Pharmacology, Biochemistry and Behavior 187 (2019) 172800

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

A single coadministration of subeffective doses of ascorbic acid and ketamine reverses the depressive-like behavior induced by chronic unpredictable stress in mice

Morgana Moretti[a,*], Isabel Werle[a], Priscila Batista da Rosa[a], Vivian Binder Neis[a], Nicolle Platt[a], Suene V.S. Souza[b], Ana Lúcia S. Rodrigues[a]

[a] Department of Biochemistry, Center of Biological Sciences, Universidade Federal de Santa Catarina, Florianópolis, Santa Catarina, Brazil
[b] Post-Graduate Nutrition Program, Center of Health Sciences, Universidade Federal de Santa Catarina, Campus Universitário, Trindade, 88040-900 Florianópolis, SC, Brazil

ARTICLE INFO

Keywords:
Antidepressant
Ascorbic acid
Depression
Ketamine
Stress

ABSTRACT

In this study, we investigated the ability of a single coadministration of subeffective doses of ascorbic acid and ketamine to reverse the depressive-like behavior induced by chronic unpredictable stress (CUS) in mice. Moreover, we examined the effect of combined administration of ascorbic acid and ketamine on hippocampal phosphorylation of p70S6K and immunocontents of GLUA1 and PSD-95 in mice submitted to the CUS procedure. CUS procedure was applied for 21 days. Animals received a single coadministration of subeffective doses of ascorbic acid (0.1 mg/kg) and ketamine (0.1 mg/kg) and were subjected to behavioral evaluation 24 h after the treatments. Immediately after the behavioral observations the hippocampi were dissected for Western blotting analyses. Our results revealed that a single administration of subeffective doses of ascorbic acid and ketamine completely reversed the depressive-like behavior induced by CUS, however, this effect was not accompanied by changes in the phosphorylation of p70S6K and immunocontent of GLUA1 or PSD95 in the hippocampus. These findings point to a synergistic antidepressant-like effect of ascorbic acid and ketamine, paving the way for additional studies on the combined use of these compounds for the management of major depressive disorder (MDD).

1. Introduction

Major depressive disorder (MDD) is associated with profound socioeconomic burden and has a substantial negative impact on functioning and quality of life of the affected patients (Nemeroff, 2007). The management of MDD is still a challenge, and even though numerous pharmacological and psychological interventions for this mood disorder have demonstrated efficacy, a significant number of patients do not respond to standard treatment and many individuals exhibit refractory or intolerant responses, making imperative the optimization of the treatment.

There is an increasing interest in diet quality to prevent or even attenuating the symptoms of some psychiatric disorders and several studies have shown that some nutraceuticals are promising agents to be used alone or in combination with the current antidepressant agents to afford a better therapeutic profile (Manosso et al., 2013). Compounds including ascorbic acid, folic acid and zinc have neuromodulatory properties that mediate their neuroprotective and antidepressant effects (Brocardo et al., 2008; Budni et al., 2012; Manosso et al., 2015; Manosso et al., 2016; Moretti et al., 2017). Importantly, these nutraceuticals are in general safe, inexpensive and present few side effects for most individuals.

It has been reported that ascorbic acid has antidepressant-like effect in mice. Similar to the classical antidepressant fluoxetine, it reversed the depressive-like behavior induced by chronic unpredictable stress (CUS), restraint stress and pro-inflammatory cytokine administration in mice (Moretti et al., 2012a; Moretti et al., 2013; Moretti et al., 2015). The mechanism by which ascorbic acid exerts its antidepressant-like effect in mice is dependent, at least in part, on the modulation of GABAA, GABAB (Rosa et al., 2016), and opioid receptors (Moretti et al., 2018), inhibition of NMDA receptors, L-arginine-nitric oxide-cyclic guanosine monophosphate pathway (Moretti et al., 2011) and

* Corresponding author at: Department of Biochemistry, Center of Biological Sciences, Universidade Federal de Santa Catarina, Campus Universitário, Trindade, 88040-900 Florianópolis, SC, Brazil.
E-mail address: morgana.moretti@ufsc.br (M. Moretti).

https://doi.org/10.1016/j.pbb.2019.172800
Received 5 August 2019; Received in revised form 4 October 2019; Accepted 10 October 2019
Available online 01 November 2019
0091-3057/

Table 1
Schedule of stressor agents used in the 21 days of chronic stressful stimuli.

	Week 1	Week 2	Week 3
Day 1	Damp bedding (24 h)	Cage tilt (45°, 24 h)	Inescapable shock (0.7 mA, 3–3 s, 5 min)
Day 2	Paired housing (1 h)	Stroboscopic light (3 h)	Apparatus exposure, no footshock (1 h)
Day 3	Cold bath (15 °C, 20 min)	Tail pinch (10 min)	Stroboscopic light (3 h)
Day 4	Restraint stress (2 h)	Light/dark cycle inversion, removal of bedding (12 h)	Tail pinch (10 min)
Day 5	Inescapable shock (0.7 mA, 3–3 s, 5 min)	Paired housing (1 h)	Restraint stress (4 h)
Day 6	Apparatus exposure, no footshock (1 h)	Cold bath (15 °C, 20 min)	Light/dark cycle inversion, removal of bedding (12 h)
Day 7	Damp bedding (24 h)	Restraint stress (3 h)	Cage tilt (45°, 24 h)

potassium channels (Moretti et al., 2012, as well as activation of mTOR pathway in the brain (Moretti et al., 2014). Interestingly, ascorbic acid has positive effect on mood of healthy individuals (Brody, 2002) and is able to reduce depressive symptoms in pediatric and adult patients when administered alone (Cocchi et al., 1980) or combined with antidepressants (Amr et al., 2013, Aburawi et al., 2014).

