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Inhibition of GSK-3β on Behavioral Changes and Oxidative Stress in an Animal Model of Mania

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Abstract

The present study evaluated the effects of AR-A014418 on behavioral and oxidative stress parameters of rats submitted to the animal model of mania induced by ouabain (OUA). Wistar rats were submitted to stereotaxic surgery and received a single *intracerebroventricular* (ICV) injection of artificial cerebrospinal fluid (aCSF), OUA, or AR-A014418. After 7 days, the animals were submitted to open-field test. After behavioral analysis, the brains were dissected in frontal cortex and hippocampus to the evaluation of oxidative stress. The OUA induced manic-like behavior in rats, which was reversed by AR-A014418 treatment. The ICV administration of OUA increases the levels of superoxide in submitochondrial particles, lipid hydroperoxide (LPH), 4-hydroxynonenal (4-HNE), 8-isoprostane, protein carbonyl, 3-nitrotyrosine, and activity of superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR) in both structures evaluated. In general, the treatment with AR-A014418 reversed these effects of OUA on the submitochondrial particles, LPH, 4-HNE, 8-isoprostane, protein carbonyl, 3-nitrotyrosine levels, and SOD activity. Furthermore, the injection of OUA decreased the catalase activity, and AR-A014418 promoted an increase in activity of this enzyme in the brain structures. These results suggest that GSK-3β inhibition can modulate manic-like behaviors. Also, it can be suggested that inhibition of GSK-3β can be effective against oxidative stress. However, more studies are needed to better elucidate these mechanisms.

Keywords Bipolar disorder · Ouabain · Na^+K^+ATPase · GSK-3β · AR-A014418 · Oxidative stress

Introduction

Bipolar disorder (BD) is a chronic and recurrent mood disorder, characterized by mood alterations, alternating between episodes of mania and depression [1–3]. Evidence suggests that sodium and potassium-activated adenosine triphosphatase (Na+K+ATPase) is involved in BD pathophysiology [4]. Besides, BD patients present increased levels of endogenous digitalis-like compounds (DLCs), which are involved in regulation of Na^+K^+ATPase activity [5, 6]. It is interesting that ouabain (OUA), a DLC, induces inhibition of Na^+K^+ATPase activity [6–9], and its intracerebroventricular (ICV) administration in rats mimics specific manic-like symptoms, such as hyperactivity, risk-taking behavior, and stereotypic behaviors. These manic-like symptoms can be reversed by standard mood stabilizers administration, such as lithium and valproate. Therefore, the ICV administration of OUA in rodents has been considered a suitable animal model of mania [10–14].

A previous study from our research group showed changes in glycogen synthase kinase 3 (GSK-3) signaling in the animal model of mania induced by OUA, suggesting an involvement of this enzyme with Na^+K^+ATPase alterations [15]. However, this interaction has not been clarified. GSK-3 is a serine/threonine kinase, constantly active within the cell and, when phosphorylated by other enzymes, such as Akt, has its activity

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inhibited [16, 17]. GSK-3 has been described to have a role in synaptic plasticity, transcription factor modulation, and other functions, besides interacting with various signaling pathways, such as MAPK, PI3K, and Wnt [18–22]. Li and Jope [23] showed that BD patients in mania present increased GSK-3β levels when compared to healthy controls, suggesting that inhibition of GSK-3β can be a therapeutic target for BD. Besides, the selective GSK-3β inhibitor, AR-A014418, can modulate behavioral and biochemical changes, which can be through the modulation of PI3K/Akt/GSK-3β signaling pathway. Previous studies have suggested that AR-A014418 may have antimanic-like and antidepressant-like effects [15, 24–26].

Another molecular system that has been demonstrated in BD patients and animal models of mania is the oxidative stress (OS) [27–34]. It is interesting that previous studies showed that GSK-3β has an essential role in neurogenesis, inflammation, and oxidative stress [35–39]. The oxidative stress occurs when the levels of reactive oxygen species (ROS) exceed the antioxidant defense mechanisms, which may lead to protein oxidation, lipids peroxidation, and cell death. Mitochondria have a pivotal role in the cell energy and ROS production; therefore, its dysfunction as well as oxidative stress parameters are widely assessed in BD studies [27–34]. Nitric oxide (NO) and peroxynitrite (ONOO-) are examples of reactive nitrogen species (RNS), which can also induce damage to biomolecules [40, 41]. Besides ROS, the RNS, such as NO and peroxynitrite (ONOO), can equally induce damage to biomolecules [42].

Glutathione peroxidase (GPx) and glutathione reductase (GR) are essential antioxidant enzymes. GPx removes H_2O_2, using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG). In turn, GR regenerates GSH from GSSG, with nicotinamide adenine dinucleotide phosphate (NADPH) as a source of reducing power [43–45]. Studies suggested that superoxide dismutase (SOD), catalase (CAT), and glutathione promotes effects on the primary antioxidant defense system in psychiatric disorders, such as BD and schizophrenia [30].

Therefore, this study aimed to evaluate the effects of AR-A014418 on behavioral and oxidative stress parameters of rats submitted to the animal model of mania induced by OUA.

Materials and Methods

Animals

Adult male Wistar rats, approximately 60 days old, from the breeding colony, maintained at the Universidade do Extremo Sul Catarinense were used. The animals were housed five per cage under controlled conditions of temperature 22 ± 1 °C, relative humidity 45–55%, and day/light cycle 12:12 h (light on at 06:00 h). Rat chow (standard diet for laboratory animals—NUVILAB CR-1®, Brazil) and tap water were available ad libitum. All experimental procedures were carried out following the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC). The local ethics committee approved this study, Comissão de Etica no Uso de Animais da Universidade do Extremo Sul Catarinense. It is important to emphasize that all efforts were made to minimize animal suffering and to reduce the number of animals used.

Surgical Procedure

Animals were intramuscularly anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg). In a stereotaxic apparatus, the skin of the rat skull was removed and a 27-gauge 9-mm guide cannula was placed at 0.9 mm posterior to bregma, 1.5 mm right from the midline, and 1.0 mm above the lateral brain ventricle. Through a 2-mm hole made at the cranial bone, a cannula was implanted 2.6 mm ventral to the superior surface of the skull and fixed with dental acrylic cement. To minimize animal suffering, rats received the intramuscular injection of tramadol 10 mg/kg, after surgery. Tramadol is used to treat moderate to moderately severe pain. Animals recovered from surgery within 3 days.

Experimental Design of Protocol

Animals [$n = 28$ (seven animals each group)] received a single ICV injection of 4 μl of OUA 10^{-3} M dissolved in artificial cerebrospinal fluid (ACSF) or 4 μL of ACSF alone on the fourth day following surgery [10, 47]. A 30-gauge cannula was placed into the guide cannula and connected by a polyethylene tube to a microsyringe. The tip of the cannula infusion protruded 1.0 mm beyond the cannula guide aiming at the right lateral brain ventricle. Five minutes after OUA or ACSF infusion, 1 μL of AR-A014418 (1.2 μM) or 1 μL ACSF into the lateral ventricle was delivered. It is important to note that the ICV administration of the drugs was made slowly, approximately for 2 min, because the cerebroventricular compartment is very sensitive to pressure changes. There were four groups: (1) ACSF + ACSF, (2) ACSF + AR-A014418, (3) OUA + ACSF, and (4) OUA + AR-A014418. Locomotor activity 7 days after the ICV administration of drugs was measured (see Scheme 2).

Note 1: OUA is an inhibitor of Na+K+ATPase; therefore, it modulates the action potential in excitable cells. If administered by peripheral via, such as intraperitoneally,

OUA can lead to cardiorespiratory arrest through alterations in action potential in the heart [46, 47]. Therefore, to evaluate the changes only in the brain's Na^+K^+ATPase, without harming other systems, the OUA was administered via ICV.

Note 2: The AR-A014418 dose used in the present study (1.2 mM) was based on a previous study by Gould and colleagues [43]. Gould and colleagues demonstrated that administration AR-A014418 at the dose of 30 mmol/kg results in approximately 1.2 mM brain concentrations, which reversed the hyperactivity induced by amphetamine in rats [24].

