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Anxiolytic-like Effect of Chrysin on Female Zebrafish is Likely Mediated by α_5 subunits of $GABA_A$ Receptors

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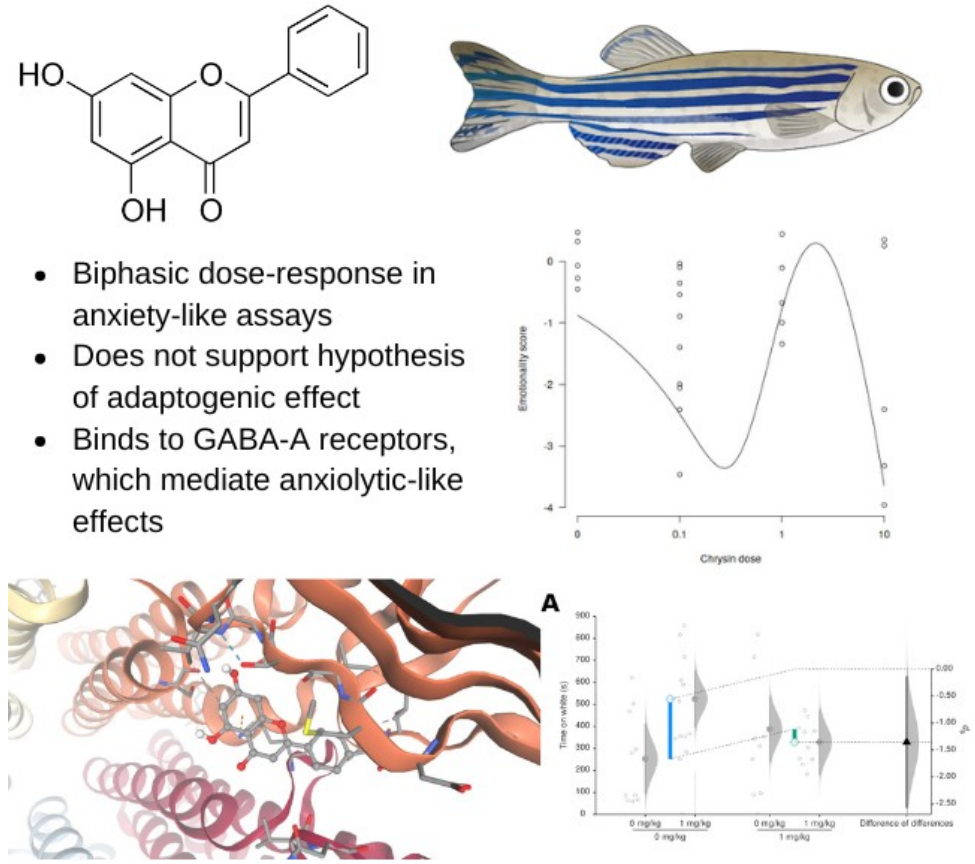
Abstract



Chrysin (5,7-dihydroxyflavone) is a natural flavonoid with potential anxiolytic-like effects in preclinical models. Acute treatment with this molecule (0 – 10 mg/kg) produced a biphasic dose-response in the zebrafish light/dark test (LDT), with anxiolytic-like effect at low doses and anxiogenic-like effects at high doses. Chrysin (1 mg/kg) decreased anxiety-like behavior in the zebrafish novel tank test (NTT), but did not prevent the anxiogenic effects of acute stress. The anxiolytic-like effects of chrysin (1 mg/kg) in the LDT were blocked by pretreatment with picrotoxin, suggesting interaction with γ-aminobutiric acid A ($GABA_A$) receptors. Molecular modeling suggested that chrysin interacts with α_5 subunits at residues lining the ion channel pore. These results demonstrate one of the possible mechanisms of action of chrysin, reinforcing the anxiolytic potential of this molecule.

Keywords: Drug discovery; Chrysin; $GABA_A$ receptors; Neurotransmitters; Zebrafish

1 Introduction

Benzodiazepines (BZDs) are a class of psychoactive drugs widely used in the control of anxiety disorders, but they also produce many side effects, including sedation, myorelaxation, ataxia, and

tolerance (1). As a result, the search for better anxiolytic drugs is ongoing. Among those, flavonoids emerge as a complementary therapeutic approach to psychiatric disorders, either as main choices of treatment or as adjuvants to reduce BZD dose (2–5), considering the amount of preclinical data that suggest that those compounds decrease anxiety with effects that are similar to those of BZDs (4,6). In this context, chrysin (5,7-dihydroxyflavone) is a natural flavonoid found in passionfruit flowers (*Passiflora caerulea* and *P. incarnata*), chamomile (including *Matricaria chamomilla* and *Chamaemelum nobile*), *Pleurotus ostreatus* mushrooms, propolis and honey (7,8). Chrysin has been described as possessing anti-inflammatory, antioxidant, anti-proliferative, anti-apoptotic, neuroprotective, and putative anxiolytic effects (9). This profile of mechanisms position chrysin as a potential adaptogen (i.e., a molecule which modifies an organism's nonspecific resistance to stress, increasing its ability to adapt to stressors; (10).

