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Anhedonic- and anxiogenic-like behaviors and neurochemical alterations are abolished by a single administration of a selenium-containing compound in chronically stressed mice

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ABSTRACT

Despite the severity and the high prevalence of depression and anxiety and the efforts that have been done to improve their treatment, the available pharmacotherapy still has several limitations. Therefore, the investigation of novel agents and the characterization of the molecular signaling pathways underlying their effects are needed. The organoselenium compound 3-[(4-chlorophenyl)selanyl]-1-methyl-1*H*-indole (CMI) has emerged as a promising antidepressant and anxiolytic molecule in several animal models of depression through the modulation of neuroinflammation and oxidative stress. In light of this, the present study aimed to dive into the mechanism of action of CMI in ameliorating anhedonic- and anxiogenic-like behaviors induced by repeated corticosterone administration in mice. A single administration of CMI (1 mg/kg, i.g.) abrogated the behavioral alterations induced by corticosterone in the open field test, splash test, and elevated plus maze test. Additionally, CMI treatment decreased the levels of reactive species and lipid peroxidation in the plasma of corticosterone-treated mice and normalized the expression of *GR*, *BDNF*, *synaptophysin*, *GSK*-3β, Nrf_2, and *IDO* in the hippocampi of stressed mice. Noteworthy, the pre-treatment of mice with LY294002 (PI3K inhibitor) and rapamycin (mTOR inhibitor) abrogated the anti-anhedonic- and anxiolytic-like effects elicited by CMI in corticosterone-treated mice, while ZnPP (HO-1 inhibitor) counteracted the anxiolytic-like effect of CMI. These findings suggest that CMI might ameliorate behavioral and biochemical alterations in the depression-anxiety comorbidity induced by corticosterone, highlighting the potential of CMI as a possible adjuvant therapy.

1. Introduction

Major depression affects 322 million people worldwide and is often present with other neuropsychiatric disorders, especially anxiety [1,2]. While the molecular mechanisms responsible for the onset and progression of these disorders remain elusive, hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis has been reported as one of the most common neurobiological alterations in depressive [3] and anxious [4] patients.

In response to physiological or psychological stress, activation of the HPA axis results in the secretion of glucocorticoids from the adrenal cortex into the bloodstream. The secretion of glucocorticoids (cortisol in humans and corticosterone in rodents) is the primary endocrine response to stress and has a vital role in the survival of the species [5]. However, the hypersecretion of glucocorticoids can impair glucocorticoid receptor (GR) signaling and promote glucocorticoid resistance [6]. While acute exposure to glucocorticoids has inhibitory effects on the immune system, chronic exposure can lead to increased neutrophil proliferation and generation of proinflammatory cytokines and reactive species (RS) [7]. The resulting increased peripheral inflammation and oxidative stress have been reported in depressed [8] and anxious [9] patients.

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Abbreviations

Akt/PKB	protein kinase B
BDNF	brain-derived neurotrophic factor
CMI	3-[(4-chlorophenyl)selanyl]-1-methyl-1*H*-indole
DMSO	dimethyl sulfoxide
EPM	elevated plus maze
GR	glucocorticoid receptor
GSK3-β	glycogen synthase kinase 3 beta
HC	hippocampi
HO-1	heme-oxygenase 1
HPA	hypothalamic-pituitary-adrenal axis
ICV	intracerebroventricular
IDO	indoleamine 2,3-dioxygenase
IG	intragastrically
iNOS	inducible nitric oxide synthase
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
mTOR	mammalian target of rapamycin
NFκB	nuclear factor kappa B
Nrf_2	nuclear factor erythroid 2-related factor 2
OFT	open field test
PDB	protein data bank
PI3K	phosphoinositide 3-kinase
qRT-PCR	quantitative real-time polymerase chain reaction
RS	reactive species
TBARS	thiobarbituric acid reactive species
TNF-α	tumor necrosis factor alpha
TrkB	tropomyosin receptor kinase B

In the central nervous system, the hippocampus is particularly vulnerable to the effects of hypercortisolemia due to the high expression of the glucocorticoid receptor (GR) and its role in the HPA axis negative feedback [3,6]. GR resistance can also be induced by proinflammatory cytokines and p38 mitogen-activated protein kinase (p38 MAPK) in the context of neuroinflammation [10]. One of the main effects of chronic exposure to glucocorticoids in the brain is the negative impacts on hippocampal neurogenesis and neuroplasticity [11]. Upon binding to the receptor tropomyosin-related kinase B (TrkB), the brain-derived neurotrophic factor (BDNF) activates several intracellular pathways, including the phosphatidylinositol 3-kinase (PI3K) to promote cell survival [12]. The mammalian target of rapamycin (mTOR) acts downstream of BDNF/TrkB/PI3K, increasing the transcription and the translation of proteins involved in synaptogenesis, including synaptophysin [13]. Activation of the BDNF/TrkB/PI3K/mTOR signaling pathway has been implicated in the fast and persistent antidepressant effects of ketamine [14,15].

Besides, activation of PI3K promotes protein kinase B (Akt)-mediated phosphorylation and inhibition of glycogen kinase synthase 3 beta (GSK-3β). GSK-3β is upregulated in response to neuroinflammation, oxidative stress, and hypercortisolemia, and contributes to glucocorticoid resistance [16] and impaired antioxidant defense. The latter is partially mediated by GSK-3β decreasing the nuclear translocation of the nuclear factor erythroid 2-related factor 2 (Nrf_2) and reducing the expression of antioxidant enzymes, such as heme oxygenase 1 (HO-1) [17]. HO-1 expression increases in response to oxidative stress and is involved in the behavioral responses of several antioxidant compounds with antidepressant-like activity, such as ascorbic acid and creatine [14]. Further, activation of GSK-3β induces the nuclear factor kappa B (NFκB) activity and the synthesis of pro-inflammatory cytokines [18] that activate indoleamine 2,3-dioxygenase (IDO) and inducible nitric oxide synthase (iNOS), contributing to the reduction of the serotonergic neurotransmission and impaired redox homeostasis [19].

Despite the efforts toward alleviating the symptomatology, 40% of depressed patients (Institute for Quality and Efficiency in Ref. [20] and more than 15% of anxious patients [21] do not respond to the currently available pharmacotherapy, stimulating the search for better therapeutic strategies. The 3-[(4-chlorophenyl)selanyl]-1-methyl-1*H*-indole (CMI), a synthetic selenium-containing compound, has emerged as a promising molecule with *in vitro* and *in vivo* antioxidant activity [22–24] and antidepressant- and anxiolytic-like effects in models of acute and chronic inflammation, acute stress, and in tumor-bearing mice [25–28]. Importantly, CMI does not cause hepato-, nephro-, and neurotoxicity to mice [22], further encouraging the studies with this molecule.

In light of the above, the present study was aimed at investigating whether a single dose of CMI would ameliorate behavioral and biochemical alterations in a model of anhedonia (a core symptom of depression) and anxiety induced by chronic administration of corticosterone and whether the behavioral response to CMI involves the PI3K/mTOR signaling and transcriptional modulation of related genes in the hippocampi.

2. Materials and methods

2.1. Animals

Male Swiss mice (2 months old; 20–25 g), maintained at 22–25 °C with water and food *ad libitum*, under a 12:12 h light/dark cycle provided by Animal Facility of the Federal University of Pelotas (UFPel) (Pelotas, Brazil) were used. The studies were performed in accordance with protocols approved by the Committee on the Care and Use of Experimental Animal Resources (UFPel - 2208).