Open-Field Task

The effects of stimulants on behavior have been widely used as an animal model of mania because they can induce psychomotor agitation, which is commonly observed during manic episodes. Manic-like behaviors, which include hyperactivity, are easily evaluated in the open-field test [48]. The locomotor activity (crossings and rearings), risk-taking (visits to the center of the open-field), and increased stereotypic behaviors (sniffing and grooming) were assessed 7 days after ICV injection of OUA or ACSF, using the open-field task. This task was carried out in 40 × 60-cm open field surrounded by 50-cm-high walls made of brown plywood with a frontal glass wall. The floor of the open-field was divided into nine equal rectangles by black lines. The animals were gently placed to explore the arena for 5 min. The following behavioral parameters were assessed in the open-field test: crossings, the total number of square crossings during the entire test period; rearings, the total number of erect postures during the whole test period [49]; visits to center, total number of visits to the center of open-field (a center square of 30 cm × 30 cm was defined as the "center" area of the field); grooming, the total time (in s) of grooming behavior during the entire test period; and sniffing, the time (in s) of sniffing behavior during the test period. Rat sniffs the environment in moving (walking + rearing) [49]. It is important to note that a 5-min test is a short test and represents one aspect of motor activity—the initial phase of novelty exploration [50, 51].

Evaluation of Oxidative and Nitrosative Stress Parameters in the Rat Brains

Brain Samples

The rats were killed by decapitation immediately after the open-field test. The frontal cortex and hippocampus from the rat's brains were then dissected, rapidly frozen, and stored at − 70 °C until assayed. The samples, taken from the frontal cortex and hippocampus of the rats, were homogenized in KCl KH_2PO_4 (12 mM KCl, 0.038 mM KH_2PO_4, pH = 7.4).

Mitochondrial Isolation

Rat brain homogenates were centrifuged at 700×*g* for 10 min to discard nuclei and cell debris, and the pellet was washed to enrich the supernatant that was centrifuged at 7000×*g* for 10 min. The obtained pellet, washed, and resuspended in the same buffer was considered to consist mainly of intact mitochondria able to carry out oxidative phosphorylation. The operations were carried out at 0–2 °C. Submitochondrial particles (SMP) were obtained by freezing and thawing (three times) of isolated mitochondria. For superoxide production measurements, SMP was washed twice with 140 mM KCl and 20 mM Tris–HCl (pH 7.4) and suspended in the same medium [52].

Superoxide Production in Submitochondrial Particles of the Rat Brain

Superoxide production was determined in washed brain SMP using a spectrophotometric assay based on superoxide-dependent oxidation of epinephrine to adrenochrome at 37 °C (λ 480 nm = 4.0 mM^{-1} cm^{-1}). The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl (pH 7.4), SMP (0.3–1.0 mg protein/ml), 0.1 μM catalase, and 1 mM epinephrine. NADH (50 μM) and succinate (7 mM) were used as substrates, and rotenone (1 μM) and antimycin (1 μM) were added as specific inhibitors, respectively, to assay O_2^- production at the NADH dehydrogenase and at the ubiquinone–cytochrome b region. SOD was used at 0.1–0.3 μM final concentration to give assay specificity [53].

Measures of Lipid Peroxidation

Three separate measures of lipid peroxidation were analyzed: 4-hydroxynonenal (4-HNE) (Cell Biolabs, Inc., San Diego, CA, USA; STA-338) and 8-isoprostane (Cayman; Item No. 516351) following manufacturer's instructions. 4-HNE protein adducts to lysine, histidine, or cysteine were quantified by standard sandwich ELISA using an enzyme immunoassay. 8-isoprostane was quantified using ACETM Competitive EIAs with 8-isoprostane-acetylcholinesterase conjugate as a tracer and 8-isoprostane-specific rabbit antiserum. As 8-isoprostane and the tracer compete for limited antiserum binding, the color intensity caused by tracer binding was inversely proportional to the amount of 8-isoprostane. Lipid hydroperoxide (LPH) levels were measured using the assay kit by Cayman Chemical. The LPH was isolated from homogenized samples of frontal cortex and hippocampus taken

from the rats using a solution of methanol in chloroform (0 °C, 1500×*g*, 5 min). Afterward, the samples were incubated at 21 °C with 0.1 units per unit chromogen mixture of chloroform extract. The samples were then applied to plates to read their absorbance at 500 nm. Their absorbance was compared with a standard curve to determine the amount LPH peroxidation of the samples.

Protein Carbonyl Content

Oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhydrazine (DNPH), as previously described [54]. Proteins were precipitated by the addition of 20% trichloroacetic acid and were redissolved in DNPH; the absorbance was read at 370 nm.

3-Nitrotyrosine

3-Nitrotyrosine quantitation was performed using the assay kit by Cell Biolabs. After incubating the sample for a short period, the anti-3-nitrotyrosine antibody was added, followed by a secondary HRP-conjugated antibody. The quantity of 3-nitrotyrosine in the samples was determined by comparison to a standard curve.

Activity of Antioxidant Enzymes

Catalase

CAT activity was assayed using a double-beam spectrophotometer with temperature control. This method is based on the disappearance of H2O2 at 240 nm in a reaction medium containing 20 mM H2O2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and 0.1–0.3 mg protein/ml [55]. One CAT unit is defined as 1 mol of hydrogen peroxide consumed per minute, and the specific activity is reported as units per milligram of protein.

Superoxide Dismutase

This method for the assay of SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on O2–2, a substrate for SOD [56]. The inhibition of autoxidation of this compound thus occurs when SOD is present, and the enzymatic activity can be then indirectly assayed spectrophotometrically at 420 nm, using a double-beam spectrophotometer with temperature control. A calibration curve was generated using purified SOD as the standard to calculate the specific activity of SOD present in the samples. A 50% inhibition of pyrogallol autoxidation is defined as 1 unit of SOD, and the specific activity is represented as units per milligram of protein.

Glutathione Peroxidase

GPx activity was measured using the assay kit by Cayman Chemical (Paulinia, SP, Brazil). Oxidized glutathione is produced via the reduction of hydrogen peroxide by GPx and is recycled into its reduced state by GR and oxidized nicotinamide adenine dinucleotide phosphate (NADP+). A decreased absorbance of light follows the oxidation of NADPH to NADP+ at 340 nM. One unit of GPx is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to NADP+ per minute at 25 °C.

Glutathione Reductase

GR activity was measured using the assay kit by Cayman Chemical (Paulinia, SP, Brazil). Through this kit, it was possible to measure the rate of oxidation of NADPH into NADP+ , which is followed by a decrease in absorbance at 340 nM. One unit of GR is determined by the amount of enzyme that caused the oxidation of 1.0 nmol of NADPH to NADP+ per minute at 25 °C.

Statistical Analysis

Results are presented as the means ± standard error (SE). The variables were analyzed according to their distribution through Shapiro Wilk's test for normality. The Levene test assessed the homogeneity of variances among groups. Differences among experimental groups were determined by two-way ANOVA followed by Tukey's post hoc test. A value of $p \leq 0.05$ was considered significant. Correlations were analyzed using the Pearson's correlation test. Pearson's correlation coefficient was used to analyze the strength of the relationship between continuous variables.

Results

The effects of AR-A014418 treatment on the manic-like behavior elicited by ICV OUA administration in rats are demonstrated in Fig. 1. Administration of OUA increased locomotor (crossings) and exploratory activity (rearings) (Fig. 1a), risk-taking behavior (visits to the center of open-field) (Fig. 1b), and stereotypy-like behavior (grooming and sniffing) (Fig. 1c), as compared to the control group. AR-A014418 reversed the behavioral alterations induced by OUA. AR-A014418 per se did not alter spontaneous locomotion and exploratory activity of rats, indicating that the effects of the GSK-3β inhibitor on OUA-treated rats were not associated with sedation.

