Original research paper

Differential effects of acute and chronic treatment with the flavonoid chrysin on anxiety-like behavior and Fos immunoreactivity in the lateral septal nucleus in rats

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The aim of this study was to compare the effects of acute (a single injection) and chronic (21 consecutive days) treatments with chrysin 2, 4, and 8 µmol kg^{-1} on anxiety-like behavior and Fos immunoreactivity in the lateral septum nucleus (LSN), a structure that is involved in the regulation of anxiety, in male Wistar rats. These effects were compared with the clinically effective anxiolytic diazepam 7 µmol kg^{-1}. The results showed that acute, but not chronic treatment, with 4 µmol kg^{-1} chrysin exerted anxiolytic- and anti-depressant-like effects with these effects being similar to that of diazepam. Also, none of the above-mentioned treatments did alter Fos immunoreactivity in the LSN, but a tendency towards the reduction of this variable was detected with chrysin 4 µmol kg^{-1} and diazepam 7 µmol kg^{-1}. Altogether, results suggest that chrysin exerts anxiolytic-like effects, however, it can produce pharmacological tolerance after repeated use, similar to benzodiazepines.

Keywords: chrysin, anxiolytic, elevated plus maze, Fos protein, lateral septal nucleus

Flavonoid chrysin (5,7-dihydroxyflavone) is a partial agonist of γ-aminobutyric acid-A (GABA$_A$) receptors (1). Acute injection of a low dose (4 µmol kg^{-1}) of this flavone produces anxiolytic-like effects in mice, without producing significant changes in spontaneous loco-motor activity (1). Higher doses of chrysin (20 and 80 µmol kg^{-1} *p.o.*, daily, for 28 days) exerted antidepressant-like effects in mice that were exposed to chronic unpredictable mild stress and forced swim test (2). The repeated use of anxiolytic drugs, such as benzodiazepines (*e.g.*, diazepam), reduces its pharmacological actions in the long term (3). Also, no comparative studies have explored the acute and chronic actions of similar doses of chrysin on anxiety- and depression-like behavior. We sought to compare the acute and chronic effects of chrysin

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on anxiety- and depression-like behavior and the activation of a brain structure that is involved in the modulation of anxiety and depression (*i.e.*, the lateral septal nucleus [LSN]) to gain a better understanding of its potential therapeutic use in the long term.

The LSN is involved in many emotional and affective disorders, such as anxiety and depression. For example, the activation of LSN neurons may contribute to the inhibition of fear-associated behaviors, and neuronal inhibition of the LSN may be related to anxiolytic-like effects (4). An increase in Fos immunoreactivity in the LSN was observed in rats that were exposed to stressful situations (*i.e.*, the forced swim test) and situations that produce anxiety, such as the light-dark box test (5). Treatment with anxiolytic drugs, such as diazepam, reduced Fos immunoreactivity in the LSN in rats that were exposed to stressful situations (6). These data support the participation of the LSN in anxiety and the mechanism of action of anxiolytic drugs.

The present study investigates the effects of acute and chronic treatment with three doses of chrysin in Wistar rats that were subjected to a behavioral test battery composed of the elevated plus maze (EPM), locomotor activity test (LAT), and forced swim test (FST). We have also performed the Fos protein labeling to explore the relationship between the behavioral effects of chrysin and activity of the LSN.

EXPERIMENTAL

Animals

Ninety adult male Wistar rats, 3 months old and weighing 250–300 g at the beginning of the experiments, were used. The rats were housed in Plexiglas cages (five rats per cage) under a 12 h/12 h light/dark cycle (light on at 7:00 AM) at an average temperature of 25 ± 1 °C with *ad libitum* access to purified water and food (Nutri-cubos Purina, Agribrands Purina México, México).

The experimental procedures considered the international and national ethical guidelines, the National Institutes of Health Guide for the Care and Use of Laboratory Animals (7) and the official Mexican guidelines *Especificaciones Técnicas para la Producción, Cuidado y Uso de Animales de Laboratorio* (8). All efforts were made to minimize animal discomfort during the study.

