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Involvement of GABAergic system in the antidepressant-like effects of chrysin (5,7-dihydroxyflavone) in ovariectomized rats in the forced swim test: comparison with neurosteroids

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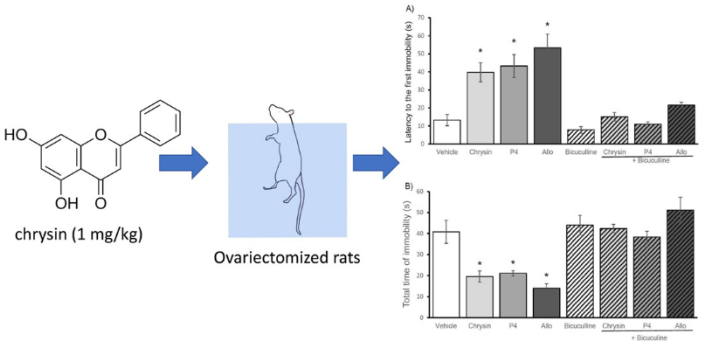
GRAPHICAL ABSTRACT



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ABSTRACT

Rationale: The absence of ovarian hormones that is characteristic of natural and surgical postmenopause in women is frequently related to such disorders as depression and anxiety. Chronic treatment with the flavonoid chrysin was previously shown to exert antidepressant-like effects in rodents subjected to validate behavioral models. Chrysin has also been shown to have anxiolytic-like properties, but its antidepressant-like effects and mechanism of action in the absence of ovarian hormones remain unknown.

Objectives: To compare the effects of the flavonoid chrysin with the effects of the neurosteroids progesterone and allopregnanolone on depression-like behavior in ovariectomized rats and evaluate the participation of γ-aminobutyric acid-A ($GABA_A$) receptors in these actions.

Methods: Ovariectomized female Wistar rats were subjected to the locomotor activity test and forced swim test. The animals were assigned to eight treatment groups: vehicle, chrysin (1 mg/kg), progesterone (1 mg/kg), allopregnanolone (1 mg/kg), bicuculline (1 mg/kg), and pretreatment with bicuculline followed by chrysin,

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progesterone or allopregnanolone, respectively. After the treatments, the rats underwent the behavioral tests.
Results: Chrysin, progesterone, and allopregnanolone increased the latency to the first immobility and decreased the total immobility time in the forced swim test. The number of crossings and the time spent rearing and grooming decreased from the pretest to test sessions in the locomotor activity test. Chrysin, progesterone, and allopregnanolone only prevented the decreases in rearing and grooming. Bicuculline blocked the effects of chrysin, progesterone, and allopregnanolone in both behavioral tests.
Conclusions: These results show that the GABA-binding site at $GABA_A$ receptors participates in the acute antidepressant-like effects of chrysin, similar to neurosteroids, in ovariectomized rats.

1. Introduction

The neurobiology of depression involves neurotransmitters and hormonal functions. The prevalence of depression is higher in women than in men, indicating sexual dimorphism [1], which is partially attributable to cultural factors but also biological effects of sexual hormones (i.e., estradiol, progesterone, and their reduced metabolites, such as allopregnanolone). Sexual hormones can interact with neurotransmitters systems, the stress response axis, and neuronal plasticity [2]. The steroid hormones progesterone and allopregnanolone produce time-dependent effects on neurotransmitter pathways via both direct and indirect actions. Such indirect actions can modulate the γ-aminobutyric acid (GABA)ergic system. Low brain concentrations of steroid hormones can decrease GABAergic neurotransmission and produce anxiety- and depression-like behavior [3]. The premenstrual period, postpartum period, and natural or surgical menopause are characterized by low concentrations of ovarian hormones that can trigger symptoms of depression and anxiety, which evolve into depression and anxiety disorders in many cases [4,5]. Steroid hormones have been proposed as potential therapeutic agents to treat symptoms of anxiety and depression.

Chemical compounds that are derived from plants represent also a potential therapeutic alternative to ameliorate the emotional and affective symptoms of menopause. Such plants as *M. tomentosa*, *M. grandiflora*, and *Hypericum perforatum*, among others, have been used as traditional medicines for the treatment of anxiety and depression symptoms. Metabolites that are contained in plant extracts have been proposed to interact with the GABAergic system [6]. For example, some flavonoids have been shown in preclinical studies to exert potential anxiolytic-like effects through actions on $GABA_A$ receptors [7].