One of the most important discovery in the field of neurobiology of depression is the rapid and sustained antidepressant effect elicited by ketamine, an NMDA receptor antagonist that has been used to treat refractory MDD and suicidal ideation (Zarate et al., 2006; Ballard et al., 2014). In accordance with clinical findings, studies have shown that a single dose of ketamine rapidly reverses the depressive-like behavior and synaptic deficits in pyramidal neurons in the medial prefrontal cortex of rats subjected to 3 weeks of chronic stress exposure (Li et al., 2010; Li et al., 2011). The fast antidepressant response of ketamine is associated with modulation of the glutamatergic system, mTOR pathway and increased synaptogenesis (Li et al., 2010; Duman et al., 2012), a similar molecular pathway modulated by ascorbic acid (Moretti et al., 2011; Moretti et al., 2014). Considering that the use of ketamine for the treatment of MDD is restricted by its psychotomimetic and dissociative side effects as well as abuse potential, efforts are under way to develop ketamine-like treatments with reduced side effects. This scenario encourages more studies that aim at comparing the mechanisms associated with the antidepressant-like effect of ascorbic acid and ketamine and to investigate possible synergistic antidepressant effect after administration of these compounds in animal models of depression. Our main hypothesis is that a single administration of subeffective doses (that not exhibit antidepressant effect *per se*) of ascorbic acid plus ketamine would promote behavioral and neurochemical changes in mice submitted to a model of depression induced by CUS.

2. Methods

2.1. Animals

Considering that MDD is roughly twice prevalent in women compared to men (Wong and Licinio, 2001) and that females are reported to be more susceptible to stress than males (Yoshimura et al., 2003), this study was performed using female Swiss mice (30–35 g, 60–70 days of age) from the Central Animal Care Facility of the Federal University of Santa Catarina (Florianópolis, SC, Brazil). The animals were maintained at 20–22 °C with free access to water and food, under a 12/12 h light-dark cycle (lights on at 07:00 h). Mice were caged in groups of 10–12 in a 41x34x16 cm cage. All manipulations were made between 9:00 and 17:00 h. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Experimental protocols were approved by the Institutional Ethics Committee and all efforts were made to minimize animal suffering.

2.2. Chemicals

Ascorbic acid and ketamine were obtained from Sigma Chemical Co., St. Louis, U.S.A. The antibodies anti-phospho-P70S6K (#9205S), anti-total-P70S6K (#9202S), anti-PSD95 (#2507S), anti-GluA1 (#13185S) and anti-β-actin (#4970S), as well as LumiGLO reagent (luminol chemiluminescent substrate) were purchased from Cell Signaling Technology (Beverly, MA, USA). The secondary antibody mouse anti-rabbit IgG HRP (horseradish peroxidase, sc-2357) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Acrylamide, bis-acrylamide, β-mercaptoethanol, Hybond™ nitrocellulose, Amersham Hyperfilm™, sodium dodecyl sulfate (SDS) and Tris were obtained from GE Healthcare Life Sciences (Piscataway, NJ, USA).

2.3. Chronic unpredictable stress (CUS) protocol

CUS protocol was adapted from Mineur et al. (2006) and consisted of a variety of stressors randomly applied at different times of day for 21 days to prevent habituation (Table 1). Mice were divided into control (non-stressed) and stressed groups; control mice were kept in their home cages according to conditions previously reported. Control and stressed mice were weighed once a week during the twenty-one days of procedure.

2.4. Pharmacological treatment

Two hours after the last stress exposure (day 21), control and stressed animals received a single coadministration of subeffective doses of ascorbic acid (0.1 mg/kg) and ketamine (0.1 mg/kg) and were subjected to behavioral evaluation 24 h after the treatments. The subeffective doses of ascorbic acid and ketamine were chosen based on previous studies of our group (Binfare et al., 2009; Rosa et al., 2016); we demonstrated that, *per se*, these doses of ascorbic acid or ketamine (acutely administered) do not reduce the immobility time in the tail suspension test. Ascorbic acid was dissolved in distilled water and administered orally (p.o.). Ketamine was dissolved in isotonic saline solution (NaCl 0.9%) and administered by intraperitoneal route (i.p.). Appropriate vehicle-treated groups were also assessed simultaneously. Ascorbic acid and ketamine solutions were freshly prepared before administration. Fig. 1 shows a schematic representation of the treatment protocol and behavioral evaluation.

2.5. Behavioral analysis

Tail suspension test, open-field test and splash test were performed in the same group of animals, 10 min apart. Data from our laboratory do not show significant differences in the performance of control animals subsequently exposed to these tests compared with mice evaluated in independent groups.