As occurs with other flavones, chrysin appears to produce acute anxiolytic-like effects through the mediation of GABAergic receptors (5,6,11–14). Chrysin displaces [^{3}H]flunitrazepam binding to central benzodiazepine receptors (15,16) which are located as an allosteric site in $GABA_A$ receptors. In adult zebrafish and male rats, acute chrysin reduced anxiety-like behavior, while the flavone backbone altered measures of risk assessment but not the main endpoints of anxiety-like behavior (17). In male mice, chrysin isolated from *P. caerluea* L. (Botanic family: *Passifloraceae*) produced acute anxiolytic-like effects in the elevated plus-maze, an effect that is blocked by pretreatment with the specific BZD site antagonist Ro 15-1788 (18); similarly, the anxiolytic-like effects of acute chrysin treatment in male rats was blocked by pretreatment with another BZD antagonist, flumazenil (19). This molecule also appears to interact indirectly with $GABA_A$ receptors; in long-term ovariectomized female rats, chrysin reduces anxiety-like behaviors, an effect that is blocked by picrotoxin, a chloride channel blocker that impedes the activation of $GABA_A$ receptors without interacting with either the orthosteric site or the BZD allosteric site (20). Chrysin also produces antidepressant-like effects in the forced swimming test in ovariectomized female rats, an effect that was blocked by bicuculline, an antagonist that binds to the orthosteric site (21). Reinforcing the idea that chrysin can interact with the neurosteroidergic modulation of $GABA_A$ receptors, chrysin prevents the increased anxiety-like behavior that is observed in female rats during the metestrus-diestrus phases of the estrous cycle, an effect that was prevented by pretreatment with picrotoxin (22). During proestrus and diestrus, microinjection of chrysin in the dorsal hippocampus produces opposite effects, increasing anxiety-like behavior during proestrus and decreasing it in diestrus; both effects are prevented by picrotoxin, bicuculline, and flumazenil (23).

The use of complementary model organisms represents a good strategy in behavioral pharmacology, helping to determine whether a given behavioral effect is likely to be conserved across species and therefore be translatable to humans (24,25). Zebrafish (*Danio rerio*) has been used in several fields of behavioral pharmacology and neuroscience to screen novel targets and to understand mechanisms of action (26–28), including its use in natural products research (29–31). Advantages of this model include its intermediate physiological complexity and ease of manipulation in the laboratory (32), as well as the availability of behavioral assays (24,33). Moreover, findings from zebrafish that are also observed in rodents (common overlapping mechanisms) are likely to represent more evolutionarily ancient and fundamental mechanisms (24,25). Previous work suggested that chrysin produces similar anxiolytic-like effects in both zebrafish and rats (17), suggesting that this effect is conserved across vertebrates; however, whether these effects are mediated by similar mechanisms is still unknown.

Building on previous research that showed that acute chrysin produced effects that are consistent with an anxiolytic-like profile in zebrafish (17)(German-Ponciano et al., 2020), it is necessary explore the effects of this flavonoid specifically in females, and to understand the involvement of specific subunits of $GABA_A$ receptor in its anxiolytic-like effects. Therefore, here we report on the effects of this flavonoid on acute toxicity endpoints and behavior in the light/dark test (34) in female zebrafish. The use of female animals is of interest, given the potential for chrysin to mimic the effects of ovarian neurosteroids (21–23) and evidence of sex differences on the effects of diazepam in zebrafish (35,36). We also report a participation of $GABA_A$/chloride channels in the putative anxiolytic-like effects of chrysin in this species which could be related to interactions with α_5 subunits of this receptor. However, we did not observe adaptogenic effects of chrysin.

2 Materials and methods

2.1 Animals and housing

Experiments were made at Laboratório de Neurociências e Comportamento “Frederico Guilherme Graeff” (LaNeC) at Unifesspa/Brazil. For all experiments, a total of 85 adult female zebrafish (*Danio rerio*, Hamilton 1822) of the longfin phenotype were used. This phenotype has been reported to display higher anxiety-like behavior in the novel tank test (37), suggesting its utility in behavioral screens for anxiolytic-like effects. Animals were acquired from a commercial vendor (PowerFish Aquicultura e Ranário, Itaguaí/RJ) and kept in the lab for at least 14 days before experiments began. Animals were housed in 50 L tanks filled with dechlorinated water at a density of 5 zebrafish/L (38) under optimized keeping conditions, with constant aeration and chemical-mechanical filtration at a temperature of 26 ± 2º C, pH 7.0 ± 1, hardness 75- 200 mg/L $CaCO_3$,

salinity of 0.25-0.75 dissolved oxygen ~7,8 mg/L at 28 ºC, and nitrite <0.01 ppm (39). Lighting was provided by fluorescent lamps under a 10:14 light/dark cycle (lights on at 8AM), providing light levels of 307 ± 96.7 lux. Animals were fed daily with commercial feed. Since no recirculation system was used, tanks were fitted with individual filters, and water was periodically changed, and water parameters (pH, hardness, dissolved oxygen, ammonia, and nitrite levels) assessed weekly. Experiments were executed after approval by the Institutional Review Board of Universidade do Estado do Pará under Protocol 06/18 and followed the Brazilian guidelines for the use of animals in research (40).