2.2. Drugs

CMI (Fig. 1A) was synthesized at the Laboratory of Clean Organic Synthesis at Federal University of Pelotas [29]. A dose of 1 mg/kg of body weight of CMI was prepared in canola oil (non-polar substance) and administered intragastrically (i.g.) in a constant volume of 10 mL/kg. Corticosterone was purchased from Sigma Aldrich (St Louis, Missouri, USA), dissolved in distilled water with 2% Tween 80 and 0.2% dimethyl sulfoxide (DMSO) and administered i.g. at 20 mg/kg/day [30]. Rapamycin solution, an inhibitor of mammalian target of rapamycin (mTOR); LY294002, an inhibitor of phosphoinositide 3-kinase (PI3K); and protoporphyrin IX zinc (II) (ZnPP), an inhibitor of heme-oxygenase 1 (HO-1) were purchased from Sigma Aldrich (St Louis, Missouri, USA). Rapamycin (0.2 nmol/site) was dissolved in DMSO [30]. LY294002 (10 nmol/site) and ZnPP (10 μg/mouse) were dissolved in saline (0.9% NaCl) at a final concentration of 1% DMSO [14]. All inhibitors were administered by the intracerebroventricular route (i.c.v) at a volume of 3 μL/mouse. I.c.v. injections were performed using the "free hand" method under isoflurane anesthesia according to procedures previously described [31]. The RNA extraction reagents were purchased from Ambion (Life Technology, USA) and oligonucleotides were synthesized by Exxtend Biotecnologia Ltda (Campinas, Brazil). The other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.3. Behavioral tests

2.3.1. Open field test (OFT)

The OFT was performed to assess the locomotor activity of mice [32]. Animals were individually placed in the center of a box (30 × 30 × 15 cm) divided into nine quadrants with equal areas. During 5 min, the

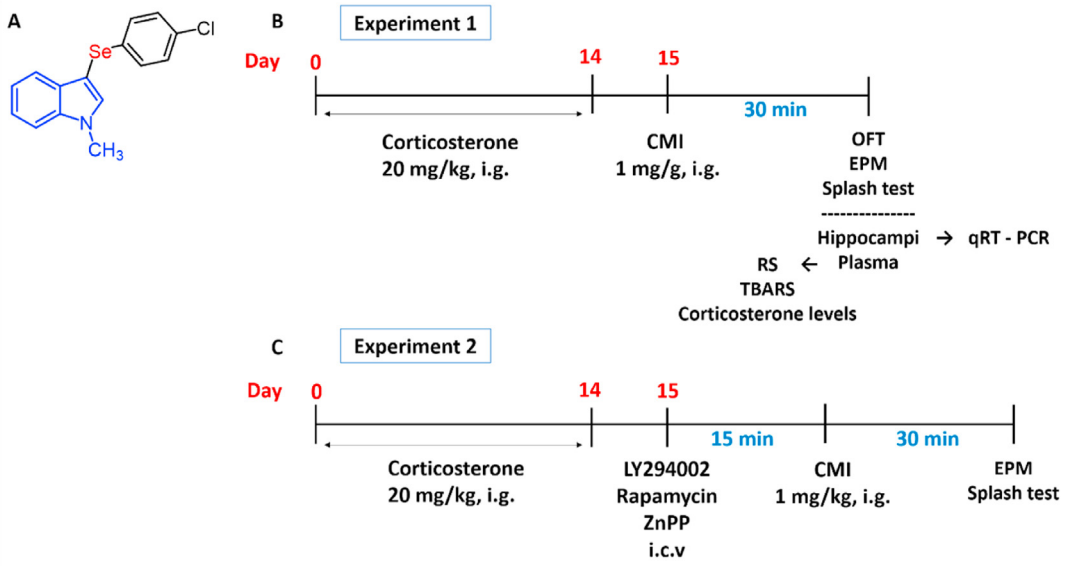

Fig. 1. Diagram of the experimental schedule. CMI chemical structure (A), experimental design for experiment 3.2.1, (B), and for experiment 3.2.2 (C). CMI: 3-[(4-chlorophenyl)selanyl]-1-methyl-1*H*-indole; i.g.: intragastrically; OFT: open field test; EPM: elevated plus maze test; RS: reactive species; TBARS: thiobarbituric acid reactive species assay; qRT-PCR: quantitative real-time polymerase chain reaction.

locomotor activity was assessed by the number of crossings and rearings. Additionally, the anxiogenic-like behavior was assessed by the number of groomings and entries in the central square.

2.3.2. Splash test

The splash test was carried out to assess the anti-anhedonic-like behavior of mice [33]. A 10% sucrose solution was squirted on the dorsal coat of each mouse. During 5 min, the grooming time (nose/face grooming, head washing, and body grooming) was recorded manually. The grooming behavior is a measurement of motivational and self-care activities that are decreased in depressive behavior.

2.3.3. Elevated plus maze (EPM) test

The EPM test was carried out to assess the anxiogenic-like behavior of mice [34]. The apparatus is plus-shaped with two open and two enclosed arms (arm length: 100 cm; arm width: 10 cm) elevated 40 cm above the floor. For the test, each animal was placed in the center of the apparatus and the number of head dips, number of entries, and time spent in the open arms were scored manually for 5 min.

2.4. Experimental design

2.4.1. Experiment 1 – anti-anhedonic- and anxiolytic-like effects of CMI

This experiment was carried out to address whether CMI would improve anhedonic- and anxiogenic-like behaviors in stressed mice and modulate markers of oxidative stress and neuroinflammation. Animals were randomly assigned to four experimental groups (n = 6 mice/group). Two groups received corticosterone (20 mg/kg) and two groups received corticosterone vehicle, i. e. distilled water with 2% Tween 80 and 0.2% DMSO, orally once a day. The administration lasted 14 days, and on the 15th day, CMI (1 mg/kg) or its vehicle (canola oil) were administered (Fig. 1B). The behavioral tests were performed 30 min after CMI or vehicle administration (i.g.), as previously described [22,25,26]. The locomotor activity was evaluated in the open field test (OFT), the anti-anhedonic-like behavior was assessed in the splash test, and the anxiogenic-like behavior was assessed in the elevated plus-maze (EPM) test and OFT. The behavioral tests were carried out immediately after each other in the following order: OFT, EPM, and splash test. By following this order, we assessed anxiety-related behavior before depression-like behavior since anxiety tests can be influenced by experience. The order of behavioral testing should be taken into consideration when recovery time or testing on different days is not possible [35]. Also, this test sequence allowed the effects of CMI to be tested between 30 and 45 min after its administration [22], avoiding any confounding factor arising from observations carried out in distinct time points. All observations were done by observers blinded to the treatments. The protocol chosen for this study, *i.e.*, period of corticosterone treatment and time of inhibitors administration, is extensively used and well established for anhedonic- and depressive-like behaviors in mice [14,15]. After the behavioral evaluations, mice were anesthetized (inhalation of isoflurane) for blood collection by cardiac puncture. Following that, mice were euthanized by an overdose of isoflurane inhalation, and the death was confirmed by cervical dislocation. Finally, the brain was removed and the hippocampi (HC) were isolated for analysis.

2.4.2. Experiment 2 – behavioral effects of CMI in the presence of PI3K, mTOR, and HO-1 inhibitors

This experiment was carried out to address whether the behavioral effects of CMI require PI3K, mTOR, and HO-1 signaling. Animals were randomly assigned to eight experimental groups (n = 6 mice/group). Four groups received corticosterone (20 mg/kg) and four groups received its vehicle orally once a day. The administration lasted 14 days and on the 15th day, mice were anesthetized with isoflurane followed by i.c.v injections of the inhibitors (LY294002, 10 nmol/site; rapamycin, 0.2 nmol/site; ZnPP, 10 μg/mouse) or its respective vehicles. CMI (1 mg/kg) or its vehicle were administered after 15 min and, after further 30 min, the behavioral tests were carried out (Fig. 1C). The behavioral tests followed the protocol described in Experiment 1 (item 2.3.1). After the behavioral tests, mice were euthanized by an overdose of isoflurane and no biological samples were collected for analysis.

I.c.v. injections were performed using the "free hand" method under isoflurane anesthesia according to procedures previously described [31]. Briefly, the i.c.v. administration was performed using a microsyringe (25 μL, Hamilton) connected to a 26-gauge stainless-steel needle that was inserted perpendicularly 2 mm through the skull to the midline point equidistant from each eye of mice anesthetized with isoflurane. Mice were under isoflurane anesthesia for no longer than 10 min and they started to recover 2 min after the anesthesia cessation. The behavioral analyses were carried out 45 min after the isoflurane anesthesia, a time in

which the animals did not present locomotor impairment (as assessed in the OFT) and, therefore, did not impact the subsequent behavioral analyses.