The flavonoid chrysin (5,7-dihydroxyflavone) has multiple biological effects. Chronic treatment with chrysin (5 or 20 mg/kg for 28 days) exerted antidepressant-like effects in cycling female mice that were exposed to chronic unpredictable stress and then evaluated in the tail suspension test [8]. Chrysin treatment at the same doses for 14 days in an olfactory bulbectomy model exerted antidepressant-like effects in the forced swim test (FST) [9]. These effects were similar to fluoxetine and associated with neuroplasticity that involved an increase in brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in the hippocampus and prefrontal cortex in mice [10]. These mechanisms imply changes in neurotrophin levels and plastic changes that are related to chronic treatment with chrysin for 14-28 days. Chrysin has also been shown to exert anxiolytic-like effects in male mice and ovariectomized female rats at lower doses [11–15]. These effects were related to activation of the $GABA_A$ receptor system [7]. Such actions on $GABA_A$ receptors were observed at the first administration of chrysin, which produced immediate effects, in contrast to the chronic effects of chrysin that were previously reported. However, unknown are the antidepressant-like effects of chrysin in female rats with the absence of ovarian hormones that is caused by ovariectomy.

Ovariectomy is used in rats as a surgical menopause model [16]. From 1-week post-surgery, ovariectomized (OVX) rats exhibited anxiety-like behavior in the elevated plus maze compared with rats in the proestrus-estrus phase, and this effect was even greater at 12 and 15 weeks post-ovariectomy [16,17]. The administration of 17β-estradiol [18,19] and the phytoestrogen genistein [20] significantly reduced anxiety-like behavior in OVX rats through actions on the estrogen β receptor, and these effects were similar to diazepam [17]. Interestingly, some neurosteroids (e.g., progesterone and allopregnanolone) that act on $GABA_A$ receptors also exerted anxiolytic- and antidepressant-like effects when they were intraperitoneally injected or microinjected in the lateral septal nucleus, hippocampus, and nucleus *accumbens* in rats [21–26]. Moreover, N,N-dihexyl-2-(4-fluorophenyl)indole-3-acetamide (FGIN-1-27), a 18-kDa translocator protein agonist that induces neurosteroidogenesis, was shown to produce anxiolytic-like effects in non-mammalian models [27]. Therefore, these neurosteroids act acutely as natural anxiolytic and antidepressant agents.

GABAergic compounds, including neurosteroids and plant-derived flavonoids, exert anxiolytic- and antidepressant-like effects through actions on $GABA_A$ receptors, and chrysin has been reported to be a GABAergic compound with anxiolytic properties, but the antidepressant-like effects of chrysin in female rats with the absence ovarian hormones are unknown. The present study investigated the mechanism of action of chrysin to either support or refute its potential use to ameliorate clinical symptoms of depression that are associated with low ovarian hormones, such as during natural or surgical menopause in women. Menopause is a physiological state that is characterized by changes in the sensitivity of serotonergic, dopaminergic, and GABAergic receptors [28,29]. The present study compared the effects of acute chrysin administration with the effects of progesterone and allopregnanolone on depression-like behavior in ovariectomized rats. We evaluated the participation of the GABA binding site on $GABA_A$ receptors in the acute antidepressant-like effects of chrysin using the competitive antagonist bicuculline.

2. Material and Methods

2.1. Ethics

All of the experimental procedures were strictly performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals - National Research Council [30] and Norma Oficial Mexicana para el Cuidado y Uso de Animales de Laboratorio - NOM-062-ZOO [31]. All efforts were made to minimize animal discomfort during the study.

2.2. Animals

Sixty-three adult female Wistar rats, weighing 200-250 g at the beginning of the experiments, were used. The rats were housed four per cage in Plexiglas cages (44 cm width × 33 cm length × 20 cm height). The housing conditions included a 12 h/12 h light/dark cycle (lights on at 7:00 AM), an average temperature of 25 °C ± 2 °C, and *ad libitum* access to purified water and food (Nutri-cubos Purina, elaborated by Agribrands Purina México, México City, México).