Data from the two-way ANOVA revealed significant effects of ICV OUA administration [Crossing: $F(1.20) = 53.11$, $p < 0.001$; Rearing: $F(1.20) = 9.02$, $p = 0.007$; Risk-

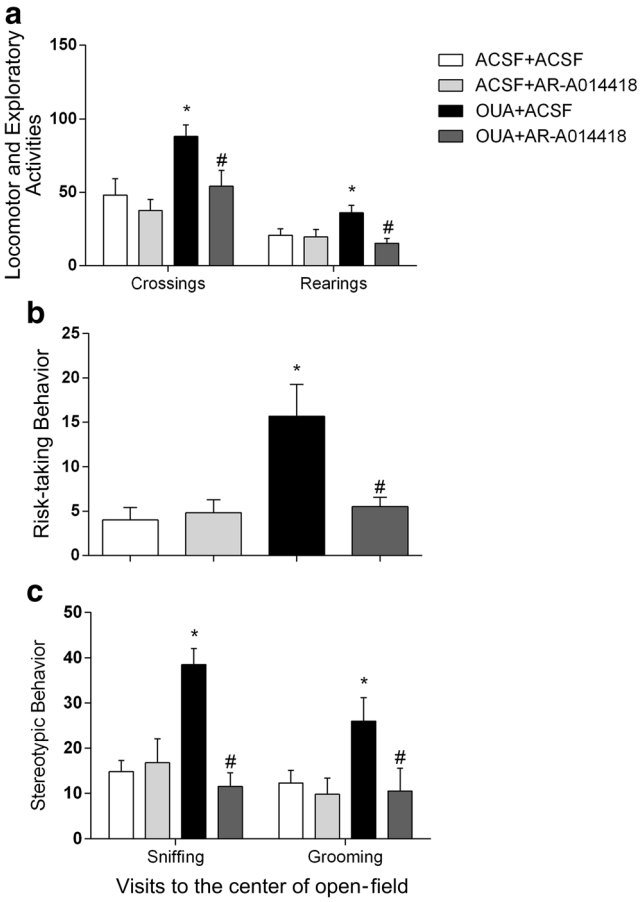


Fig. 1 Effects of administration of AR-A0144818 on the number of crossings and rearings (**a**), visits to center of open-field (**b**), and sniffing and grooming (**c**) in animals submitted to OUA-induced animal model ($n = 10$ per group). Data were analyzed by two-way analysis of variances followed by the Tukey test when F was significant. Values are expressed as mean ± SE. *$p \leq 0.05$ compared to ACSF group. #$p \leq 0.05$ compared to OUA group

taking: $F(1.20) = 49.78$, $p < 0.001$; Grooming: $F(1.20) = 16.85$, $p < 0.001$; Sniffing: $F(1.20) = 36.18$, $p < 0.001$] and treatment [Crossing: $F(1.20) = 32.94$, $p < 0.001$; Rearing: $F(1.20) = 35.56$, $p < 0.001$; Risk-taking: $F(1.20) = 28.50$, $p < 0.001$; Grooming: $F(1.20) = 26.58$, $p < 0.001$; Sniffing: $F(1.20) = 67.28$, $p < 0.001$] and a significant OUA administration × treatment interaction [Crossing: $F(1.20) = 8.92$, $p = 0.007$; Rearing: $F(1.20) = 27.52$, $p < 0.001$; Risk-taking: $F(1.20) = 39.60$, $p < 0.001$; Grooming: $F(1.20) = 13.86$, $p = 0.001$; Sniffing: $F(1.20) = 90.53$, $p < 0.001$].

Figure 2 shows the effects of OUA and AR-A014418 treatment on the levels of superoxide in submitochondrial particles in the frontal cortex and hippocampus of rats. OUA increased the levels of superoxide levels in both structures evaluated. Besides, AR-A014418 reversed OUA-induced increased in the levels of superoxide in submitochondrial particles both in the frontal cortex and hippocampus.

Data from the two-way ANOVA revealed significant effects of ICV OUA administration [frontal cortex: $F(1.20) = 30.90$, $p < 0.001$; hippocampus: $F(1.20) = 33.82$, $p < 0.001$] and treatment [frontal cortex: $F(1.20) = 23.28$, $p < 0.001$; hippocampus: $F(1.20) = 20.33$, $p < 0.001$] and a significant OUA administration × treatment interaction [frontal cortex: $F(1.20) = 24.86$, $p < 0.001$; hippocampus: $F(1.20) = 14.94$, $p < 0.001$].

In the Fig. 3, the effects of OUA on the oxidative damage parameters to lipid, by assessing the amount of LPH (Fig. 3a), 4-HNE (Fig. 3b), and 8-isoprostane (Fig. 3c), can be observed. The OUA administration increased LPH, 4-HNE, and 8-isoprostane levels in both structures evaluated. The treatment with AR-A014418 reversed the increased LPH levels in both brain structures assessed. The treatment with AR-A014418 reversed the 4-HNE increased induced by OUA in the hippocampus but partially reversed this oxidative marker in the frontal cortex. The AR-A014418 also reversed the OUA-induced 8-isoprostane increased in the frontal cortex but not in the hippocampus.

Data from the two-way ANOVA revealed effects of ICV OUA administration [LPH: frontal cortex: $F(1.20) = 17.03$, $p < 0.001$; hippocampus: $F(1.20) = 11.73$, $p = 0.002$; 4-HNE: frontal cortex: $F(1.20) = 63.40$, $p < 0.001$; hippocampus: $F(1.20) = 35.62$, $p < 0.001$; 8-isoprostane: frontal cortex: $F(1.20) = 92.33$, $p < 0.001$; hippocampus: $F(1.20) = 34.85$, $p < 0.001$] and treatment [LPH: frontal cortex: $F(1.20) = 16.51$, $p < 0.001$; hippocampus: $F(1.20) = 7.95$, $p = 0.01$; 4-HNE: frontal cortex: $F(1.20) = 10.84$, $p = 0.003$; hippocampus: $F(1.20) = 18.26$, $p < 0.001$; 8-isoprostane: frontal cortex: $F(1.20) = 84.96$, $p < 0.001$; hippocampus: $F(1.20) = 15.40$, $p < 0.001$] and a OUA administration × treatment interaction [LPH: frontal cortex: $F(1.20) = 19.58$, $p < 0.001$; hippocampus: $F(1.20) = 6.37$, $p = 0.02$; 4-HNE: frontal cortex: $F(1.20) = 37.97$, $p < 0.001$; hippocampus: $F(1.20) = 22.20$, $p < 0.001$; 8-isoprostane: frontal cortex: $F(1.20) = 85.39$, $p < 0.001$; hippocampus: $F(1.20) = 8.59$, $p = 0.008$].

Figure 4 demonstrates a significant increase in carbonyl (Fig. 4a) and 3-nitrotyrosine levels (Fig. 4b) after administration of OUA in the frontal cortex and hippocampus of rats. The treatment with AR-A014418 reversed these alterations caused by OUA.

Data from the two-way ANOVA revealed a significant effect of ICV OUA administration [Carbonyl: frontal cortex: $F(1.20) = 25.79$, $p < 0.001$; hippocampus: $F(1.20) = 20.51$, $p < 0.001$; 3-nitrotyrosine: frontal cortex: $F(1.20) = 27.56$, $p < 0.001$; hippocampus: $F(1.20) = 10.68$, $p = 0.003$] and treatment [Carbonyl: frontal cortex: $F(1.20) = 13.37$, $p = 0.001$; hippocampus: $F(1.20) = 8.36$, $p = 0.009$; 3-nitrotyrosine: frontal cortex: $F(1.20) = 13.51$, $p = 0.001$; hippocampus: $F(1.20) = 3.84$, $p = 0.06$] and a OUA administration × treatment interaction [Carbonyl: frontal cortex: $F(1.20) = 5.99$, $p = 0.02$; hippocampus: $F(1.20) = 3,52$, $p = 0.07$; 3-nitrotyrosine: frontal cortex: $F(1.20) = 8.89$, $p = 0.007$; hippocampus: $F(1.20) = 7.18$, $p = 0.014$].

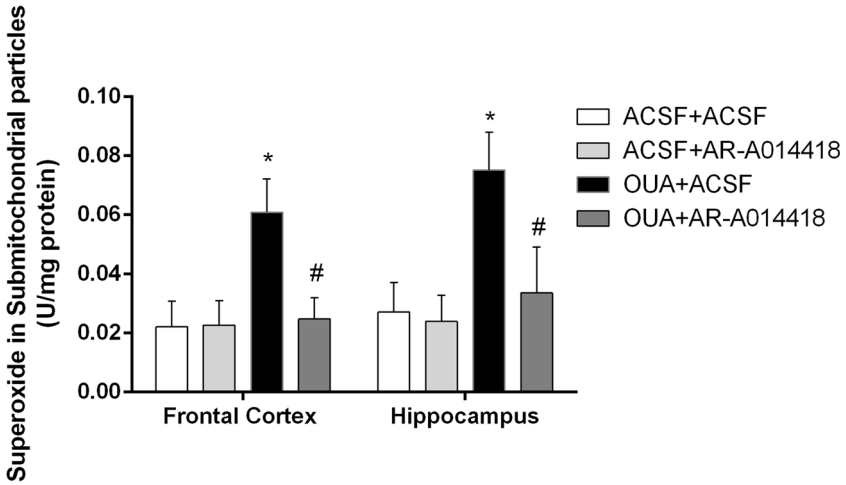


Fig. 2 Effects of administration of AR-A0144818 on the production of superoxide in submitochondrial particles in the frontal cortex and hippocampus of rats submitted to OUA-induced animal model ($n = 6$ per group). Data were analyzed by two-way analysis of variances followed by the Tukey test when F was significant. Values are expressed as mean ± SE. *$p \leq 0.05$ compared to ACSF group. #$p \leq 0.05$ compared to OUA group