2.2 Drug treatment

Chrysin (CAS #480-40-0; Sigma-Aldrich, C80105) and picrotoxin (CAS #124-87-8; Sigma-Aldrich P1675) were dissolved in 5% dimethylsulfoxide (DMSO). While this concentration of DMSO has been shown to affect zebrafish behavior during bath exposure and chronic treatments (41,42), it apparently does not change behavior during acute treatment, especially at shorter post-administration intervals (42); our results (Section 3.1) also did not reveal acute lethality or sublethal toxic effects up to 96 h after injection. In all treatments, drugs were injected intraperitoneally following the protocol described by Kinkel et al. (43); briefly, after being anesthetized in ice-cold water (temperature 12 ºC – 14 ºC), animals were transferred to a water-soaked sponge and the drug was injected intraperitoneally with a microsyringe (Hamilton, model Microliter 701N 80300), using a standard volume of 5 µl. After the injection, animals were transferred to a recovery tank for observation; animals that did not recover after more than 2 minutes were removed from the study. Following previous work, an interval of 30 min between injection and the behavioral test was used (17). The order with which animals were treated, as well as their distribution on drug versus vehicle groups, was randomized using a random number generator tool (http://www.randomization.com/). The vials containing each drug and the respective vehicle were identified using letter and number codes, blinding experimenters until the end of the statistical analysis.

2.3. Behavioral assays

2.3.1. Light/dark test

The LDT was made following the protocol proposed by Maximino et al. (34) and modified by Maximino (44). Briefly, 30 min after the last injection animals were individually transferred to the central compartment of a half-black, half-white tank (15 cm height × 10 cm width × 45 cm length) for a 3-min acclimation period, after which the doors that delimit the compartment were removed and the animal was free to explore the tank for 15 min. Experiments were recorded using a

digital video camera positioned obliquely at the tank top, and later spatiotemporal and ethological variables were extracted from the videos with the help of TheRealFishTracker (v. 0.4.0; https://www.dgp.toronto.edu/~mccrae/projects/FishTracker/); software used to record scototaxis, erratic swimming, and speed or X-Plo-Rat 2005 (https://github.com/lanec-unifesspa/x-plo-rat); software used to record thigmotaxis and risk assessment. The following endpoints were registered:

- Scototaxis (main endpoint): The time spent in the white compartment, in seconds (s);
- Risk assessment: The frequency of risk assessment events, defined either as a fast (<1 s) entry in the white compartment followed by reentry, or as a partial entry into the white compartment (when the pectoral fin does not cross the midline)
- Erratic swimming: The absolute turn angle, in degrees.
- Swimming speed: The average swimming speed, in cm/s.

2.3.2 Novel tank test

After being subjected to the pharmacological treatment, animals were immediately transferred to the NTT apparatus, a transparent glass tank (15 cm width × 25 cm length × 20 cm height) filled with 5 L of tank water. The animal was free to explore the tank for 6 min., during which its behavior was recorded using a video camera positioned in the front of the tank. The following endpoints were extracted using TheRealFishTracker:

- Geotaxis (main endpoint): The total time spent in the bottom third of the tank (s).
- Surfacing: The total time spent in the top third of the tank (s).
- Erratic swimming: The absolute turn angle (in degrees), reflecting accelerated and randomly directed swimming movements.
- Freezing duration (s): The time (in s) that the animal spent at a speed < 0.5 cm/s.
- Swimming speed: The average swimming speed, in cm/s.

2.4. Behavioral experiments

2.4.1. Experiment 1: Dose-response curve and acute toxicity

To determine both the dose-response curve for the light/dark test and whether the range of doses used produced any type of acute toxicity, animals (n = 6-10/group) were treated with doses of 0, 0.1, 1 and 10 mg/kg of chrysin and subjected first to the test, and then to observation on holding tanks to assess toxicity (45). For all experiments, animals from the control groups were taken from the same tanks as those from the other groups, and injected with vehicle. Anxiety-like behavior was assessed 30 min. after injection using the light/dark test. While scototaxis is the main endpoint of

the LDT, other endpoints are likely to be affected by chrysin as well (17); thus, instead of using only the main endpoint to produce the dose-response curve, we summarized results across groups in the form of an *emotionality score*. This was calculated by extracting Z-scores from scototaxis, thigmotaxis, risk assessment, and erratic swimming and averaging these values (Guilloux et al., 2011); positive values indicate increased emotionality/anxiety in relation to controls (i.e., anxiogenic-like effects), while negative values indicate decreased anxiety (i.e., anxiolytic-like effects). Z-scores for each endpoint were calculated with the formula:

$$Z = \frac{X - \mu}{\sigma},$$

where X represents the individual value for that subject, and μ and σ represent the mean and standard deviation for the control group. Since scototaxis is an "inverse" endpoint (i.e., increased time on white indicates an anxiolytic-like effect), its additive inverse Z-score was used. After averaging all Z-scores in a composite emotionality score, a Cedergreen-Ritz-Streibig model with 5 parameters was fitted to the data, using the R package drc (46).

