcrowding [15 rats per cage] + Persistent light 24 h

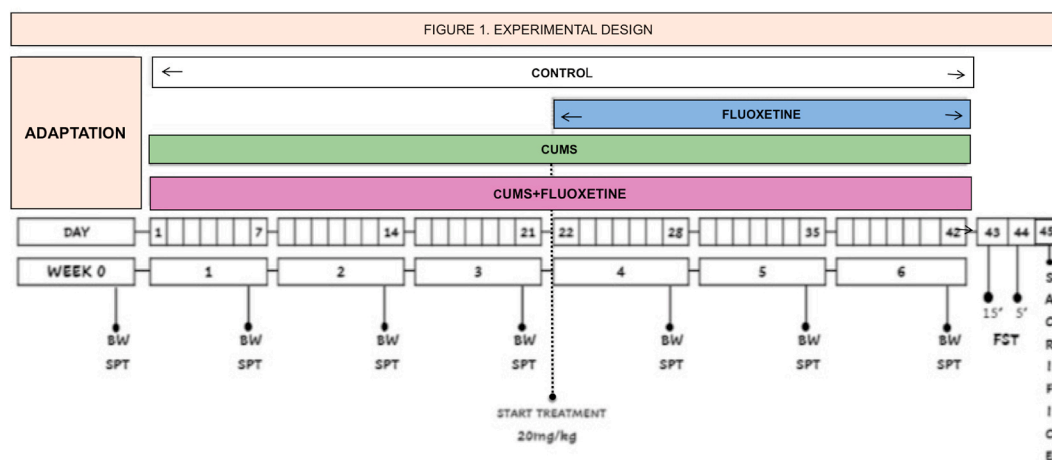


Fig. 1. Experimental design. The rats were assigned to the different experimental groups (CON, FLX, CUMS and CUMS + FLX). Both VCUG and VCUG + FLX groups were subjected to stress for 42 days (6 weeks). One day after the end of CUMS or FLX administration (day 43). The rats in each experimental group underwent FST for 15 min. After a period of 24 h (day 44), the test was repeated for 5 min. Finally, 72 h after completing CUMS (day 45), the rats were sacrificed by decapitation to obtain blood serum and dissect three brain structures: the hypothalamus, the hippocampus and the prefrontal cortex, to analyze the concentrations of DA, NA and 5-HT. The expression of miR-16, -21, -144, -155, -146a and -223 and the levels of CORT were analyzed in plasma and serum, respectively.

recorded every week.

2.3. Determination of monoamines

The animals of all groups were euthanized by decapitation at the onset of the dark phase, between 9 and 11 a.m. The brain areas of interest were dissected as described in previous reports, with some modifications [55–57]. Immediately after euthanasia, an incision was made along the midline of the head skin. Then a small incision was made in the upper part of the skull, beginning in the caudal area at the point of the parietal bone, taking care not to graze the brain. Next, the dorsal cranial bones and the brain were removed, quickly but gently, from the cranial cavity. The brains were rinsed immediately in Milli-Q water treated with ice-cold DEPC to remove blood from the surface. Once the hypothalamus, hippocampus, and prefrontal cortex were dissected, samples were placed in liquid nitrogen for preservation and stored at -80°C for later analysis of the monoamines by reversed-phase high-performance liquid chromatography (RP-HPLC).

2.4. Reverse-phase high-performance liquid chromatography (RP-HPLC)

For NA, DA, and 5-HT extraction, 400 μl of buffer containing 5% ascorbic acid, 200 mM sodium phosphate, 2.5 mM L-cysteine, and 2.5 mM EDTA was added. To precipitate the protein 100 μl of 0.4 M perchloric acid was added, with an incubation at 20°C for 20 min. Then, after centrifugation at 12,000 rpm for 10 min (4°C) the collected supernatants were filtered by 0.22 μm then used for the evaluation of NA, DA, and 5-HT by RP-HPLC in a system that consisted of a PU-2089-plus pump (Jasco, Inc.), an AS-2057-plus autosampler (Jasco, Inc.), and an X-LCTM3120FP fluorescence detector (Jasco, Inc.). ChromNav software (Jasco, Inc.) was used to control all instruments. Chromatographic runs were performed using a Júpiter C₁₈ column (300 Å, 5 μm , 4.6 \times 250 mm, Phenomenex®) at 30°C . The column was equilibrated with mobile phase A containing 0.1% trifluoroacetic acid in water. Then a linear gradient was performed from minute 5 to minute 20 with mobile phase B containing 0.1% trifluoroacetic acid in acetonitrile at a flow rate of 0.8 ml/min. The fluorescence detector was set to a gain of 1000, an attenuation of 32, a response time of 20 s, and 280 nm and 315 nm for excitation and emission, respectively. Using 50 μl as sample injection volume.

