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Ketamine Exhibits Different Neuroanatomical Profile After Mammalian Target of Rapamycin Inhibition in the Prefrontal Cortex: the Role of Inflammation and Oxidative Stress

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Abstract Studies indicated that mammalian target of rapamycin (mTOR), oxidative stress, and inflammation are involved in the pathophysiology of major depressive disorder (MDD). Ketamine, an *N*-methyl-D-aspartate (NMDA) receptor antagonist, has been identified as a novel MDD therapy; however, the antidepressant mechanism is not fully understood. In addition, the effects of ketamine after mTOR inhibition have not been fully investigated. In the present study, we examined the behavioral and biochemical effects of ketamine in the prefrontal cortex (PFC), hippocampus, amygdala, and nucleus accumbens after inhibition of mTOR signaling in the PFC. Male adult Wistar rats received pharmacological mTOR inhibitor, rapamycin (0.2 nmol) or vehicle into the PFC and then a single dose of ketamine (15 mg/kg, i.p.). Immobility was assessed in forced swimming tests, and then oxidative stress parameters and inflammatory markers were evaluated in the brain and periphery. mTOR activation in the PFC was essential to ketamine's antidepressant-like effects. Ketamine increased lipid damage in the PFC, hippocampus, and amygdala. Protein carbonyl was elevated in the PFC, amygdala, and NAc after ketamine administration. Ketamine also increased nitrite/nitrate in the PFC, hippocampus, amygdala, and NAc. Myeloperoxidase activity increased in the hippocampus and NAc after ketamine administration. The activities of superoxide dismutase and catalase were reduced after ketamine administration in all brain areas studied. Inhibition of mTOR signaling pathways by rapamycin in the PFC was required to protect against oxidative stress by reducing damage and increasing antioxidant enzymes. Finally, the TNF-α level was increased in serum by ketamine; however, the rapamycin plus treatment group was not able to block this increase. Activation of mTOR in the PFC is involved in the antidepressant-like effects of ketamine; however, the inhibition of this pathway was able to protect certain brain areas against oxidative stress, without affecting inflammation parameters.

Keywords mTOR · Oxidative stress · Inflammation · Ketamine · Animal model · Major depressive disorder

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Introduction

Major depressive disorder (MDD) is a significant public health threat, accounting for 65.5 million disability-adjusted

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life years and ranking third among the leading causes of global disease burden [1]. The standard of care for the last 50 years has focused on monoamine neurotransmitters, including selective serotonin reuptake inhibitors (SSRIs) and serotonin–norepinephrine reuptake inhibitors (SNRIs) as treatments [2]. Although there are medications that alleviate depressive symptoms, these have serious limitations [3]. Most notably, available treatments require weeks or months to produce a therapeutic response, and only about one third of patients respond to the first medication prescribed [4]. Novel treatments for MDD have the potential to ameliorate both individual suffering and public health cost burden [5]. Recent research has highlighted the glutamatergic system, as a potential novel target for antidepressant drugs, particularly for those patients suffering from treatment-resistant depression (TRD) and who are less likely to benefit from additional monoamine treatments.

Ketamine is a noncompetitive, high affinity N-methyl-D-aspartate (NMDA) antagonist that prevents excess calcium influx and cellular damage [6]. Several preclinical studies found that ketamine showed antidepressant-like behaviors in various animal models of depression [7–11]. In addition, in patients with MDD, ketamine demonstrated a rapid (within 1 day), robust (across a variety of symptoms), and relatively sustained (approximately 7 days) antidepressant efficacy at sub-anesthetic intravenous doses (only 0.5 mg/kg over 40 min, compared with the 2-mg to 3-mg/kg dosage used in anesthesia over much shorter time periods) [12, 13].

Experimental and human studies are now exploring the cellular and molecular mechanisms associated to ketamine's antidepressant response, specially the mammalian target of rapamycin (mTOR) pathway. mTOR is a multi-effector serine/threonine protein kinase involved in translation control and long-lasting synaptic plasticity [14]. The dysregulation of mTOR signaling cascade has been hypothesized to be a common pathophysiological feature of neuropsychiatric disorders [15]. In fact, clinical findings from postmortem studies demonstrated that MDD individuals had reduced levels of mTOR protein and p70S6K, a target of mTOR, in the prefrontal cortex (PFC), when compared to healthy controls [16].

Studies using animal models of depression suggest a role of the PFC in the pathophysiology of depression and antidepressant responses [17]. The adaptive organization of goal-directed behavioral responses is supported by the PFC [18, 19] and its appropriate connectivity with other brain structures, as the hippocampus and the amygdala [20–23]. It is argued that neuropsychiatric disorders involve alterations to the behavioral control system supported by the PFC and its connectivity with other brain systems [24–26]. In support of this idea, it has been shown that patients suffering from these disorders display alterations of PFC-dependent cognitive functions like cognitive flexibility [27], working memory [28, 29], and fear extinction [30]. In the same line of evidence,

functional alterations of the PFC have been observed in patients suffering from neuropsychiatric disorders [31–35]. Also, these patients display altered functional connectivity in the PFC-hippocampus and the PFC-amygdala pathway [36–38]. These evidences suggest that such disorders are related to the maladaptive accommodation of behavioral responses supported by the PFC and its connection with other structures. Moreover, the PFC may be an important region for investigating both the underlying pathophysiology of MDD and the antidepressant mechanism actions [17]. However, there are no studies in the literature that show the effects caused by the inhibition of mTOR pathway and treatment with ketamine in the PFC in other brain structures related to depression, such as hippocampus, amygdala, and nucleus accumbens.