After the end of behavioral testing, animals were transferred to an observation tank and left undisturbed for 96 h. During this interval, animals were observed in the following intervals post-injection: 5 h, 24 h, 40 h, 48 h, 54 h, 78 h, and 96 h. Follow the protocol described by Costa et al. (45), mortality was counted and should not exceed 1 fish in the control group, and water quality parameters should not fall below the critical values for zebrafish. Sublethal endpoints were also recorded, following the protocol, in the following domains: distribution of animals in the tank; vertical distribution; abnormal behaviors; appearance, and abnormalties in provoked behaviors (45).

2.4.2. Experiment 2: Netting stress

30 min. after injection of either vehicle or 1 mg/kg chrysin, 40 zebrafish (n = 10/group) were subjected to a single net handling stress (47), in which animals in the stress (S+) groups were individually netted out of the tank and suspended in a net above water for 30 s. 15 min after the stressor (an interval sufficient to achieve bodily cortisol levels 9 times higher than control levels, and an interval used in other stressors, such as chasing; e.g. (48), animals were transferred to the NTT, and their behavior was recorded as described in Section 2.3.2, above. Animals in the S- group were left undisturbed for the same time period, and later injected with either vehicle (controls) or chrysin.

2.4.3 Experiment 3: Participation of $GABA_A$ receptors

To assess the role of the $GABA_A$ receptor in the effects of chrysin, animals were pre-treated with either vehicle or picrotoxin (1 mg/kg), a chloride channel blocker, and, 15 min later, injected with either vehicle or chrysin (1 mg/kg). 30 min after the last injection, the animal was transferred to the LDT, as described in Section 2.3.1, above.

2.4.4. Statistical analysis

Data for the dose-response curve were estimated by fitting a Cedergreen-Ritz-Streibig model with 5 parameters was fitted to the data, using the R package drc (46), with robust estimation through least trimmed squares. Since no lethal or sublethal endpoints were observed after chrysin treatment for any dose or time interval, no statistical analysis is reported. For Experiments 2 and 3, data were analyzed using 2-way (stress X drug in Experiment 2, or picrotoxin X drug in Experiment 3) analyses of variance (ANOVAs), followed by Tukey's post-hoc tests when p-values were lower than 0.05. Data were represented using Cumming estimation plots, with raw data plotted in the upper axes and mean differences (Hedges' *g*) plotted on the lower axes as a bootstrap sampling distribution (49).

2.5. Molecular docking of chrysin with the human $GABA_A$ receptor

2.5.1. Ligand and target preparation for docking

The structure coordinates of chrysin (PubChem CID: 5281607) were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) in SMILES format. The initial coordinates of the target human $GABA_A$ receptor (chimaeric beta3-alpha5 $GABA_A$ receptor in complex with nanobody Nb25 and pregnanolone, PDB: 5O8F) was obtained from the RCSB PDB database (https://www.rcsb.org/). SwissDock (50) was used to prepare the ligand and the target for docking. The target was optimized in SwissDock using AutoDock Vina (51,52) to remove all water molecules and the Nb25 nanobody in the original structure and adding Kollman charges and nonpolar hydrogens.

2.5.2. Grid preparation and molecular docking

AutoDock Vina was used for the preparation of the grid box on SwissDock. A search space was prepared using a 20 × 20 × 20 Å (X = 49.0, Y = 294.0, Z = 376.0). Molecular docking as

performed using the SwissDock webserver, using both AutoDock Vina to calculate an affinity constant and the Attracting cavities algorithm (53,54).

3 Results

3.1 Dose-response curve and acute toxicity

Fitting a Cedergreen-Ritz-Streibig model with 5 parameters was fitted to the data suggested that chrysin has a hormetic dose-response profile, with lower doses decreasing anxiety-like behavior in the LDT, and the highest dose increasing it. Calculated parameters can be found in Table 1. No lethal or sublethal events were observed in the interval of 96 h after injection in any of the chrysin doses.

Table 1 - Regression parameters for a Cedergreen-Ritz-Streibig 5-parameter model on the effects of chrysin on emotionality scores in the LDT

$R^2_{[df = 20]}$	0.368
b (no direct interpretation)	0.479 ± 0.472
c (Lower horizontal asymptote)	-26.2 ± 24.65
d (Upper horizontal asymptote)	-0.0007 ± 0.129
e (no direct interpretation)	11.14 ± 29.34
f (Size of the hormesis effect)	19.66 ± 3.89