2.5.1. Tail suspension test (TST)

The TST was performed 24 h after drug administrations. The total duration of immobility induced by tail suspension was measured according to the method described by Steru et al. (1985). Briefly, 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

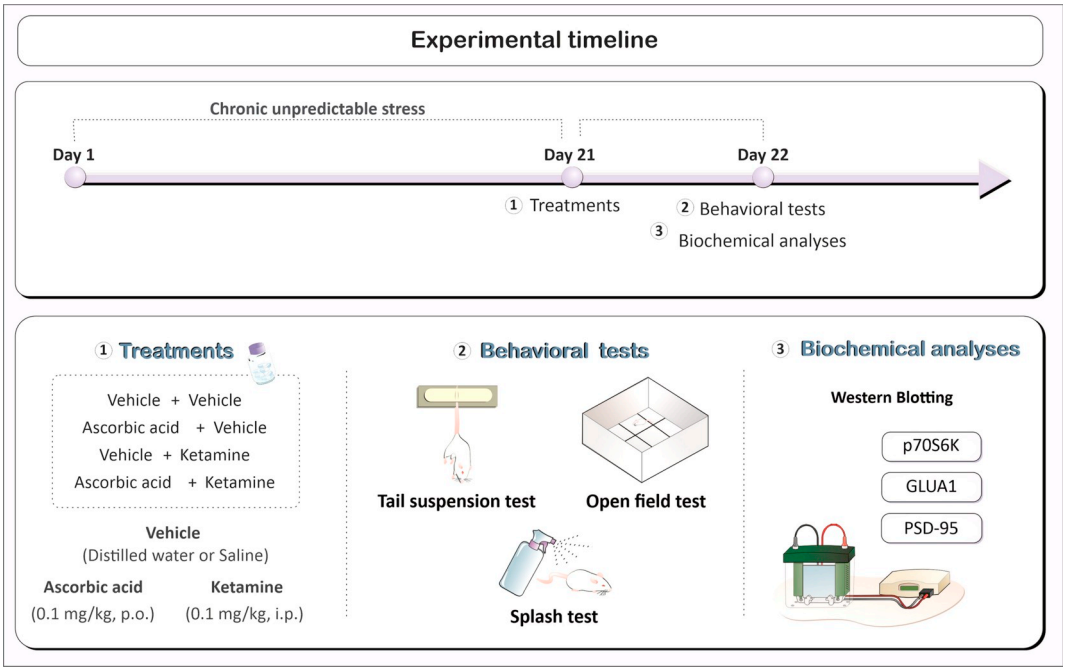

Fig. 1. Schematic representation of the treatment protocol, behavioral and biochemical evaluations.

completely motionless. Immobility time was manually recorded during a 6-min period by an experienced observer. The observer was in the room where the test was performed and was blind to the animal condition (Kaster et al., 2012). The animals were placed in the experimental room 24 h before the test for acclimatization.

2.5.2. Open-field test

Five minutes after the TST, mice were evaluated in the open-field paradigm, as previously described (Moretti et al., 2011b). Animals were individually placed in a wooden box measuring 40 × 60 × 50 cm high, with the floor of the arena divided into 12 rectangles. The number of rectangles crossed with all paws (crossing) was manually counted in a 6-min session. The apparatus was cleaned with a solution of 10% ethanol between tests in order to hide animal clues.

2.5.3. Splash test

Ten minutes after the open-field test the splash test was carried out. This test was carried out as described by Moretti et al., 2012a, 2012b and consists of squirting a 10% sucrose solution on the dorsal coat of a mouse placed individually in clear Plexiglas boxes (9 × 7 × 11 cm). Because of its viscosity, the sucrose solution dirties the mouse fur and animals initiate grooming behavior. After applying sucrose solution, the time spent grooming was manually recorded for a period of 5 min as an index of self-care and motivational behavior, considered to be parallel with some symptoms of depression such as apathetic behavior. The apparatus was cleaned with a solution of 10% ethanol between tests in order to hide animal clues.

2.6. Estrous cycle identification

Immediately after behavioral observations, vaginal secretion of mice was collected, and the estrous cycle phase of each animal was determined by cytology. The determination of each phase is based on the proportion among three types of cells observed in the vaginal smear: epithelial cells, cornified cells and leukocytes. A proestrus smear consists of a predominance of nucleated epithelial cells; an estrous smear primarily consists of anucleated cornified cells; a metestrus smear consists of the same proportion among leukocytes, cornified, and nucleated epithelial cells, and a diestrus smear primarily consists of a predominance of leukocytes (Byers et al., 2012).

2.7. Sample preparations and Western blotting analyses

Immediately after the estrous cycle characterization, mice were decapitated, the hippocampi were dissected and placed in liquid nitrogen and stored at −80 °C until used for Western blotting analyses, as described previously (Moretti et al., 2015; Neis et al., 2016). Briefly, samples were mechanically homogenized in 400 µl of TRIS 50 mM pH 7.0, EDTA 1 mM, NaF 100 mM, PMSF 0.1 mM, Na_3VO_4 2 mM, Triton X-100 1%, glycerol 10%, Sigma Protease Inhibitor Cocktail (P2714), and then incubated for 10 min in ice. Lysates were centrifuged (10,000 ×*g* for 10 min, at 4 °C) to eliminate cellular debris. The supernatants were diluted 1/1 (v/v) in TRIS 100 mM pH 6.8, EDTA 4 mM, SDS 8% and boiled for 5 min. Thereafter, sample dilution (40% glicerol, TRIS 100 mM, bromophenol blue, pH 6.8) in the ratio 25:100 (v/v) and β-mercaptoethanol (final concentration 8%) were added on the samples. Protein content was estimated at 620 nm wavelength and the concentration calculated using a standard curve with bovine serum albumin as standard (Peterson, 1977).

The same amount of protein (60 µg per lane) for each sample was electrophoresed in SDS–PAGE minigels (10% acrylamide) and 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). To verify transfer efficiency process, gels were stained with Coomassie blue and membranes with Ponceau S.

