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# Antidepressant-like effect of ascorbic acid is associated with the modulation of mammalian target of rapamycin pathway

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ABSTRACT

The present study investigated the involvement of the PI3K, GSK-3β, heme oxygenase-1 (HO-1) and mTOR in the antidepressant-like effect of ascorbic acid in the tail suspension test (TST). Male Swiss mice were pretreated with ascorbic acid (1 mg/kg, p.o.) or vehicle and 45 min after, LY294002 (10 μg/site, i.c.v., reversible PI3K inhibitor), rapamycin (0.2 nmol/site, i.c.v., selective mTOR inhibitor), zinc protoporphyrin (ZnPP – 10 ng/site, i.c.v., HO-1 inhibitor) or vehicle was administered. We also investigated the synergistic effect of ascorbic acid (0.1 mg/kg, p.o., sub-effective dose in the TST) with lithium chloride (10 mg/kg, p.o., non-selective GSK-3β inhibitor), AR-A014418 (0.01 μg/site, i.c.v., selective GSK-3β inhibitor) or cobalt protoporphyrin (CoPP – 0.01 μg/site, i.c.v., HO-1 inducer) in the TST. The antidepressant-like effect of ascorbic acid (1 mg/kg, p.o.) was prevented by the treatment of mice with LY294002, rapamycin or ZnPP. In addition, sub-effective doses of lithium chloride, AR-A014418 or CoPP, combined with a sub-effective dose of ascorbic acid produced a synergistic antidepressant-like effect. We also demonstrated that 1 h after its administration, ascorbic acid increased the phosphorylation of p70S6K and the immunocontent of PSD-95 in the hippocampus of mice. These results indicate that the antidepressant-like effect of ascorbic acid in the TST might be dependent on the activation of PI3K and mTOR, inhibition of GSK-3β as well as induction of HO-1, reinforcing the notion that these are important targets for antidepressant activity and contributing to better elucidate the mechanisms underlying the antidepressant-like effect of ascorbic acid.



## 1. Introduction

Worldwide, depression is a common, recurrent and incapacitating psychiatric disorder associated with significant morbidity and mortality (Nemeroff, 2007). Treatment of depression has usually focused on alleviating symptoms and preventing recurrence of episodes, however a considerable number of patients exhibit partial, refractory or intolerant responses to the pharmacological treatment and less than one third of them achieve remission after 10–14 weeks of treatment with a standard antidepressant (Trivedi et al., 2006). The recognition of the clear need for the development of new, effective, and better-tolerated therapeutic approaches with a more rapid onset of action has conducted to the investigation of the putative roles of intracellular signaling cascades and non-monoaminergic systems in the pathophysiology and treatment of depression (Coyle and Duman, 2003).

Modulation of glutamatergic neurotransmission has emerged as a promising strategy for next-generation fast-acting antidepressants. Of particular significance is the demonstration that low doses of the N-Methyl-D-aspartate (NMDA) receptor antagonist, ketamine, induces a rapid (within hours), long lasting (up to 1 week), and robust antidepressant response in treatment-resistant cases of depression in humans (Berman et al., 2000; Zarate et al., 2006). Accordingly, it was demonstrated that a single dose of NMDA receptor antagonists (ketamine or Ro 25-6981) completely reversed the behavioral deficits in a rat 21-day chronic unpredictable stress model (Li et al., 2011), reinforcing the antidepressant potential of these compounds.

Although the mechanisms underlying the fast and robust effects of ketamine are not completely understood, recent animal studies showed that they are mediated by rapid, but transiently activation of the mammalian target of rapamycin (mTOR) pathway (Li et al., 2010). Activation of mTOR signaling stimulates mRNA translation

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and new protein synthesis by activating p70 S6 kinase (p70S6K) and by inhibiting eukaryotic initiation factor 4E-binding protein 1. In rats, it was demonstrated that mTOR signaling results in rapid and sustained elevation of synapse-associated proteins and spine number in the prefrontal cortex (Li et al., 2010). The rapid antidepressant action of ketamine was also dependent on the inhibition of glycogen synthase kinase-3β (GSK-3β) activity through its phosphorylation at Ser9 residue. It was shown that a subthreshold dose of ketamine was potentiated by a single dose of lithium chloride (a non-selective GSK-3β inhibitor) or by a preferential GSK-3β inhibitor, an effect associated with rapid activation of the mTORC1 signaling pathway and increased phosphorylation of GSK-3β (Liu et al., 2013). Conversely, inhibition of mTOR attenuated GSK-3β phosphorylation and increased its kinase activity in lipopolysaccharide (LPS)-stimulated cells (Wang et al., 2011). Corroborating the notion that the activation of mTOR pathway and inhibition of GSK-3β by phosphorylation are implicated in the antidepressant responses of rapid antidepressants, the behavioral responses to ketamine were blocked in mice expressing constitutively active GSK-3β (Beurel et al., 2011). Stimulation of protein kinase B (PKB/Akt), possibly by activity-dependent release of brain-derived neurotrophic factor (BDNF), is probably implicated in the increased phosphorylation of GSK-3β induced by ketamine (Jourdi et al., 2009). Of note, the inactivation of GSK-3β induced by phosphorylation at Ser9 is linked to activation of the NF-E2-related factor 2 (Nrf2). This is a nuclear transcription factor which controls redox homeostasis by regulating several stress responsive genes, including heme oxygenase-1 (HO-1), which plays a pivotal role in cell protection against inflammatory insult and oxidative/nitrosative stress (Surh et al., 2009).