In addition, there are several reports showing that MDD can increase reactive oxygen species (ROS) generation in several brain areas involved in the regulation of mood [39] and antidepressant treatment generally reverses these effects [40–42]. In fact, a previous study demonstrated that ketamine at dose of 15 mg/kg reversed the oxidative damage and, still, increased the activities of antioxidant enzymes superoxide dismutase and catalase in the brain of deprived rats [10]. In contrast, a previous study showed that ketamine at dose of 25 mg/kg was able to increase thiobarbituric acid reactive substances (TBARS) and carbonyl protein in some brain areas, such as prefrontal cortex (PFC), hippocampus, amygdala, and striatum [43]. In this way, the results of the effects of ketamine on the oxidative parameters in studies are still not well elucidated.

Unlike other widely prescribed antidepressants such as imipramine or fluoxetine, activation of the mTOR pathway and its associated increase in the synaptic spine density within the PFC is one unique mechanism underlying ketamine's antidepressant-like effects [44]. mTOR pathway dysregulation is associated with MDD, but the inflammation and oxidative damage has also been suggested to play a role in the pathophysiology of this disorder as well [45–48]. In addition, the effects of ketamine after mTOR inhibition in inflammation and oxidative damage have not been investigated. Thus, the aim of this study was to evaluate the effects of the administration of ketamine on the forced swimming test, inflammatory markers in periphery, and oxidative stress parameters in the PFC, hippocampus, amygdala, and nucleus accumbens, after the inhibition of mTOR signaling in the PFC.

Material and Methods

Animals

Male adult (60 days old) Wistar rats, weighing between 250 and 300 g, were housed five to a cage with food and water

available ad libitum and were maintained on a 12-h light/dark cycle (lights on at 7:00 a.m.). In vivo studies were performed in accordance with the National Institutes of Health guidelines and also with the approval of the ethics committee from Universidade do Extremo Sul Catarinense (UNESC) under protocol 031-2014-01.

Experimental Design and Treatment

First, all rats ($n = 39$ total) underwent a surgical procedure to receive a single dose of a pharmacological inhibitor of mTOR (rapamycin) (from Sigma-Aldrich, Brazil) at a dose of 0.2 nmol/site, according with Li et al. [3], in the PFC or vehicle (control group that received saline), in the PFC. Rapamycin was dissolved in 100 % DMSO and administered in the PFC, in a volume of 3 μl per rat. Ketamine was obtained from For Dodge (Brazil), and the dose was based on previous study [49]. The animals were then divided into four experimental groups: (1) vehicle + saline; (2) rapamycin + saline; (3) vehicle + ketamine 15 mg/kg; and (4) rapamycin + ketamine 15 mg/kg. The intraperitoneal administration of ketamine (from Fort Dodge, Brazil) was performed at 30 min after the injection of rapamycin inhibitor, and 30 min after the administration of ketamine, the animals were submitted to the forced swimming test [3].

Surgical Procedure

The animals were anesthetized with chloral hydrate (from Vetec, Brazil) 40 mg/kg and xylazine (from Bayer, Brazil) 30 mg/kg intraperitoneally. Using a stereotaxic surgical procedure, the skin and scalp of the rat in the skull region were removed. A 1-mm length cannula was placed through the brain tissue at the following coordinates: 4.2 mm behind the bregma and 3.0 mm to the right of the bregma, with the cannula being inserted 3.4 mm deep into the PFC [50]. The fixation of the cannula tube was made using acrylic cement. The PFC injection of rapamycin (3 μl) or vehicle occurred 72 h after surgery.

Forced Swimming Test (FST)

The FST was conducted according to previous reports [51–53]. The test involves two individual exposures to a cylindrical tank filled with water, in which the rats cannot touch the bottom of the tank or escape. The tank is made of transparent Plexiglas, 80 cm tall, 30 cm in diameter, and filled with water (22–23 °C) to a depth of 40 cm. For the first exposure, rats without drug treatment were placed in the water for 15 min (pretest session). Twenty-four hours later, rats were once again placed in the water for a 5-min session (test session) and the immobility time, a passive behavior characterized by the absence of movements except for those necessary for the animal's snout to remain above the water level, swimming time, horizontal movements across the water surface and climbing time, and vertical movements against walls. The time of immobility, swimming and climbing were recorded in seconds. Rats were treated with ketamine or saline only 30 min before the second exposure to the cylindrical tank of water (test session). During the test session, immobility time was recorded.

Cytokine Analysis

Immediately after the forced swimming test, under anesthesia, blood was collected for subsequent analyses of cytokine levels ($n = 4$–5). The serum was diluted in extraction solution containing PBS. The concentration of cytokines (IL-1β, IL-10, and TNF-α) was determined by ELISA kit (from Sigma-Aldrich, Brazil). All samples were assayed in duplicate. Briefly, the capture antibody (13 ml, contains 0.1 % sodium azide) was diluted in phosphate-buffered saline (PBS), added to each well, and left overnight at 4 °C. The plate was washed four times with PBS and 0.05 % Tween 20 % (Sigma, St. Louis, MO, USA). The plate was blocked with 1 % bovine serum albumin and incubated for 1 h at room temperature before washing four times with PBS and 0.05 % Tween 20 %. The samples and standards were added, and the plate was incubated overnight at 4 °C. After washing the plate, a detection antibody (concentration provided by the manufacturer) diluted in PBS was added. The plate was incubated for 2 h at room temperature. After washing the plate, streptavidin (DuoSet R&D Systems, Minneapolis, MN, USA) was added and the plate was incubated for 30 min. Finally, a color reagent, phenylenediamine (Sigma, St. Louis, MO, USA), was added to each well and the reaction was allowed to develop in the dark for 15 min. The reaction was stopped with the addition of 1 M H2SO4 to each well. The absorbance was read on a plate reader at 492 nm wavelengths (Emax, Molecular Devices, Minneapolis, MN, USA). The samples were placed in the plate for the ELISA analysis. For IL-1B, the overall intra- and inter-assay coefficient of variation was <10 %. For TNF-α and IL-6, the overall intra-assay coefficient of variation was <5 % and the overall inter-assay coefficient of variation was <10 %.