2.3. Ovariectomy

Before ovariectomy, we obtained daily vaginal smears as previously reported [32]. Only females with three continuous regular cycles (4-5

days) were included in the study. The surgical procedure was performed in rats at 3 months of age. An abdominal ventral incision was made under deep anesthesia with sodium pentobarbital (60 mg/kg, i.p., Cheminova de México, México City, México, México Reg. SAGARPA Q-7048-044) and atropine sulfate (0.05 mg/kg, i.p., Sigma-Aldrich, St. Louis, MO, USA) as previously described [33]. Both ovaries were removed, and the surgical area was carefully cleaned with saline solution and benzalkonium chloride (Medipharm, San Luis Río Colorado, Sonora, México). The muscle and skin were sutured separately. To minimize postsurgical pain, metamizole (Dipirona50, 50 mg/kg, i.m., Virbac Animal Health, Guadalajara, México) was administered for 4 days after surgery. After surgery and during the entire experimental protocol, all of the rats were examined daily to detect health anomalies, including changes in water and food intake, eye orbital tightening, nose/cheek flattening, ear position, vibrissae position, hair bristling, and changes in coat color and texture. Some of these evaluations were based on the Rat Grimace Scale [34]. When we detected any of these characteristics, the rat was removed from the study and euthanized to avoid suffering. After 2 weeks post-ovariectomy, the rats were randomly assigned to each experimental group that received their respective treatments and underwent the behavioral tests.

2.4. Drugs

Chrysin (5,7-dihydroxyflavone, < 97% purity), progesterone (4-pregnane-3,20-dione, ≥ 99% purity), allopregnanolone (5α-pregnan-3α-ol-20-one), bicuculline (< 97% purity), propylene glycol, Tween-80, and 2-hydroxypropyl-β-cyclodextrin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Injectable water was purchased from PiSA Farmacéutica (Guadalajara, Jalisco, México).

2.5. Experimental groups and treatments

A total of 56 rats were included in the study and assigned to eight independent groups ($n = 7$ rats/group): (*i*) vehicle group (vehicle-1 [in which bicuculline was dissolved] + vehicle-2 [in which chrysin, progesterone, and allopregnanolone were dissolved]), (*ii*) chrysin (Chry) group (vehicle-1 + 1 mg/kg chrysin), (*iii*) progesterone (P4) group (vehicle-1 + 1 mg/kg progesterone), (*iv*) allopregnanolone (Allo) group (vehicle-1 + 1 mg/kg allopregnanolone), (*v*) bicuculline (Bic) group (1 mg/kg bicuculline + vehicle-2), (*vi*) 1 mg/kg Chry + 1 mg/kg Bic, (*vii*) 1 mg/kg P4 + 1 mg/kg Bic, and (*viii*) 1 mg/kg Allo + 1 mg/kg Bic. All of the drugs were injected intraperitoneally in a volume of 1 ml/kg. P4 and Allo were used as reference antidepressant compounds with GABAergic activity. Vehicle-1 (85% water, 14% propylene glycol, and 1% Tween-80, in which Bic was dissolved) and Bic were administered 75 min before the behavioral tests. Vehicle-2 (35% 2-hydroxypropyl-β-cyclodextrin, in which Chry, P4, and Allo were dissolved), Chry, P4, and Allo were administered 60 min before the behavioral tests. The dose of chrysin (1 mg/kg) was based on dose-response curves from previous studies, in which this dose produced anxiolytic-like effects through GABAA receptors [11,12,15]. The rationale for using this anxiolytic dose of Chry to explore its antidepressant-like effect was based on previous studies in which other GABAergic anxiolytic drugs, such as neurosteroids, also produced antidepressant-like effects in the FST [21,22,25,26]. The doses of P4 and Allo were based on previous studies, in which these doses produced antidepressant-like effects in the FST [21,25]. The dose of Bic (1 mg/kg) and administration schedule were based on previous studies, in which it effectively blocked the antidepressant-like effects of GABAergic compounds in the FST [22]. The selection of doses from previous works is consistent with the 3Rs (refine, reduce, replace) of ethical recommendations for preclinical research [35]. The effects of the treatments were evaluated in the locomotor activity test (LAT) and then in the FST.