The discovery of compounds that can produce ketamine-like effects with a safer side-effect profile and decreased abuse liability may represent an important advance in the field of depression. Our group has demonstrated that ascorbic acid, a water-soluble vitamin with neuroprotective and antioxidant properties (Rice, 2000), exhibits an antidepressant-like effect in the mouse tail suspension test (TST), an animal model predictive of antidepressant activity (Binfaré et al., 2009). Additionally, this compound was able to reverse the depressive-like behavior and brain oxidative damage induced by acute and chronic unpredictable stress in mice (Moretti et al., 2012b, 2013). Considering that the antidepressant properties of ascorbic acid are mediated, at least in part, by inhibition of NMDA receptors (Moretti et al., 2011), we hypothesized that this compound could exhibit ketamine-like biochemical effects. Therefore, the goal of this study was to examine if the antidepressant-like effect of ascorbic acid in the TST is dependent on the modulation of mTOR and its down and upstream signaling targets: phosphatidylinositol 3-kinase (PI3K), GSK-3β, HO-1, p70S6K and PSD95.

## 2. Material and methods

### 2.1. Animals

The experiments were conducted using male adult Swiss mice (2 months, 30–40 g), maintained at 20–22 °C with free access to water and food, under a 12:12 h light/dark cycle, with lights on at 7:00 a.m. The animals were caged in groups of 15 in a 41 × 34 × 16 cm cage and were placed in the experimental room 24 h before the test for acclimatization. All behavioral tests were carried out between 09.00 a.m. and 04.00 p.m. The animals were used according to the NIH Guide for the Care and Use of Laboratory Animals. The protocol and experiments were approved by the local Ethical Committee of Animal Research (CEUA/UFSC). All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

### 2.2. Drugs

The following drugs were used: ascorbic acid, LY294002, AR-A014418, zinc protoporphyrin (ZnPP), cobalt protoporphyrin (CoPP) (Sigma Chemical Co., St Louis, USA) and lithium chloride (MERCK, Darmstadt, Germany). Lithium chloride and ascorbic acid were dissolved in distilled water. These solutions, freshly prepared before administration, were given orally (p.o.) by gavage in a volume of 10 ml/kg body weight. LY294002 and AR-A014418 were dissolved in saline at a final concentration of 1% DMSO and administered by i.c.v. route, in a volume of 5 μl per mouse. Rapamycin was dissolved in 100% DMSO and administered by i.c.v. route, in a volume of 3 μl per mouse. ZnPP and CoPP were dissolved in final concentration of 0.1% DMSO and administered by i.c.v. route, in a volume of 5 μl per mouse, respectively. Appropriate vehicle-treated groups were also assessed simultaneously.

The i.c.v. injections were performed by employing a "free hand" method under light ether anesthesia according to the procedure described previously (Kaster et al., 2012). Briefly, a 0.4 mm external diameter hypodermic needle attached to a cannula, which was linked to a 25 μl Hamilton syringe, was inserted perpendicularly through the skull (no more than 2 mm into the brain of each mouse). The drugs were then administered into the left lateral ventricle. The injection was given over 30 s, and the needle remained in place for another 30 s in order to avoid the reflux of the substances injected. The injection site was 1 mm to the left from the mid-point on a line drawn through to the anterior base of the ears. I.c.v. injections were performed by an experienced investigator, and after dissection of the brain of the animal, the success of the injection was examined, macroscopically, discarding results from mice presenting misplacement of the injection site or any sign of cerebral hemorrhage (<5%).

### 2.3. Pharmacological treatment

To investigate the involvement of PI3K in the antidepressant-like effect of ascorbic acid, mice were pre-treated with ascorbic acid (1 mg/kg, p.o., active dose in the TST) or vehicle 45 min before i.c.v. administration of LY294002 (PI3K inhibitor, 10 nmol/site) or vehicle. The TST was carried out 15 min after LY294002 administration.

To test the hypothesis that the antidepressant-like effect of ascorbic acid could be mediated by the inhibition of GSK-3β activity, mice were treated with a sub-effective dose of ascorbic acid (0.1 mg/kg, p.o.) or vehicle and immediately after, a sub-effective dose of lithium chloride (10 mg/kg, p.o., a non-selective GSK-3β inhibitor) or vehicle was administered. The TST was carried out 60 min later. In another set of experiments mice were treated with a sub-effective dose of ascorbic acid (0.1 mg/kg, p.o.) or water and 45 min after, were injected with a sub-effective dose of the selective GSK-3β inhibitor, AR-A014418 (0.01 μg/site, i.c.v.) or vehicle. A further 15 min were allowed to elapse before the animals were tested in the TST.

To investigate the involvement of HO-1 in the antidepressant-like effect of ascorbic acid in the TST, mice received an active dose of ascorbic acid (1 mg/kg, p.o.) or vehicle and after 45 min were treated with the HO-1 inhibitor ZnPP (10 ng/site, i.c.v.) or vehicle. In another set of experiments mice were treated with a sub-effective dose of ascorbic acid (0.1 mg/kg, p.o.) or water and 45 min after, received a sub-effective dose of the HO-1 inducer CoPP (0.01 μg/site, i.c.v.) or vehicle. Animals were tested in the TST 15 min after the i.c.v. administrations.

To evaluate the participation of mTOR in the anti-immobility effect of ascorbic acid in the TST, mice were treated with an active dose ascorbic acid (1 mg/kg, p.o.) or vehicle and after 45 min

received an i.c.v injection of rapamycin (0.2 nmol/site, a selective mTOR inhibitor). The TST was carried out 15 min after rapamycin administration.

For Western blot analysis, animals were treated with ascorbic acid (1 mg/kg, p.o., active dose in the TST) or water and 1 h later were killed and had their hippocampi dissected. These mice were not submitted to behavioral evaluations.

### 2.4. Behavioral tests

#### 2.4.1. Tail suspension test (TST)

The total duration of immobility induced by tail suspension was measured according to the method described by Steru et al. (1985). Mice both acoustically and visually isolated were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Mice were considered immobile only when they hung passively and completely motionless. Immobility time was manually recorded during a 6-min period by an experienced observer. The observer was in the room where experiments were performed and was blind to the animal condition.