2.5. RNA isolation from extracellular vesicles

Blood samples were collected, and 350 μL of plasma were centrifuged 12000g for 8 min to further isolate RNA from extracellular vesicles with the exoRNeasy serum/plasma midi kit (adding 1.6×10^8 copies of synthetic cel-miR-39 control in the RNA isolation step) (Qiagen). Immediately after isolation, RNA was retrotranscribed (see below).

2.6. Detection of miRNAs by RT-qPCR

miRNAs levels were measured by RT-qPCR, 1.5 μL of isolated RNA was retrotranscribed with the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems). The RT reaction comprised a single cycle of 30 min at 16°C , 30 min at 42°C , and 5 min at

85 °C. Each miRNA was amplified from 2 µL of the RT (using specific primers) with the specific TaqMan probes (Applied Biosystems). Using the LightCycler TaqMan Master Kit reagent, PCR was carried out on a LightCycler 480 II thermocycler (Roche Applied Science, Basel, Switzerland). The PCR conditions included an enzyme activation step of 10 min at 95 °C, followed by 45 cycles of 95 °C for 15 s, at 60 °C for 60 s, and at 72 °C for 1 s. Relative miRNA levels were determined using the $2^{-\text{Target miRNA Ct} - \text{cel-miR-39 Ct}}$ equation.

2.7. Drugs

Fluoxetine hydrochloride (Prozac) was dissolved in a freshly-prepared 0.9% saline solution and administered by gavage [58] at a volume of 0.1 mL/100 g of body weight. This dose was chosen due to its effect in the FST (49).

2.8. Statistical analyses

All results are expressed as mean \pm SEM for each group. Between-group differences in monoamines, CORT levels, FST results, and miRNAs were analyzed by a one-way analysis of variance (ANOVA), while body weight and consumption preference on the sucrose test were analyzed using a two-way ANOVA, followed by a Tukey test. A P-value <0.05 indicated statistically-significant differences.

3. Results

3.1. Body weight and SPT

As mentioned above, the average initial body weights of the rats in grams were CTRL = 346, FLX = 345, CUMS = 340, and CUMS + FLX = 341. The FLX group reduced its average body weight by 14.08% from the first week of treatment ($p < 0.05$). The CUMS and CUMS + FLX groups also significantly reduced their body weights, by 8.97% and 13.77%, respectively, after 5 weeks of stress ($p < 0.05$), compared to CTRL (Fig. 2).

Fig. 3, panels A and B, show the results for sucrose preference (SP) as a percentage of the rats' consumption of the sucrose solution (Panel A) and water (B) in ml in 1 h. SP decreased in FLX by week 6. In CUMS and CUMS + FLX, SP decreased significantly ($p < 0.05$) from week 4 of subjection to stress, compared to CTRL. This decrease in sucrose consumption in the CUMS + FLX group was maintained until week 6, compared to CUMS and CTRL ($p < 0.01$). The reduction in SP revealed an increase in water consumption in the rats in both the CUMS and CUMS + FLX groups, neither one of which discriminated between water and sucrose.

3.2. FST and CORT levels

On the FST, the CUMS group significantly increased immobility behavior ($p < 0.05$) and reduced ($p < 0.01$) swimming, but with no effect on climbing. The opposite effect was observed in the FLX group, as it reduced immobility but increased swimming, also with no effect on climbing ($p < 0.05$). The CUMS + FLX group reduced immobility but increased swimming ($p < 0.05$) (Fig. 4A). Fig. 4 panel B, shows the results of the effect of CUMS, FLX, and CUMS + FLX on plasma CORT levels. The CUMS group experienced a significant increase in CORT concentrations, but this was re-established when FLX was administered at the beginning of week 4 of stress in the CUMS + FLX group ($p < 0.05$). FLX had no effect on CORT concentrations.

3.3. Levels of monoamines in the hypothalamus, hippocampus, and prefrontal cortex

Results show an increased NA concentration in the prefrontal cortex in the CUMS + FLX group compared to CTRL ($p < 0.05$), though no changes were seen in the hypothalamus. In the hippocampus, NA decreased in the CUMS and CUMS + FLX groups compared to the CTRL and FLX groups (Fig. 5 Panel A) ($p < 0.05$). DA increased in the prefrontal cortex in the CUMS and CUMS + FLX groups compared to the CTRL and FLX groups ($p < 0.05$). For the hippocampus, DA decreased only in the CUMS + FLX group with respect to

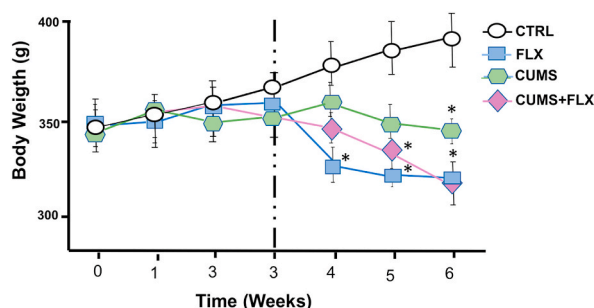


Fig. 2. Effects of CUMS and FLX on weekly body weight gain. Body weight gain in the CUMS + FLX, CUMS, and FLX groups was significantly less than in the CTRL group from the 4th week of the study. Data expressed as Means \pm Standard Error ($n = 6$ per group), analyzed by a two-way ANOVA with a post hoc Tukey test. * $p < 0.05$ compared to CTRL; + $p < 0.05$ vs. FLX, ‡ $p < 0.05$ vs. CUMS.