2.6. Behavioral tests

Before any pharmacological treatments, all of the rats were subjected to a 5-min pretest session in the LAT and then a 15-min pretest session in the FST. The results of the pretest session in the LAT were considered basal motor activity of the rat. Data from the pretest session in the FST were discarded in the behavioral analysis because the animals in this experimental procedure were subjected to a stressful aversive situation, represented by forced swimming that triggers the development of behavioral despair in the following session [36]. Twenty-four hours after the FST pretest session, the rats received the respective treatments and then were subjected to a 5-min test session in the LAT and then a 5-min test session in the FST to evaluate the effects of the treatments.

2.6.1. Locomotor activity test

To evaluate the effects of the treatments on spontaneous motor activity, the rats were individually subjected to the LAT. The apparatus was an opaque Plexiglas housing cage (44 cm × 33 cm base, 20 cm high walls; Circulo ADN, Mexico City, Mexico), which has been validated as a reliable tool to evaluate general motor activity in rats [15,16,20,32]. The floor was delineated into 12 squares (11 cm × 11 cm). General motor activity was examined to determine whether the treatments caused hypoactivity, hyperactivity, or no motor changes, which could interfere with behavioral activity in the FST. At the onset of the test, the rats were gently placed in one of the corners of the cage, and the following variables were measured: (*i*) number of squares crossed (i.e., when an animal passed from one square to another with its hind legs), (*ii*) time (in seconds) spent rearing (i.e., when the rat acquired a vertical posture relative to the cage floor), and (*iii*) time (in seconds) spent grooming (i.e., paw licking, nose/face grooming, head washing, body grooming/scratching, leg licking, and tail/genital grooming according to Kalueff and Tuohimaa, 2005 [37]. No other variables (e.g., time spent in the center or periphery) were evaluated because of the relatively small size of the cage. After the LAT session, the rat was subjected to the FST. Approximately 2 min elapsed between each test.

After each test session, the locomotor activity cage was carefully cleaned with a 10% ethanol solution to remove the scent of the previous rat, which can influence spontaneous behavior of the subsequent rat [38]. Five minutes elapsed between each test to allow the odors and cleaning solutions to dissipate.

2.6.2. Forced swim test

In this test, the rats were individually forced to swim in a rectangular pool (50 cm × 30 cm × 60 cm) with 24 cm deep water (25 °C ± 1 °C), which has been validated for detecting antidepressant-like effects of clinically effective antidepressant drugs e.g., clomipramine, desipramine, and fluoxetine [39,40], neurosteroids e.g., progesterone and allopregnanolone [21,25], and plant extracts [41,42]. The following variables were evaluated: latency to first immobility and total immobility time (i.e., when the rat floated without making vigorous movements that led to displacements and only maintained its head above the water surface for more than 2 s). These parameters have been reliably used in the FST to detect antidepressant-like effects of antidepressants drugs.

A digital video camera (Sony DCR-SR42, 40× optical zoom, Carl Zeiss lens) was installed above the LAT cage, and another digital video camera was installed in front of the FST pool. Two blind independent observers measured the behavioral variables using *ex profeso* software to record the number and time (in seconds) of each evaluated behavioral variable until > 95% agreement was reached among observers.

2.7. Statistical analysis

The LAT data were analyzed using two-way repeated-measures analysis of variance (ANOVA), with session (pretest and test) as the

within-subjects factor and treatment as the between-subjects factor. The FST pretest data were excluded from the analysis because such a pretest session is used only to induce despair-like behavior [36]. Therefore, only the data from the test session were analyzed using one-way ANOVA, with treatment as the factor. Values of $p \leq 0.05$ in the ANOVA were followed by the Bonferroni *post hoc* test. All of the analyses were performed using Sigmastat 3.5 software. The data are expressed as mean ± standard error.

3. Results

Five rats were removed from the study because they had irregular ovarian cycles. Another two rats were removed from the study because they had evident signs of deteriorating health after ovariectomy. Therefore, the present results correspond to a total of 56 rats that were distributed into eight groups ($n = 7$/group).

3.1. Locomotor activity test

The effects of the treatments on the number of crossings, rearing time, and grooming time in the LAT pretest and test sessions are shown in Table 1. No effect of treatment on the number of crossings was found ($F_{7,48} = 0.647$, $p = 0.715$), but a significant effect of session (pretest, test) was observed ($F_{7,48} = 114.559$, $p < 0.001$). The *post hoc* test showed that the number of crossings was lower in the test session (18.92 ± 0.8) than in the pretest session (36.55 ± 1.4). No significant treatment × session interaction was found ($F_{7,48} = 1.133$, $p = 0.359$).