The membranes were blocked with 5% skim milk in TBS (TRIS 10 mM, NaCl 150 mM, pH 7.5). Proteins were detected using specific antibodies incubated overnight diluted in TBS-T (Tris 10 mM, NaCl 150 mM, 0.1% Tween-20, pH 7.5) containing 2.5% BSA in the dilutions 1:1.000. All membranes were incubated with rabbit anti-β-actin (1:2000) antibody to verify that equal amounts of proteins were loaded on the gel. Next, the membranes were incubated with peroxidase-linked secondary antibody (1:2500) 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 the Image Lab™ Software. The phosphorylation levels of P70S6K were

determined as a ratio of the O.D. of the phosphorylated band and the O.D. of the total band. The immunocontent of PSD95 and GLUA1 as well as the total immunocontent of P70S6K were determined from the relationship between the O.D. of these protein bands and the O.D. of the β-actin band. Data were expressed as percentage of the control (considered as 100%).

2.8. Statistical analysis

The Kolmogorov–Smirnov test was used to evaluate the normality assumption of behavioral and biochemical data. All variables in the present study showed a normal distribution. Comparisons between experimental and control groups were performed by two-way ANOVA followed by Duncan's multiple range post hoc test. Repeated measures ANOVA was used to analyze the weight gain through the 3-week protocol. Statistica 7.0 software was used to perform the analysis. GraphPad Prism 6 software was used to create the artwork. All experimental results are given as the mean + S.E.M. A value of $p < .05$ was considered to be significant.

3. Results

3.1. Immobility time in TST

Fig. 2A shows the effect of combined administration of subeffective doses of ascorbic acid and ketamine on the immobility time in the TST in control and stressed mice. The two-way ANOVA revealed significant differences for CUS procedure and for treatments, but not for CUS procedure × treatments interaction, as shown in Table 2. In control (non-stressed) animals, post hoc analysis indicated that treatment with a subeffective dose of ascorbic acid combined with a subeffective dose of ketamine decreased the immobility time in the TST, whereas the administration of drugs alone presented no effect. Exposure to different stressors for 21 days significantly increased the immobility time in the TST as compared to control mice. In stressed mice, combined treatment with ascorbic acid and ketamine produced a synergistic antidepressant-like effect, since it reversed the increased immobility time induced by CUS.

3.2. Locomotor activity

The open field test was carried out to investigate the effect of CUS and/or treatments in the locomotor activity of mice. As detailed in Table 2, the two-way ANOVA revealed significant differences for CUS procedure, but not for treatments or CUS procedure × treatments interaction. Post-hoc analysis showed that CUS protocol increased the locomotor activity of mice, independent on drug administration (Fig. 2B).

3.3. Splash test

Fig. 2C shows the effect of combined administration of subeffective doses of ascorbic acid and ketamine on total time spent grooming in control and stressed mice. The two-way ANOVA revealed a main effect for CUS procedure, but not for treatments or CUS procedure × treatments interaction, as demonstrated in Table 2. However, post-hoc analysis did not indicate significant differences between CUS and control groups.

3.4. Body weight

The repeated measures ANOVA revealed a significant main effect for CUS procedure, but not for treatments or CUS procedure × treatments interaction. Post-hoc analysis showed that CUS exposure reduced the mice weight gain, independent on drug administration (Fig. 3A). Fig. 3B shows the total weight gain of mice over the 21 days of our experimental protocol. The two-way ANOVA showed a significant main effect for CUS procedure, but not for treatments or CUS procedure × treatments interaction (Table 2). Post-hoc analysis showed that stressed mice had a decreased weight gain, independent on drug administration.

3.5. P70S6K phosphorylation and immunocontent of GLUA1 and PSD95

Fig. 4 shows representative Western blots of the effect of the combined treatment with ascorbic acid and ketamine on hippocampal immunocontent of proteins involved in synaptogenesis after CUS exposure. Densitometric analysis revealed no changes in P70S6K phosphorylation as well as total P70S6K immunocontent (Fig. 3A, Table 2) The two-way ANOVA also did not find significant differences in the immunocontent of GLUA1 (Fig. 4B, Table 2) and PSD95 (Fig. 4C, Table 2).

4. Discussion

Considering that ascorbic acid acts as a neuromodulator, induces an antidepressant-like behavior in the TST (a predictive test for depression) (Binfare et al., 2009; Moretti et al., 2012; Moretti et al., 2014) and improves mood in some clinical studies (Rice, 2000; Brody, 2002; Amr et al., 2013), our group has focused on the investigation of its antidepressant mechanisms in animal models. In line with the newest discoveries in the field of neurobiology of depression and the rapid antidepressant effect elicited by ketamine, we are particularly interested in the comparative investigation of behavior and brain signaling pathways associated with rapid antidepressant effect after ketamine and ascorbic acid administration. The results presented herein show that a single coadministration of subeffective doses of ascorbic acid and ketamine abrogated the depressive-like behavior induced by CUS in female mice.