The activities of enzymes CAT and SOD are shown in Fig. 5. The ICV administration of OUA induced a significant decrease in CAT activity in frontal cortex and hippocampus of rats (Fig. 5a). The treatment with AR-A014418 reversed these

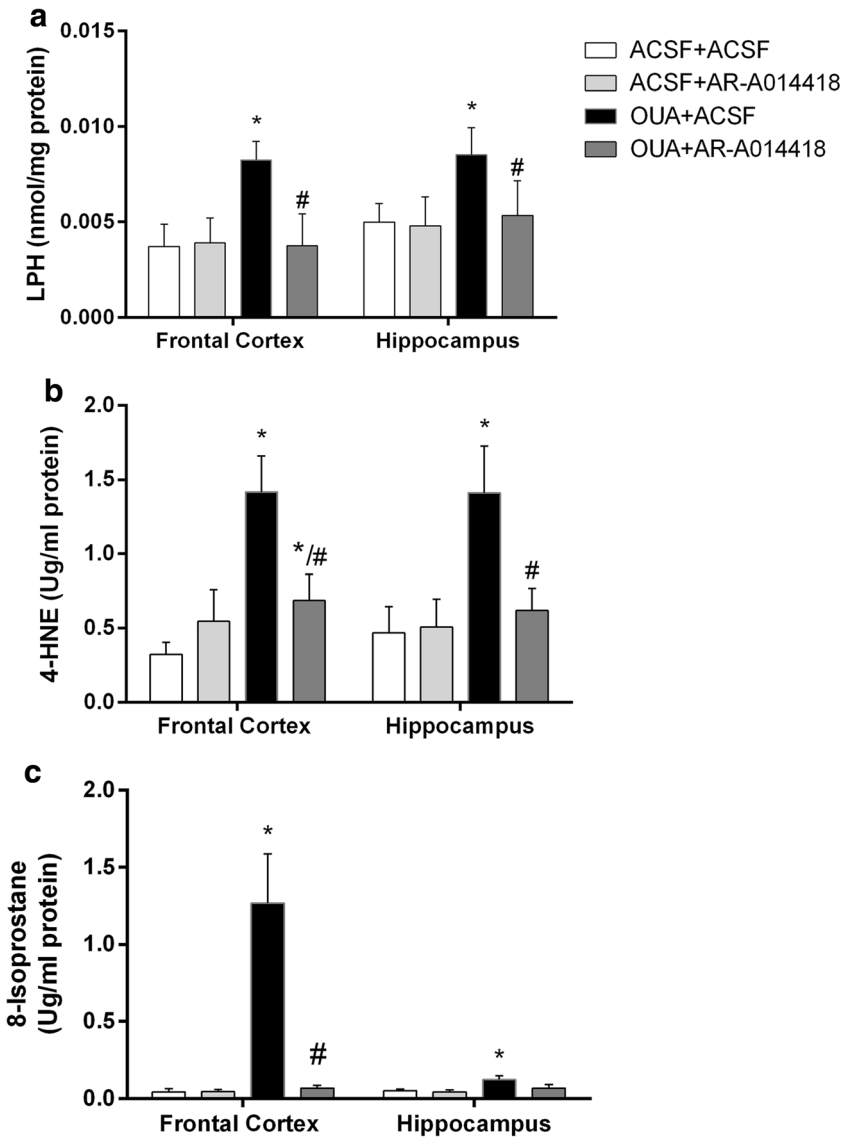


Fig. 3 Effects of administration of AR-A0144818 on the levels of LPH (**a**), 4-HNE (**b**), and 8-isoprostane (**c**) in the frontal cortex and hippocampus of rats submitted to OUA-induced animal model ($n = 6$ per group). Data were analyzed by two-way analysis of variances followed by the Tukey test when F was significant. Values are expressed as mean ± SE. *$p \leq 0.05$ compared to ACSF group. #$p \leq 0.05$ compared to OUA group

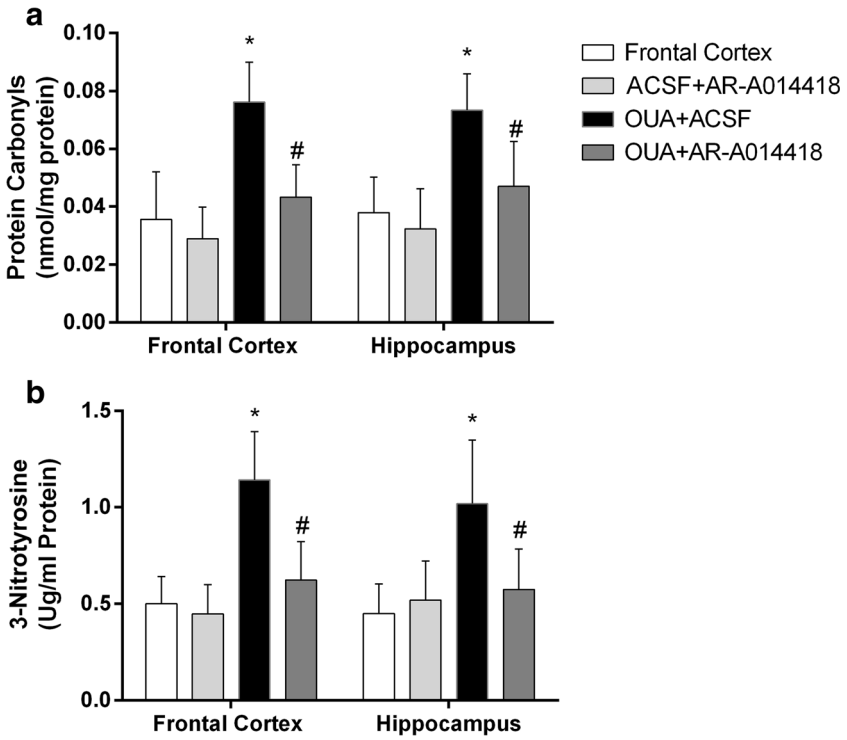


Fig. 4 Effects of administration of AR-A0144818 on the levels of protein carbonyl (**a**) and 3-nitrotyrosine (**b**) in the frontal cortex and hippocampus of rats submitted to OUA-induced animal model ($n = 6$ per group). Data were analyzed by two-way analysis of variances followed by the Tukey test when F was significant. Values are expressed as mean ± SE. *$p \leq 0.05$ compared to ACSF group. #$p \leq 0.05$ compared to OUA group

alterations induced by OUA in both structures evaluated. The activity of SOD was increased after ICV administration of OUA in frontal cortex and hippocampus of rats (Fig. 5b). The administration of AR-A014418 partially reversed the OUA-induced SOD rising in the hippocampus but not in the frontal cortex.

Data from the two-way ANOVA revealed a significant effect of ICV OUA administration [CAT: frontal cortex: $F(1.20) = 13.94$, $p = 0{,}001$; hippocampus: $F(1.20) = 10.30$, $p = 0.004$; SOD: frontal cortex: $F(1.20) = 39.67$, $p < 0.001$; hippocampus: $F(1.20) = 85.92$, $p < 0.001$] and treatment [CAT: frontal cortex: $F(1.20) = 17.51$, $p < 0.001$; hippocampus:

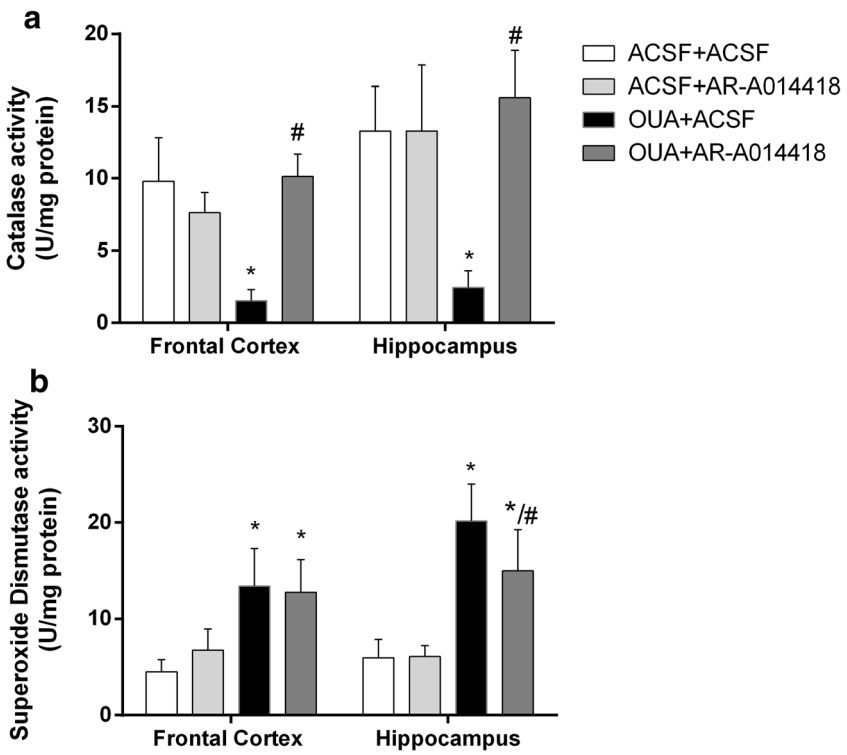


Fig. 5 Effects of administration of AR-A0144818 on the activity of catalase (**a**) and superoxide dismutase (**b**) in the frontal cortex and hippocampus of rats submitted to OUA-induced animal model ($n = 6$ per group). Data were analyzed by two-way analysis of variances followed by the Tukey test when F was significant. Values are expressed as mean ± SE. *$p \leq 0.05$ compared to ACSF group. #$p \leq 0.05$ compared to OUA group

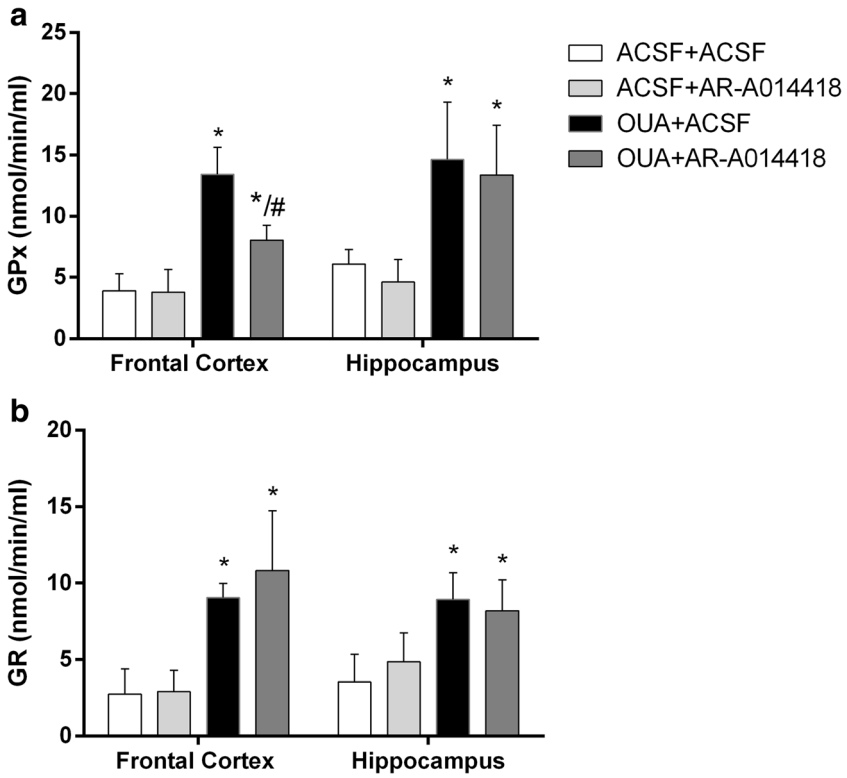


Fig. 6 Effects of administration of AR-A0144818 on the activity of glutathione peroxidase (**a**) and glutathione reductase (**b**) in the frontal cortex and hippocampus of rats submitted to OUA-induced animal model ($n = 6$ per group). Data were analyzed by two-way analysis of variances followed by the Tukey test when F was significant. Values are expressed as mean ± SE. *$p \leq 0.05$ compared to ACSF group. #$p \leq 0.05$ compared to OUA group

$F(1.20) = 24.51$, $p < 0.001$; SOD: frontal cortex: $F(1.20) = 0.48$, $p = 0.49$; hippocampus: $F(1.20) = 4.10$, $p = 0.06$] and a OUA administration × treatment interaction [CAT: frontal cortex: $F(1.20) = 49.10$, $p < 0.001$; hippocampus: $F(1.20) = 24.52$, $p < 0.001$; SOD: frontal cortex: $F(1.20) = 1.48$, $p = 0.23$; hippocampus: $F(1.20) = 4.47$, $p = 0.047$].

Figure 6 shows the effects of OUA on the activity of GPx (Fig. 6a) and GR (Fig. 6b). The ICV injection of OUA increased the activity of GPx and GR in frontal cortex and hippocampus of rats when compared to control group. The administration of AR-A014418 decreased this alteration induced by OUA on the GPx activity in the frontal cortex but not in the hippocampus.

Data from the two-way ANOVA revealed a significant effect of ICV OUA administration [GPx: frontal cortex: $F(1.20) = 96.52$, $p < 0.001$; hippocampus: $F(1.20) = 41.42$, $p = 0.001$; GR: frontal cortex: $F(1.20) = 57.95$, $p < 0.001$; hippocampus: $F(1.20) = 32.85$, $p < 0.001$] and treatment [GPx: frontal cortex: $F(1.20) = 15.57$, $p < 0.001$; hippocampus: $F(1.20) = 1.05$, $p = 0.3$; GR: frontal cortex: $F(1.20) = 1.09$, $p = 0.3$; hippocampus: $F(1.20) = 0.13$, $p = 0.7$] and a OUA administration × treatment interaction [GPx: frontal cortex: $F(1.20) = 14.16$, $p = 0.001$; hippocampus: $F(1.20) = 0.00$, $p = 0.93$; GR: frontal cortex: $F(1.20) = 0.74$, $p = 0.39$; hippocampus: $F(1.20) = 1.78$, $p = 0.19$].

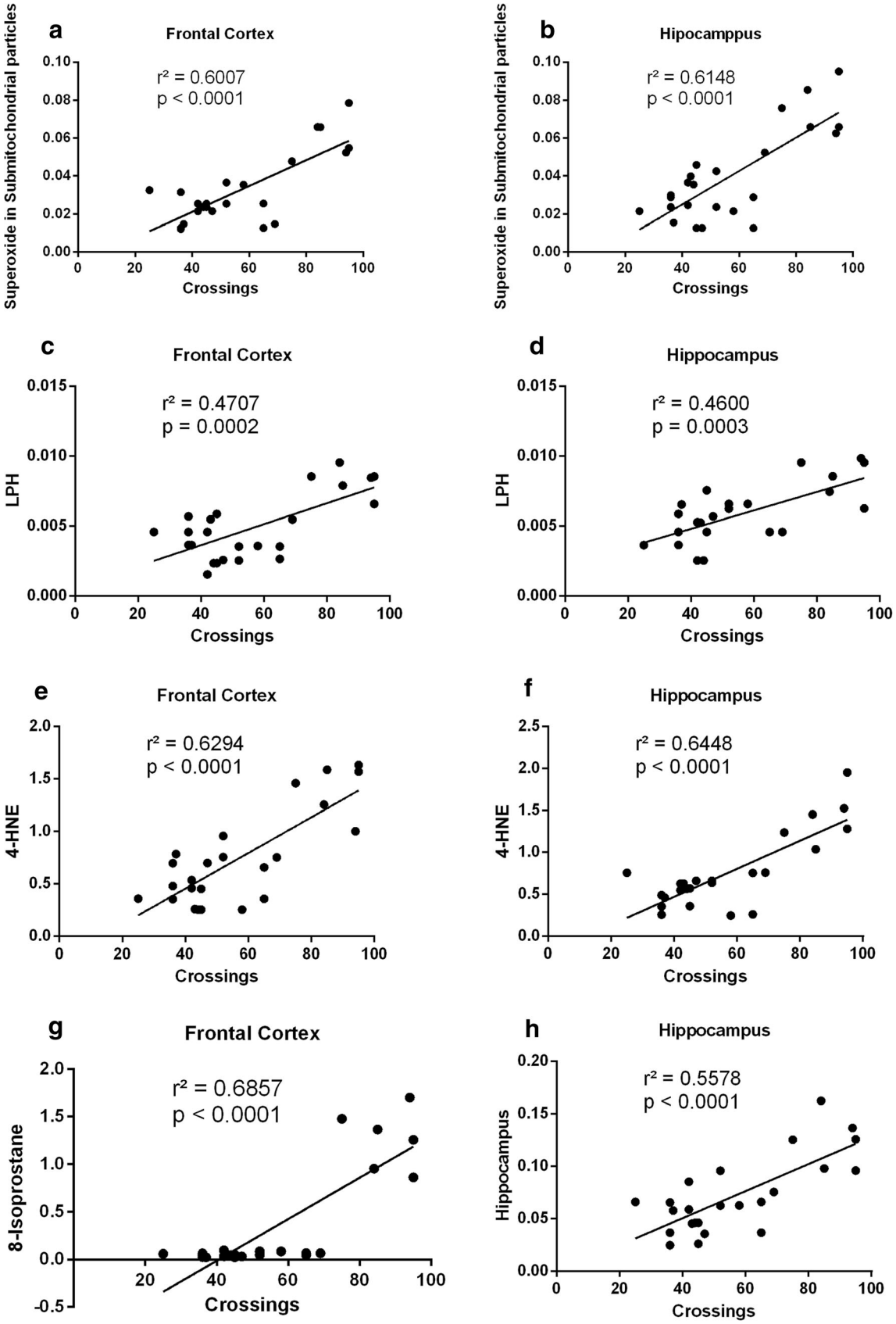
Figure 7 shows the correlation between locomotor activity and production of superoxide in submitochondrial particles (Fig. 7a, b), LPH (Fig. 7c, d), HNE (Fig. 7e, f), or 8-isoprostane (Fig. 7g, h) levels. Frontal cortex and hippocampus of rats were analyzed. Locomotor activity was positively correlated with all oxidative damage parameters and in both structures evaluated.