#### 2.4.2. Open–field test

Ten minutes after the TST, the locomotor activity was assessed in an open-field test as previously described (Moretti et al., 2012b). The apparatus consists of a wooden box measuring 40 × 60 × 50 cm high. The floor of the arena was divided into 12 equal squares. The number of squares crossed with all paws (crossings) was manually counted in a 6-min session. The light was maintained at minimum to avoid anxiety behavior. The apparatus was cleaned with a solution of 10% ethanol between tests in order to hide animal clues.

### 2.5. Western blot

Hippocampi were dissected, mechanically homogenized in 500 ml of sample buffer (200 mM Tris, 40 mM EDTA, 4% SDS, pH 6.8) and immediately boiled for 5 min. Sample dilution solution (1:4 vol/vol; 40% glycerol, 50 mM Tris and minimal bromophenol blue) and β-mercaptoethanol were added to each sample for a final concentration of 5%. Protein content was quantified by the method of Peterson (1977), using bovine serum albumin as a standard. The samples containing 60 mg of total protein per track were separated by SDS-PAGE (miniVE Vertical Electrophoresis SystemTM, GE Healthcare Life Sciences, Piscataway, NJ, USA) using 10% gel and the proteins were transferred to nitrocellulose membranes using a tank transfer system at 100 V and 270 mA for 1 h (Mini-PROTEAN Tetra cell Electrophoresis System, Bio-Rad, Hercules, CA) (Cordova et al., 2012). The membranes were blocked (1 h) with 5% skim milk in TBS (10 mM Tris, 150 mM NaCl, pH 7.5). p70S6K total and phosphorylated forms, PSD-95, and β-actin immunocontent were detected using specific antibodies (obtained from Cell Signaling Technology, Inc. and diluted by a factor of 1:1000) incubated overnight diluted in TBS-T (10 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5) containing 2.5% BSA. Next, the membranes were incubated with a mouse anti-rabbit peroxidase-linked secondary antibody (Santa Cruz Biotechnology, inc. – 1:5000) for 1 h and the reactions developed by chemiluminescence (LumiGLOH, Cell Signaling, Beverly, MA, USA). All blocking and incubation steps were followed by three washes (5 min) of the membranes with TBS-T. The optical density (O.D.) of the bands was quantified using Scion ImageTM (Frederick, MD, USA). The phosphorylation levels p70S6K was determined as a ratio of O.D. of the phosphorylated band over O.D. of the total band. The immunocontent of PSD-95 was determined as a ratio of O.D of the PSD-95 band over O.D. of the β-actin band. Results are expressed as absolute values.

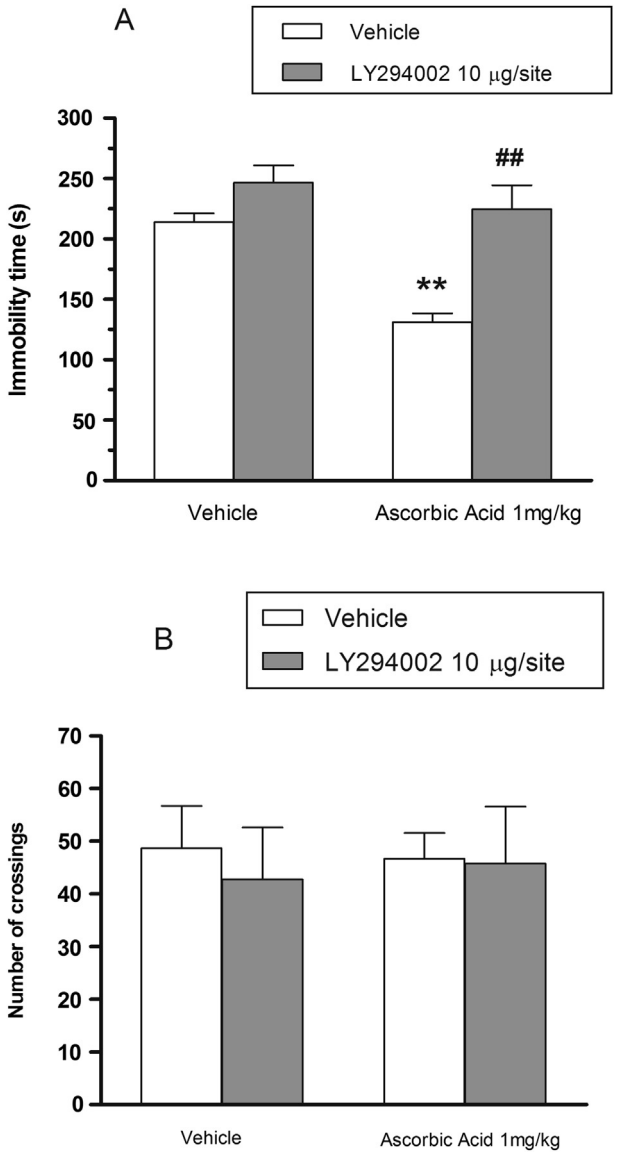


**Fig. 1.** Effect of the treatment of mice with LY294002 (10 μg/site, i.c.v.) on the ascorbic acid-induced (1 mg/kg, p.o.) antidepressant-like effect in the TST (panel A) and on locomotor activity in the open-field test (panel B). Values are expressed as mean + S.E.M of 7–8 mice. $^{**}p < 0.01$ compared with the vehicle-treated control group. $\#\#p < 0.01$ compared with ascorbic acid-treated group.

### 2.6. Statistical analysis

All data are presented as mean ± S.E.M. Differences among experimental groups were determined by two-way ANOVA followed by Newman–Keuls post hoc test for behavioral tests and by Student's *t*-test for Western blot experiments. A value of $p < 0.05$ was considered to be significant.