Although MDD is a multifactorial and heterogeneous disorder, it is well known that stress is an environmental risk factor associated with the onset of the disease (Krishnan and Nestler, 2008). Accordingly,

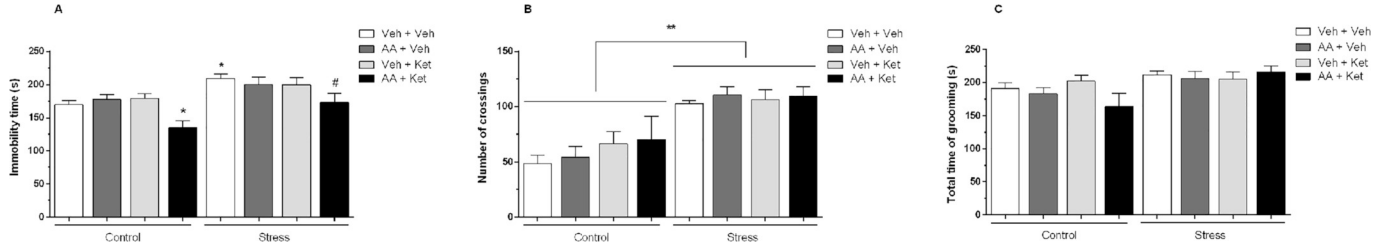

Fig. 2. Effect of combined administration of subeffective doses of ascorbic acid (0.1 mg/kg, p.o.) and ketamine (0.1 mg/kg, i.p) in animals subjected to CUS. Panel A shows the immobility time in the mouse TST, panel B shows the locomotor activity in the open field test and panel C shows the time spent grooming in mice submitted to CUS procedure. Each column represents the mean + S.E.M. of 6–9 animals. Statistical analysis was performed by two-way ANOVA, followed by Duncan's test. $^{*}p < .05$ and $^{**}p < .01$ compared with the control group. $^{\#}p < .05$ compared with the CUS + vehicle group; $^{\#\#}p < .01$ compared with non-stressed group.

Table 2
Significance results of the two-way ANOVA.

	CUS	Treatments	CUS × treatments interaction
Tail suspension test	[F(1,56) = 18.825, $p < .01$]	[F(3,56) = 6.152, $p < .01$]	[F(3,56) = 0.525, $p > .05$]
Open field test	[F(1,56) = 45.848, $p < .01$]	[F(3,56) = 0.771, $p > .05$]	[F(3,56) = 0.425, $p > .05$]
Splash test	[F(1,56) = 4.920, $p < .05$]	[F(3,56) = 1.626, $p > .05$]	[F(3,56) = 1.546, $p > .05$]
Δ Body weight	[F(1,56) = 35.293, $p < .01$]	[F(3,56) = 1.285, $p > .05$]	[F(3,56) = 1.008, $p > .05$]
p-P70S6K	[F(1,16) = 0.111, $p > .05$]	[F(1,16) = 0.438, $p > .05$]	[F(1,16) = 0.012, $p > .05$]
t-P70S6K	[F(1,16) = 0.06, $p > .05$]	[F(1,16) = 0.178, $p > .05$]	[F(1,16) = 0.239, $p > .05$]
GLUA1	[F(1,11) = 0.105, $p > .05$]	[F(1,11) = 0.003, $p > .05$]	[F(1,11) = 0.296, $p > .05$]
PSD95	[F(1,12) = 0.528, $p > .05$]	[F(1,12) = 0.001, $p > .05$]	[F(1,12) = 0.059, $p > .05$]

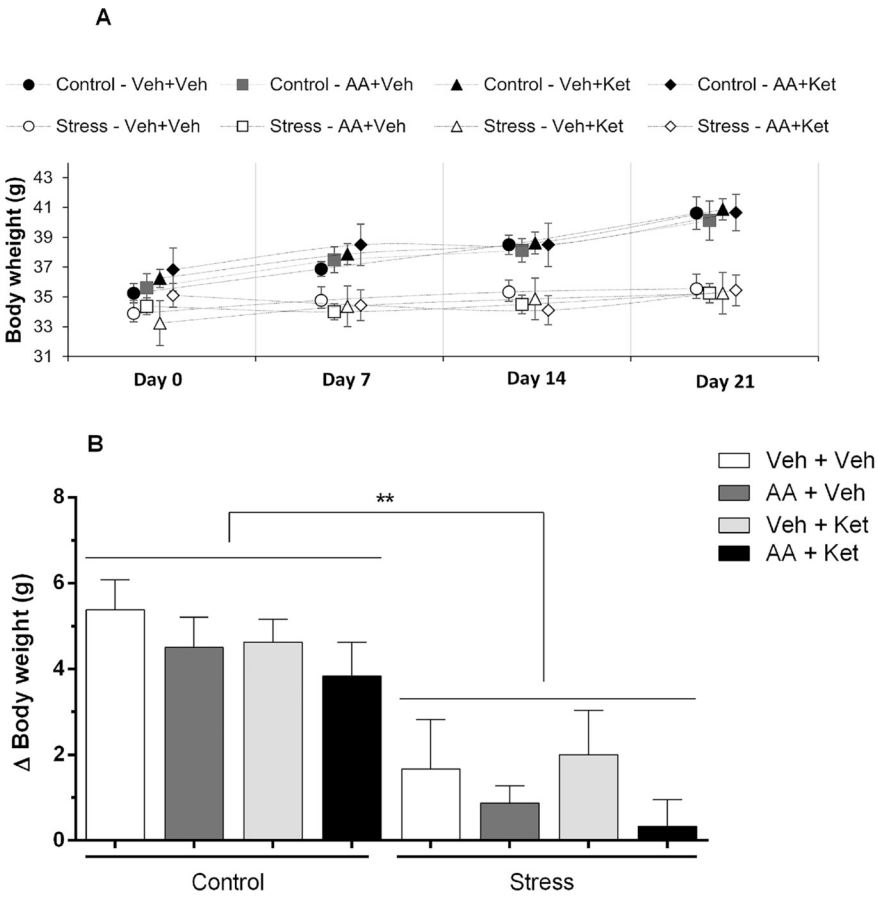

Fig. 3. Effect of combined administration of subeffective doses of ascorbic acid (0.1 mg/kg, p.o.) and ketamine (0.1 mg/kg, i.p) in animals subjected to CUS. Panel A shows the body weight of mice at days 0, 7, 14 and 21 and panel B shows the total weight gain of animals. Statistical analysis was performed by repeated measures ANOVA (Panel A) or two-way ANOVA (Panel B), followed by Duncan's test. Each point or column represents the mean + S.E.M. of 6–9 animals. $^{**}p < .01$ compared with non-stressed group.