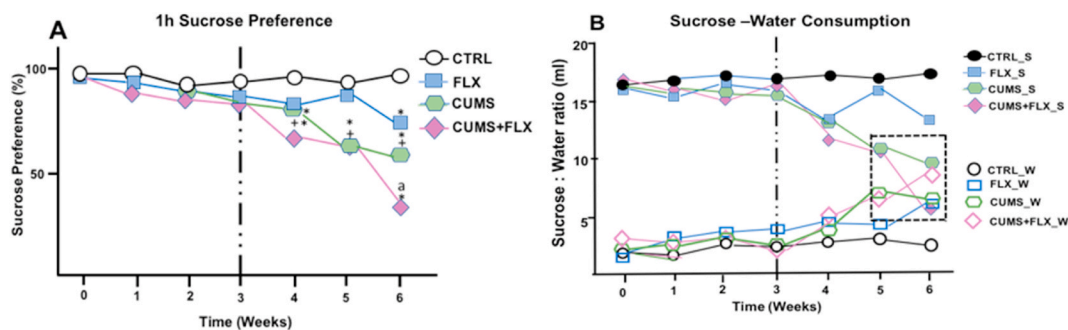


Fig. 3. Effects of CUMS and FLX on the sucrose preference test showing (A) the percentage of the sucrose solution consumed in 1 h; and (B) consumption of water and sucrose. The preference rate for sucrose and total ingestion of liquid decreased significantly in the CUMS and FLX rats compared to the CTRL and FLX groups. During the final 2 weeks of stress, no difference was observed between water and sucrose consumption in the CUMS and CUMS + FLX groups. Data expressed as Means \pm Standard Error (n = 6 per group), analyzed by a two-way ANOVA with a post hoc Tukey test. *p < 0.05 compared to CTRL; +p < 0.05 vs. FLX, ^ap < 0.05 vs. CUMS.

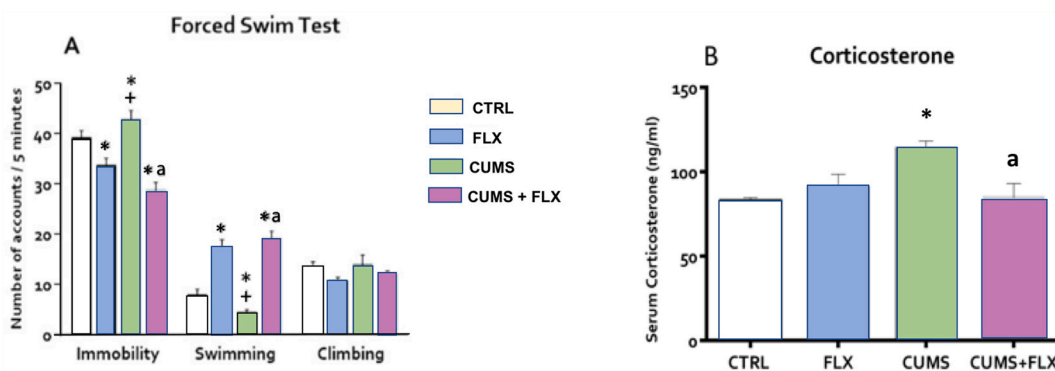


Fig. 4. Effect of CUMS and FLX on the FST (A) and serum CORT levels (B). CUMS showed an increase in immobility behavior with a decrease in swimming behavior. Treatment with FLX reduced immobility behavior and increased swimming behavior. CORT increased due to the effect of CUMS, and decreased with FLX treatment. Data expressed as Means \pm Standard Error (n = 6 per group). ANOVA followed by a Tukey test. *p < 0.05 compared to CTRL; +p < 0.05 vs. FLX, ^ap < 0.05 vs. CUMS.

the FLX group (Fig. 5 Panel B). Finally, 5-HT levels increased in the prefrontal cortex in the CUMS + FLX group compared to the FLX group. In the hippocampus, 5-HT decreased in the CUMS + FLX group compared to the CTRL and FLX groups (Fig. 5 Panel C).