## 3. Results

### 3.1. Behavioral observations

It has been increasingly indicated that signaling through the mTOR pathway is activated by some compounds that modulate glutamatergic neurotransmission (Dwyer et al., 2012; Li et al., 2010). In this study, we initially used a sequence of pharmacological tools to determine if the signaling events upstream of mTOR,

specifically PI3K and GSK-3β, are able to influence the anti-immobility effect of ascorbic acid in the TST.

Fig. 1A shows the effect of inhibition of the upstream Akt activator PI3K by LY294002 (10 nmol/site, i.c.v.) in the antidepressant-like effect of ascorbic acid (1 mg/kg, p.o.) in the TST. The two-way ANOVA revealed significant differences for ascorbic acid treatment [$F(1,26) = 24.967$; $p < 0.01$], LY294002 treatment [$F(1,26) = 17.376$; $p < 0.01$] and ascorbic acid treatment × LY294002 interaction [$F(1,26) = 5.821$; $p < 0.05$]. Post-hoc analysis showed that the antidepressant-like effect of ascorbic acid was completely prevented by treatment of animals with the PI3K inhibitor LY294002. Fig. 1B shows that the administration of LY294002 alone or in combination with ascorbic acid was devoid of effect in the open-field test (ascorbic acid treatment [$F(1,27) = 0.156$; $p > 0.05$], LY294002 treatment [$F(1,27) = 0.003$; $p > 0.05$], ascorbic acid treatment × LY294002 interaction [$F(1,27) = 0.086$; $p > 0.05$]).

Fig. 2A illustrates the effect of the co-administration of sub-effective doses of ascorbic acid (0.1 mg/kg, p.o.) and the non-selective GSK-3β inhibitor, lithium chloride (10 mg/kg, p.o.) in the TST. The two-way ANOVA revealed significant differences for ascorbic acid treatment [$F(1,28) = 15.762$; $p < 0.01$], lithium chloride treatment [$F(1,28) = 4.287$; $p < 0.05$] and ascorbic acid treatment × lithium chloride interaction [$F(1,28) = 5.245$; $p < 0.05$]. Post-hoc analysis indicated that treatment with a sub-effective dose of ascorbic acid produced a synergistic antidepressant-like effect with a sub-effective dose of lithium chloride in the TST. Fig. 2B shows that the administration of lithium chloride alone or in combination with ascorbic acid did not modify the locomotor activity of mice in the open-field test (ascorbic acid treatment [$F(1,25) = 0.378$; $p > 0.05$], lithium chloride treatment [$F(1,25) = 1.226$; $p > 0.05$] and ascorbic acid treatment × lithium chloride interaction [$F(1,25) = 0.065$; $p > 0.05$]).

To confirm the hypothesis that GSK-3β is involved in the antidepressant-like effect of ascorbic acid in the TST, we treated the animals with sub-effective doses of ascorbic acid (0.1 mg/kg, p.o.) and the selective inhibitor of GSK-3β, AR-A014418 (0.01 μg/site, i.c.v.). The two-way ANOVA revealed significant differences for ascorbic acid treatment [$F(1,28) = 18.221$; $p < 0.01$], AR-A014418 treatment [$F(1,28) = 6.583$; $p < 0.05$] and ascorbic acid treatment × AR-A014418 interaction [$F(1,28) = 5.424$; $p < 0.05$]. As depicted in Fig. 2C, post-hoc analysis indicated that the combined administration of sub-effective doses of ascorbic acid and AR-A014418 produced an antidepressant-like effect in the TST. Fig. 2D shows that the administration of AR-A014418 alone or in combination with ascorbic acid did not affect locomotor activity of mice in the open-field test (ascorbic acid treatment [$F(1,27) = 0.062$; $p > 0.05$], AR-A014418 treatment [$F(1,27) = 0.593$; $p > 0.05$], ascorbic acid treatment × AR-A014418 interaction [$F(1,27) = 0.373$; $p > 0.05$]).

Considering previous studies which demonstrated that ketamine administration upregulates the expression of HO-1 (Helmer et al., 2006; Suliburk et al., 2009) and the possible regulation of NRF2/HO-1 by GSK-3β (Surh et al., 2009), we investigated the involvement of HO-1 in the antidepressant-like effect of ascorbic acid in the TST. Fig. 3A shows the effect of inhibition of HO-1 by ZnPP (10 ng/site, i.c.v.) on the antidepressant-like effect of ascorbic acid (1 mg/kg, p.o.) in the TST. The two-way ANOVA revealed significant differences for ascorbic acid treatment [$F(1,27) = 14.361$;