The ANOVA revealed significant effects of treatment ($F_{7,48} = 5.110$, $p < 0.001$) and session ($F_{7,48} = 168.674$, $p < 0.001$) on rearing time, with a significant treatment × session interaction ($F_{7,48} = 4.389$, $p < 0.001$). The *post hoc* test showed that the vehicle-treated group exhibited a significant decrease in rearing time in the test session compared with the pretest session (i.e., after the 15-min stress session in the FST), and this decrease was prevented by Chry, Allo, and P4, which maintained a longer rearing time compared with the vehicle-treated group. Interestingly, the effects of Chry, Allo, and P4 were partially blocked by pretreatment with Bic.

The ANOVA revealed significant effects of treatment ($F_{7,48} = 6.807$, $p < 0.001$) and session ($F_{7,48} = 92.088$, $p < 0.001$) on grooming time, with a significant treatment × session interaction ($F_{7,48} = 9.142$, $p < 0.001$). The *post hoc* test showed that the vehicle-treated group exhibited a significant decrease in grooming time in the test session (i.e., after the 15-min stress session in the FST) compared with the pretest session, and this decrease in grooming time was prevented by Chry, Allo, and P4, which maintained higher grooming time compared with the vehicle-treated group. The effects of Chry, Allo, and P4 on grooming time were blocked by pretreatment with Bic.

3.2. Forced swim test

The ANOVA revealed a significant effect of treatment on the latency to the first immobility ($F_{7,48} = 16.183$, $p < 0.001$). The *post hoc* test showed that the Chry-treated group exhibited a longer latency to the first immobility compared with the vehicle-treated group, an effect that was similar to the Allo- and P4-treated groups. The effects of Chry, Allo, and P4 on the latency to the first immobility were prevented by pretreatment with Bic (Fig. 1).

The ANOVA revealed a significant effect of treatment on total immobility time ($F_{7,48} = 13.246$, $p < 0.001$). The *post hoc* test showed that the Chry-treated group had a shorter immobility time compared with the vehicle-treated group. This effect of Chry was similar to the effects of Allo and P4 in the FST. The effects of Chry, Allo, and P4 on the total immobility time were prevented by pretreatment with Bic (Fig. 2).

4. Discussion

The present study explored the mechanism of action of the antidepressant-like effects of the flavonoid Chry compared with P4 and Allo as reference GABAergic compounds with proven antidepressant-like effects in the FST. In OVX rats, Chry exerted behavioral effects that were similar to P4 and Allo. Furthermore, Bic blocked the antidepressant-like effects of Chry, P4, and Allo, thus supporting participation of the GABAergic system in the pharmacological actions of Chry. These findings are relevant to the development of new antidepressant treatments for women with low levels of steroid hormones.

The FST is a valid model for investigating substances with potential antidepressant-like effects. In this test, rodents are forced to swim without any possibility of escape, which increases immobility, an indicator of low levels of motivation and interpreted as depressive-like behavior [36,43]. In the present study, a single injection of Chry, P4, or Allo alone was sufficient to prevent the establishment of immobility in OVX rats, suggesting an antidepressant-like effect as occurs with clinically effective antidepressant drugs desipramine and fluoxetine in the FST [36,44]. Moreover, no gross locomotor effects (crossing), were observed in the locomotor activity test, suggesting that the effects of chrysin in the FST were not attributable to motors effects. The latency to the first immobility is considered an indicator of the magnitude of the first effort that rats expend to cope with the stressful situation of being forced to swim [39,45]. A short latency to the first immobility is considered a complementary indicator of despair-like behavior. A longer latency is associated with an antidepressant-like effect that is produced by some antidepressant drugs, such as desipramine, imipramine, fluoxetine, and duloxetine [39,40,46]. We found that the latency to the first immobility was increased by the treatments, thus supporting the antidepressant-like effects of Chry that were previously reported in male mice [8,9] and extending these findings to OVX rats. These effects

Table 1
Effect of treatments on crossing, rearing and grooming in the LAT.