Experimental groups and treatments

Acute effects of chrysin and diazepam. – Forty-five rats were assigned to five independent groups (*n* = 9 rats per group). The control group received the vehicle (a mixture of 14 % propylene glycol, 1 % Tween 80 and 85 % injectable sterilized water) in which chrysin and diazepam were dissolved. The other three groups received 2, 4 and 8 µmol kg^{-1} chrysin (Sigma-Aldrich, USA). The last group received 7 µmol kg^{-1} diazepam (Valium solution injectable, Productos Roche, México). These doses were selected because 4 µmol kg^{-1} chrysin was shown to produce anxiolytic-like effects at the preclinical level in mice (1). To generate a dose-response curve, we included two additional doses of chrysin (0.5× and 2× the effective dose that produces anxiolytic-like effects). The dose of diazepam was based on its anxiolytic-like effects in experimental models of anxiety, including the EPM (9). The injections were given intraperitoneally (*i.p.*) in a volume of 1 mL kg^{-1} in a single injection (acute treatment).

Sixty minutes after the injection, the rats were evaluated in the behavioral test battery, including elevated plus maze (EPM), locomotor activity test (LAT) and forced swim test (FST). Ninety minutes after the last behavioral test, five rats from each group were euthanized and perfused to obtain brains to immunohistochemically analyze Fos protein levels in the LSN.

Chronic effects of chrysin and diazepam. – Another 45 rats were assigned to five experimental groups (*n* = 9 rats per group) that received the same drugs and doses used for the acute treatment, except that the rats received the treatments once daily for 21 consecutive days (chronic treatment). Sixty minutes after the last injection, the rats were evaluated in the EPM, LAT and FST. Ninety minutes after the last behavioral test (day 21 of treatment), five rats from each group were euthanized and perfused to obtain brains to immuno-histochemically analyze Fos protein levels in the LSN.

Behavioral tests

The rats were evaluated in a behavioral test battery that began with the EPM (5 min), followed by the LAT (5 min) and FST (6 min). Approximately 3 min elapsed between tests. Two blinded observers measured the behavioral variables in each behavioral test using *ex profeso* software until at least 95 % agreement was reached between observers. We used a battery of behavioral tests to reduce the number of animals that were used in this study in agreement with the ethical recommendations of the 3R principles in preclinical research (10).

Elevated plus maze. – The apparatus consisted of two opposite open and closed arms that were set in a plus configuration. The apparatus was illuminated at 40 lux as described by Contreras *et al.* (9). A digital video camera (DCR-SR42, 40× optical zoom, Carl Zeiss lens, Sony, Japan) was installed above the apparatus to record behavioral activity. The following variables were evaluated: (*i*) time spent on the open arms, (*ii*) number of entries into the open arms, (*iii*) total number of arm entries (open arms + closed arms), and (*iv*) percentage of open arm entries ([(open arm entries)/(total arm entries)] × 100). Additionally, as an integrative measure, the anxiety index (AI) was calculated according to the following equation. After the EPM, the rats were evaluated in the LAT.

$$AI = 1 – \{[(\text{open arm time/test duration}) + (\text{open arm entries/total number of entries})]/2\}$$

Locomotor activity test. – To evaluate pharmacological effects on spontaneous locomotor activity, grooming, and rearing, the rats were individually subjected to a 5-min LAT. An opaque Plexiglas cage (44 cm × 33 cm) with 20 cm high walls was used to identify or discard locomotor changes (*i.e.*, hypoactivity, hyperactivity, or no change) that are associated with treatments and could interfere with behavior in the EPM (9). The cage floor was delineated into 12 squares (11 cm × 11 cm). A digital video camera (DCR-SR42) was installed above the cage to record spontaneous behavioral activity. The following variables were evaluated: (*i*) number of squares crossed (crossings), (*ii*) time spent rearing (in seconds), and (*iii*) time spent grooming (in seconds), including paw licking, nose/face grooming, head washing, body grooming/scratching, leg licking, and tail/genital grooming. After each test session, the EPM and locomotor activity apparatus were carefully cleaned with 15 % ethanol to remove the scent of the previously evaluated rat. After the LAT, the rats were evaluated in the FST.