Brain Samples and Biochemical Analysis

After the behavioral tests were complete and blood collected, the animals were killed by decapitation, the skulls were removed, then the whole brain was removed and placed in a petri plate that was on ice, and then the PFC, hippocampus, nucleus accumbens, and amygdala were quickly isolated by hand dissection using a magnifying glass, a spatula, and a thin brush by a qualified researcher. In addition, the dissection was based on the histological distinctions described by Paxinos

and Watson [50]. After the removal of the structures, they were placed in Eppendorf tube and stored in a freezer at 70 °C for posterior biochemical analysis. The brain tissues were used for cytokine analysis and oxidative stress parameters. For biochemical analysis, we used a total of $n = 4$–5 per group for cytokine analysis (one hemisphere for each), and TBARS, protein carbonyls, nitrite/nitrate concentration, myeloperoxidase activity, superoxide dismutase activity, and catalase activity $n = 4$–5 per group (one hemisphere for each).

Tissue and Homogenate Preparation

The PFC, hippocampus, amygdala, and nucleus accumbens were homogenized (1:10, w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 IU/ml heparin). The homogenates were centrifuged at $800 \times g$ for 10 min at 4 °C and supernatants kept at −70 °C until they were used for enzyme activity determination. The maximum period between homogenate preparation and enzyme analysis was always less than 5 days. Protein content was determined by the method described by Lowry et al. [54], using bovine serum albumin as a standard.

Oxidative Stress Parameters

Myeloperoxidase (MPO) Activity

Neutrophil infiltrate in tissues was measured by MPO activity [55]. Brain tissues were homogenized (50 mg/ml) in 0.5 % hexadecyltrimethylammonium bromide and centrifuged at $15,000 \times g$ for 40 min. The suspension was then sonicated three times for 30 s. An aliquot of supernatant was mixed with a solution of 1.6 mM tetramethylbenzidine and 1 mM H_2O_2. Activity was measured spectrophotometrically as the change in absorbance at 650 nm at 37 °C. Data were expressed as milliunits per milligram of protein.

Thiobarbituric Acid Reactive Species Formation (TBARS)

Lipid peroxidation was measured by formation of TBARS [56]. After brain dissection, brain structures were washed with PBS, harvested, and lysed. Thiobarbituric reactive species, obtained by acid hydrolysis of 1,1,3,3-tetra-ethoxy-propane (TEP), was used as the standard for the quantification of TBARS. TBA 0.67 % was added to each tube and the tubes were vortexed. The reaction mixture was incubated at 90 °C for 20 min, and the reaction was stopped by placing the samples on ice. The optical density of each solution was measured in a spectrophotometer at 535 nm. Data were expressed as nanomole of malondialdehyde (MDA) equivalents per milligram of protein.

Carbonyl Protein Formation

Protein carbonyl content was measured in brain homogenates using 2,4-dinitrophenylhydrazine (DNPH) in a spectrophotometric assay [57]. Briefly, sample tissues were sonicated in ice-cold homogenization buffer containing phosphatase and protease inhibitors (200 nM calyculin, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM sodium orthovanadate, and 1 μM microcystin-LR) and centrifuged at $1000 \times g$ for 15 min to sediment insoluble material. Three hundred microliter aliquots of the supernatant containing 0.7–1.5 mg of protein were treated with 300 μl of 10 mM DNPH, dissolved in 2 M HCl, and compared with 2 M HCl alone (reagent blank). Samples then were incubated for 1 h at room temperature in the dark and stirred every 10 min. Samples were precipitated with trichloroacetic acid (final concentration of 20 %) and centrifuged at $16,000 \times g$ at 4 °C for 15 min. The pellet was washed three times with 1 ml of ethanol/ethyl acetate (1:1 v/v). Each time, the pellet was lightly vortexed and left exposed to the washing solution for 10 min before centrifugation ($16,000 \times g$ for 5 min). The final pellet was dissolved in 1 ml of 6 M guanidine and 10 mM phosphate buffer–trifluoroacetic acid, pH 2.3, and the insoluble material was removed by centrifugation at $16,000 \times g$ for 5 min. Absorbance was recorded in a spectrophotometer at 370 nm for both DNPH-treated and HCl-treated samples. Protein carbonyl levels were expressed as nanomole of carbonyl per milligram of protein.

Measurement of Nitrite/Nitrate Concentration

Total nitrite concentrations were measured using the Griess reaction, by adding 100 μl of Griess reagent 0.1 % (w/v) naphthyl ethyl endi amide dihydrochloride in H_2O and 1 % (w/v) sulfanilamide in 5 % (v/v) concentrated H_3PO_4, vol. [1:1] to the 100-μl sample. Absorbance was recorded in a spectrophotometer at 550 nm [58]. Data were expressed as nanomole of nitrite/nitrate concentration per milligram of protein.

Superoxide Dismutase (SOD) Activity

SOD estimation was performed based on its ability to spontaneously inhibit oxidation of adrenaline to adrenochrome [59]. A combination of 2.78 ml of sodium carbonate buffer (0.05 mM; pH 10.2), 100 μl of EDTA (1.0 mM), and 20 μl of the supernatant or sucrose (blank) was incubated at 30 °C for 45 min. Thereafter, the reaction was initiated by adding 100 μl of adrenaline solution (9.0 mM). The change in the absorbance was recorded at 480 nm for 8 min. Temperature was maintained at 30 °C throughout the assay procedure. One unit of SOD produced approximately 50 % of auto-oxidation of adrenaline. Results were expressed as units per milligram of protein.