Variable/Groups	Pre-test	Test
Crossing (n)		
Vehicle	40.14 ± 5.5	14.0 ± 2.1
Chry	33.57 ± 2.2	20.71 ± 2.4
P4	33.85 ± 3.0	19.57 ± 2.8
Allo	36.57 ± 4.6	18.71 ± 1.7
Bic	40.28 ± 3.1	22.57 ± 2.7
Chry + Bic	38.71 ± 3.3	21.14 ± 2.3
P4 + Bic	31.42 ± 5.6	19.85 ± 2.0
Allo + Bic	37.85 ± 4.2	14.85 ± 1.7
Rearing (s)		
Vehicle	28.34 ± 3.1	8.59 ± 0.7**
Chry	34.32 ± 2.2	30.13 ± 3.8^{aa,bb,dd,ee}
P4	30.93 ± 1.7	21.04 ± 1.9**, ^{a}
Allo	32.02 ± 3.2	24.45 ± 1.5*^{,aa,b,dd,e}
Bic	32.84 ± 2.9	11.68 ± 2.0**
Chry + Bic	31.76 ± 2.9	19.85 ± 3.0**
P4 + Bic	29.59 ± 3.5	10.99 ± 1.4**
Allo + Bic	30.66 ± 1.9	12.44 ± 1.3**
Grooming (s)		
Vehicle	30.02 ± 4.1	5.86 ± 2.6**
Chry	29.65 ± 2.3	32.87 ± 3.2^{aa,bb,cc,dd,ee}
P4	33.59 ± 6.6	28.77 ± 2.2^{aa,bb,cc,dd,ee}
Allo	26.39 ± 3.0	28.78 ± 2.3^{aa,bb,cc,dd,ee}
Bic	31.87 ± 2.8	3.23 ± 0.9**
Chry + Bic	29.01 ± 2.5	8.71 ± 2.3**
P4 + Bic	30.49 ± 5.0	2.48 ± 0.9**
Allo + Bic	27.34 ± 6.3	3.36 ± 1.0**

All of the evaluated drugs were administered in a dose of 1 mg/kg. Chry, chrysin; Allo, allopregnanolone; P4, progesterone; Bic, bicuculline. *p < 0.01, **p < 0.001 vs pretest session of the same group; single letter p < 0.01; double letter p < 0.001 (a vs Vehicle, b vs Bic, c vs Cry + Bic, d vs P4 + Bic, e vs Allo + Bic; in the test session). Two-way ANOVA for repeated measures, Bonferroni´s post-hoc test.

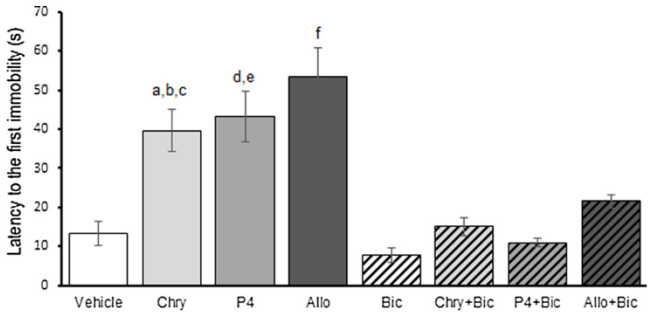

Fig. 1. Latency to the first immobility. Chrysin, progesterone, and allopregnanolone increased the latency to the first immobility. Pretreatment with bicuculline alone had no effects in the forced swim test but blocked the effects of chrysin, progesterone, and allopregnanolone. All of the drugs were administered at a dose of 1 mg/kg. Chry, chrysin; P4, progesterone; Allo, allopregnanolone; Bic, bicuculline. $^{a}p < 0.005$, *vs.* chrysin + bicuculline; $^{b}p < 0.002$, *vs.* vehicle; $^{c}p < 0.001$, *vs.* bicuculline and progesterone + bicuculline; $^{d}p < 0.05$, *vs.* allopregnanolone + bicuculline; $^{e}p < 0.001$, *vs.* vehicle, bicuculline, chrysin + bicuculline, and progesterone + bicuculline; $^{f}p < 0.001$, *vs.* vehicle, bicuculline, chrysin + bicuculline, progesterone + bicuculline, and allopregnanolone + bicuculline (one-way ANOVA followed by Bonferroni's *post hoc* test).