Forced swim test. – For this experimental protocol, the method described by Borsini was used (11), with modifications of the shape and size (26 cm × 29 cm base, 50 cm height) of the tank. After the LAT session, each rat was placed in a glass tank that was filled with water to a depth of 30 cm at a temperature of 25 ± 1 °C. The rat was subjected to a single 6-min forced swim session, and the total time of immobility was recorded only during the last 4 min (11). The total immobility time was the sum of all immobility periods that were > 2 s during the last 4 min of the session. Immobility was assumed when the rat made only the minimum movements that were necessary to keep its head above the water surface, without leading to water displacements that moved the rat's body around the tank, which is considered a measure of depression-like behavior. After the FST, five rats from each group were anesthetized to extract their brains and process them for immunohistochemical study and quantify Fos immunoreactivity in the LSN.

Fos immunoreactivity in the LSN

Ninety minutes after the FST, the rats were anesthetized with an overdose of sodium pentobarbital (Sedalpharma, Pet's Pharma, México; 0.4 mmol kg^{-1}, *i.p.*) and transcardially perfused with 0.9 % saline, followed by 4 % paraformaldehyde in phosphate buffer (PB, 0.1 mol L^{-1}, pH 7.4). Brains were immediately removed, cryoprotected in serial dilutions of sucrose (10, 20 and 30 %) in PB, and coronally sectioned at 50 µm with a cryostat (Microm HM 505N, Microm International GmbH, Germany) at –22 °C. Serial sections were collected
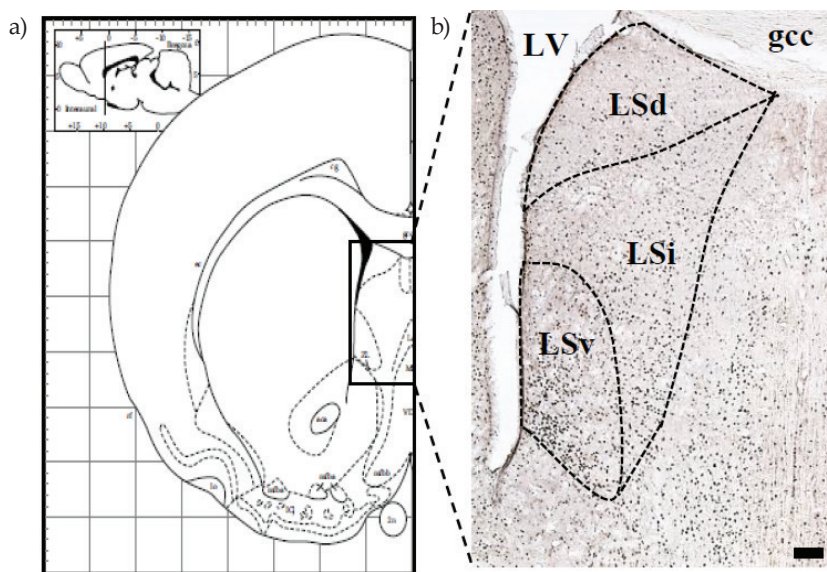

Fig. 1. Lateral septal nucleus: a) black rectangle indicates the LSN, adapted from Paxinos and Watson (13), b) Photomicrograph representing the division of the LSN where Fos immunoreactivity was measured as indicated in methods (LV – lateral ventricle, gcc – genu of the corpus callosum, LSd – dorsal lateral septum, LSi – intermediate lateral septum, LSv – ventral lateral septum). Black dots: Fos immunoreactivity, scale bar = 100 µm.

in incubation boxes with PB. Fos immunohistochemistry was performed using a free--floating technique. One of every four sets of sections was used for Fos immunoreactivity as described previously (12).

To quantify Fos immunoreactivity in the LSN, the sections were examined under bright-field illumination with an Olympus BX41 microscope (Alta Tecnología en Laboratorios, México) at 10× and 20× magnifications. The LSN was analyzed bilaterally, including the dorsal (75,000 μm^2), intermediate (200,000 μm^2), and ventral (150,000 μm^2) areas. For quantification and statistical analysis, the number of Fos-immunoreactive cells in the LSN was counted in the following stereotaxic coordinates: interaural 9.70 mm, bregma 0.70 mm (13) using a 10 × 10 grid (500 × 500 μm). The average number of Fos-expressing cells was calculated for each experimental group (Fig. 1).