2.5. Biochemical evaluation

2.5.1. Sample processing

After euthanasia, HC were isolated, immersed in TRIzol, and maintained at −80 °C for the quantitative real-time polymerase chain reaction (qRT-PCR). The blood was collected in heparinized tubes, centrifuged for 10 min at 12,000×*g* and 4 °C and the plasma fraction was used to determine the levels of endogenous corticosterone, reactive species (RS), and thiobarbituric acid reactive species (TBARS).

2.5.2. Determination of plasma corticosterone levels

For the determination of corticosterone levels [28,36], aliquots of plasma were incubated with chloroform and centrifuged for 5 min at 2500×*g*, followed by addition of 0.1 M NaOH and another round of centrifugation. After the addition of H_2SO_4 and ethanol 50%, samples were centrifuged (5 min at 10,000×*g*) and incubated at room temperature for 2 h. Fluorescence intensity emission, corresponding to plasma corticosterone levels, was recorded at 540 nm (with 247 nm excitation) and corticosterone levels were expressed as ng/mL.

2.5.3. Determination of reactive species (RS) levels

The RS levels were quantified by incubating aliquots of plasma with 1 mM dichloro-dihydro-fluorescein diacetate (DCHF-DA) and 10 mM Tris-HCl pH 7.4 [37]. In the presence of RS, DCFH-DA is oxidized to fluorescent dichlorofluorescein (DCF). The fluorescence intensity emission was measured at 520 nm (with 480 nm excitation) and RS levels were expressed as fluorescence units.

2.5.4. Determination of thiobarbituric acid reactive species (TBARS) levels

The lipid peroxidation was measured by detecting the levels of malondialdehyde (MDA), an end-product of lipid peroxidation. Aliquot of plasma were incubated with 8.1% SDS, 0.8% TBA and acetic acid/HCl (pH 3.4) during 2 h at 95 °C [38]. Absorbance was measured by spectrophotometry at 532 nm and the results were expressed as nmol of MDA/mL.

2.5.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total mRNA was extracted from the HC using TRIzol (Invitrogen™, Carlsbad, USA) followed by DNase treatment with DNA-free® kit (Ambion™, USA) and mRNA quantification. The cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems™, UK) according to the manufacturer's protocol. The cDNA amplification was made with UltraSYBR Mix (COWIN Bioscience Co., Beijing, China) using the Stratagene Mx3005P and the oligonucleotides were obtained from Exxtend Biotecnologia Ltda, Campinas, Brazil. Gene expressions were normalized using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a reference gene and the conditions for the reaction involved 95 °C for 15 s, 60 °C for 60 s and 72 °C for 30 s. The 2ΔΔCT (Delta-Delta Comparative Threshold) method was used to normalize the fold change in gene expressions. The following genes were analyzed: *GR* (fwd 5′-ACA GCA ACG GGA CCA CCT C-3′, rev 5′-ATG GCA TCC CGA AGC TTC -3′), *GSK-3β* (fwd 5′-CGG GAC CCA AAT GTC AAA CT-3′, rev 5′-TCC GAG CAT GTG GAG GGA TA-3′), *IDO* (fwd 5′-AAT CAA AGC AAT CCC CAC TG-3′, rev 5′-AAA AAC GTG TCT GGG TCC AC-3′), *BDNF* (fwd 5′-CCA TAA GGA CGC GGA CTT GTA C-3′, rev 5′-AGA CAT GTT TGC GGC ATC CAG G-3′), *Nrf*$_2$ (fwd 5′-GTC TTC ACT GCC CCT CAT C-3′, rev 5′-TCG GGA ATG GAA AAT AGC TCC-3′), *synaptophysin* (fwd 5′-TGT GTT TGC CTT CCT CTA CTC-3′, rev 5′-TCA GTG GCC ATC TTC ACA TC-3′), and *GAPDH* (fwd 5′-AGG TCG GTG TGA ACG GAT TTG-3′, rev 5′-TGT AGA CCA TGT AGT TGA GGT CA-3′).

2.6. Molecular docking

The interaction of CMI with the enzymes p38 MAPK (PDB: 1ZZL) and GSK-3β (PDB: 1Q4L) was predicted by the software Autodock Vina 1.1.2 [39]. The crystal structure of the proteins was retrieved from the Protein Data Bank (http://www.pdb.org/pdb/) and optimized with CHIMERA 1.5.3. The grid box size covering the residues in the active sites of proteins was implemented by Autodock Tools 1.5.6. CMI was designed and optimized in the software Avogadro 1.1.1. The docking poses of CMI with the active site of the enzymes were visualized using Accelrys Discovery Studio 3.5.

2.7. Statistical analysis

The assumption of data normality was assessed with the D'Agostino-Pearson test. All data are presented as mean ± standard error of the mean (SEM). Comparisons in experiment 3.2.1 (±corticosterone × ± CMI) were analyzed by two-way analysis of variance (ANOVA). The Newman-Keuls post-hoc test was performed for between-group comparisons when ANOVA revealed significant interaction. Comparisons in experiment 3.2.2 (±corticosterone × ± CMI × ± inhibitors) were analyzed by three-way ANOVA. The Newman-Keuls post-hoc test was performed for between-group comparisons when ANOVA revealed significant interaction. Values equal to or less than 0.05 ($p \leq 0.05$) were considered statistically significant. The statistical analyzes were performed using GraphPad Prism version 8.0 for Windows, GraphPad Software (San Diego, CA, USA).

3. Results

3.1. Anhedonic- and anxiogenic-like behaviors and peripheral levels of oxidative markers in CMI-treated mice

In the present study, neither corticosterone nor CMI treatment changed the number of crossings ($F_{(1,20)} = 1.87$, $p = 0.19$; Fig. 2A) and rearings ($F_{(1,20)} = 0.21$, $p = 0.65$; Fig. 2B) in the OFT. Regarding the anxiogenic-like behavior, the two-way ANOVA revealed a statistically significant corticosterone × CMI interaction for the number of groomings in the OFT ($F_{(1,20)} = 6.27$, $p = 0.02$; Fig. 2C) with CMI decreasing the number of groomings in corticosterone-treated mice ($p < 0.01$). Additionally, the two-way ANOVA showed a statistically significant corticosterone × CMI interaction ($F_{(1,20)} = 7.50$, $p = 0.01$) for the number of entries in the central square of the open field apparatus (Fig. 2D). The post hoc analysis indicated that CMI treatment increased the number of entries in the central square in corticosterone-treated mice ($p < 0.05$).

The ability of CMI to abrogate anhedonic-like behavior induced by corticosterone administration in the splash test is depicted in Fig. 2E. The two-way ANOVA showed a statistically significant corticosterone × CMI interaction for the grooming time in the splash test ($F_{(1,20)} = 7.38$, $p = 0.01$). A post hoc analysis showed that CMI abolished the decreased grooming time in corticosterone-treated mice ($p < 0.01$).

The effect of CMI in anxiogenic-like behavior was further evaluated in the EPM test (Fig. 2F–H). A two-way ANOVA revealed a statistically significant corticosterone × CMI interaction for the number of entries in the open arms ($F_{(1,20)} = 4.25$, $p < 0.01$; Fig. 2F), for the time spent in the open arms ($F_{(1,20)} = 8.79$, $p = 0.008$; Fig. 2G) and for the number of head dips ($F_{(1,20)} = 14.13$, $p = 0.001$; Fig. 2H). Importantly, CMI increased the number of entries ($p \leq 0.05$) and the time spent in the open arms ($p < 0.01$) while decreased the number of head dips ($p \leq 0.05$).