Data from Pearson's correlation of crossings versus production of superoxide in submitochondrial particles were as follows: [frontal cortex ($n = 24$; $r^2 = 0.6007$; $p < 0.0001$), hippocampus ($n = 24$; $r^2 = 0.6148$; $p < 0.0001$)], LPH [frontal cortex ($n = 24$; $r^2 = 0.4707$; $p = 0.0002$), hippocampus ($n = 24$; $r^2 = 0.4600$; $p = 0.0003$)], 4-HNE [frontal cortex ($n = 24$; $r^2 = 0.6294$; $p < 0.0001$), hippocampus ($n = 24$; $r^2 = 0.6448$; $p < 0.0001$)], and 8-isoprostane [frontal cortex ($n = 24$; $r^2 = 0.6857$; $p < 0.0001$), hippocampus ($n = 24$; $r^2 = 0.5578$; $p < 0.0001$)].

Figure 8 shows the correlation between locomotor activity and protein damage, measured through carbonyl (Fig. 8a, b) or 3-nitrotyrosine (Fig. 8c, d) levels in the frontal cortex and hippocampus of rats. The oxidative and nitrosative protein damage was positively correlated with locomotor activity.

Data from Pearson correlation of crossings versus carbonyl were as follows: [frontal cortex ($n = 24$; $r^2 = 0.6195$; $p < 0.0001$), hippocampus ($n = 24$; $r^2 = 0.6280$; $p < 0.0001$)], 3-nitrotyrosine [frontal cortex ($n = 24$; $r^2 = 0.4307$; $p = 0.0005$), hippocampus ($n = 24$; $r^2 = 0.3132$; $p = 0.0045$)].

Fig. 7 Correlations between locomotor activity (number of crossings) ▶ and production of superoxide in submitochondrial particles (**a** and **b**). Correlations between locomotor activity (number of crossings) and LPH levels (**c** and **d**). Correlations between locomotor activity (number of crossings) and 4-HNE levels (**e** and **f**). Correlations between locomotor activity (number of crossings) and 8-isoprostane levels (**g** and **h**) in frontal cortex and hippocampus of animals submitted to OUA-induced animal model. Results were assessed using the Pearson's correlation test


a
Frontal Cortex
r² = 0.6007
p < 0.0001
Superoxide in Submitochondrial particles
Crossings
b
Hipocamppus
r² = 0.6148
p < 0.0001
Superoxide in Submitochondrial particles
Crossings
c
Frontal Cortex
r² = 0.4707
p = 0.0002
LPH
Crossings
d
Hippocampus
r² = 0.4600
p = 0.0003
LPH
Crossings
e
Frontal Cortex
r² = 0.6294
p < 0.0001
4-HNE
Crossings
f
Hippocampus
r² = 0.6448
p < 0.0001
4-HNE
Crossings
g
Frontal Cortex
r² = 0.6857
p < 0.0001
8-Isoprostane
Crossings
h
Hippocampus
r² = 0.5578
p < 0.0001
Hippocampus
Crossings

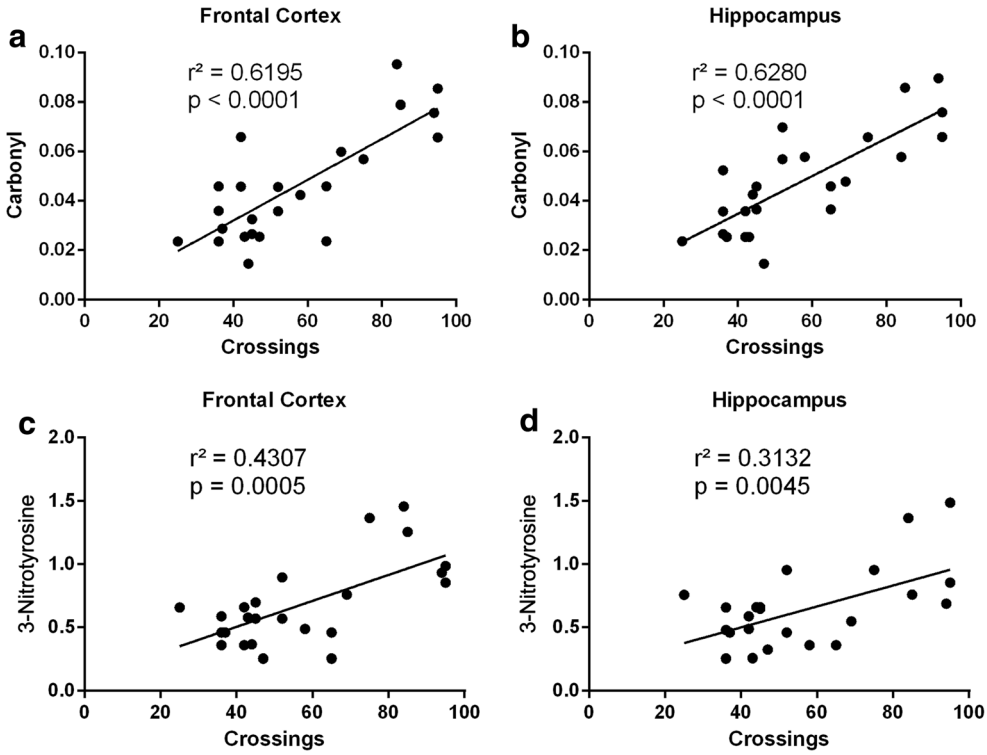


Fig. 8 Correlations between locomotor activity (number of crossings) and levels of carbonyl (**a** and **b**). Correlations between locomotor activity (number of crossings) and levels of 3-nitrotyrosine (**c** and **d**) in frontal cortex and hippocampus of animals submitted to OUA-induced animal model. Results were assessed using the Pearson's correlation test

The correlation between the activities of antioxidant enzymes and locomotor activity is demonstrated in Fig. 9. It is possible to observe that CAT activity was negatively correlated with locomotor activity in frontal cortex (Fig. 9a) and hippocampus (Fig. 9b) of rats. However, the activities of SOD (Fig. 9c, d), GPx (Fig. 9e, f), and GR (Fig. 9g, h) were positively correlated with locomotor activity in both brain structures evaluated.