3.4. Expression of miRNAs in plasma EVs

Fig. 6 shows the effect of CUMS, FLX, and CUMS + FLX on the expression of miRNAs. miR-146a was not modified by the effect of CUMS or FLX, but miR-155 decreased in the CUMS (p < 0.01) and CUMS + FLX (p < 0.05) groups compared to the CTRL and FLX groups, respectively (Fig. 6 Panel A and B). Similarly, miR-21 decreased significantly in the CUMS group (p < 0.01) with respect to CTRL, while FLX administration in the CUMS group induced an increase in miR-21 with respect to the CUMS (p < 0.01) and FLX groups (p < 0.05) (Fig. 6 Panel C). MiR-144 and miR-16 (p < 0.0001) decreased in the FLX, CUMS and CUMS + FLX groups (p < 0.05) compared to CTRL (Fig. 6 Panel D and E), but miR-16 increased in the CUMS + FLX group compared to CUMS. Likewise, miR-223 decreased significantly in the FLX and CUMS groups compared to CTRL (p < 0.01) (Fig. 6 Panel F).

4. Discussion

In the present study, we analyzed the therapeutic antidepressant effect of FLX on circulating miRNAs in EVs, the FST, corticosterone levels, and sucrose consumption using the CUMS animal depression model. Evidence suggests that stress plays an important role in the development of depression. After 42 days of CUMS, the rats showed reduced sucrose consumption, decreased weight gain, increased swimming behavior on the FST, and higher CORT levels with alterations in monoamine concentrations and reduced expression of miR-16, -223, -21, and -144a, four miRNAs that are modulated in inflammatory states. All these findings support the use of CUMS as an animal model of depression [7,59,60]. FLX at a dose of 20 mg/kg inhibited the increase in stress-induced CORT levels and re-established miRNAs expression and the behavioral response on the FST that had decreased due to the impact of CUMS. However, it did not restore monoamine concentrations or NA and DA levels on the SPT. Moreover, FLX worsened some behavioral characteristics

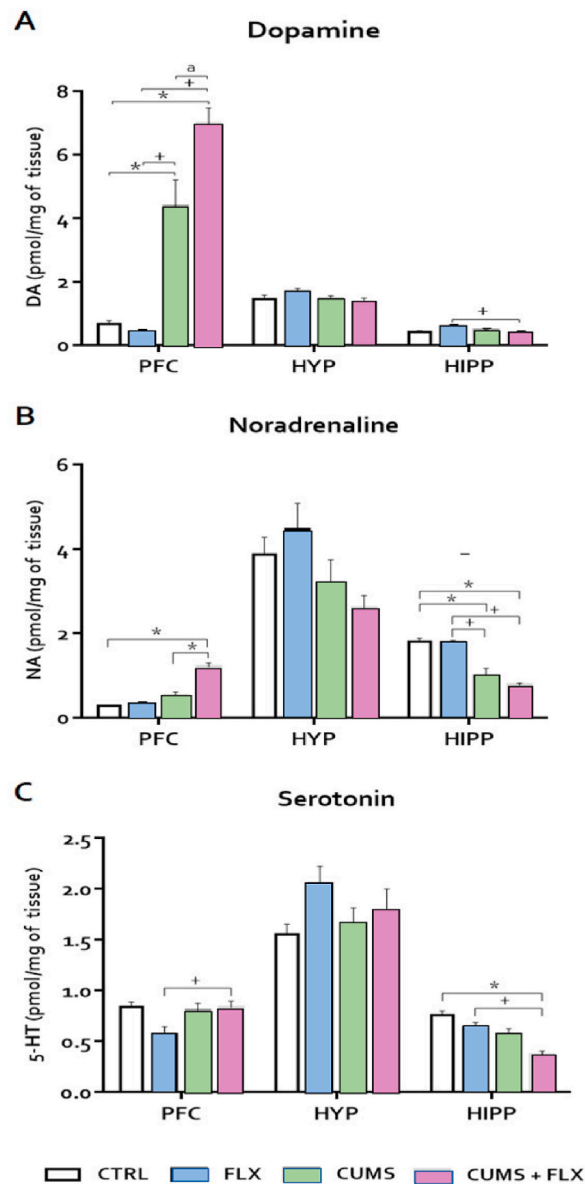


Fig. 5. Effect of CUMS and FLX on DA (A), NA (B), and 5-HT (C) levels. CUMS and FLX induced neurochemical changes in all the brain structures analyzed. An increased in the levels of NA and DA in the PFC in the CUMS and CUMS + FLX groups compared to the CTRL and FLX group. Data expressed as means \pm standard error ($n = 6$ per group). Statistical analysis was performed by an ANOVA followed by a Tukey test. * $p < 0.05$ compared to CTRL; + $p < 0.05$ vs. FLX, ^a $p < 0.05$ vs. CUMS. NA = noradrenalin; DA = dopamine; 5-HT = serotonin. HYP=Hypothalamus, HIPP= Hipocampus, PFC= Prefrontal Cortex.

and body weight in the intact and CUMS groups. These results are suggestive of FLX' therapeutic effect.