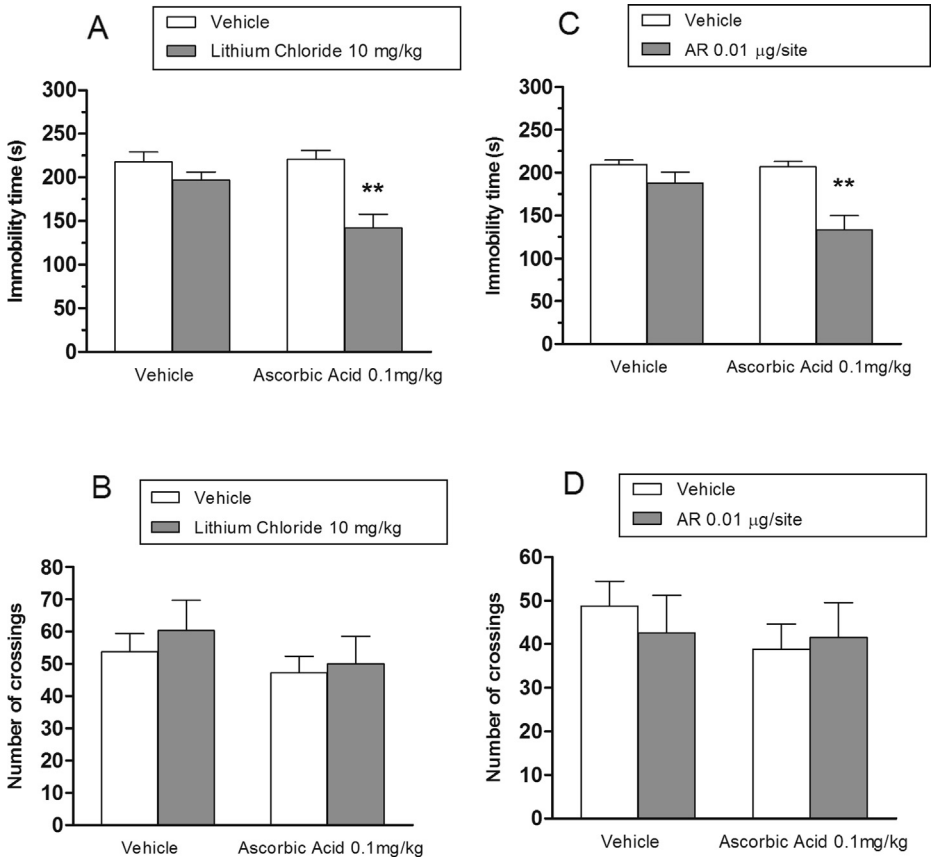


**Fig. 2.** Effect of the treatment of mice with lithium chloride (10 mg/kg, p.o.) or AR-A014418 (0.01 μg/site, i.c.v.) in combination with a sub-effective dose of ascorbic acid (0.1 mg/kg, p.o.) in the TST (panel A and C) and in the open-field test (panel B and D). Values are expressed as mean + S.E.M of 7–8 mice. $^{**}p < 0.01$ compared with the vehicle-treated control group.

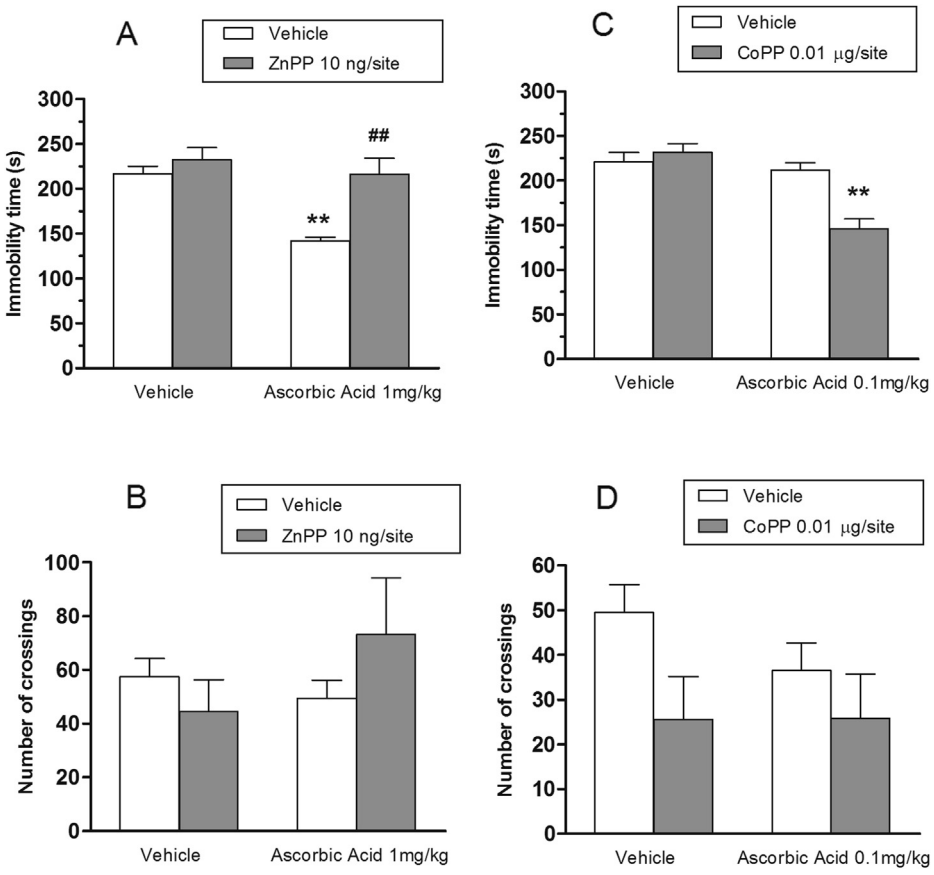


**Fig. 3.** Panel A and B show the effect of the treatment of mice with ZnPP (10 ng/site, i.c.v.) on the ascorbic acid-induced (1 mg/kg, p.o.) antidepressant-like effect in the TST and on locomotor activity in the open-field test, respectively. Panel C and D illustrate the effect of the treatment of mice with CoPP (0.01 μg/site, i.c.v.) in combination with a sub-effective dose of ascorbic acid (0.1 mg/kg, p.o.) in the TST and in the open-field test, respectively. Values are expressed as mean + S.E.M of 6–8 mice. $^{**}p < 0.01$ compared with the vehicle-treated control group. $\#\#p < 0.01$ compared with ascorbic acid-treated group.

$p < 0.01$], ZnPP treatment [$F(1,27) = 14.680$; $p < 0.01$] and ascorbic acid treatment × ZnPP interaction [$F(1,27) = 6.064$; $p < 0.05$]. Post-hoc analysis showed that the antidepressant-like effect of ascorbic acid was completely prevented by treatment of animals with the HO-1 inhibitor ZnPP. Fig. 3B shows that the administration of ZnPP alone or in combination with ascorbic acid was devoid of effect in the open-field test (ascorbic acid treatment [$F(1,25) = 0.206$; $p > 0.05$], ZnPP treatment [$F(1,25) = 0.789$; $p > 0.05$], ascorbic acid treatment × ZnPP interaction [$F(1,25) = 2.477$; $p > 0.05$]).