Data from Pearson's correlation of crossings versus CAT activity were as follows: [frontal cortex ($n = 24$; $r^2 = 0.4335$; $p = 0.0005$), hippocampus ($n = 24$; $r^2 = 0.6214$; $p < 0.0001$)], SOD [frontal cortex ($n = 24$; $r^2 = 0.3518$; $p = 0.0023$), hippocampus ($n = 24$; $r^2 = 0.5320$; $p < 0.0001$)], GPx [frontal cortex ($n = 24$; $r^2 = 0.6233$; $p < 0.0001$), hippocampus ($n = 24$; $r^2 = 0.4268$; $p = 0.0005$)], GR [frontal cortex ($n = 24$; $r^2 = 0.1929$; $p = 0.0318$), hippocampus ($n = 24$; $r^2 = 0.3473$; $p < 0.0024$)].

Figure 10 shows the correlation between production of superoxide in submitochondrial particles and lipid damage, evaluated through LPH (Fig. 10a, b). Besides, Fig. 10 shows the correlation between production of superoxide and protein damage, assessed through carbonyl (Fig. 8c, d) levels in the frontal cortex and hippocampus of rats. The protein damage was positively correlated with production of superoxide.

Data from Pearson's correlation of LPH versus superoxide in submitochondrial particles production were as follows: [frontal cortex ($n = 24$; $r^2 = 0.6095$; $p < 0.0001$), hippocampus ($n = 24$; $r^2 = 0.3778$; $p = 0.0014$)], carbonyl [frontal cortex ($n = 24$; $r^2 = 0.6098$; $p < 0.0001$), hippocampus ($n = 24$; $r^2 = 0.4024$; $p = 0.0002$)].

Discussion

Studies have demonstrated the importance of Na^+K^+ATPase in BD pathophysiology [57–59]. The OUA, a Na^+K^+ATPase inhibitor, induces manic-like behavior in rats, and it is considered a good animal of mania. In the present study, the manic-like behavior in rats was observed by the increase in the number of crossing and rearing (locomotor and exploratory activity), risk-taking (center visits), and stereotypic (grooming and sniffing) behaviors. Furthermore, the administration of AR-A014418, a GSK-3β inhibitor, reversed these behavioral alterations induced by OUA. Similar results were observed in another study of our research group, which demonstrated that AR-A014418 prevented OUA-induced locomotor and exploratory increase in Wistar rats. However, the present study is the first to evidence the effects of AR-A014418 on the risk-taking and stereotypic behavior in an animal model of mania induced by OUA. In the previous study, it was suggested that the manic-like effects of OUA are associated with the activation of GSK-3β and that Li and VPA exert protective effects against OUA by inhibition of the GSK-3β pathway [42]. Besides, several studies have reported the involvement of GSK-3β in manic-like behaviors induced by psychostimulants, such as amphetamine [60–62]. The previous study showed that the increased of DA neurotransmission results in inactivation of Akt and concomitant activation of GSK-3α and GSK-3β. Furthermore, pharmacological or genetic inhibition of GSK-3 significantly reduces DA-dependent locomotor behaviors [62]. Sui and colleagues [63] demonstrated by electrochemical experiments that ICV

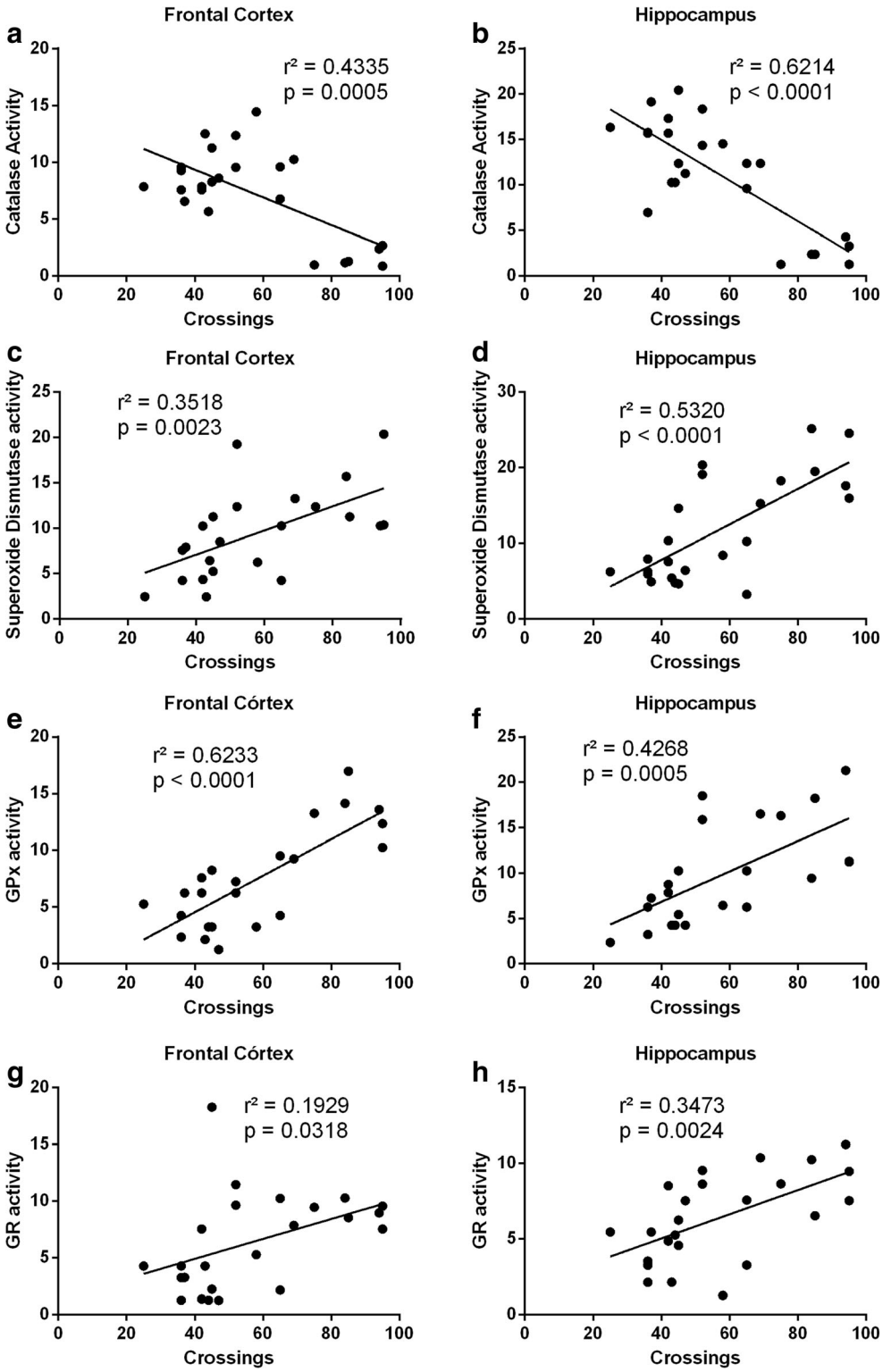


Fig. 9 Correlations between locomotor activity (number of crossings) and activity of antioxidant enzymes in frontal cortex and hippocampus of rats submitted to OUA-induced animal model: catalase (**a** and **b**), superoxide dismutase (**c** and **d**), glutathione peroxidase (**e** and **f**), and glutathione reductase (**g** and **h**). Results were assessed using the Pearson's correlation test

administration of OUA enhanced dopamine release in the brain of rats. Therefore, the inhibition of GSK-3β, and consequently modulation of the dopaminergic system can be the mechanism by which AR-A014418 is acting on the manic-like behavior in this study. It is important to emphasize that lithium and valproate, which are a gold standard for BD treatment, inhibit GSK-3β [64–68]. Together these studies suggest that GSK-3β is an important target for BD treatment.