numerous studies have demonstrated that mice or rats subjected to chronic stress have increased immobility time in the TST and forced swimming test (Lu et al., 2006; Kumar et al., 2011; Moretti et al., 2012), which is suggestive of depressive like-behavior. The present data agree with these previous findings by showing a depressive-like behavior in mice under chronic and unpredictable stress. The effects of CUS on behavior are typically reversed by the chronic administration of classical antidepressant, such as fluoxetine (Moretti et al., 2012), or by a single administration of subanesthetic and effective dose of the fast-acting antidepressant ketamine (Neis et al., 2016). Here we showed for the first time that the depressive-like behavior induced by CUS was reversed by a single coadministration of subeffective doses of ascorbic acid and ketamine. Previous studies from our group have demonstrated that ascorbic acid shares with ketamine some antidepressant mechanisms, including the activation of mTOR signaling and GABAA receptors as well as inhibition of GABAB receptors (Moretti et al., 2014; Rosa et al., 2016). Furthermore, a double-blind, randomized, placebo-controlled study showed that a single oral administration of ascorbic acid produced a significant anxiolytic effect in subjects with high trait anxiety (Moritz et al., 2017), an interesting finding considering that anxiety and depressive symptoms are frequently comorbid conditions (Kessler et al., 2003).

It is worth to mention that the antidepressant-like effect of ascorbic acid has been previously shown in models of depression induced by tumor necrosis factor-α administration (Moretti et al., 2015), acute restraint stress (Moretti et al., 2013) and a shorter CUS protocol (14 days of stress) (Moretti et al., 2012). Moreover, we have showed that a sub-effective dose of ascorbic acid exerts a synergistic antidepressant-like effect when administered with subeffective doses of the classical antidepressants fluoxetine, imipramine and bupropion (Moretti et al., 2015). The present data revealed that subeffective doses of ascorbic acid and ketamine also elicits an antidepressant-like effect in control animals (not submitted to CUS), a finding that extend the previous data from our group and further suggests similarities between ascorbic acid and ketamine. It is interesting to mention that Dossat et al. (2018) showed that proestrus female mice present a behavioral sensitivity to the antidepressant-like effect of ketamine. In our study we have similar percentage of animals in the same phase of the estrus cycle in