3.2 Netting stress

Medium-sized main effects of stressor ($F[1, 36] = 4.59$, $p = 0.039$; $\omega^2 = 0.08$) and drug ($F[1, 36] = 4.29$, $p = 0.046$; $\omega^2 = 0.07$) were found for geotaxis, but no significant interaction effect was found ($F[1, 36] = 0.31$, $p = 0.578$, $\omega^2 = -0.01$). Thus, the acute net stressor increased geotaxis, chrysin decreased it, but chrysin did not block the effects of netting stress (Figure 1A). No main effects of stressor ($F[1, 36] = 2.04$, $p = 0.161$, $\omega^2 = 0.02$) nor drug ($F[1, 36] = 1.81$, $p = 0.187$, $\omega^2 = 0.02$) were found for top-dwelling (Figure 1B); no significant interaction effect was found ($F[1, 36] = 1.39$, $p = 0.245$, $\omega^2 = 0.01$). Similarly, no main effects of stressor, drug, or interaction were found for erratic swimming ($F[1, 36] < 1.65$, $p > 0.207$, $\omega^2 = -0.03 – 0.02$; Figure

1C). Finally, no main effects of stressor, drug, or interaction were found for swimming speed (F[1, 36] < 1.09, p > 0.302, ω^2 = -0.02 – 0.02; Figure 1D).

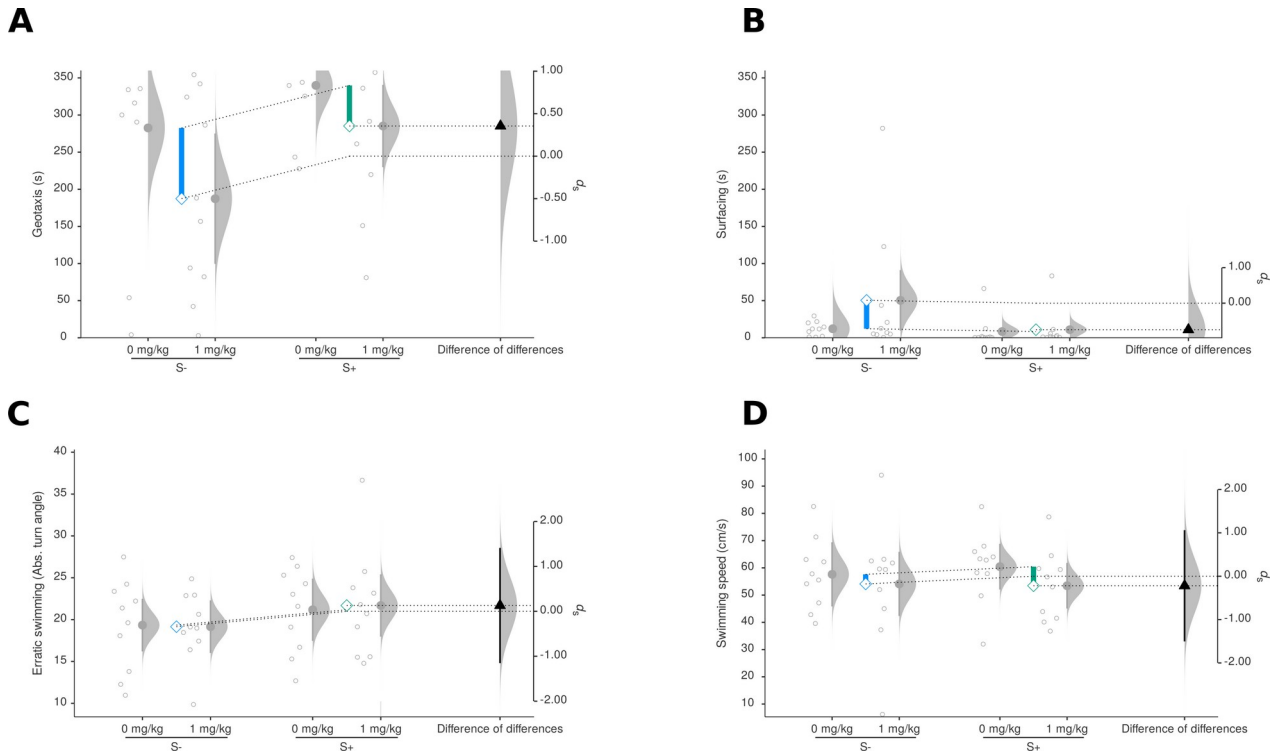


Figure 1: Effects of an acute netting stressor (S-: no stressor; S+: stressor) and chrysin (0 and 1 mg/kg) on anxiety-like behavior in the novel tank test. (A) Geotaxis. (B) Surfacing. (C) Erratic swimming. (D) Swimming speed. Individual points are shown in the panels in the left, with mean ± 95% confidence interval; the panel in the right shows the effect size for the interaction (d_s).