Catalase (CAT) Activity

The CAT activity was measured by the method that employs hydrogen peroxide (H_2O_2) to generate H_2O and O_2 [60]. Brain tissue was sonicated in 50 mmol/l phosphate buffer (pH 7.0), and the resulting suspension was centrifuged at $3000 \times g$ for 10 min. The sample aliquot (20 μl) was added to 980 μl of the substrate mixture. The substrate mixture contained 0.3 ml of hydrogen peroxide in 50 ml of 0.05 M phosphate buffer (pH 7.0). Initial and final absorbencies were recorded at 240 nm after 1 and 6 min, respectively. A standard curve was established using purified catalase (Sigma, MO, USA) under identical conditions.

Statistical Analysis

All data are presented as mean ± SEM. Differences among experimental groups in the assessment of behavior, oxidative stress parameters, and energy metabolism were determined by one-way ANOVA, followed by Tukey's post hoc test when ANOVA was significant; p values <0.05 were considered to be statistical significant.

Results

Effects of Ketamine After the Inhibition of mTOR Pathway in the PFC with Rapamycin in Rats Submitted to the Forced Swimming Test

Figure 1 illustrates the effects of ketamine after the inhibition of mTOR in the PFC with rapamycin in the forced swimming test. Ketamine at a dose of 15 mg/kg reduced the immobility time, compared to the vehicle group treated with saline, and the administration of rapamycin inhibitor blocked the ketamine effects ($F_{(3-40)} = 4.419$; $p = 0.009$; Fig. 1), in the forced swimming test. We did not observe any alterations for either ketamine or rapamycin groups on climbing ($F_{(3-40)} = 3.070$;
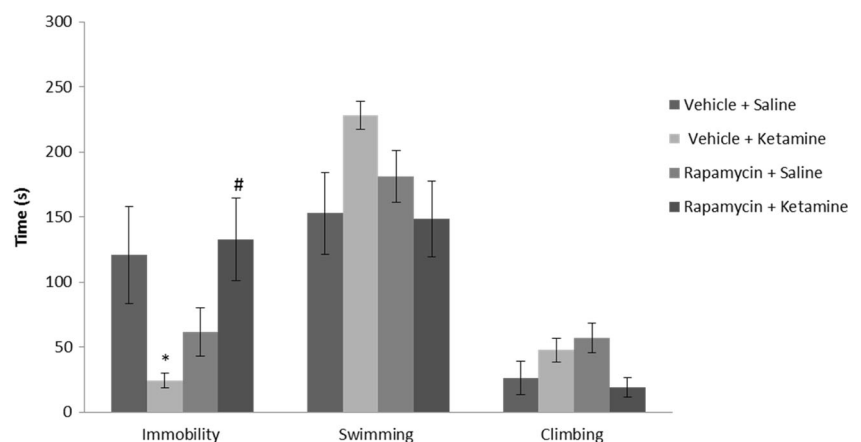
$p = 0.05$; Fig. 1) and swimming time ($F_{(3-40)} = 2.708$; $p = 0.059$; Fig. 1).

Effects of Ketamine After the Inhibition of mTOR Pathway in the PFC with Rapamycin on TBARS, Carbonyl Protein Formation, Nitrite/Nitrate Concentration, and Myeloperoxidase Activity in Brain Structures

Figure 2 shows the effect of ketamine, after the inhibition of mTOR in the PFC with rapamycin, on TBARS, carbonyl protein formation, nitrite/nitrate concentration, and myeloperoxidase activity in the PFC, hippocampus, amygdala, and nucleus accumbens. The TBARS levels were increased in the PFC with ketamine treatment when compared with the vehicle group treated with saline ($F_{(3-19)} = 5.891$; $p = 0.007$; Fig. 2a). In the hippocampus, the TBARS levels were increased in the ketamine treatment group when compared with the vehicle group treated with saline and was diminished in the rapamycin groups when compared to with ketamine treatment group, demonstrating that rapamycin was able to abolished the lipid peroxidation produced by ketamine ($F_{(3-19)} = 21.444$; $p < 0.001$; Fig. 2a). In the amygdala, the TBARS levels were increased in the ketamine treatment group and in the rapamycin plus ketamine treatment group when compared with the vehicle group treated with saline, however, were diminished in the rapamycin group when compared to with ketamine treatment group ($F_{(3-17)} = 7.111$; $p = 0.004$; Fig. 2a). Finally, in the nucleus accumbens, the rapamycin group and the rapamycin plus ketamine treatment group decreased the TBARS levels, when compared with the vehicle group treated with saline ($F_{(3-19)} = 6.459$; $p < 0.05$; Fig. 2a).