To validate the involvement of the HPA axis in our experiments, plasma levels of corticosterone were measured in mice treated with exogenous corticosterone. The results presented in Fig. 2I show that the chronic corticosterone treatment-induced increase in plasma corticosterone was significantly reversed by CMI (corticosterone × CMI interaction; $F_{(1,18)} = 13.62$, $p = 0.002$) while the administration of CMI to control mice had no effect. One sample from the corticosterone + vehicle

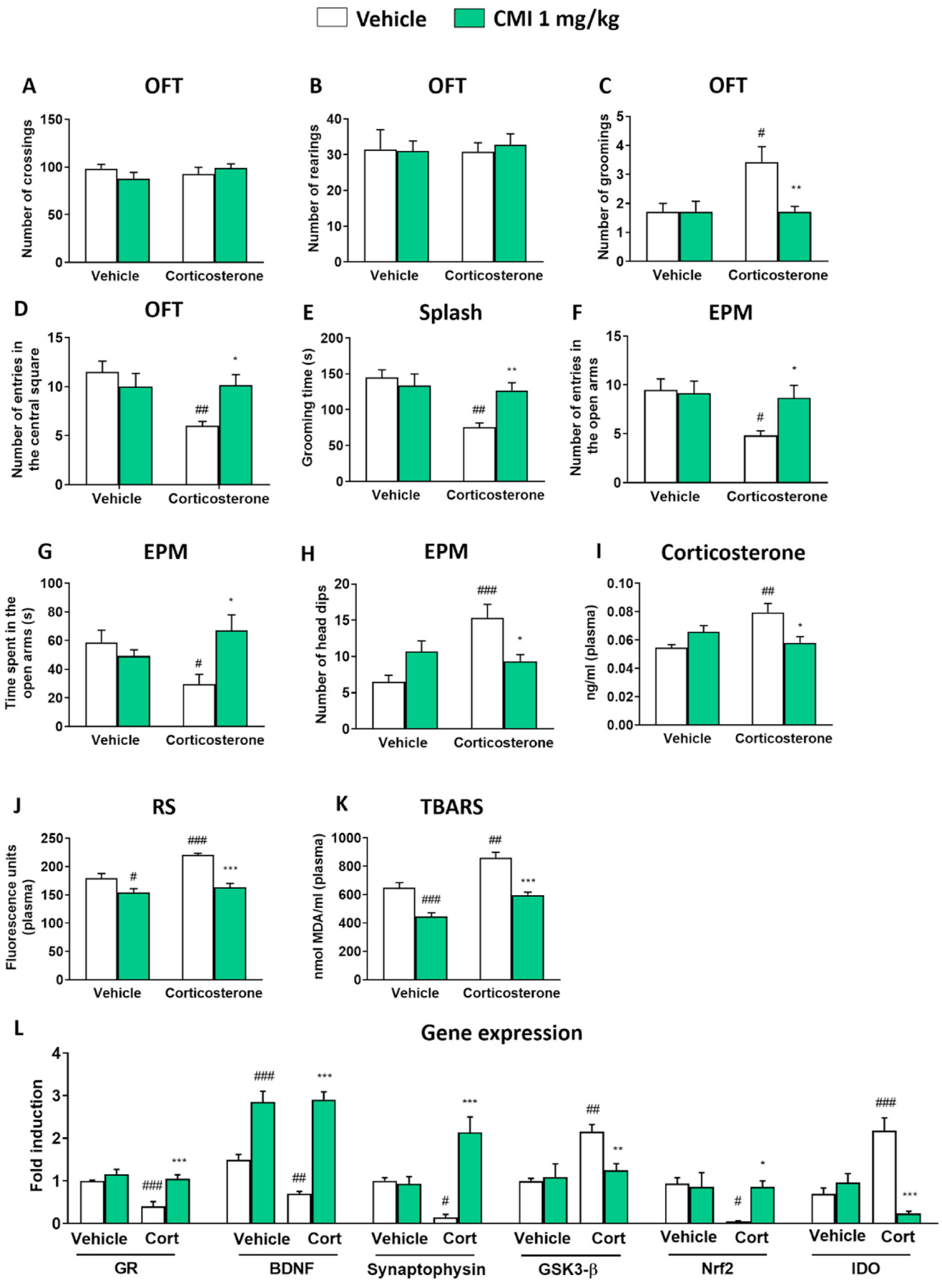

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Fig. 2. Effect of acute administration of CMI (1 mg/kg, i.g.) on behavioral tasks and biochemical parameters. Number of crossings (A), rearings (B), and groomings (C) and number of entries in the central square (D) of the open field box. Grooming time in the splash test (E). Number of entries (F) and time spent (G) in the open arms of the EPM apparatus and number of head dips in the EPM test (H). Plasmatic levels of corticosterone (I), RS (J), and lipid peroxidation (K). Gene expression in the hippocampi of mice (L). Data were analyzed by a two-way ANOVA followed by Newman-Keuls post hoc test and are expressed as mean ± SEM of 6 independent animals. ($^{\#}$) $p \leq 0.05$, ($^{\#\#}p < 0.01$, and ($^{\#\#}$) $p < 0.001$ when compared to vehicle-treated group. (*) $p \leq 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ when compared to corticosterone-treated group.

group and one sample from the corticosterone + CMI group were excluded from the analyses because the corticosterone levels were not detected.

The two-way ANOVA revealed that CMI treatment reversed the increased levels of RS in the plasma of corticosterone-treated mice. Administration of corticosterone increased the levels of RS in the plasma, showing a significant corticosterone × CMI interaction ($F_{(1,20)} = 6.03$, $p = 0.02$; Fig. 2J). Regarding lipid peroxidation, the two-way ANOVA revealed a main effect for the CMI treatment ($F_{(1,20)} = 36.17$, $p < 0.001$, Fig. 2K) and for the corticosterone-treatment ($F_{(1,20)} = 60.41$, $p < 0.001$) in TBARS. A post hoc analysis showed that CMI *per se* reduced the levels of RS ($p < 0.05$) and TBARS ($p < 0.001$). Administration of corticosterone increased RS ($p < 0.001$) and TBARS ($p < 0.01$) levels in the plasma of mice while CMI treatment reversed it ($p < 0.001$).

The statistical analysis showed that the administration of CMI *per se* (*i.e.,* group not treated with corticosterone) did not alter the behavior of mice and the corticosterone plasma levels, which is in agreement with previous data indicating that CMI exerts its anhedonic-, antidepressant- and anxiolytic-like effects only in mice with behavioral alterations induced by an exogenous stimulus as opposed to control mice [22, 25–28]. However, CMI is an antioxidant molecule and, therefore, modulates the plasma levels of RS and TBARS in control mice (not stressed).

3.2. Transcription analysis in the HC of mice after CMI treatment

The effects of CMI on gene expression in the HC of mice is depicted in Fig. 2L. The two-way ANOVA revealed a significant corticosterone × CMI interaction ($F_{(1,20)} = 7.18$, $p = 0.01$) for the *GR* expression. The post hoc analysis showed that corticosterone decreased the *GR* expression ($p < 0.001$) when compared to the control group, while CMI increased the *GR* expression in corticosterone-treated mice ($p < 0.001$). Administration of CMI in non-stressed mice did not alter *GR* expression.

Similarly, a two-way ANOVA revealed that *BDNF* expression was downregulated in the HC of mice treated with corticosterone and the CMI treatment reversed it (corticosterone × CMI interaction, $F_{(1,20)} = 6.37$, $p = 0.02$). A post hoc analysis showed that corticosterone administration decreased *BDNF* expression ($p < 0.01$) when compared to the control group, while CMI increased *BDNF* expression in vehicle-treated ($p < 0.001$) and corticosterone-treated mice ($p < 0.001$). The two-way ANOVA showed a statistically significant corticosterone × CMI interaction ($F_{(1,20)} = 24.70$, $p < 0.001$) for *synaptophysin* expression. The post hoc analysis revealed that corticosterone treatment downregulated *synaptophysin* expression in the HC of corticosterone-treated mice ($p \leq 0.05$) while the single administration of CMI upregulated the expression of synaptophysin ($p < 0.001$). Administration of CMI in non-stressed mice did not alter *synaptophysin* expression.

The two-way ANOVA showed a statistically significant corticosterone × CMI ($F_{(1,20)} = 6.66$, $p = 0.02$) interaction for the *GSK-3β* expression in the HC of mice. The post hoc analysis indicated that corticosterone treatment upregulated *GSK-3β* expression ($p < 0.01$) and treatment with CMI restored it ($p < 0.01$). HC from stressed mice presented downregulated Nrf_2 expression ($p \leq 0.05$), and a single administration of CMI ($p \leq 0.05$) abolished it (corticosterone × CMI interaction, $F_{(1,20)} = 5.35$, $p = 0.03$). Administration of CMI in non-stressed mice did not alter *GSK-3β* and Nrf_2 expression.