3.3. Participation of $GABA_A$ receptors

No main effects of chrysin (F[1,41] = 2.05, p = 0.16, ω^2 = 0.02) nor pretreatment (F[1, 41] = 0.17, p = 0.684, ω^2 = -0.02) were found for scototaxis (Figure 2A), but a medium-sized significant interaction was found (F[1,41] = 4.99, p = 0.031, ω^2 = 0.08). Post-hoc analyses (Tukey's HSD) showed that chrysin decreased scototaxis in vehicle-pretreated animals (p = 0.045, *d* = -1.11, 0 mg/kg PTX + 0 mg/kg CHR vs. 0 mg/kg PTX + 1 mg/kg CHR), an effect that was blocked by picrotoxin pretreatment (p = 0.948, *d* = 0.24, 0 mg/kg PTX + 1 mg/kg CHR vs. 1 mg/kg PTX + 1 mg/kg CHR). No main effects of drug were found for risk assessment (F[1, 41] = 3.73, p = 0.06, ω^2 = 0.05), but a large significant main effect of pretreatment was found (F[1, 41] = 6.26, p = 0.016, ω^2 = 0.09)(Figure 2B); a medium-sized interaction effect was found for this endpoint (F[1, 41] = 4.87, p = 0.033, ω^2 = 0.07). Post-hoc analyses showed that chrysin increased

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risk assessment in vehicle-pretreated animals (p = 0.019, *d* = -1.25, 0 mg/kg PTX + 0 mg/kg CHR vs. 0 mg/kg PTX + 1 mg/kg CHR), an effect that was blocked by picrotoxin pretreatment (p = 0.006, *d* = 1.43, 0 mg/kg PTX + 1 mg/kg CHR vs. 1 mg/kg PTX + 1 mg/kg CHR). No main effects of chrysin (F[1, 41] = 0.13, p = 0.722, ω^2 = -0.02) nor pretreatment (F[1, 41] = 0.25, p = 0.62, ω^2 = -0.02) were found for erratic swimming, and an interaction effect was also absent (F[1, 41] = 0.64, p = 0.428, ω^2 = -0.01; Figure 2C). No main effects of chrysin (F[1, 41] = 0.62, p = 0.435, ω^2 = -0.01) nor pretreatment (F[1, 41] = 0.06, p = 0.812, ω^2 = -0.02) were found for swimming speed, and an interaction effect was also absent (F[1, 41] = 0.0, p = 0.947, ω^2 = -0.02; Figure 2D).

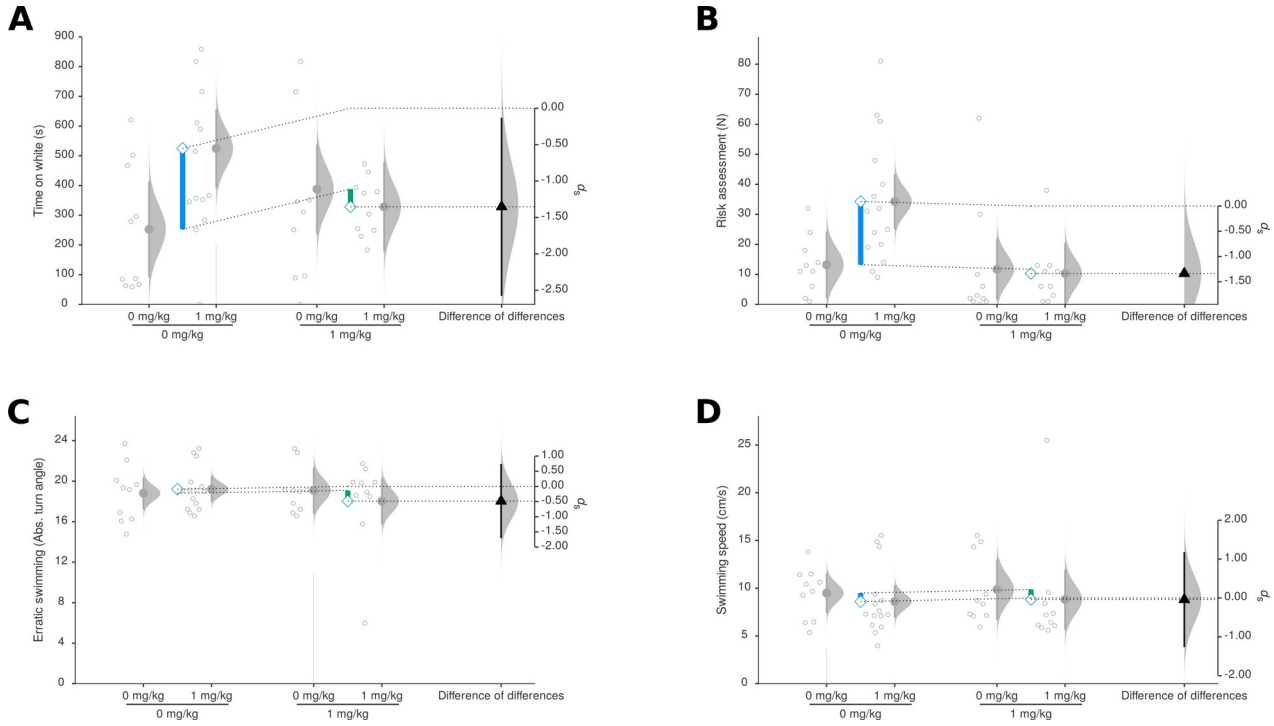

Figure 2: Effects of picrotoxin (0 and 1 mg/kg) and chrysin (0 and 1 mg/kg) on anxiety-like behavior in the light/dark test. (A) Time on white. (B) Risk assessment. (C) Erratic swimming. (D) Swimming speed. Individual points are shown in the panels in the left, with mean ± 95% confidence interval; the panel in the right shows the effect size for the interaction (d_s).