Figure 2b demonstrates that in the PFC ($F_{(3-19)} = 7.655$; $p = 0.002$; Fig. 2b) and in the nucleus accumbens ($F_{(3-19)} = 17.449$; $p < 0.001$; Fig. 2b), there was an increase on carbonyl protein formation after the infusion of ketamine treatment when compared with the vehicle group treated with saline. There was a decrease in the rapamycin groups when
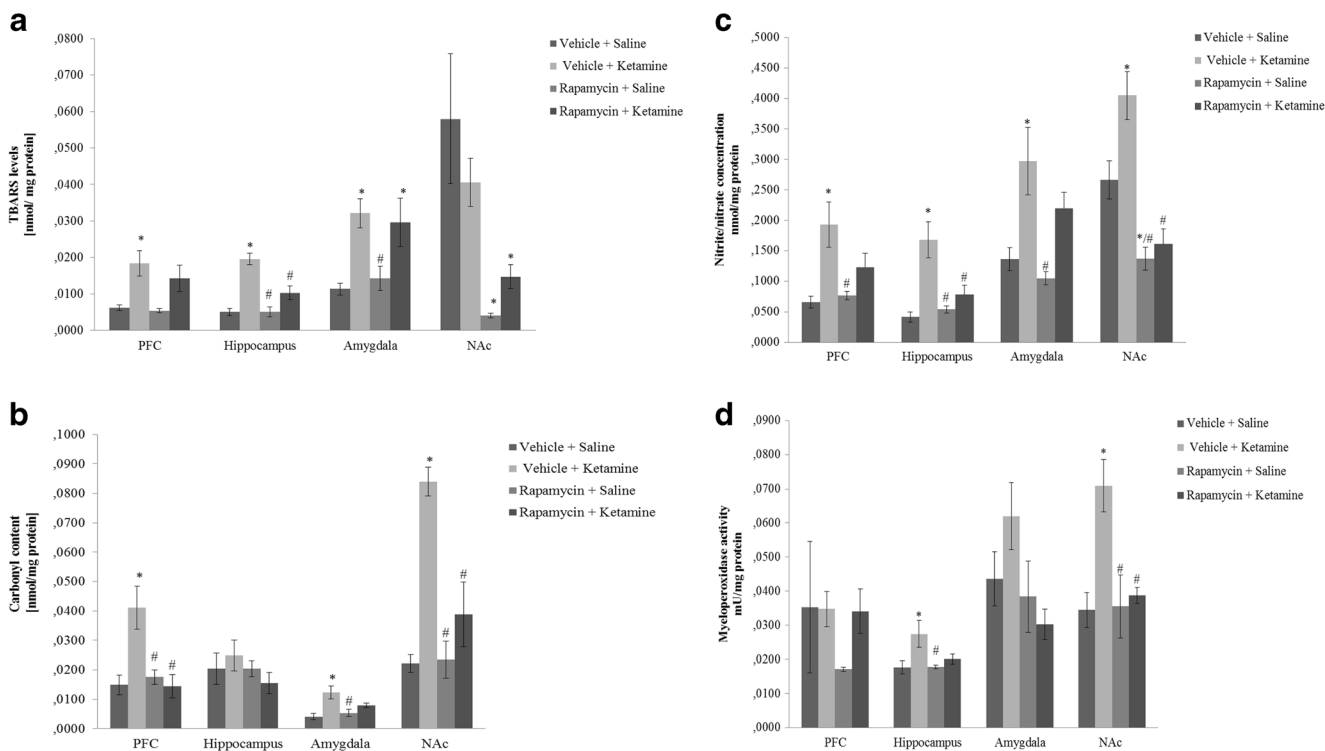

Fig. 1 The effects of ketamine administration after the infusion of inhibitor rapamycin in the PFC on immobility, swimming, and climbing times in the forced swimming test (**a**) ($n = 39$ animals). *Bars* represent mean ± standard deviation. *$p < 0.05$ vs. vehicle + saline; #$p < 0.05$ vs. vehicle + ketamine according to ANOVA followed by Tukey's post hoc test

Fig. 2 The effects of ketamine administration after the infusion of inhibitor rapamycin in the PFC on TBARS levels (**a**), carbonyl protein levels (**b**), nitrite/nitrate concentration (**c**), and myeloperoxidase activity (**d**) in the prefrontal cortex, hippocampus, amygdala, and nucleus accumbens. *Bars* represent mean standard deviation ($n = 4$–5 per group). $*p < 0.05$ vs. vehicle + saline; $#p < 0.05$ vs. vehicle + ketamine according to ANOVA followed by Tukey's post hoc test

compared to the ketamine treatment group, demonstrating that rapamycin was able to abolish the effects of ketamine on carbonyl protein formation. In the amygdala, the ketamine treatment group increased the carbonyl protein formation when compared with the vehicle group treated with saline and the rapamycin (alone) group decreased the carbonyl protein formation when compared to ketamine group ($F_{(3–17)} = 6.429$; $p = 0.006$; Fig. 2b). In the hippocampus, we did not observe any statistical significance on carbonyl protein formation ($F_{(3–19)} = 0,773$; $p = 0.526$; Fig. 2b).

Figure 2c illustrates that there was an increase on nitrite/nitrate concentration after ketamine treatment when compared with the vehicle group treated with saline and there was a decrease with the rapamycin (alone) group when compared to ketamine treatment group, in the PFC ($F_{(3–19)} = 6.541$; $p = 0.004$; Fig. 2c) and amygdala ($F_{(3–17)} = 6.270$; $p = 0.006$; Fig. 2c). In the hippocampus, the ketamine treatment group increased the nitrite/nitrate concentration when compared with the vehicle group treated with saline the rapamycin groups decreased the nitrite/nitrate concentration when compared to with ketamine treatment group, demonstrating that rapamycin blocked the ketamine effects on nitrite/nitrate concentration ($F_{(3–19)} = 10.616$; $p < 0.001$; Fig. 2c). Finally, in the nucleus accumbens, the ketamine treatment group increased and the rapamycin (alone) group decreased the nitrite/nitrate concentration, when compared with

the vehicle group treated with saline, and still, the rapamycin groups decreased the nitrite/nitrate concentration when compared to ketamine treatment group, showing that rapamycin blocked this ketamine effects ($F_{(3–18)} = 16.691$; $p < 0.001$; Fig. 2c). Figure 2d shows that in the hippocampus ($F_{(3–17)} = 4.366$; $p < 0.023$; Fig. 2d) and in the nucleus accumbens ($F_{(3–17)} = 7.377$; $p = 0.003$; Fig. 2d), there was an increase on myeloperoxidase activity after the infusion of ketamine treatment when compared with the vehicle group treated with saline and there was a decrease in rapamycin (alone) group when compared to ketamine treatment group ($F_{(3–17)} = 4.366$; $p < 0.023$; Fig. 2d); however, just in the nucleus accumbens, rapamycin blocked the ketamine effects on the myeloperoxidase activity. Still, we did not observe any alteration on myeloperoxidase activity in the PFC ($F_{(3–18)} = 0.672$; $p = 0.582$; Fig. 2d) and amygdala ($F_{(3–15)} = 2.071$; $p = 0.158$; Fig. 2d).