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA), with treatment as the independent factor. Non-parametric data were analyzed using the Kruskal-Wallis test. Values of $p \leq 0.05$ in the ANOVA were followed by the Student-Newman-Keuls *post hoc* test. The results are expressed as mean ± standard error.

RESULTS AND DISCUSSION

Acute effects of chrysin and diazepam

Elevated plus maze. – The analysis of the time spent and the percentage of entries into the open arm showed significant differences ($F_{4,40}$ = 3.859, p = 0.010; $F_{4,40}$ = 3.197, p = 0.023, resp.) between treatments. Chrysin (4 $\mu mol\ kg^{-1}$) significantly increased both variables, compared with the vehicle group, and this effect was similar to diazepam (7 $\mu mol\ kg^{-1}$) (Figs. 2a,b). An increase in these two variables is considered as a principal indicator of an anxiolytic-like effect (14). Clinically effective anxiolytic drugs (*e.g.*, diazepam), and some secondary metabolites of plants with anxiolytic potential (*e.g.*, flavonoids and phytoestrogens) increase the time spent and the percentage of entries into the open arms (1, 9) supporting an anxiolytic-like effect.

The analysis of the anxiety index confirmed the anxiolytic-like effect of chrysin in the EPM. The anxiety index was significantly affected by the treatment ($F_{4,40}$ = 3.988, p = 0.008): chrysin (4 $\mu mol\ kg^{-1}$) and diazepam (7 $\mu mol\ kg^{-1}$) significantly decreased this variable compared with the vehicle group (Fig. 2c). This effect has been reported in rats treated with clinically effective anxiolytic drugs (15), supporting the anxiolytic-like effect of acute treatment with chrysin.

In regard to the total number of arm entries and number of entries into the open arms, no significant difference was observed between the vehicle and either of the test groups. Therefore, in the present study, we were able to discard non-specific motor effects of the treatments and confirm the anxiolytic-like effect of chrysin and diazepam.

Locomotor activity test. – The LAT was performed in order to discard or identify motor changes that were associated with the treatments and could interfere with the interpretation of the behavior displayed by the rats in the EPM. In this study, no significant differ-
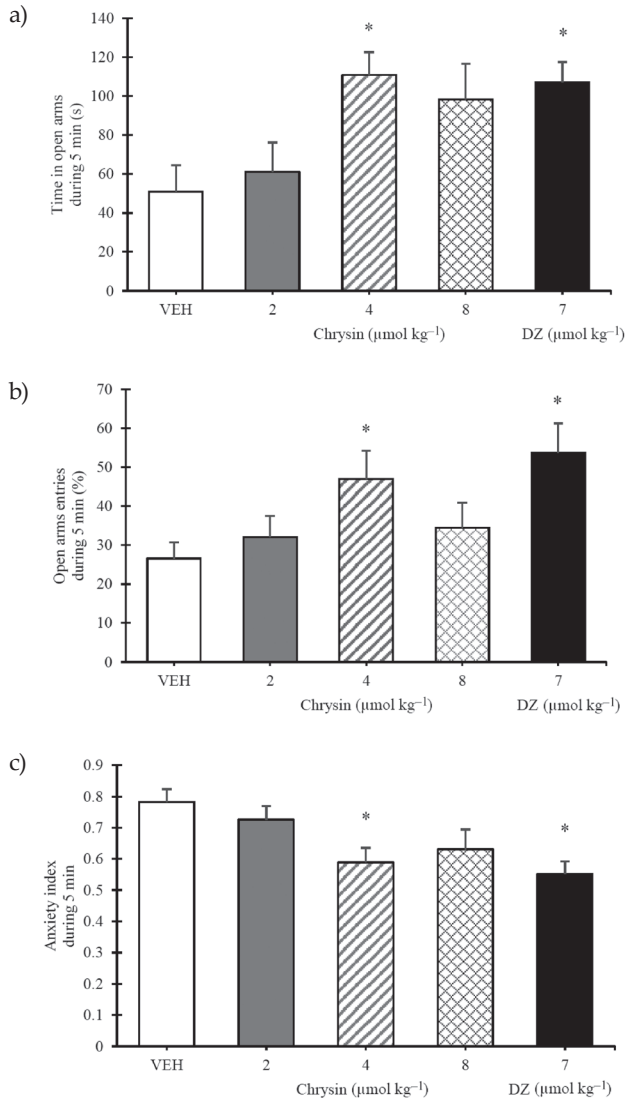
Fig. 2. Acute effect of chrysin in the elevated plus maze: a) time spent in the open arms, b) percentage of entries into the open arms, c) anxiety index. Significant difference *vs.* vehicle group: *$p < 0.05$. VEH – vehicle, DZ – diazepam.