3.4. Molecular docking

Chrysin forms cation-π interactions with LYS282 and hydrophobic contacts with ARG141 α5-subunit, PRO184, LEU183, and VAL50 of the α_5-subunit of the human $GABA_A$, with a binding energy of -6.562 kcal/mol, equivalent to an affinity constant of 0.011 µM (Figure 3).

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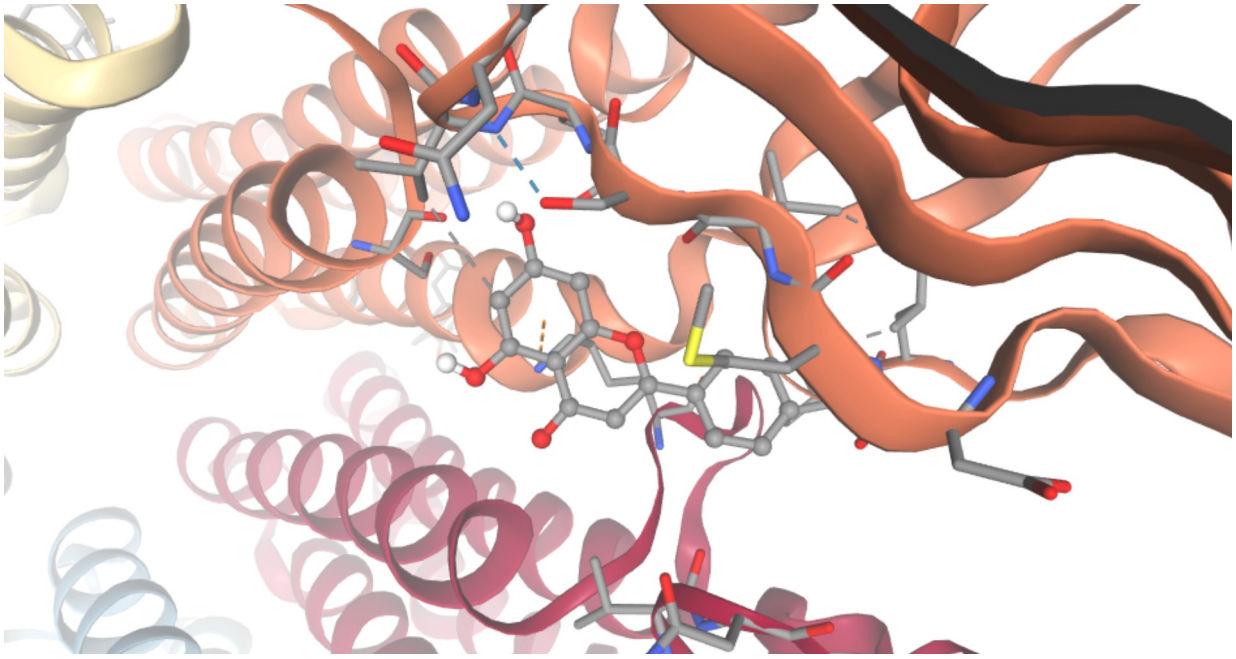

Figure 3: Molecular interactions between chrysin and a model of the human $GABA_A$ receptor (PDB: 5O8F). The protein chain that is depicted is part of the α_5 subunit. Blue dashed lines: hydrophobic interactions; orange dashed lines: cation-π interactions; grey dashed lines: hydrophobic contacts.

Discussion

The present work described the effects of acute chrysin treatment on several parameters of anxiety-like behavior in adult female zebrafish. We found that acute chrysin (0.1 – 10 mg/kg) produces an hormetic dose-response profile, decreasing emotionality in the LDT at lower doses and increasing it at higher doses. Chrysin was found not to produce acute signs of toxicity in this dose range. Although chrysin (1 mg/kg) produced anxiolytic-like effects in the NTT, it was not able to block the anxiogenic-like effects of acute netting stress in this assay. Finally, the anxiolytic-like effects of chrysin (1 mg/kg) in the LDT were blocked by pretreatment with picrotoxin, supporting the participation of chloride channels in these behavioral effects that could be associated with interactions on α_5 subunits of the $GABA_A$ receptor, as suggested by the results from molecular docking.

Acute treatment with chrysin has been shown to produce anxiolytic-like effects in male rats (17,19), mice (18), and zebrafish (17). Here we extend these findings in female zebrafish by producing a full dose-response curve, showing that chrysin produces a biphasic dose-response profile, decreasing anxiety at low doses and increasing it at higher doses. This may be due to chrysin acting at different mechanisms that are depending on the treatment duration and dose. Indeed, it has been proposed that chrysin acutely administered can interact at multiple sites of the

$GABA_A$ receptor – including indirectly, by stimulating the production of neurosteroids – as well as modulate oxidative stress (13,14,20–23,55), while the use of high doses require a long-term to stablish neuroplasticity as consequence of activation of BDNF, NGF, and serotonergic pathways related to its antidepressant-like effects (56). Moreover, in the present study, we extended previous findings using the zebrafish LDT by showing that chrysin also exerts an anxiolytic-like effect in the NTT.