To confirm the hypothesis that HO-1 is involved in the antidepressant-like effect of ascorbic acid in the TST, we treated the animals with sub-effective doses of ascorbic acid (0.1 mg/kg, p.o.) and the HO-1 inducer CoPP (0.01 μg/site, i.c.v.). The two-way ANOVA revealed significant differences for ascorbic acid treatment [$F(1,28) = 7.687$; $p < 0.01$], CoPP treatment [$F(1,28) = 22.814$; $p < 0.01$] and ascorbic acid treatment × CoPP interaction [$F(1,28) = 14.775$; $p < 0.01$]. As represented in Fig. 3C, post-hoc analysis indicated that the combined administration of sub-effective doses of ascorbic acid and CoPP produced an antidepressant-like effect in the TST. Fig. 3D shows that the administration of CoPP alone or in combination with ascorbic acid did not produce alterations in the locomotor activity of mice (ascorbic acid treatment [$F(1,27) = 4.601$; $p < 0.05$], CoPP treatment [$F(1,27) = 0.629$; $p > 0.05$], ascorbic acid treatment × CoPP interaction [$F(1,27) = 0.664$; $p > 0.05$]).

Considering that PI3K and GSK-3β, upstream targets of mTOR, are required for the antidepressant-like effects of ascorbic acid, we decided to examine whether the behavioral effect of this compound in the TST is dependent on the activation of mTOR. As shown in Fig. 4A, the treatment of mice with a selective mTOR inhibitor, rapamycin (0.2 nmol/site, i.c.v.) totally prevented the antidepressant-like effect elicited by ascorbic acid ([$F(1,27) = 30.863$; $p < 0.01$ for ascorbic acid treatment, ([$F(1,27) = 13.683$; $p < 0.01$ for rapamycin treatment and [$F(1,27) = 11.625$; $p < 0.01$] for ascorbic acid treatment × rapamycin interaction). Fig. 4B illustrates the effects of administration of ascorbic acid and rapamycin in the locomotor activity of mice. None of the compounds, alone or in combination, modified the locomotion of the animals in the open-field test (ascorbic acid treatment [$F(1,27) = 0.048$; $p > 0.05$], rapamycin treatment [$F(1,27) = 0.098$; $p > 0.05$], ascorbic acid treatment × rapamycin interaction [$F(1,27) = 0.534$; $p > 0.05$]).

### 3.2. Biochemical observations

Using the Western blot analysis, we investigated if the hippocampal phosphorylation of p70S6K (a downstream target of mTOR) and the immunocontent of PSD-95 (a protein required for synaptic plasticity associated with NMDA receptor signaling) were affected 1 h after an acute administration of ascorbic acid (1 mg/kg, p.o., dose that produces an antidepressant-like effect in the TST). As determined by Student's *t* test, 1 h after administration, ascorbic acid was able to increase the phosphorylation of p70S6K ($p < 0.05$) and the immunocontent of PSD-95 ($p < 0.05$) in the hippocampus of mice, as illustrated in Fig. 5A and B, respectively.

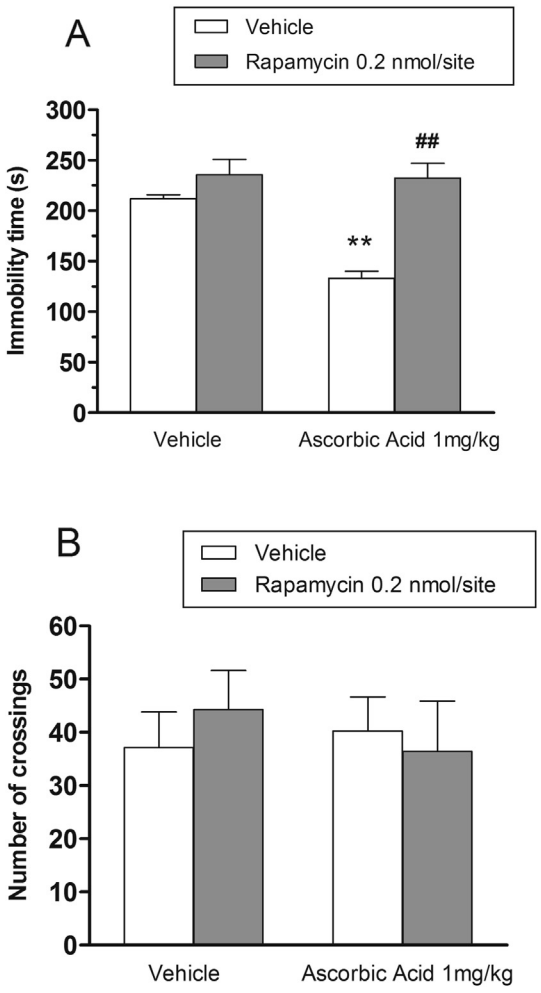


**Fig. 4.** Effect of the treatment of mice with rapamycin (0.2 nmol/site, i.c.v.) on the ascorbic acid-induced (1 mg/kg, p.o.) antidepressant-like effect in the TST (panel A) and on locomotor activity in the open-field test (panel B). Values are expressed as mean + S.E.M of 7–8 mice. $^{**}p < 0.01$ compared with the vehicle-treated control group. $\#\#p < 0.01$ compared with ascorbic acid-treated group.

## 4. Discussion

In the present study, we provided the first evidence that the antidepressant-like effect of ascorbic acid in the TST involves PI3K activation, GSK-3β inhibition, increased mTOR signaling, as well as HO-1 activation. Moreover, the antidepressant effect elicited by ascorbic is associated with increased hippocampal phosphorylation of p70S6K and increased levels of PSD-95 in mice.