Additionally, a two-way ANOVA revealed a statistically significant corticosterone × CMI interaction for *IDO* expression ($F_{(1,20)} = 31.48$, $p < 0.001$). Corticosterone-treated mice showed upregulated *IDO* expression in the HC ($p < 0.001$), while the single administration of CMI reversed it ($p < 0.001$). Administration of CMI in non-stressed mice did not alter *IDO* expression.

3.3. Behavioral effects of CMI in the presence of PI3K inhibitor

A single administration of CMI in the presence of LY294002, a PI3K inhibitor, did not reverse the anhedonic- and anxiogenic-like effects induced by corticosterone (Fig. 3), suggesting that PI3K is required for the behavioral effects of CMI. A three-way ANOVA showed a statistically significant corticosterone × CMI × LY294002 interaction for the grooming time ($F_{(1,40)} = 9.12$, $p = 0.004$) in the splash test (Fig. 3A). The post hoc analysis revealed that CMI administration exerted an anti-anhedonic-like effect in the absence ($p \leq 0.05$) of LY294002, but this effect was not observed in the presence of LY294002. A three-way ANOVA showed statistically significant corticosterone × CMI × LY294002 interaction for the number of entries in the open arms ($F_{(1,40)} = 31.84$, $p < 0.001$) in the EPM apparatus (Fig. 3B). The post hoc analysis revealed that CMI treatment did not exert the anxiolytic-like effect in the presence of LY294002, but this effect was observed in the absence ($p \leq 0.05$) of this inhibitor. Likewise, a three-way ANOVA showed a statistically significant corticosterone × CMI × LY294002 interaction for the time spent in the open arms ($F_{(1,40)} = 4.68$, $p = 0.04$) of the EPM apparatus (Fig. 3C). The anxiolytic-like effect of CMI was observed in the absence ($p \leq 0.05$) of LY294002, but it was not observed in the presence of the inhibitor. Regarding the number of head dips in the EPM test (Fig. 3D), a three-way ANOVA showed a statistically significant corticosterone × CMI × LY294002 interaction ($F_{(1,40)} = 5.00$, $p = 0.03$). The post hoc analysis revealed that CMI treatment presented an anxiolytic-like effect in the absence ($p < 0.01$) of LY294002 but not in its presence. Administration of CMI and LY294002 in non-stressed mice did not cause behavioral alterations.

3.4. Behavioral effects of CMI in the presence of mTOR inhibitor

A single administration of CMI in corticosterone-treated animals in the presence of rapamycin, an mTOR inhibitor, did not show the effects previously suggesting that mTOR is required for the behavioral effects of CMI. The results regarding the splash test are depicted in Fig. 3E. The three-way ANOVA showed a statistically significant corticosterone × CMI × rapamycin interaction for the grooming time ($F_{(1,40)} = 5.78$, $p = 0.02$). The post hoc analysis showed that CMI did not exert the anti-anhedonic-like effect in the presence of rapamycin, while this effect was observed in the absence ($p \leq 0.05$) of the inhibitor. The three-way ANOVA revealed no statistically significant corticosterone × CMI × rapamycin interaction for the number of entries in the open arms (Fig. 3F) in the EPM. However, main effects for corticosterone ($F_{(1,40)} = 24.7$, $p = 0.0001$) and CMI administration ($F_{(1,40)} = 6.93$, $p = 0.0120$) were found. The post hoc analysis showed that CMI treatment was only effective in the absence of rapamycin ($p \leq 0.05$). Similarly, no significant interaction was found for the time spent in the open arms of the EPM apparatus (Fig, 3G), but the main effect for corticosterone ($F_{(1,40)} = 6.40$, $p = 0.0072$) was revealed by the three-way ANOVA. The post hoc analysis did not show any significant statistically difference. Regarding the number of head dips (Fig. 3H) in the EPM test, no significant interaction was found. However, main effects were found for the corticosterone ($F_{(1,40)} = 13.90$, $p = 0.0006$) and CMI ($F_{(1,40)} = 9.76$, $p = 0.0033$) treatments. The post hoc analysis revealed that a single administration of CMI

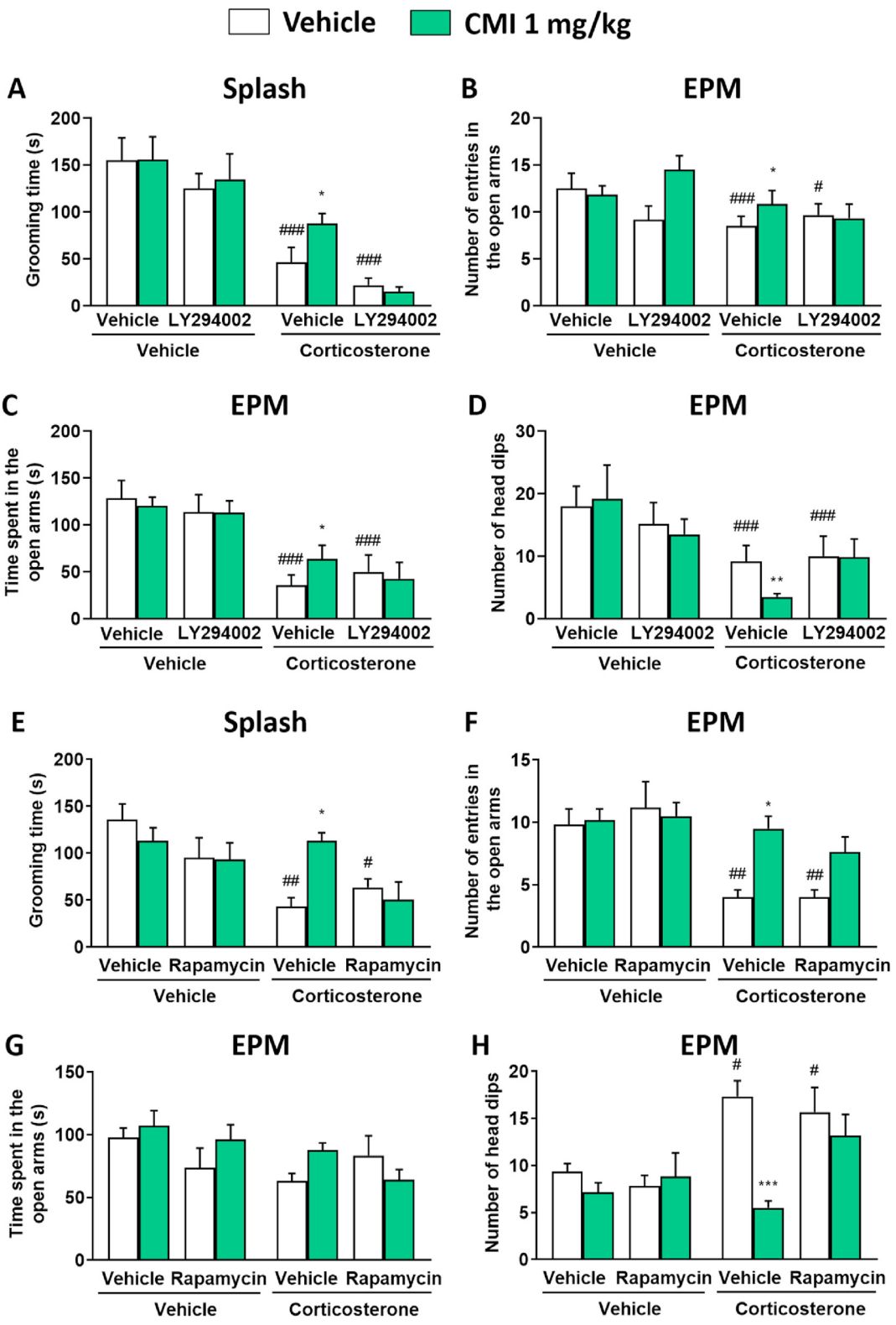

Fig. 3. Effect of acute administration of CMI (1 mg/kg, i.g.) in corticosterone-treated mice in combination with PI3K and mTOR inhibitors. Effect of CMI and LY294002 (10 nmol/site, i.c.v.) on the grooming time in the splash test (A), number of entries (B) and time spent (C) in the open arms of the EPM apparatus, and number of head dips in the EPM test (D). Effect of CMI and rapamycin (0.2 nmol/site, i.c.v.) on the grooming time in the splash test (E), number of entries (F) and time spent (G) in the open arms of the EPM apparatus, and number of head dips in the EPM test (H). Data were analyzed by a three-way ANOVA followed by Newman-Keuls post hoc test and are expressed as mean ± SEM of 6 independent animals. ($^{\#}$) $p \leq 0.05$, ($^{\#\#}$) $p < 0.01$, and ($^{\#\#\#}$) $p < 0.001$ when compared to vehicle-treated group. (*) $p \leq 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ when compared to corticosterone-treated group.

in the absence of rapamycin exerted anxiolytic-like effect ($p < 0.001$), an effect that was not observed in the presence of the inhibitor. Administration of CMI and rapamycin in non-stressed mice did not cause behavioral alterations.