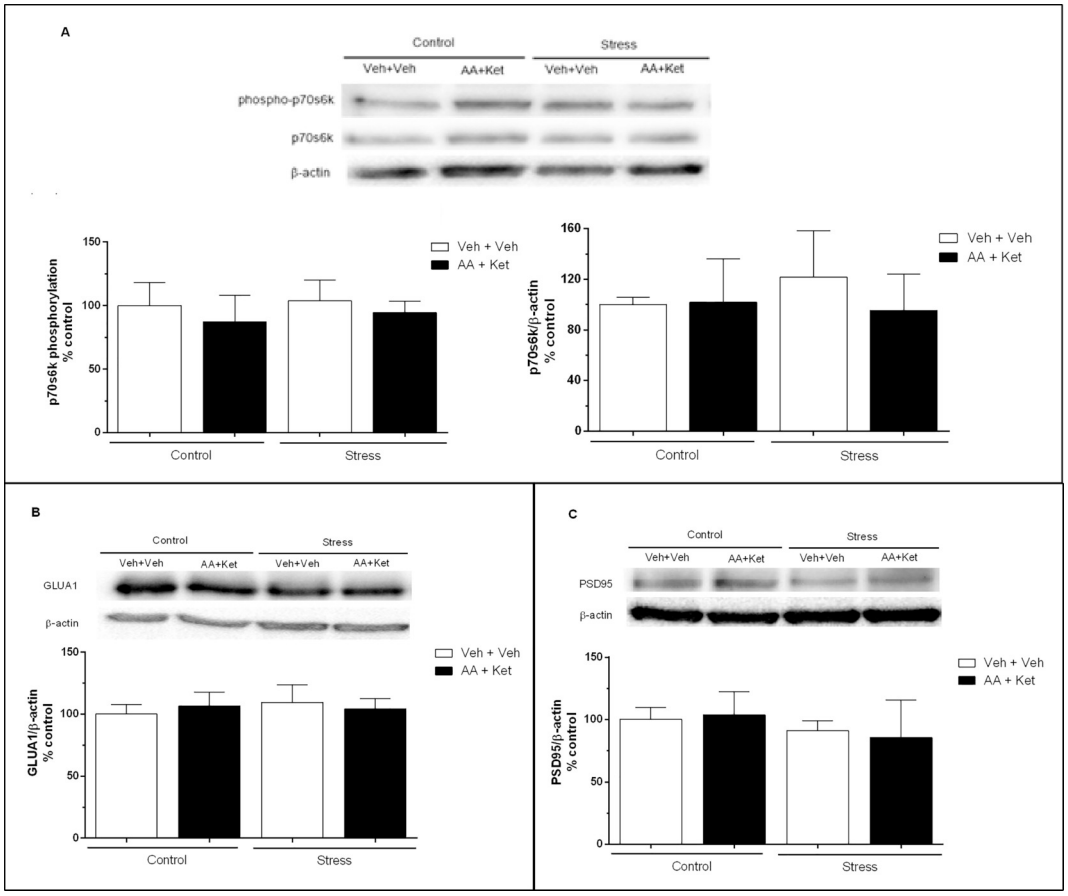

Fig. 4. Effect of combined administration of subeffective doses of ascorbic acid (0.1 mg/kg, p.o.) and ketamine (0.1 mg/kg, i.p) in mice subjected to CUS. Panels A, B and C show the hippocampal phosphorylation of p70S6K and immunocontents of GLUA1 and PSD-95, respectively. Each column represents the mean + S.E.M. of 3–5 animals. Statistical analysis was performed by two-way ANOVA.

the stressed (diestrus = 76.5%/metaestrus = 17.7/estrus = 5.8%) and non-stressed (diestrus = 75%/metaestrus = 19.4/estrus = 5.6%) groups. Moreover, we have no animal in the proestrus phase, which indicate that the antidepressant-like effect of the combined administration of ascorbic acid + ketamine probably is not due to an increased sensibility induced by hormonal changes. The investigation of a possible fast-acting antidepressant mechanism of ascorbic acid administered alone in robust models of depression such as CUS remains to be further explored, but the current results point to a synergistic antidepressant-like effect of ascorbic acid and ketamine, paving the way for additional studies on the combined use of these compounds for the treatment of resistant MDD. The use of ascorbic acid as adjunctive treatment would allow reducing the dose of ketamine, which could improve its tolerability.

Along with alterations in the immobility time in the TST, it is known that CUS exposure usually induces lower sucrose consumption (Willner et al., 1987; Garcia et al., 2009; Li et al., 2011) and decreases self-care and motivational behavior in rodents (Isingrini et al., 2010; Moretti et al., 2012), suggested to reflect anhedonia, a core symptom of MDD. However, in the present study we observed a lack of effect of CUS on anhedonic behavior, as evidenced by the result of splash test. The reasons for this are not completely understood but it can be associated with inbreeding of our mouse colony, which can influence individual responses and behaviors (Jacobson and Cryan, 2007) and be linked to a partial resistance to stress. Recent studies from our group using the same mouse colony showed that a 14-day protocol of chronic unpredictable stress (Neis et al., 2016) as well as chronic corticosterone administration (21 days) (Olescowicz et al., 2018) have failed to produce anhedonic behavior in female Swiss mice, as opposed to previous finding from our group (Moretti et al., 2012; Rosa et al., 2014). Moreover, studies using CUS as a model of depression present numerous variations in experimental conditions, including the duration and intensity of stressors, age, sex and lineage of animals (Gupta et al., 2014; Chen et al., 2017), factors that may account for different results reported in literature. Understanding how these factors are connected to behavioral responses may improve the reliability of behavioral findings among studies. Therefore, future studies are required to elucidate the effect of ascorbic acid and ketamine in behavioral paradigms capable of detecting anhedonic behavior.

Our results also indicated that stress increased the locomotion and decreased de weight gain of mice. Importantly, the hyperlocomotion induced by stress was not modified by any treatment, so this effect probably did not account for the antidepressant-like effect induced by the combined administration of ascorbic acid and ketamine. Moreover, our results are in accordance with previous findings showing that mice subjected to models of depression display a significant increase in locomotor activity (Machado et al., 2012). Stress also caused a reduction in mice weight gain, as also found by a study using a similar CUS protocol (Kaster et al., 2015). However, our treatments were not able to alter this parameter.

As mentioned before, the antidepressant-like effect of both ascorbic acid and ketamine depends on the activation of mTOR pathway (Li et al., 2010; Moretti et al., 2014). The downstream effects of mTOR are mediated by phosphorylation of p70S6K and eukaryotic initiation factor 4E-binding protein 1 (4E-BP-1), which promotes the initiation of protein translation for the synthesis of synaptic proteins, including synapsin I and PSD95 (Li et al., 2010). Moreover, mTOR activation is likely the upstream signaling of ketamine-induced increase of GluA1

expression, which is linked to enhanced AMPA-type glutamate receptor phosphorylation and surface abundance, a key mechanism for the antidepressant effect of ketamine (Zhang et al., 2016; Zhang et al., 2017). Reinforcing the importance of these targets in the neurobiology of depression, a reduced phosphorylation of the mTOR signaling pathway was found in the amygdala of rats exposed to chronic stress (Chandran et al., 2013) and in the prefrontal cortex of individuals with MDD (Jernigan et al., 2011) Additionally, a reduced PSD95 protein level was found in the prefrontal cortex of depressed subjects relative to controls (Feyissa et al., 2009). Curiously, our data revealed no alterations in the hippocampal phosphorylation of p70S6K and immunocontent of GLUA1 and PSD95 in mice subjected to CUS and/or treated with ascorbic acid and ketamine. Although some studies have suggested that these targets are involved in the neurobiology of depression, our data agrees with previous findings showing that a 14-day protocol of unpredictable stress induces a depressive phenotype in mice but does not alter cortical GLUA1 and PSD95 immunocontents (Neis et al., 2016). Similarly, corticosterone-induced model of depression was not associated with changes in hippocampal GLUA1 and PSD95 protein levels (Neis et al., 2016). On the other hand, we have previously demonstrated that the antidepressant-like effect of an effective dose (1 mg/kg) of ascorbic acid in the mouse TST depends on the activation of the mTOR signaling pathway. Moreover, 1 h after the administration, ascorbic acid increased the phosphorylation of p70S6K and the immunocontent of PSD-95 in the hippocampus of mice (Moretti et al., 2014). Here we used samples collected 24 h after the treatment with subeffective doses ascorbic acid and/or ketamine, so it is plausible to suggest that the synergic antidepressant-like effect of ascorbic acid and ketamine may involve the modulation of other pharmacological targets. Another hypothesis is that modulation of p70S6K and synaptic proteins may contribute to the behavioral effect associated with CUS or the synergistic antidepressant effect of ascorbic and ketamine, however these modifications can occur in specific hippocampal sub-regions or in time points not evaluated in this study.