The first reports demonstrating antidepressant properties of ascorbic acid are derived from clinical studies. A case-report showed that high intravenous doses of ascorbic acid (50 mg/kg/day) relieved adrenocorticotropic hormone-induced depression in a child (Cocchi et al., 1980). After that, it was reported that this compound was able to decrease scores in a Beck Depression Inventory in healthy young adults (Brody, 2002), an indicative of mood improvement. High dietary intake of vitamin C was also associated with lower depressive symptoms in an elderly population (Oishi et al., 2009). Moreover it was recently demonstrated that orally administered vitamin C as an adjunct to fluoxetine administration leads to significantly greater decrease in depressive symptoms compared to fluoxetine treatment alone (Amr et al., 2013). Consistent with these findings, decreased plasma ascorbic acid levels in major depressive patients were described (Khanzode et al., 2003), suggesting that low levels of endogenous ascorbate may be associated with the pathogenesis of depression. In preclinical models, the administration of ascorbic acid elicited an antidepressant-like effect in the TST (Binfaré et al., 2009), a model of despair universally used to assess the antidepressant activity of new drugs and compounds (Steru et al., 1985). Ascorbic acid administration also reversed the depressive-like behavior and brain oxidative damage induced by acute and chronic stress in mice, reinforcing the antidepressant properties of this vitamin (Moretti et al., 2012b, 2013).

Ascorbate is maintained at elevated concentrations in neurons, where it facilitates the release of some neurotransmitters, such as noradrenaline and acetylcholine (Kuo et al., 1978) and inhibits neurotransmitter binding to glutamatergic receptors, including NMDA (Majewska et al., 1990; Rebec and Pierce, 1994; Rosa et al., 2005; Zuo et al., 2006). Accordingly, the mechanism by which ascorbic acid exerts its antidepressant-like effect in mice is dependent, at least in part, on the NMDA receptor inhibition (Moretti et al., 2011). Furthermore, our group also reported that the antidepressant-like effect of ascorbic acid in the TST involves the modulation of monoaminergic systems (Binfaré et al., 2009), L-arginine-nitric oxide-cyclic guanosine monophosphate pathway (Moretti et al., 2011) and potassium channels (Moretti et al., 2012a). The present study extends these data regarding the antidepressant-like effect of ascorbic acid by exploring, for the first time, the intracellular pathways involved in its antidepressant properties, adding new contributions to this particular topic of investigation.

The PI3Ks are known for regulating signaling cascades mainly associated with cellular growth and survival (Cantley, 2002). Once activated, PI3K catalyzes the production of phosphatidylinositol-3,4,5-triphosphate, which allows the recruitment of signaling proteins, including Akt (Kennedy et al., 1997). Phosphorylated Akt, in turn inactivates GSK-3β, a constitutively active kinase that is inhibited by the phosphorylation of the Ser9 residue. The PI3K/Akt/GSK-3β pathway is implicated in the pathophysiology of depression and in the antidepressant effect of different compounds. For instance, a decrease in the expression and activity of PI3K was observed in patients who committed suicide (Dwivedi et al., 2008). Additionally, post-mortem samples from major depressive patients showed an increased GSK-3β activity in ventral prefrontal cortex area as well as a higher hippocampal level of GSK-3β mRNA (Karege et al., 2007; Oh et al., 2010).

Regarding the involvement of PI3K/Akt/GSK-3β pathway in the antidepressant therapy, it was reported that trefoil factor 3, folic acid and guanosine have their antidepressant-like effect dependent on activation of PI3K/Akt signaling pathway and/or inhibition of GSK-3β (Bettio et al., 2012; Budni et al., 2012; Shi et al., 2012). In agreement with these findings, rodents treated with GSK-3 inhibitors showed reduced duration of immobility when exposed to the forced swimming test (Gould et al., 2004; Kaidanovich-Beilin et al., 2004; Rosa et al., 2008), pointing to a potential role of GSK-3 inhibitors as antidepressants. Outstandingly, it was demonstrated that GSK-3 inhibition potentiates the synaptogenic and antidepressant-like effects of subthreshold doses of ketamine (Liu et al., 2013) and that inhibition of GSK-3β is necessary for the rapid antidepressant effect of this compound in mice (Beurel et al., 2011). Our results are in line with literature data, since it consistently demonstrated that the antidepressant-like of ascorbic acid involves the activation of PI3K/Akt and inhibition of GSK-3β. It is important to mention that vitamin C supplementation in rats was previously reported to restore decreased levels of phosphorylated Akt induced by diabetes (Badr et al., 2012), a finding which is in agreement with our results.

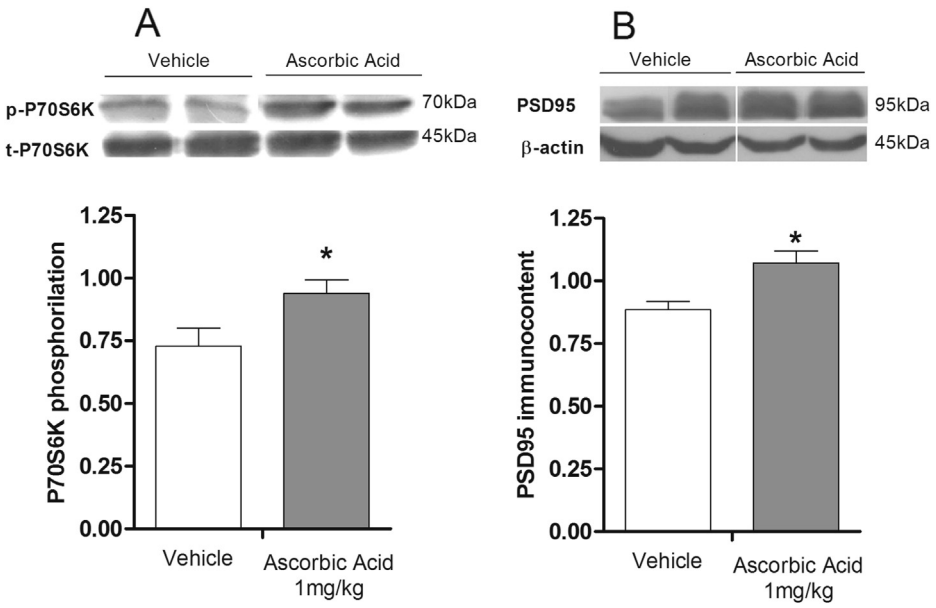


**Fig. 5.** Effect of treatment with ascorbic acid (1 mg/kg, p.o.) on p70S6K phosphorylation (panel A) and PSD-95 immunocontent (panel B) in hippocampus of mice. The results are expressed as absolute values and expressed as mean + S.E.M of 5–8 mice. $^{*}p < 0.05$ compared with the vehicle-treated control group.