3.5. Behavioral effects of CMI in the presence of HO-1 inhibitor

A single administration of CMI in corticosterone-treated animals in the presence of ZnPP, an HO-1 inhibitor, did not show the effects previously observed in the EPM test (Fig. 4). The three-way ANOVA revealed no significant corticosterone × CMI × ZnPP interaction for the number of entries in the open arms (Fig. 4A) of the EPM apparatus. However, a significant corticosterone × CMI interaction ($F_{(1,40)} = 6.06$, $p = 0.02$) and main effects for corticosterone treatment ($F_{(1,40)} = 63.06$, $p < 0.001$) and CMI administration ($F_{(1,40)} = 8.07$, $p = 0.007$) were found. The post hoc analysis showed that CMI treatment was effective only in the absence of ZnPP ($p < 0.01$). A three-way ANOVA showed a statistically significant corticosterone × CMI × ZnPP interaction for the time spent in the open arms ($F_{(1,40)} = 7.48$, $p = 0.009$) (Fig. 4B) of the EPM test. The post hoc analysis revealed that CMI administration exerted an anxiolytic-like effect only in the absence ($p < 0.01$) of ZnPP. The three-way ANOVA revealed no significant corticosterone × CMI × ZnPP interaction for the number of head dips (Fig. 4C). However, the three-way ANOVA analysis showed a significant corticosterone × CMI interaction ($F_{(1,40)} = 36.91$, $p < 0.001$) and main effects for corticosterone treatment ($F_{(1,40)} = 30.57$, $p < 0.001$) and CMI administration ($F_{(1,40)} = 15.14$, $p < 0.001$). The post hoc analysis showed that CMI treatment presented anxiolytic-like effect ($p < 0.01$) in both conditions, indicating that ZnPP is not required to modulate the number of head dips. Administration of CMI and ZnPP in non-stressed mice did not cause behavioral alterations.

The final concentration of ZnPP increased the number of grooming in the splash test (data not shown), and therefore, the results were not considered for analysis as they would not properly indicate the CMI effect.

3.6. Predicted interaction of CMI with the active site of p38 MAPK and GSK-3β

Bearing in mind the role of inflammation and activation of p38 MAPK in the GR resistance seen in the response to chronic glucocorticoid exposure, we predicted *in silico* whether CMI would interact with the active site of p38 MAPK. The optimal binding sites for CMI with p38 MAPK are depicted in Fig. 5A and B. Significant interactions were formed between CMI and the residues VAL38, ALA51, LYS53, LEU75, LEU86, LEU104, THR106, HIS107, ALA157, and LEU167. In addition, other residues, such as VAL30, TYR35, ILE84, VAL105, LEU108, MET109, and GLY110 played important roles for the interaction between CMI and p38 MAPK (binding free energy (Δ_G) of −8.4 kcal/mol).

Considering the relationship of GSK-3β with inflammation and oxidative stress, Fig. 5C and D shows the best binding position of CMI with GSK-3β (Δ_G of −7.6 kcal/mol), which involved a carbon-hydrogen bond with ASN186, a pi-donor hydrogen bond with CYS199, and non-covalent interactions with ILE62, VAL70, LYS85. In addition, the residues GLY65, LEU132, THR134, VAL135, ARG141, GLN185, LEU188, and ASP200 also contributed to the interaction of CMI with the active site of GSK-3β.

4. Discussion

The present study showed that a single administration of CMI

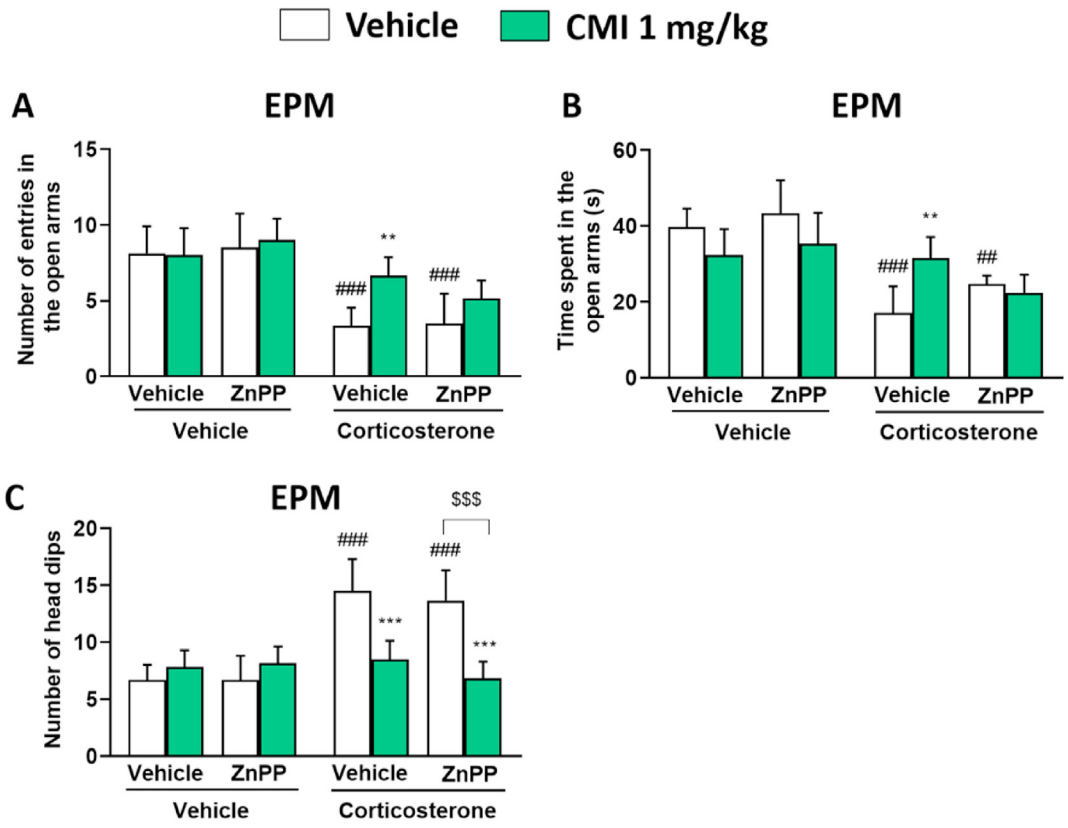

Fig. 4. Effect of acute administration of CMI (1 mg/kg, i.g.) in corticosterone-treated mice in combination with ZnPP (10 μg/mice, i.c.v.) on the grooming time in the splash test (A), number of entries (B) and time spent (C) in the open arms of the EPM apparatus, and number of head dips in the EPM test (D). Data were analyzed by a three-way ANOVA followed by Newman-Keuls post hoc test and are expressed as mean ± SEM of 6 independent animals. ($^{\#\#}$) $p < 0.01$ and ($^{\#\#\#}$) $p < 0.001$ when compared to vehicle-treated group. (**) $p < 0.01$ and (***) $p < 0.001$ when compared to corticosterone-treated group. ($^{\$\$\$}$) $p < 0.001$ when comparing corticosterone- and ZnPP-treated mice in the absence and presence of CMI.