ences were evidenced between treatments (vehicle and test groups) in regard to number of crossings during 5 min, time spent rearing, and time spent grooming, thus confirming that the increase in the time and percentage of entries into the open arms in the EPM is associated with emotional, but not motor effects (16).
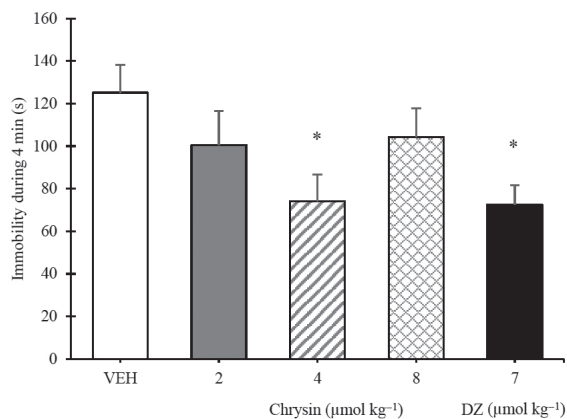
Fig. 3. Acute effect of chrysin and diazepam in the forced swim test. Significant difference *vs.* vehicle group: *$p < 0.05$. VEH – vehicle, DZ – diazepam.

Forced swim test. – In this test, substances with potential antidepressant-like effects (*e.g.*, antidepressants and anxiolytics) significantly reduce immobility time (17). In the present study, the total immobility time was significantly different between the groups ($F_{4,40} = 2.898$, $p = 0.034$). Chrysin (4 µmol kg⁻¹) significantly ($p < 0.05$) decreased total immobility time compared to the vehicle group, an effect that was comparable to that of diazepam (7 µmol kg⁻¹) (Fig. 3), suggesting an antidepressant-like effect. However, this rapid effect does not necessarily represent an antidepressant-like effect because clinically effective antidepressants usually require at least several days of treatment to produce therapeutic effects. Therefore, a protective effect of chrysin and diazepam against stress-induced behavioral changes can be suggested, which could be related to anxiolytic-like effects detected in the EPM.

Fos immunoreactivity in the LSN

The analysis of Fos immunoreactivity in the LSN (dorsal, intermediate and ventral areas) revealed no significant differences among treatments. Therefore, we performed a global analysis of the total number of Fos-immunoreactive cells in the LSN in an area of 425,000 µm².

The evidence indicates that the LSN plays a critical role in the regulation of emotional states, including anxiety and depression (4). The analysis of Fos immunoreactivity in the whole LSN did not reveal a significant difference between groups. However, a marked tendency toward a decrease in this variable was observed in rats which were treated with 2 and 4 µmol kg⁻¹ chrysin and 7 µmol kg⁻¹ diazepam compared with the vehicle group ($p = 0.056$), an effect that was not observed with 8 µmol kg⁻¹ chrysin (Fig. 4). Considering the aforementioned, a previous study reported that higher dose of diazepam (20 µmol kg⁻¹) was required to significantly reduce Fos immunoreactivity in the LSN in anxious rats exposed to stressful situations (6, 18). This would explain why the acute and chronic doses of chrysin and diazepam (Fig. 5) used in the present study did not reach a
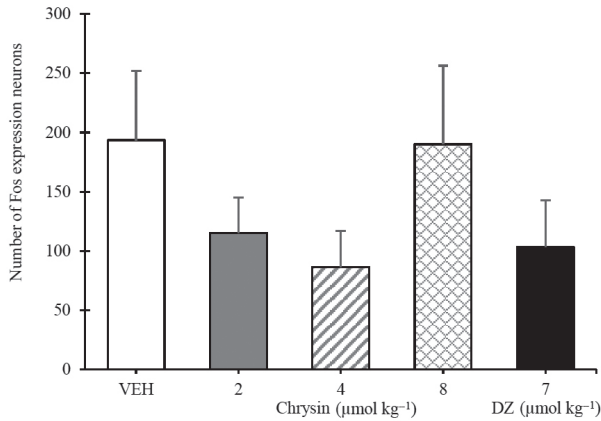
Fig. 4. The number of Fos immunoreactive cells in the LSN in acute chrysin and diazepam (area evaluated: 425,000 μm^2). VEH – vehicle, DZ – diazepam.
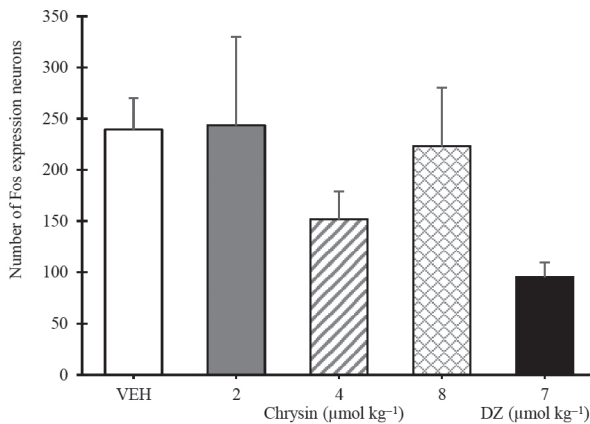