An investigation of the molecular mechanisms of chrysin suggested that chloride channels are responsible, at least in part, for the anxiolytic-like effects of 1 mg/kg chrysin in the LDT. Previously, it has been shown that pretreatment with picrotoxin blocks the anxiolytic-like effects of chrysin in females in metestrus and diestrus phase of the ovarian cycle in rats (22,23), a hormonal state in which anxiety-like behavior is consistently elevated. Picrotoxin also blocks the antidepressant-like effects of a low dose of chrysin (but not a high dose) in male rats (57) and blocks the anxiolytic-like effect of chrysin in a surgical menopause model in female rats (20). Thus, at least some of the effects of chrysin are attributable to its modulation of $GABA_A$ receptors. Indeed, our molecular model suggests that chrysin interacts with residues in the α5 subunit of the $GABA_A$ receptor that line the ion channel pore, although at sites different from both pregnanolone (58) and diazepam (59). Consistent with this observation, it was found that picrotoxin blocked the decrease in scototaxis (preference for dark environments), the main endpoint in the LDT. Interestingly, picrotoxin pretreatment also blocked increased risk assessment observed after chrysin treatment. Risk assessment in the LDT is increased by anxiogenic treatments and decreased by anxiolytic treatments (60), and thus the effects of chrysin in this variable are paradoxical. It has been previously shown that, in the rat open-field test, chrysin increases rearing (17), which could be interpreted as increased risk assessment; however, in that study chrysin did not increase measures of risk assessment in the rat elevated plus-maze, nor did it change risk assessment in the zebrafish LDT. A possible explanation for that discrepancy is sex differences: while German-Ponciano et al. (17) were not able to determine the sex of the zebrafish, in the present experiments only females were used. Regardless of these differences, both the effects of chrysin on scototaxis and risk assessment were blocked by picrotoxin in the present experiments, suggesting a common mechanism of action.

In the present experiments, chrysin was unable to block the anxiogenic-like effects of netting stress in the NTT. Previous experiments with female mice exposed to unpredictable chronic stress showed that chrysin prevented the depressive-like phenotype induced by stress (56), suggesting that, at least in some cases, chrysin can produce an adaptogenic effect. The multiple effects of

chrysin on neuronal targets (e.g., increased GABAergic inhibition, modulation of serotonergic signaling, anti-inflammatory effects, and promotion of neurotrophic factors; (5) suggest that this molecule could increase adaptability in stress (see Panossian (10) for a review of characteristics of plant adaptogens). The lack of effects of chrysin in the present results seem to weaken that hypothesis. An alternative hypothesis is that the short duration of both treatment and stressor could not fully reveal the adaptogenic potential of this molecule. Further studies with chronic stress could help unravel these hypotheses.

While the mediation of the acute behavioral effects of chrysin by $GABA_A$ receptors has been shown in rodents before, the fact that this drug also produces its effects by acting on the same receptor is zebrafish is novel, and suggests conservation of mechanisms of action. It has been suggested that similarity of effects across distantly-related species increases the probability of shared mechanisms between these species and humans, underscoring the need to use alternative model organisms such as zebrafish in behavioral pharmacology (24,25). Our results on molecular modeling suggest that chrysin binds to the α_5 subunit of the $GABA_A$ receptor; while the expression of this receptor in the adult zebrafish brain is unknown, in larvae its expression is restricted to regions of the pallium, hypothalamus, cerebellum, and Mauthner cells (61). The zebrafish α_5 subunit associates with $\beta_3\gamma_2$ and β_3 subunits to induce GABA-elicited currents at very low GABA concentrations (62). This is consistent with the localization of these subunits at extrasynaptic sites in mammals, where the GABA concentration is low (63). Thus, chrysin could exert its acute anxiolytic-like effects by increasing the inhibitory tone on areas associated with defensive behavior.

In summary, the present experiments showed that an acute treatment of female zebrafish with chrysin produces anxiolytic-like effects, at low doses, in different behavioral screens, and that these anxiolytic-like effects are mediated by $GABA_A$ receptors, probably through interactions with α_5 subunits of the $GABA_A$ receptor. Whether or not the effects of high doses are mediated by this receptor is still unknown. Moreover, while chrysin has a profile of mechanisms that is consistent with an adaptogenic effect, we were not able to show that this flavonoid promotes adaptation to an acute, short-lasting stressor. Whether or not this effect is present for longer-lasting stressors remains to be analyzed.

Data availability statement

The data that support the findings of this study are openly available in GitHub at DOI: 10.5281/zenodo.2597622, https://github.com/lanec-unifesspa/chrysin/tree/master/mechanisms .

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