4.1. Effect of FLX on body weight and SPT

FLX administration once the stress has begun to damage an organism acts on SERT. Its antidepressant effect involves various mechanisms that seek to improve symptoms of depression [61]. In rats and mice, SPT is recognized as the best method for evaluating anhedonia [62]. Observations of our CUMS-exposed rats showed a significant decrease in sucrose intake compared to CTRL after four weeks that continued through the six weeks of the CUMS protocol, indicating an anhedonic-like state [63]. These results are consistent with previous studies [64–66] and confirm earlier reports [60,64]. Some reports indicate that FLX administration at a dose of 2.1 mg/kg in rats subjected to CUMS improves levels of sucrose consumption on the SPT [67]. This effect, however, was not detected at the dose of 20 mg/kg. Some researchers have suggested that reductions in body weight following CUMS contribute to lower sucrose intake

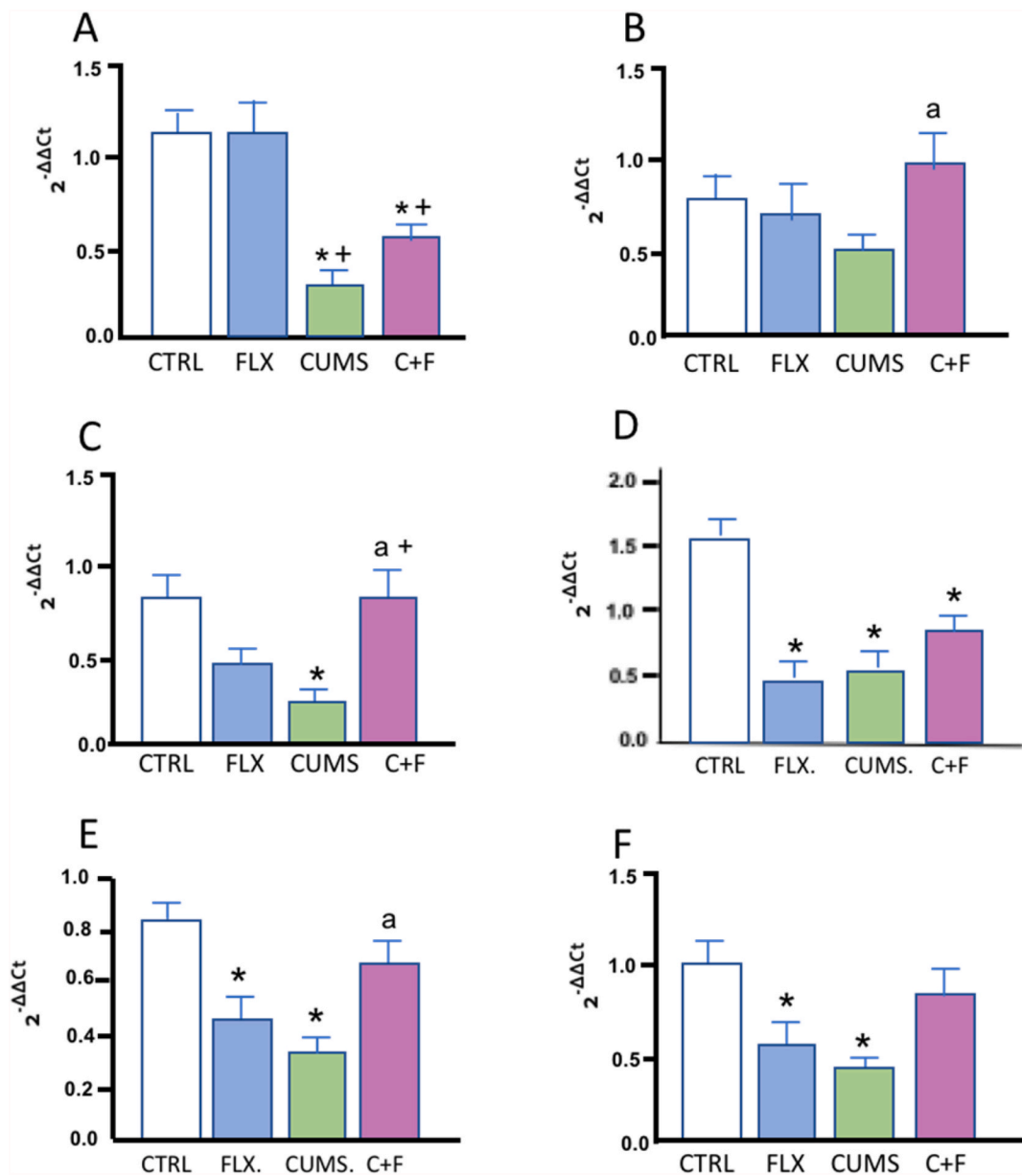


Fig. 6. Effect of CUMS and FLX on levels of miRNA expression. FLX induced changes in the expression of miRNAs in CUMS rats. Data expressed as Means \pm Standard Error ($n = 6$ per group). Statistical analysis was performed by an ANOVA followed by a Tukey test. * $p < 0.05$ compared to CTRL; + $p < 0.05$ vs. FLX, ^a $p < 0.05$ vs. CUMS.