Fig. 5. The number of Fos immunoreactive cells in the LSN in chronic chrysin and diazepam administrations (area evaluated: 425,000 μm^2). VEH – vehicle, DZ – diazepam.

statistical significance. These data suggest a partial relationship between behavioral effects of chrysin and diazepam and a reduction of cellular activity in the LSN.

Chronic effects of chrysin and diazepam

Neither of the parameters in EPM (time spent on the open arms, number of entries into the open arms, total number of arm entries nor percentage of open arm entries), LAT (number of crossings, time spent rearing and time spent grooming), total immobility time in FST, nor the anxiety index was significantly different between treatments (vehicle and test groups) during chronic treatments.

Fos immunoreactivity in the LSN

The analysis of the global number of Fos immunoreactive cells in the LSN showed no significant difference between treatments. However, this variable was significantly lower in rats which were treated with 4 µmol kg^{-1} chrysin and 7 µmol kg^{-1} diazepam compared with the vehicle group. This effect was not observed in rats that were treated with 2 and 8 µmol kg^{-1} chrysin (Fig. 5).

In the present study, none of the chronic doses of chrysin or diazepam significantly affected the behavioral response in the EPM, LAT, FST, nor Fos immunoreactivity in the LSN. The lack of effects of chronic treatment may be associated with the development of pharmacological tolerance. Chrysin acts on GABA$_A$ receptor (1), and the repeated use of GABAergic substances (*i.e.*, benzodiazepines) has been suggested to desensitize this receptor, thus causing the loss of their pharmacological efficacy in the long term (3). This may partially explain the loss of anxiolytic- and antidepressant-like effect of chronic treatment with chrysin and diazepam. Nonetheless, this possibility requires to be explored in specific studies.

Finally, no dose-response effects were detected with different doses of chrysin, but a biphasic effect was observed. Low and high doses of chrysin did not affect the evaluated variables; only the intermediate dose produced anxiolytic- and antidepressant-like effects, the same dose that produces anxiolytic-like effects in other studies (1). In the first case, it could be associated with insufficient availability of chrysin in the brain, whereas in the second case, high doses of the flavonoid could saturate the receptor and inactivate it. Similar findings were reported with several GABAergic compounds (*i.e.* neurosteroids, benzodiazepines, barbiturates, and plant extract containing flavonoids) whose action was established on the GABA$_A$ receptor (19, 20), the same receptor in which chrysin exerts its pharmacological effects (1).

CONCLUSIONS

Acute, but not chronic treatment with the flavonoid chrysin produced anxiolytic-like and protective effects against behavioral changes that were produced by a stressful situation, and these effects were similar to diazepam. Moreover, the effects of chrysin were partially related to cellular changes in the LSN. Similar to anxiolytic benzodiazepine drugs, chrysin treatment appears to produce pharmacological tolerance after repeated use, which should be considered in future experimental protocols to confirm or discard this possibility.

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