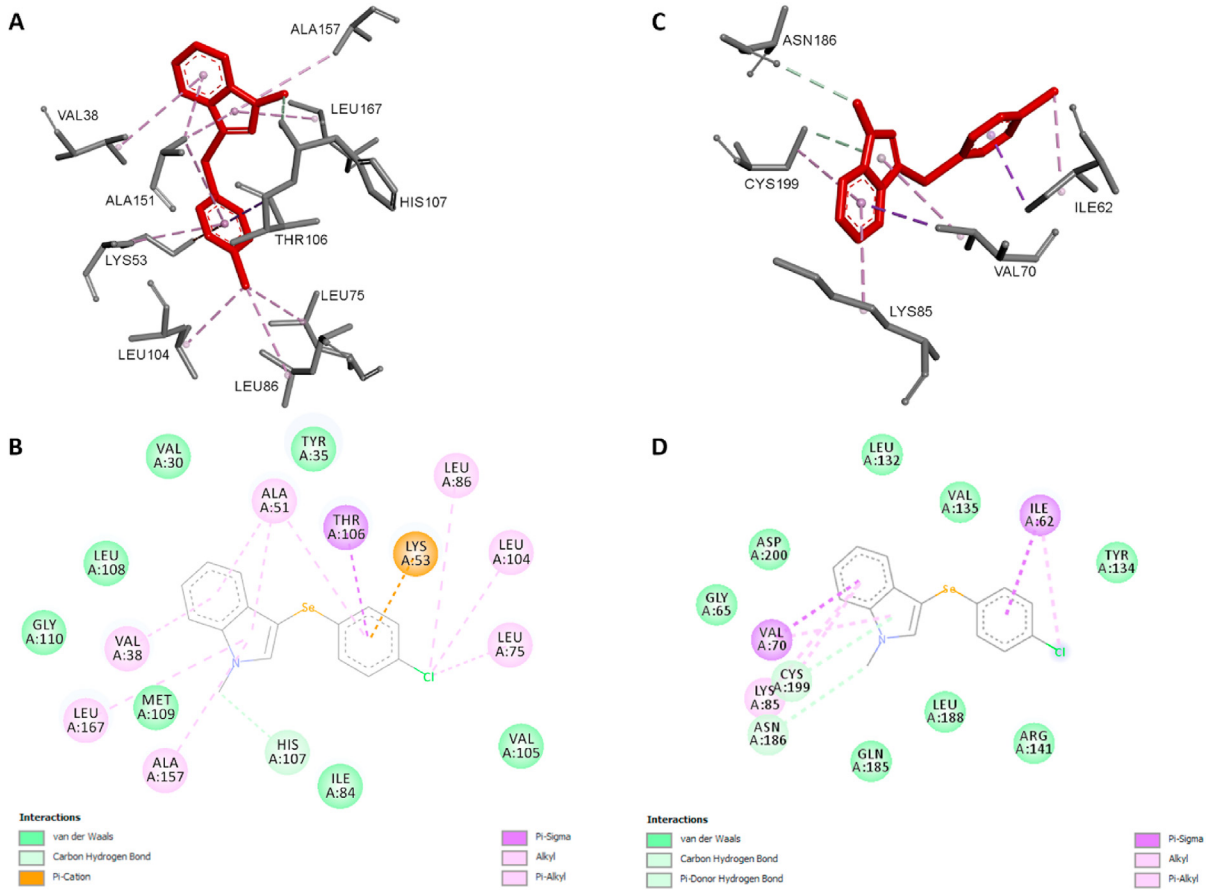

Fig. 5. Molecular docking of CMI in the binding pocket of Hs p38 MAPK (A and B) and Hs GSK-3β (C and D).

ameliorated behavioral and biochemical alterations elicited by a chronic administration of corticosterone in mice. Whilst numerous reports are investigating the antidepressant- and anxiolytic-like effects of CMI [22, 25–28], the mechanism involved in its fast-action was not explored. Here, we reported for the first time that the behavioral response of CMI involves, at least in part, the PI3K/mTOR signaling pathway since the presence of PI3K and mTOR inhibitors abolished the anti-anhedonic- and anxiolytic-like effects of CMI on stressed mice. Moreover, we demonstrated that a single administration of CMI reduced markers of neuroinflammation and oxidative stress in corticosterone-treated mice. Finally, we complemented our experimental data with an *in silico* approach, suggesting a predicted binding mode of CMI with the active site of p38 MAPK and GSK-3β.

We aimed at investigating the influence of PI3K/mTOR pathway in the anti-anhedonic- and anxiolytic-like effects of CMI because of the antidepressant-like effect of the new fast-acting drugs ketamine and esketamine is mediated by the activation of this pathway [13,40]. The assessment of the influence of oxidative stress and neuroinflammation in CMI's effect was prompted by the increasing literature showing that persistent oxidative stress and neuroinflammation are involved with the etiology of depression and anxiety [41]. Although several studies have reported the influence of these signaling pathways in the antidepressant-like effect of novel molecules [15,30], they do not take into consideration that 59% of depressed patients suffer from anxiety episodes [1,2]. On the other hand, our study strengths the intrinsic relationship of depression-anxiety comorbidity by showing that chronic corticosterone treatment-induced anhedonic- and anxiogenic-like behaviors, which were ameliorated by a single administration of CMI (Fig. 6).

Administration of corticosterone is a widely used preclinical model of depression based on the hyperactivation of the HPA axis, promoting behavioral and neurochemical alterations similar to those found in depressive patients [42]. Here, we showed that a single administration of CMI ameliorated the anhedonic- and anxiogenic-like behavior induced by repeated administration of corticosterone in the OFT, splash test, and EPM test. By the time CMI improved the behavioral alterations, it also reduced the circulating levels of endogenous corticosterone and peripheral levels of RS and lipid peroxidation. Indeed, increased levels of oxidative stress markers in the periphery have been studied as biomarkers of depression since they can be reduced following treatment with conventional antidepressants. Despite the significance of these results showing that CMI acts 30 min after its administration to ameliorate behavioral and biochemical alterations in a chronic model of stress in mice, it is possible that in the current behavioral design we are observing the acute effect of CMI. Future studies should address the long-lasting effects of CMI (*i.e.,* conduct behavioral analyses after 24 and 48 h of CMI administration) to further validate its rapid ability to abrogate depressive-, anhedonic- and anxiogenic-like behaviors.

In addition to its impacts on the periphery, chronic glucocorticoid exposure has been shown to damage hippocampal neurons and decrease the expression of *BDNF* by inducing GR resistance [11]. Activation of p38 MAPK as a consequence of immune activation, for example, can lead to GR phosphorylation and resistance [10]. More specifically, the hippocampus is highly sensitive to glucocorticoids due to the important role of this limbic structure in the integration of the stress response [43]. In the present study, the molecular docking investigation predicted that CMI might bind to the active site of p38 MAPK similarly to the crystalized inhibitor triazolopyridine [44]. Notably, mice submitted to the chronic unpredictable mild stress, a well-validated protocol of stress-induced behavioral alterations, presented reduced expression of hippocampal