In the present study, the administration of OUA increases superoxide levels in submitochondrial particles in frontal cortex and hippocampus of rats. According to our results, other authors also showed that OUA increases superoxide in

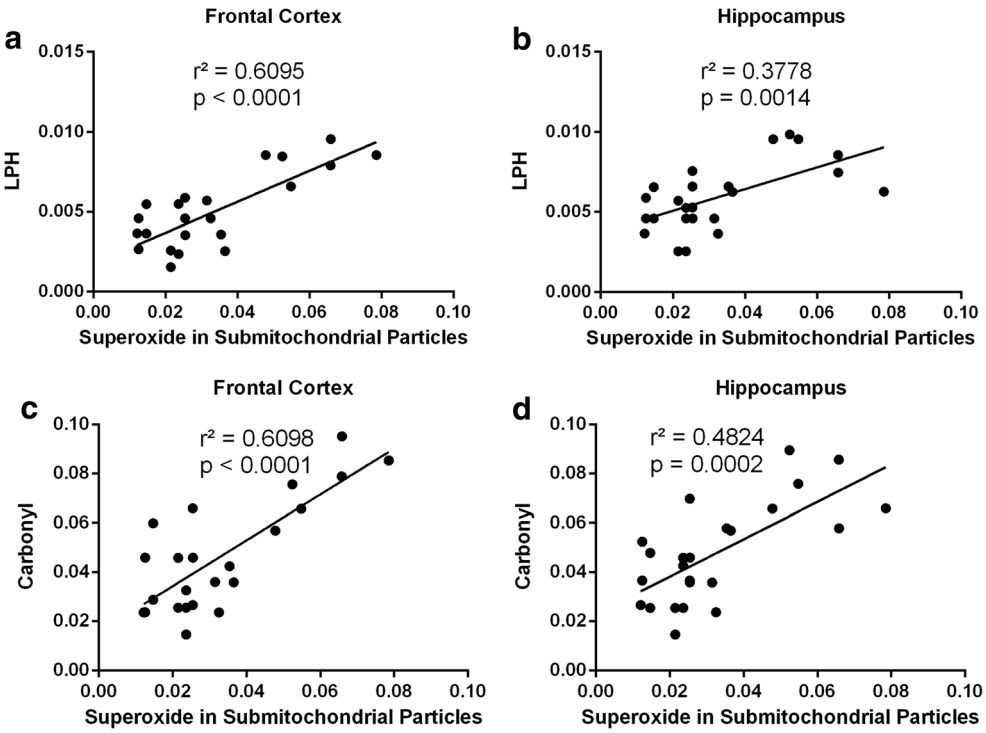


Fig. 10 Correlations between production of superoxide in submitochondrial particles and levels of LPH (**a** and **b**) and protein carbonyl (**c** and **d**) in frontal cortex and hippocampus of animals submitted to OUA-induced animal model. Results were assessed using the Pearson's correlation test

submitochondrial particles [13, 48, 69]. The increase in submitochondrial superoxide induced by OUA can explain, at least in part, the oxidative damage induced in the lipid and protein observed in the present study. Herein, there was a positive correlation between oxidative damage to protein or lipid and superoxide production in both brain structures evaluated. According to these results, previous studies demonstrated that OUA increases the levels of thiobarbituric acid reactive species (TBARS) and carbonyl protein, markers of lipid and protein oxidation [13, 70]. Together, these previous results and the present data suggest that the OUA-induced production of superoxide and damage to mitochondrial membrane affect the entire cell, which can lead to tissue damage.

Besides, we found that the treatment with GSK-3β inhibitor, AR-A014418, decreased the submitochondrial superoxide increase induced by OUA. Therefore, it can be suggested that there is a relationship between Na+K+ATPase alterations and mitochondrial damage, both observed in BD patients. In addition to being found in the cytosol, the GSK-3β is also present in the cell nucleus and mitochondria [71–73]. Yan and colleagues [74] demonstrated that GSK-3β-dependent dynamin-related protein 1 (Drp1) phosphorylation at Ser40 and Ser44 increases Drp1 GTPase activity, inducing mitochondrial fragmentation. In the same study, Yan and colleagues [74] demonstrated that the inhibition of GSK-3β by AR-A014418 decreases the mitochondrial fragmentation in the brain of rats. It is well described in the literature that mitochondria alterations have an essential role in the oxidative stress process [75, 76]. Therefore, it can be suggested that AR-A014418 can be protecting the mitochondria and, consequently, decreasing the levels of submitochondrial superoxide induced by OUA.

The treatment with AR-A014418 decreases LPH and HNE levels in both brain structures evaluated and 8-isoprostane only in the frontal cortex. It is well described in the literature that lithium and valproic acid, which are GSK-3β inhibitors, also have antioxidant properties [77, 78]. Previous studies have demonstrated that the treatment with lithium decreases the levels of 8-isoprostane and HNE in the brain of animals submitted to the animal model of mania induced by sleep deprivation [77]. It is important to note that lithium and valproate have other mechanisms of action; however, it can be suggested that the antioxidant effects of these drugs could be, at least in part, through inhibition of GSK-3β. An in vitro study demonstrated that GSK-3β inhibition decreases oxidative stress in the L02 liver cells submitted to H_2O_2-induced oxidative damage, leading to increased cell survival [79]. In the present study, the inhibition of GSK-3β, besides providing antimanic effects, also protects the brain against oxidative damage to lipids. It was emphasized by the Pearson correlation, which demonstrated a positive correlation between levels of superoxide, LPH, HNE, 8-isoprostane, carbonyl, or 3-nitrotyrosine and manic-like behavior.

Herein, it can be observed that OUA induces important alterations in the antioxidant enzymes. OUA decreased the activity of CAT and increased the levels of SOD, GPx, and GR in the frontal cortex and hippocampus of rats. In the present study, there was an association between enzyme alterations and manic-like behaviors. It can be observed that there

is a negative correlation between CAT activity and crossings; however, there was a positive correlation between SOD, GPx, or GR and locomotor activity. According to our results, previous studies demonstrated that OUA decreased the activity of CAT and increased the activity of SOD in the hippocampus and frontal cortex of rats submitted to this animal model of mania [13, 80, 81]. In contrast to our data, Souza and colleagues demonstrated that GPx activity was decreased after ICV administration of OUA. However, it is important to note that Souza and colleagues evaluated the effects of OUA immediately after the ICV injection, and the present study evaluated the effects of OUA 7 days after a single ICV injection. Brüning and colleagues [81] also find opposite results to ours, demonstrating no alterations in GPX and GR activity after OUA administration. Brocardo and colleagues [82] find that GPx and GR activities decrease after ICV OUA administration. In the same way, these opposite results can be explained by methodological differences.

It is known that GPx and GR are among the major antioxidant enzymes, which are directly involved in the neutralization of ROS. GPx removes H_2O_2, using it to oxidize reduced GSH into GSSG [43–45]. Regarding the antioxidant enzymes SOD and CAT, SOD has its action scavenge the O_2^- by catalyzing its dismutation to H_2O_2 and CAT metabolizes the excess of H_2O_2, producing $O_2 + H_2O$, decreasing the intracellular redox status [83]. In the present study, the increase of SOD activity induced by OUA can be explained by the increased mitochondrial superoxide levels and, consequently, increased GPx activity. It is important to highlight that OUA increased the GR activity, which regenerates GSH from GSSG, with NADPH as a source of reducing power. However, we demonstrated that OUA induces decreased CAT activity in the hippocampus and frontal cortex of rats. Although OUA increases the levels of SOD, GPx, and GR, these enzymes were not able to reverse the lipid and protein oxidative damage induced by OUA. It is interesting to note that AR-A014418 prevented decreasing of CAT activity induced by OUA but, in general, not alters the increased of SOD, GPx, and GR induced by OUA. Therefore, it can be suggested that the antioxidant effects of AR-A014418 against OUA may be associated with the modulation of CAT activity in the hippocampus and frontal cortex of rats. It seems that all evaluated enzymes work together to reverse the lipid and protein damage induced by OUA.

Conclusion

OUA induces manic-like behaviors in rats, oxidative damage to lipid and protein, and change the activity of antioxidant enzymes. The administration of AR-A014418 besides providing antimanic effects also protects the brain against oxidative stress. These results suggest that GSK-3β inhibition can modulate the manic-like behaviors and protect the brain of the animals submitted to the animal model of mania induced by OUA. Therefore, it can be suggested that GSK-3β can be an important target for bipolar mania.

Limitations of study: (1) Although OUA administration in rats is considered as a suitable animal model of mania, ICV administration needs a surgical procedure, which is an invasive method, involving anesthesia, surgery, and recovery, which can be stressful for the animals. Therefore, these procedures can have some influence on the results. (2) Besides, a single ICV injection of ouabain induces manic-like behavior for 7 days in the animals, mimicking an acute manic episode. Therefore, this model has little relevance to the chronic disorder. (3) There is any study showing if ouabain has binding affinity to the AR-A014418. The possibility that the co-administration of the compounds inhibits the effect of each other cannot be excluded. (3) Another critical point to be emphasized is that the ICV administration route of AR-A014418 for patients is not viable since this method is invasive. However, it was a valuable experiment to evaluate the mechanisms of action of this substance.

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Compliance with Ethical Standards

Conflict Interest This research has no conflict of interest.

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