[68,69]. Indeed, a decrease in body weight was observed in the CUMS group. This decrease is a constant finding in stressed animals, one considered a function of immaturity or stress responses. Some studies affirm that the combination of food and water deprivation influences SP and intake following CUMS [7,70,71], arguing that this interacts with exposure stressors that generate anhedonia in previously resilient animals [72]. However, administering FLX to our intact rats had undesirable effects, such as reduced body weight from the first week of administration [73,74].

Studies in humans have also shown reduced body weight due to the effect of FLX [75]. Rats that received different doses of FLX (10, 20, and 40mg/kg i.p. for 2, 4, and 12 weeks) also showed decreased body weight from week 2 of administration [76–78], with a dose-dependent effect [79]. This reduction in body weight seems to be attributable to the secondary effects of FLX, such as lower food intake [68,80], a secondary effect that could be due to undesirable impacts on the digestive system, such as diarrhea, nausea, vomiting, dyspepsia, dysphasia, taste confusion, and dry mouth [67], reduced femur length in adult rats [81], and lower weight of the reproductive organs (testes, epididymides, ventral prostate) [77,81,82]. Moreover, at a dose of 20 mg/kg FLX affects the intestinal microbiota that participates in regulating body mass [83]. Finally, the decrease in body weight in the CUMS + FLX group could be the

result of the sum of the effects that FLX exerts on body weight plus the effect of stress.

4.2. Effect of FLX on the FST

The FST is one of the best-known screening tests for evaluating depressive-like behaviors and antidepressant agents. On this test, increased immobility behavior by subjects is interpreted as indicative of behavioral despair [84]. Chronic FLX administration resulted in behavioral modifications and improved immobility behavior on the FST, similar to what has been reported previously for this test at a dose of 20 mg/kg [49]. The role of FLX in improving immobility behavior may be due to the modulation of circulating CORT levels. Studies in animals have shown that stress induces CORT to release that, in turn, impairs cortical functions (e.g., in the hippocampus) by suppressing neurogenesis [85], thus leading to depression [86]. Chronic FLX treatment normalizes CORT secretion in experimental models [87–89]. Our results show a similar effect with increased CORT levels induced by CUMS that were re-established when FLX was administered to the CUMS + FLX group. Other evidence suggests that chronic treatment with FLX restores homeostasis in the hypothalamus-pituitary-adrenal (HPA) axis in humans [90] by inducing at least a 50% reduction on the HDRS scale [91]. FLX has also shown efficacy in reducing immobility behavior on the FST in other animal models of depression [49,92]. FLX increases transcript levels of 5-HT_{1A} receptor in the brainstem, frontal cortex, and hippocampus in rats. Its delayed therapeutic action is thought to be mediated by desensitization of the presynaptic somatodendritic 5-HT_{1A} autoreceptors and nerve terminal 5-HT_{1B} autoreceptors, which modulate the firing of serotonergic neurons and 5-HT release, respectively [93]. Long-term FLX administration in rats also produces desensitization of post-synaptic 5-HT_{1A} receptors in the hypothalamus, manifested by reductions in the ACTH and CORT secretory responses to 5-HT_{1A} agonists [93,94].

Disruption of the HPA axis is an established finding in patients with anxiety and/or depression. Hyperactivity of this axis is a particularly powerful factor that triggers depressive disorders [95]. This parameter increased in the CUMS group. It has been reported that stress/depression significantly increases serum CORT levels [96] and results in the occupation of mineralocorticoid and glucocorticoid receptors (GR). Occupation of GR includes a higher response capacity of hippocampal neurons to stimulation by the 5-HT_{1A} receptor, attenuating 5-HT autoinhibition, accompanied by a permissive effect on stress-induced increases in the release of 5-HT in the hippocampus. 5-HT_{1A} receptors are also found in the pyramidal CA1 neurons of the hippocampus [97] so 5-HT can modulate the response capacity of the pyramidal cells bidirectionally [98]. Studies of the rat's hippocampus have shown that long-term exposure to high CORT levels brings an attenuation of responses to the activation of 5-HT_{1A} receptors [99–101] and a reduced function of hippocampal 5-HT_{1A} receptors during stress [102]. Adaptive modifications of the serotonergic modulation of hippocampal neuronal activity are thought to provide an important outcome of different types of antidepressant therapies [103]. In this regard, it has been proposed that FLX acts by increasing the expression of GR while inhibiting the steroid transporters localized on the BBB, in neurons, and PGP, a protein that limits the access of cortisol, thus increasing that of corticosteroids in the brain and glucocorticoid-mediated negative feedback on the HPA axis [104,105]. This also increases the function of the activity of the 5-HT_{1A} receptor by increasing activation of the BDNF receptor (TrkB) which enhances the capacity of 5-HT_{1A} receptors to activate G proteins in the hippocampus [102]. This reduces the increase in CORT in rats subjected to stress [96]. FLX also counteracts stress-induced changes mainly on presynaptic α_2 -adrenoceptors expressed in the prefrontal cortex [106], whose noradrenergic activity increases by acting on distinct α_2 -adrenoceptor subpopulations [106].