Effects of Ketamine After the Inhibition of mTOR Pathway in the PFC with Rapamycin on SOD and Catalase Activity in Brain Structures

Figure 3 demonstrates the effect of ketamine, after the inhibition of mTOR in the PFC with rapamycin, on SOD and catalase activity in the PFC, hippocampus, amygdala, and nucleus accumbens. The SOD activity was decreased in the PFC with
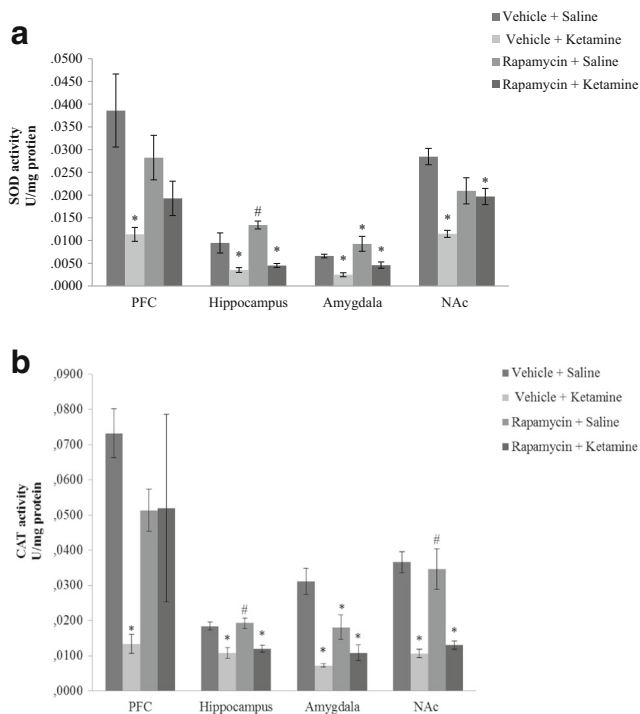
Fig. 3 The effects of ketamine administration after the infusion of inhibitor rapamycin in the PFC on SOD (**a**) and catalase (**b**) activities in the prefrontal cortex, hippocampus, amygdala, and nucleus accumbens. *Bars* represent mean standard deviation ($n = 4$–5 per group). *$p < 0.05$ vs. vehicle + saline; #$p < 0.05$ vs. vehicle + ketamine according to ANOVA followed by Tukey's post hoc test

the ketamine treatment group when compared with the vehicle group treated with saline ($F_{(3-19)} = 5.224$; $p = 0.010$; Fig. 3a). In the hippocampus, the SOD activity was decreased with the ketamine treatment group and rapamycin plus ketamine treatment group, when compared with the vehicle group treated with saline, and still, rapamycin (alone) group increased the SOD activity when compared to ketamine treatment group ($F_{(3-19)} = 13.955$; $p < 0.001$; Fig. 3a). Still in the amygdala, the SOD activity was decreased with the ketamine treatment group and rapamycin plus ketamine treatment group and increased with rapamycin group, when compared with the vehicle group treated with saline ($F_{(3-18)} = 9.351$; $p = 0.001$; Fig. 3a). Finally, in the nucleus accumbens, the SOD activity was decreased with the ketamine treatment group and rapamycin plus ketamine treatment group, when compared with the vehicle group treated with saline ($F_{(3-19)} = 12.687$; $p < 0.001$; Fig. 3a).

Figure 3b illustrates that there was a decrease on catalase activity with the ketamine treatment group when compared with the vehicle group treated with saline, in the PFC ($F_{(3-19)} = 3.093$; $p < 0.05$; Fig. 3b). In the hippocampus ($F_{(3-19)} = 11.464$; $p < 0.001$; Fig. 3b) and nucleus accumbens ($F_{(3-19)} = 17.256$; $p < 0.001$; Fig. 3b), there was a decrease on catalase activity with the ketamine treatment group and rapamycin plus ketamine treatment group when compared

with the vehicle group treated with saline, and still, there was an increase on catalase activity with rapamycin (alone) group when compared to the ketamine treatment group. Still, in the amygdala, there was a decrease on catalase activity with the ketamine treatment group and both rapamycin groups when compared with the vehicle group treated with saline ($F_{(3-18)} = 14.298$; $p < 0.001$; Fig. 3b).

Effects of Ketamine After the Inhibition of mTOR Pathway in the PFC with Rapamycin on Pro-Inflammatory Cytokines (TNF-α, IL-1β, and IL-6) Levels in Serum

Figure 4 demonstrates the effect of ketamine, after the inhibition of mTOR in the PFC with rapamycin, on TNF-α, IL-1β, and IL-6 levels in serum. The TNF-α levels were increased with the ketamine treatment group and rapamycin plus ketamine treatment group when compared with the vehicle group treated with saline, in serum ($F_{(3-21)} = 16.016$; $p < 0.001$; Fig. 4a). However, we did not observe any statistical significance in IL-1β ($F_{(3-21)} = 0.606$; $p = 0.619$; Fig. 4a) and IL-6 ($F_{(3-19)} = 1.818$; $p = 0.184$; Fig. 4a) levels in serum.