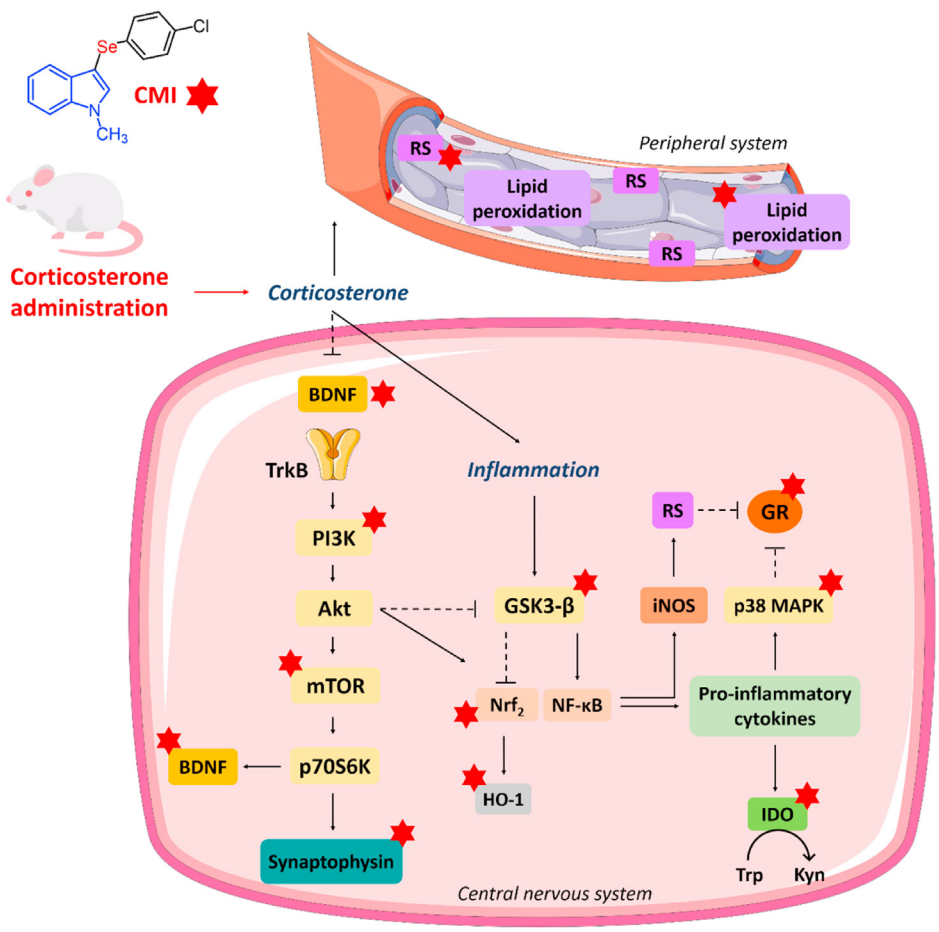

Fig. 6. Summary of the mechanisms involved with the anti-anhedonic- and anxiolytic-like effects of CMI in corticosterone-treated mice. A single administration of CMI after 14 days of corticosterone decreased the levels of endogenous corticosterone, RS, and lipid peroxidation in the plasma of mice. Meantime, CMI treatment downregulated the mRNA expression of GSK-3β and IDO and upregulated the mRNA expression of GR, BDNF, synaptophysin, and Nrf_2 in the hippocampi of corticosterone-treated mice. Furthermore, the pre-administration of PI3K, mTOR, and HO-1 inhibitors prevented the behavioral effects of CMI in corticosterone-treated mice.

GR mRNA [45]. In agreement with these data, CMI treatment counteracted the decreased *GR* expression in the HC of corticosterone-treated mice, which could contribute to the normalization of the HPA axis activity. These alterations followed by CMI treatment would help to explain its ability to increase the expression of *BDNF* in the HC of corticosterone-treated mice.

BDNF is crucial for neuronal integrity, survival, and synaptic plasticity, and its role in the pathogenesis of depression and in the mechanism of action of antidepressants has been well recognized [46,47]. BDNF-binding to TrkB activates the PI3K/mTOR signaling and increases the transcription and translation of proteins critically involved in the formation of new synapsis, such as postsynaptic density protein 95 and synaptophysin [13]. The modulation of the mTOR pathway underlies the antidepressant effects of some classical antidepressants (*e.g.* escitalopram and paroxetine) and is crucial for the fast-action of new antidepressants, such as esketamine [40]. In light of this, our study investigated whether PI3K and mTOR are required for the behavioral effects of CMI in corticosterone-treated mice. By blocking the anti-anhedonic- and anxiolytic-like effect of CMI, the PI3K and mTOR inhibitors demonstrate the relevance of this cascade to the rapid behavioral effects of CMI. Moreover, 30 min after CMI treatment, the expression of *synaptophysin* in the HC of corticosterone-treated mice was increased, suggesting the importance of synaptogenesis in the fast-acting effects of CMI. However, further studies are required to analyse the content of the proteins involved in the PI3K/mTOR pathway in response to CMI.

Following PI3K activation, Akt phosphorylates and inhibits the enzymatic activity of GSK-3β. Besides contributing to the GR resistance through its phosphorylation [16], activated GSK-3β increases the production of proinflammatory cytokines by activating NFκB [18]. Proinflammatory cytokines activate p38 MAPK and IDO, thereby impairing the serotonergic neurotransmission and contributing to the establishment of depressive-like behavior [19]. A molecular docking investigation previously predicted that CMI interacts with the active site of IDO [26], sharing the amino acid residues with the inhibitor epacadostat. The decreased expression of *GSK-3β* and *IDO* in the HC of corticosterone-treated mice could help to explain the behavioral improvement followed by CMI treatment. As revealed by the molecular docking investigation in the present study, CMI might interact with the active site of GSK-3β similarly to the inhibitor I-5 [48]. Neuroinflammation has been widely implicated in the pathophysiology of depression and anxiety, and molecules that reduce their negative impact would be advantageous in the management of psychiatric symptoms.

While the PI3K-Akt cascade promotes the translocation of Nrf_2 to the nucleus [49], activated GSK-3β blocks it [17]. Nrf_2 is a major redox regulator involved in the expression of antioxidant enzymes, such as HO-1 [49]. Indeed, the complex interplay between Nrf_2 and BDNF is under investigation, with different studies showing a bidirectional regulation [41,50]. Notably, Nrf_2 silencing in the brain increases anxiety-like behaviors in rats [51], while Nrf_2 deletion in mice resulted in depression-like behavior and reduced content of synaptophysin [50]. Here, CMI treatment increased the expression of *Nrf_2* in the HC of corticosterone-treated mice, which could be a consequence of PI3K/Akt signaling and/or reduced GSK-3β expression. Of note, the involvement of the Nrf_2 pathway was further supported by the observation that ZnPP, an HO-1 inhibitor, blocked the anxiolytic-like effect of CMI. We are aware that HO-1 activation can be induced by p38 MAPK cascade; however, the molecular docking analysis showing that CMI interacts with the active site of p38 MAPK similarly to the inhibitor triazolopyridine, suggests that activation of Nrf_2-HO-1 is a consequence of PI3K-Akt activation rather than via p38 MAPK. The antioxidant profile of CMI in

corticosterone-treated mice is in agreement with our previous data, in which a single administration of CMI was able to reduce marker of oxidative stress in the HC and prefrontal cortex of mice challenged with lipopolysaccharide [22] and submitted to the acute restraint stress [25].

Of note, the limitations of the present study should be acknowledged. Regarding the experimental design, even though we performed manual assessment of behavior, we were careful to have the same blinded observers in all experiments. Considering that depression is more prevalent in women, it would be important to address any gender differences in response to CMI effects. Moreover, despite providing behavioral evidence regarding the involvement of the PI3K/mTOR signaling pathway in the anti-anhedonic- and anxiolytic-like effects of CMI, it is imperative to investigate whether CMI modulates the content of these proteins in the HC to improve corticosterone-induced behavioral alterations.

5. Conclusions

In summary, we demonstrated herein that the anti-anhedonic- and anxiolytic-like effects of CMI in corticosterone-treated mice are partially dependent on the PI3K/mTOR signaling pathway since the presence of PI3K and mTOR inhibitors abrogated some of the behavioral effects of CMI. By decreasing peripheral levels of RS and lipid peroxidation, this study reinforces the notion that the antioxidant properties of CMI might contribute to its behavioral effects. Therefore, our results strengthen the possibility that CMI might afford rapid anti-anhedonic and anxiolytic-like effects, opening a window for a deeper investigation of CMI as a promising therapy for the management of psychiatric disorders, which are currently poorly understood and inadequately treated in several patients.

Author contribution

DAL and AMC designed the study and protocol, performed the experiments, executed analysis of data, and wrote the manuscript. MD, TAS, PTB, and MSS performed the experiments and contributed to the manuscript writing. BV and EJL synthetized CMI. FKS, TC, EJL, and LS supervised the experiments and revised the scientific content of the manuscript. All authors critically reviewed the content and approved the final version for publication.

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Declaration of competing interest

The authors declare that they have no conflict of interests.

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