4.3. Effect of FLX on monoamines in CUMS rats

Monoamines are involved in the neurobiology of depression and the action mechanisms of antidepressant agents. Monoaminergic neurons in the hypothalamus, hippocampus, and prefrontal cortex have been shown to play a pivotal role in stress response and depression-like behaviors [107,108]. Effects seen on CUMS, suggest that these depression-like behaviors couple with dopaminergic hyperfunction in the NAc and serotonergic hypofunction in the hippocampus, prefrontal cortex, and striatum [109–111], or an increase of 5-HT in the medial prefrontal cortex and striatum [43]. In rodent depression phenotypes, decreased serotonergic activity via 5-HT_{1A} receptors in the prefrontal cortex [112] suggests that CUMS impairs the monoaminergic function in structures involved in both depression and stress. Evidence indicates that 5-HT exerts a modulating effect on DA by increasing or decreasing its activity, depending on actions related to other neurotransmitters that lead to self-regulation; that is, as 5-HT increases, DA decreases [113,114].

In our results, the most significant changes detected in the CUMS group included an increase of DA in the prefrontal cortex, but a decrease in the hippocampus with a tendency towards reducing 5-HT levels. The increase of DA suggests that the modification in depression symptoms may be related to alterations in the neural encoding of action in limbic circuitry, such as the dopaminergic reward circuit that participates in processes like recognizing rewards in an environment and initiating consumption [115], and plays a crucial role in the stress response and mood disorders [116]. In fact, extracellular dopamine levels are elevated in the NAc of humans who are undergoing stress [117]. The 5-HT values, in this case, can be explained by the fact that the animals were not sacrificed at the end of the CUMS protocol, but were evaluated after the FST (that is, 72 and 48 h before sacrifice; see Fig. 1). In this regard, it is well-known that subjection, both unique and repeated, to the FST increases intracellular 5-HT and 5-HIAA levels in various brain structures in rodents [110,118,119]. Presumably, in our study, the stress of FST influenced the response in 5-HT levels in the hippocampus, especially because the rats were sacrificed 72 h after applying the battery of stressful factors and 24 h after the 5-min FST, in contrast to other experimental procedures where subjects were sacrificed upon finalizing the CUMS protocol.

It is well known that FLX binds to SERT in central neurons, thus increasing serotonin availability in the synaptic cleft [120,121]. Its effect is exerted through SERT and 5-HT_{1A} receptors. Strong experimental evidence suggests that SERT may be regulated [122] and undergo adaptive changes during its therapy that depends on treatment time. Reports on this topic have cited increases, decreases, and

the absence of changes in the density of SERT and DRN binding sites, as well as varied results for regions innervated by 5-HT, including the prefrontal cortex [123–127]. In a recent report, chronic FLX treatment induced the internalization of SERT in cellular bodies and cells, such as the axon terminal of 5-HT neurons. A differential effect with the 5-HT_{1A} autoreceptors that is present on the plasma membrane of cell bodies and dendrites is not internalized, but they are desensitized, and their re-sensitization requires several weeks after interruption of chronic FLX treatment [128]. According to our results, FLX did not modify 5-HT. There is some evidence that when FLX treatment is chronic (e.g., three weeks in rats), the increases in extracellular 5-HT concentrations may not persist in the nuclei of origin [129–131] or in the regions of 5-HT projection [130–132]. For example, chronic FLX administration (30 mg/kg, i.p.) for three days did not modify serotonin levels in the frontal cortex [133,134].