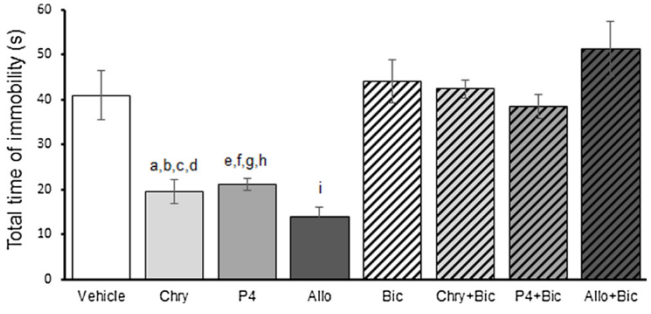

Fig. 2. Total immobility time. Chrysin, progesterone, and allopregnanolone reduced the total immobility time. Pretreatment with bicuculline alone had no effects in the forced swim test but blocked the effects of chrysin, progesterone. and allopregnanolone. All of the drugs were administered at a dose of 1 mg/kg. Chry, chrysin; P4, progesterone; Allo, allopregnanolone; Bic, bicuculline. $^{a}p < 0.02$, *vs.* progesterone + bicuculline; $^{b}p < 0.006$, *vs.* vehicle; $^{c}p < 0.002$, *vs.* chrysin + bicuculline; $^{d}p < 0.001$, *vs.* bicuculline, allopregnanolone + bicuculline; $^{e}p < 0.01$, *vs.* vehicle; $^{f}p < 0.006$, *vs.* chrysin + bicuculline; $^{g}p < 0.002$, *vs.* bicuculline; $^{h}p < 0.001$, *vs.* allopregnanolone + bicuculline; $^{i}p < 0.001$, *vs.* vehicle, bicuculline, chrysin + bicuculline, progesterone + bicuculline, and allopregnanolone + bicuculline (one-way ANOVA followed by Bonferroni's *post hoc* test).

were similar to progesterone and allopregnanolone [23,22].

In the LAT, the number of crossings is considered an indicator of overall motor activity, which is not modified by clinically effective antidepressants [40,41], neurosteroids (e.g., P4 and Allo), Bic, or picrotoxin [21,22,25]. The number of crossings also was not modified by Chry at the doses that were used in the present study [33] or at higher doses [8,9]. Rearing time and grooming time are useful behavioral indicators of the emotional state of rats when they are exposed to novel environments [47], which contrasts with the number of crossings that only reflects motor effects. In the present study, Chry, P4, and Allo seemingly increased grooming and rearing time in the LAT compared with the vehicle-treated group in the test session. However, rather than increasing these variables, Chry, P4, and Allo prevented the decreases in grooming and rearing time that were produced in the 15-min pretest session in the FST. Vehicle-treated OVX rats had the lowest level of rearing and grooming in the LAT, 24 h after the FST pretest session, suggesting that the stress of being forced to swim was sufficient to decrease these variables, which has been previously shown with other stressors in OVX rats [32,33] and in mice that were exposed to chronic unpredictable mild stress for 28 days that triggered depressive-like behavior [8,9]. Grooming can be restored in OVX rats with an injection of 17β-estradiol and genistein. In female mice, Chry and fluoxetine blocked the reduction of grooming that was triggered by unpredictable chronic stress [8,9]. These results support our findings, in which Chry blocked the decrease in grooming that was produced in the pretest session in the FST, possibly associated with the antidepressant-like effect that was detected in the test session. Notably, the effects of Chry were similar to the effects of Allo and P4. Treatment with Chry, Allo, P4, and their combinations also appeared to slightly increase the number of crossings in the LAT, but no significant differences from the vehicle-treated group were found. The lack of statistically significant effects of Chry, Allo, P4, and their combinations on the number of crossings may be attributable to the relatively small group sizes. However, previous studies showed that none of the drugs that were evaluated herein exerted significant effects (i.e., either an increase or a decrease) on the number of crossings at the tested doses using larger group sizes [15,21,22,25,48]. The lack of changes in the number of crossings confirmed the specificity of emotional effects on immobility, grooming, and rearing, thus discarding possible motor interference on these behaviors. Based on these reasons and the "3 Rs" (refine, reduce, replace) for animal research [35], we did not test a larger number of rats in our study.