Here, we also showed that the antidepressant-like effect of ascorbic acid is dependent, at least partially, on the induction of HO-1. This is the rate-limiting enzyme that degrades the pro-oxidant heme group and produces equimolecular quantities of carbon monoxide, iron, and biliverdin. Biliverdin is subsequently reduced to bilirubin by biliverdin reductase. The increased levels of these products as a consequence of improved HO activity have been related to cell protection against oxidative stress in distinct models (Dal-Cim et al., 2012; Gozzelino et al., 2010; Kocanova et al., 2007); in the brain, induction of HO-1 protected cells from oxidative damage induced by cerebral ischemia (Takizawa et al., 1998) and ethanol-induced intoxication (Ku et al., 2006). Concerning the modulation of HO-1 by ascorbic acid, it was previously demonstrated that this vitamin protects gastric epithelial cells by translating HO-1 mRNA into the active protein (Becker et al., 2003). However, to our knowledge, no study investigated whether the antidepressant-like effects of ascorbic are mediated by HO-1 activation in the brain. A recent study demonstrated that ascorbic acid attenuates methamphetamine-induced reactive oxygen species production and neurotoxicity by a mechanism dependent on induction of HO-1 (Huang et al., 2012). Our findings corroborate these data and shed light on this topic by showing that the antidepressant-like effect of ascorbic depends on the activation of HO-1, most likely via PI3K/Akt/GSK-3β/Nrf-2 pathway. In addition, our results reinforce the idea that compounds showing antioxidant properties may constitute a new strategy in the management of depressive symptoms.

In view of the prevention of the antidepressant-like effect of ascorbic acid elicited by rapamycin, our results also point to an important role for mTOR activation in the antidepressant-like effect of this vitamin. It was recently demonstrated that subjects diagnosed with major depressive disorder exhibited robust deficits in the mTOR signaling in the prefrontal cortex (Jernigan et al., 2011). Similarly, rats exposed to the chronic unpredictable stress model of depression had a significant decrease in phosphorylation levels of mTOR and its downstream signaling components in the amygdala (Chandran et al., 2013). Together, these results suggest that deficits in mTOR phosphorylation and mTOR-dependent protein synthesis are possibly implicated in the molecular alterations associated with the pathophysiology of depression. Conversely, mTOR signaling activation was demonstrated to be required for antidepressant-like effects of the NMDA receptor antagonist ketamine, the selective NMDA receptor 2B antagonist Ro 25-6981 (Li et al., 2011), the metabotropic glutamate receptor subtype 2/3 antagonists LY341495 and MGS0039 (Koike et al., 2011) and the nucleoside guanosine (Bettio et al., 2012) in animal models predictive of antidepressant activity. Altogether, these findings allow us to hypothesize that an increase in mTOR phosphorylation and subsequent enhanced mTOR-dependent protein synthesis may underlie antidepressant action of ascorbic acid. Supporting this assumption, it was previously shown that ascorbic acid can restore the down-regulated phosphorylation of the mTOR-dependent translation initiation factors S6 kinase-1 and 4E binding protein-1 in myotubes treated with a combination of LPS and interferon-γ (Frost et al., 2011).

Our data indicating that the antidepressant-like effect of ascorbic acid is dependent on activation of mTOR is reinforced by Western blot results. We showed that, 1 h after its administration, ascorbic acid was able to modulate two well-known downstream targets of mTOR: it increased the phosphorylation of p70S6K and the immunocontent of PSD-95 in the hippocampus of mice. Deficits in mTOR-dependent translation initiation, particularly via the p70S6K/eIF4B pathway, were reported in the prefrontal cortex of depressed subjects (Jernigan et al., 2011). In addition, postmortem studies have shown a decreased expression of prominent post-synaptic proteins involved in glutamate neurotransmission, including PSD-95, in the prefrontal cortex from depressed individuals (Deschwanden et al, 2011; Feyissa et al., 2009). In agreement with these observations, the antidepressant effects of ketamine and LY341495 (an $mGluR_{2/3}$ antagonist) are associated with increased phosphorylation of p70S6K and expression of synaptic protein PSD-95 (Dwyer et al., 2012; Li et al., 2010). These findings support our data and allow us to suggest an important role for mTOR pathway in the antidepressant-like effect of ascorbic acid.

Collectively, our results suggest that ascorbic acid produces biochemical alterations comparable to the ones elicited by ketamine, indicating that the antidepressant action of these agents share some common mechanisms. Besides, the results showed

herein reinforce the PI3K/Akt/GSK-3β/mTOR pathway as an important target for development of novel and efficacious antidepressant agents and extends literature data regarding the mechanisms underlying the antidepressant-like action of ascorbic acid.

## Disclosure/conflict of interest

The authors declare that no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

## Role of funding source

No external funding was used for this study.

## Contributors

Ana Lúcia S. Rodrigues and Morgana Moretti designed the study and wrote the protocol. Morgana Moretti, Josiane Budni, Andiara Espíndola Freitas and Priscila Batista Rosa administered the drugs and performed the behavioral tests. Morgana Moretti performed the biochemical analysis, undertook the statistical analysis and wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

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