Discussion

The mTOR signaling plays an important role in the regulation of cell growth, proliferation, and metabolism, which are associated with neurological diseases, cancer, metabolic disorders, and inflammation [61, 62]. Previous postmortem studies showed robust deficits in mTOR signaling in the PFC of subjects diagnosed with MDD. In fact, reduced protein expression of mTOR and its two major downstream signaling effectors, the 70-kDa ribosomal protein S6 kinase (p70S6K) and eukaryotic translation initiation factor 4B (eIF-4B), have been previously demonstrated in the PFC of depressed patients
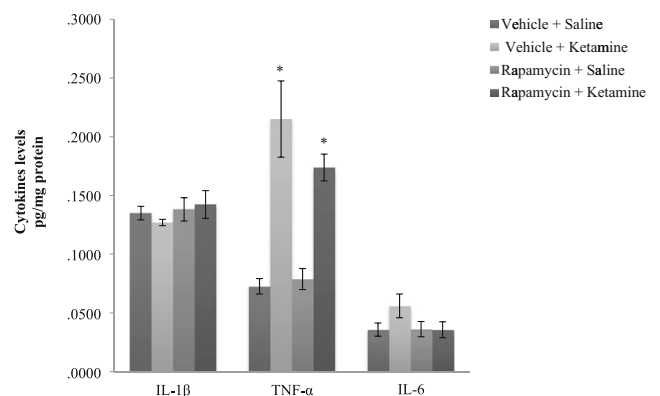

Fig. 4 The effects of ketamine administration after the infusion of inhibitor rapamycin in the PFC on cytokine levels TNF-α, IL-1β, and IL-6 in serum. *Bars* represent mean standard deviation ($n = 4$–5 per group). *$p < 0.05$ vs. vehicle + saline; according to ANOVA followed by Tukey's post hoc test

[16], suggesting that deficits in mTOR-dependent protein synthesis may contribute to the molecular and cellular pathology detected in the PFC in MDD.

The activation of postsynaptic glutamate receptors initiates a cascade which results in mTOR phosphorylation and eventually protein synthesis via the downstream effectors of mTOR [16]. Dysregulation of the glutamatergic system may ultimately lead to decreased protein synthesis [63]. In fact, a low dose of ketamine, which is reported to have antidepressant actions in behavioral models of depression [8, 11], rapidly activated the mTOR signaling pathway in the PFC of rats [3]. In addition, ketamine produced a similar rapid and transient increase in the phosphorylated and activated forms of extracellular signal-regulated kinase (ERK, including ERK1 and ERK2) and protein kinase B (PKB/Akt), growth factor signaling pathways that have been linked to activation of mTOR signaling [15]. Activation of 4E-BP1, p70S6K, mTOR, ERK, and Akt, by ketamine, was dose dependent, occurring at relatively low doses that produce antidepressant-like effects [8].

In the present study, we concurred with previous research [10, 11, 52, 53] that ketamine, at dose of 15 mg/kg, decreased the immobility time of rats subjected to the forced swimming test. However, the administration of rapamycin inhibitor blocked this ketamine effects. In accordance with the present study, Li et al. [3] also showed that a local infusion of rapamycin into the PFC complete blockade the behavioral effects of ketamine in the forced swimming test and in the novelty suppressed feeding test, suggesting that mTOR activation in the PFC was essential to ketamine's antidepressant-like effects.

Rapamycin binds to the cytosolic protein FK-binding protein 12 (FKBP12) [64]. Thereby, the rapamycin–FKBP12 complex can inhibit mTOR preventing further phosphorylation of P70S6K, 4EBP1, and, indirectly, other proteins involved in transcription and translation and cell cycle control [65]. Inhibition of mTOR leads to, among others, cell cycle arrest in tumor cells resulting in growth retardation. Anti-apoptotic signals mediated by mTOR are also antagonized by rapamycin [66]. Rapamycin has been shown to inhibit growth of melanoma cells in mouse model; additionally, as an immunosuppressor, it prevents transplant rejection in organ transplant recipients [67]. Apart from its immunosuppressive capacity, rapamycin was also recently shown to be capable of preventing coronary artery re-stenosis [68] and acts as a neuroprotective compound in various neurological diseases [64, 69–71].

In the present study, we showed that the administration of ketamine in acute dose leads to an increase on oxidative stress parameters, such as TBARS levels, carbonyl content, nitrite/nitrate concentration, and myeloperoxidase activity in some brain structures related to depression, and when we used the rapamycin inhibitor in the PFC, these effects were decreased or even abolished in some brain structures. Chiu et al. [72] showed that the injection of an acute dose of ketamine (50 mg/kg) increased lipid peroxidation. Vasconcelos et al. [73] showed that chronic administration of ketamine caused working memory deficits and led to oxidative damage in the PFC. Ketamine (25 mg/kg) increased TBARS and carbonyl protein in the PFC, hippocampus, amygdala, and striatum; however, mood stabilizers, lithium, and valproate were able to reverse oxidative stress induced by ketamine [43]. In addition, da Silva et al. [74] related that ketamine in sub-anesthetic doses induced antidepressant effects in mice submitted to the forced swimming and tail suspension tests; however, ketamine produced an oxidative damage by increasing lipid peroxidation, nitrite content, and catalase activity and decreasing glutathione (GSH), an important antioxidant. Interestingly, a positive effect of rapamycin on oxidative damage has also been shown in other study. Rotte et al. [75] demonstrated that, in dendritic cells, the lipopolysaccharides (LPS) stimulated Na+/H+ exchanger (NHE) activity, enhanced forward scatter, increased ROS formation, and triggered TNF-α release, effects all blunted in the presence of rapamycin.