4.4. Effect of FLX on miRNA expression

Stress can trigger an inflammatory response in the brain that can induce neurochemical changes and behavioral disturbances that lead to diagnoses of depression [135]. Recently, several miRNAs have been reported to be altered in the brain and peripheral tissues, such as blood, related to stress responses [136,137], depression, and/or treatment with antidepressants [27,138]. Regulating gene expression by miRNAs is considered an important factor in modulating the neurotransmission of the stress response, one that can be used as a biomarker of treatment response [139]. In the present study, the changes in the concentration of circulating miRNAs in EVs may reflect some of the systemic anti-inflammatory effects of FLX and its connection with SERT. Specifically, miR-16 regulates the expression of SERT [27,140], resilience to stress, responds to FLX administration [45], and modifies inflammation [27,28]. 5-HT also regulates key functions in peripheral tissues, such as the immune system. Several immune cells, such as T cells, macrophages, mast cells, dendritic cells, and platelets produce, store, respond, and/or transport serotonin [141–143]. On the other hand, the brain tissue of suicidal patients shows altered expressions of miR-16, as well as down-regulation of its concentration in serum [144], which may also be related to immune cell functions. Chronic FLX treatment increases miR-16 levels in the serotonergic raphe nuclei, thus reducing SERT expression [27]. In this regard, we observed a similar response with a decrease in miR-16 expression in the CUMS group and an increase after FLX treatment. Other studies indicate that the antidepressant effects of FLX appear to involve neurotransmitter synthesis and anti-inflammatory action that may promote hippocampal neuroplasticity by increasing –a protein related to neuronal plasticity in the hippocampus called GAP-43 as seen on a rat model of depression [145]– and that FLX treatment can improve the depressive behavior exhibited.

In our results, CUMS induced a reduction in the miRNAs analyzed that, in other scenarios, has been reported to promote microglial reactivity and produce neuroinflammation [153] in a process that contributes to depression. FLX may also have an effect by reducing the expression of pro-inflammatory cytokines such as IL-1 β , IFN- γ , and TNF- α [152] that could regulate the expression of miR-21, -146a, and -155, all of which are involved in microglial activation. This is because all miRNAs, except for -144, were found to increase in the stressed rats with FLX treatment (CUMS + FLX group). Paradoxically, miR-144 decreased in all the experimental groups (FLX, CUMS, CUMS + FLX). It has been reported that miR-144-3b decreased in rats exposed to CUMS but increased due to 7-chloroquinurenic acid, an NMDA receptor antagonist that is a potential rapid antidepressant [153]. miR-144 has been associated with the expression of proteins related to GABAergic, glutamatergic, and dopaminergic synapses [154,155] that control target genes or pathways involved in neurite growth, neurogenesis, and ERK and Wnt/ β -catenin signaling [156]. FLX not only inhibits SERT, but simultaneously reduces the release of glutamate and GABA [157], suggesting that these miRNAs may be altered upon FLX administration, but remained low in the FLX + CUMS group. It is important to note that our evaluation of miRNAs was performed in plasma vesicles, which may explain the contrasts with other studies that evaluated their presence in the cerebral cortex and hippocampus. It is also well known that numerous stimuli associated with depression can trigger microglial activation, such as peripheral or central inflammatory foci and stress-related conditions due to an increase in glucocorticoids via the HPA axis, which have been reported to activate microglia [143,144] or cause psychological stress that, in turn, promotes microglial activation through the release of brain alarmines [145]. CUMS increases plasma concentrations of IL-6, CRP, and TNF- α and induces depression-like behavior [144], while in animal models, cytokines induce depression-like behaviors [146,147]. In addition, miR-146a increases in microglia and disrupts the normal inflammatory response, including the NF- κ B and JAK-STAT signaling pathways, which are activated by both TLR2 and TLR [148] and overexpression of miR-146b-3p, which inhibited the production of inflammatory cytokines in microglia [149]. Moreover, miR-21 and -146a were found to be transferred to the microglia in glioma EVs where they promoted microglial activation [150,151].

5. Conclusion

Stress-induced changes in NA, DA, and 5-HT levels in the hippocampus and prefrontal cortex, structures related to higher CORT levels, decreased body weight, sucrose intake preference, and total fluid intake decreased levels of all the miRNAs evaluated. FLX remains the most important antidepressant treatment, but one that is known to have effects on numerous mechanisms. The therapeutic actions of SERT-targeted antidepressants exert their function by blocking this substance, thereby increasing 5-HT levels in the synapses of serotonergic projection terminals. In our study, FLX reduced corticosterone levels and had an antidepressant effect on the FST, but modified neurotransmitter levels only minimally and produced harmful effects on body weight. Furthermore, because FLX increased the expression of miRNAs as part of its antidepressant effect, it also regulates both neuroinflammation and serotonergic neurotransmission through miRNAs, as we determined for miR-16.

Author contribution statement

M. Maetzi Estévez-Cabrera; Gilberto Pérez-Sánchez; Adrian Hernández-Diazcouder; J. Luis Cortes-Altamirano: Performed the experiments.

Fausto Sánchez-Muñoz: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Lenin Pavón; Soria-Fregoso; Alfonso Alfaro-Rodríguez: Contributed reagents, materials, analysis tools or data.

Herlinda Bonilla-Jaime: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

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