The present study explored the participation of the GABA binding site in the $GABA_A$ receptor in the effects of the treatments. $GABA_A$ receptors appear to be involved in the anxiolytic-like actions of Chry. The binding of GABA with its receptor opens chloride ion channels to hyperpolarize the neuron and decrease neural activity. These neurophysiological actions are related to the anxiolytic effects of benzodiazepines, barbiturates, psychoactive drugs, and some neurosteroids [49]. Bic is a competitive antagonist of the GABA binding site in the $GABA_A$ receptor and blocks the anxiolytic-like effects of GABAergic compounds (e.g., 4-chlorodiazepam) and some neurosteroids (e.g., P4 and Allo) [22,50]. In the present study, pretreatment with Bic blocked the antidepressant-like effects of Chry in OVX rats, thus confirming participation of the GABAergic system in the antidepressant-like effects of Chry, similar to the mechanism of action of the neurosteroids P4 and Allo. Neurosteroids potently and selectively act as allosteric neuromodulators at $GABA_A$ receptors that contain the α4 subunit, mainly in limbic structures (e.g., amygdala and hippocampus) [51] that participate in anxiolytic and anti-stress effects (for review, see [52]). Chry is also able to exert anxiolytic effects in OVX rats by interacting with $GABA_A$ receptors [33], but these effects are slightly different from the effects of benzodiazepines, suggesting differences in affinity for the $GABA_A$ receptor. Chry was shown to act on α1, β1, and γ2 subunits of the $GABA_A$ receptor in *Xenopus laevis* oocytes [53], but the subunits that are responsible for its pharmacological effects in rodents remain to be explored. The acute effects of Chry on immobility in the FST were similar to the effects of P4 and Allo. In addition to their anxiolytic-like properties, low doses of P4 and Allo rapidly reduce immobility in the FST, and these effects are blocked by $GABA_A$ receptor antagonists, including picrotoxin and Bic [22,25,54].

In depressed patients, the therapeutic effects of antidepressants have a latency of approximately 2 weeks [55,56]. In the FST, antidepressant-like effects can be observed with high doses of selective serotonin reuptake inhibitors (10-20 mg/kg) with only three administrations within 24 h [57,58]. Short-term effects of antidepressant drugs are not seen in humans while long term effects are usually associated with neuroplasticity [59–61]. The anti-stress effects of Chry have been associated with the reestablishment of tumor necrosis factor-α, interleukin-1β, interleukin-6, caspase 3, and caspase 9 levels in the brain with chronic administration, similar to the action of fluoxetine [8]. Additionally, the upregulation of BDNF and NGF levels has been proposed to be involved in the antidepressant-like effects of Chry [10]. Such effects, however, were observed after chronic treatment (14-28

days) with a high dose of the flavonoid (20 mg/kg) [8,10]. In contrast, the present study showed that acute administration of a low dose of Chry was sufficient to elicit an antidepressant-like effect in OVX rats. These effects appeared to be mediated by the GABA binding site, which has been previously reported with neurosteroids and plant extracts [42]. For example, neurosteroids are able to reduce immobility in the FST within the first 30 min after administration, with effects that last up to 6 h, suggesting the involvement of ionotropic receptors [40]. The effects of some natural products consist of blocking the establishment of despair rather than reversing previously induced despair-like states. Consequently, the rapid effects of certain plant extracts (e.g., *Montanoa* genus plants) have been suggested to reflect protective effects against stress-induced behavioral alterations rather than an antidepressant-like effect *per se*, which requires more time to produce neuroplasticity changes [42]. Further studies are required to explore other possible actions of flavonoids to determine the specific contexts in which these natural compounds could be considered potential therapeutic alternatives to the treatment of anxiety or depression.

5. Conclusion

The present findings contribute to our understanding of the mechanisms of action of the flavonoid chrysin by demonstrating that the acute antidepressant-like actions of chrysin in the FST are regulated by the GABA binding site of the $GABA_A$ receptor in OVX rats, similar to the actions of some neurosteroids. These findings encourage additional research to explore the utility of chrysin to ameliorate symptoms of depression in humans, particularly in menopause women.

Author Contributions

JFRL and JCE conceived the project, developed the experimental design, and wrote the protocol. JFRL, JCE, FHL, and JAS performed the experiments and measured the behavioral variables. JCE, MGLM, and CM performed the statistical analysis and interpreted the results. JFR, JCE, and CM wrote the manuscript. All of the authors reviewed, discussed, and approved the final version of the manuscript.

Declaration of Competing Interest

There are no conflicts of interest to declare.

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