The present study is the first to evaluate the effects of the mTOR inhibition by rapamycin on oxidative stress parameters in the PFC and the relation of the inhibition in the PFC in others structures related to depression. Persistent oxidative stress in cells and oxidative stress have been reported to activate the pro-growth PI3K/Akt pathway, which in turn is known to activate mTOR [76, 77]. Since ROS can both activate and inhibit mTORC1 in a context-dependent manner, chronic oxidative stress that occurs in cells is expected to stimulate growth, whereas an overload of ROS results in oxidative damage and mTORC1 inhibition and therefore inhibits cell growth [78]. In addition, pharmacological agents that inhibit mTOR, such as rapamycin showed in the present study, or activate AMPK can protect neurons against dysfunction and degeneration in animal models of acute brain injury and neurodegenerative disorders [79, 80]. Still, the rats' PFC, like human PFC, projects to many limbic areas implicated in the control of affect, including the lateral septum, basolateral amygdala, insular cortex, and nucleus accumbens [81–83]. However, our study has a limitation in relation of the technique used to measure the levels of TBARS. This technique has been widely used but currently has been seen that in more complex biological systems, many compounds (including simple and complex carbohydrates, protein oxidation products, and nucleic acid oxidation products) react with thiobarbituric acid to produce colored adducts. In this sense, one cannot directly equate the measurement of TBARS with MDA or lipid

peroxidation when measured in a complex biological system.

We also demonstrated that the administration of ketamine decreased the SOD and catalase activities in the PFC, hippocampus, amygdala, and nucleus accumbens; the rapamycin plus ketamine treatment group was not able to abolish the ketamine's effect. Gazal et al. [84] demonstrated that ketamine treatment reduced the activity of the antioxidant enzymes superoxide dismutase and catalase in hippocampus; however, the pretreatment with *Cecropia pachystachya*, a plant with antioxidants and anti-inflammatory properties, or with lithium chloride prevented the pro-oxidant effects of ketamine. Hou et al. [85] showed that a repeated administration of ketamine, in an animal model of schizophrenia, increased nitric oxide (NO) and nitric oxide synthase (NOS) in PFC, hippocampus, and serum and decreased SOD in hippocampus and serum. However, depending on the time that ketamine is administered, it may have an antioxidant effect. As shown by Réus et al. [86] that the SOD activity was increased in control rats that received ketamine, for 14 days, in the PFC and nucleus accumbens and was diminished in deprived rats that received saline or ketamine in the PFC and hippocampus. And still, previous study also showed that when an acute administration of ketamine-S and the evaluation of antioxidants activity in the brain after 14 days that the treatment occurred, the activities of antioxidant enzymes SOD and catalase were reduced in the brains of rats submitted to the maternal deprivation; however, ketamine was able to reverse these changes [10]. However, rapamycin administrated alone was able to increase the SOD and catalase activities in some brain structures [85]. In a *Drosophila* model, rapamycin was able to mediate protection against oxidative damage and increase the transcription of antioxidant genes mediated by cap-n-collar (*Drosophila* ortholog of Nrf2) [87]. Various studies suggest that rapamycin acts as a neuroprotective compound in various neurological diseases [69, 70]. However, in the present study, the administration of rapamycin before ketamine treatment was not able to block the decrease on antioxidants enzymes exerted by ketamine, showing that rapamycin had an antioxidant effect only when administered alone. In addition, these antioxidant effects were only in some brain structures, such as the hippocampus, amygdala, and nucleus accumbens, suggesting that when the inhibition of mTOR pathway by rapamycin occurs in the PFC, its neuroprotective effect can become blocked in this structure.

Growing evidence supports a mutual relationship between inflammation and major depression [88]. Still, the old anesthetic ketamine has demonstrated interactions with the inflammatory response [89]. In fact, it is known that ketamine influences the immune system particularly the inflammatory reaction an immune response that largely derives from activation the innate immune mechanisms [90, 91]. In this way, the present study showed that there was an increase on TNF-α level in serum with ketamine treatment group. In accordance with our study, other studies also show that ketamine helps to avoid exacerbated pro-inflammatory reaction [92–94]. Still, the present study showed that rapamycin plus ketamine treatment was not able to block ketamine's effect. Recent data show that mTOR signaling plays an important role in the modulation of both innate and adaptive immune responses [95]. A large part of the immune response depends on the proliferation and clonal expansion of antigen-specific T cells, which depends on mTOR activation, and the pharmacological inhibition of this pathway by rapamycin, differently from our study, is therefore potently immunosuppressive. Contrary to our findings, rapamycin treatment in vivo reduced the production of IL-4 by double negative T (DN T) cells [96], which accounts for increased production of anti-DNA autoantibodies by B cells [97]. Still, rapamycin also blocked T cell activation in patients with systemic lupus erythematosus (SLE) [98] with remarkable therapeutic efficacy [99]. This is also the first study to show the effects of ketamine treatment after the mTOR inhibition in PFC on cytokine levels. The discrepancy in the results may be related to the type of the disorder that was studied and to the local infusion of rapamycin.

In conclusion, this is, to our knowledge, the first study to show the effects of ketamine after the inhibition of mTOR pathway by rapamycin on oxidative stress parameters and cytokine levels in the PFC and the relation of this inhibition in the PFC in others brain areas related to depression. We show that rapamycin was able to abolish the oxidative damage caused by ketamine; however, these effects were brain area dependent. In addition, the administration of rapamycin alone was able to improve the antioxidants effects in some brain areas. Still, rapamycin was not able to block ketamine's increase on TNF-α level in serum. Our results indicate that mTOR signaling inhibition by rapamycin could be involved, at least in part, with the mechanism of action of ketamine and presents a neuroprotective effect when reduced the oxidative stress induced by ketamine. However, further studies are necessary to clarify that ketamine and the mTOR inhibition mechanism are involved in the brain oxidative stress, inflammation, and survival signaling.

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