In conclusion, the present study extends the data about the effect of ascorbic acid and ketamine by exploring, for the first time, its synergistic antidepressant-like action in a model of depression induced by CUS. We demonstrated that a single administration of subeffective doses of these compounds completely abrogated the depressive-like behavior induced by CUS, however, this effect was not accompanied by hippocampal alterations in the phosphorylation of p70S6K and immunocontent of GLUA1 and PSD95. Considering that ascorbic acid is a very affordable and safe compound, studies dealing with other neuroplastic and neuroprotective targets in its antidepressant-like action, as well as additional comparative investigations using ascorbic acid and ketamine are needed.

Acknowledgments

This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [grant number 310113/2017-2] (Brazil); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (Brazil); International Society for Neurochemistry. ALSR is a CNPq Research Fellow.

Author contribution statement

MM and ALSR conceived and designed research. MM, IW, PBR, VBN, NP, SVSS conducted experiments. MM analyzed data and wrote the manuscript. All authors read and approved the manuscript.

Declaration of competing 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.

References

Aburawi SG, FA; Attumi, AA; Altubuly, RA; Kara AA (2014) Effect of ascorbic acid on mental depression drug therapy: clinical study. J. Psych. Psychother. 4: 131.

Amr, M., El-Mogy, A., Shams, T., Vieira, K., Lakhan, S.E., 2013. Efficacy of vitamin C as an adjunct to fluoxetine therapy in pediatric major depressive disorder: a randomized, double-blind, placebo-controlled pilot study. Nutr. J. 12, 31.

Ballard ED, Ionescu DF, Vande Voort JL, Niciu MJ, Richards EM, Luckenbaugh DA, Brutsche NE, Ameli R, Furey ML, Zarate CA, Jr. (2014) Improvement in suicidal ideation after ketamine infusion: relationship to reductions in depression and anxiety. J. Psychiatr. Res. 58: 161–166.

Binfare, R.W., Rosa, A.O., Lobato, K.R., Santos, A.R., Rodrigues, A.L.S., 2009. Ascorbic acid administration produces an antidepressant-like effect: evidence for the involvement of monoaminergic neurotransmission. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 33, 530–540.

Brocardo, P.S., Budni, J., Kaster, M.P., Santos, A.R., Rodrigues, A.L.S., 2008. Folic acid administration produces an antidepressant-like effect in mice: evidence for the involvement of the serotonergic and noradrenergic systems. Neuropharmacology 54, 464–473.

Brody, S., 2002. High-dose ascorbic acid increases intercourse frequency and improves mood: a randomized controlled clinical trial. Biol. Psychiatry 52, 371–374.

Budni, J., Lobato, K.R., Binfare, R.W., Freitas, A.E., Costa, A.P., Martin-de-Saavedra, M.D., Leal, R.B., Lopez, M.G., Rodrigues, A.L.S., 2012. Involvement of PI3K, GSK-3beta and PPARgamma in the antidepressant-like effect of folic acid in the forced swimming test in mice. J. Psychopharmacol. 26, 714–723.

Byers, S.L., Wiles, M.V., Dunn, S.L., Taft, R.A., 2012. Mouse estrous cycle identification tool and images. PLoS One 7 (4), e35538.

Chandran, A., Iyo, A.H., Jernigan, C.S., Legutko, B., Austin, M.C., Karolewicz, B., 2013. Reduced phosphorylation of the mTOR signaling pathway components in the amygdala of rats exposed to chronic stress. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 40, 240–245.

Chen, K.T., Wu, C.H., Tsai, M.H., Wu, Y.C., Jou, M.J., Huang, C.C., Wei, I.H., 2017. Antidepressant-like effects of long-term sarcosine treatment in rats with or without chronic unpredictable stress. Behav. Brain Res. 316, 1–10.

Cocchi, P., Silenzi, M., Calabri, G., Salvi, G., 1980. Antidepressant effect of vitamin C. Pediatrics 65, 862–863.

Dossat, A.M., Wright, K.N., Strong, C.E., Kabbaj, M., 2018. Behavioral and biochemical sensitivity to low doses of ketamine: influence of estrous cycle in C57BL/6 mice. Neuropharmacology 130, 30–41.

Duman, R.S., Li, N., Liu, R.J., Duric, V., Aghajanian, G., 2012. Signaling pathways underlying the rapid antidepressant actions of ketamine. Neuropharmacology 62, 35–41.

Feyissa, A.M., Chandran, A., Stockmeier, C.A., Karolewicz, B., 2009. Reduced levels of NR2A and NR2B subunits of NMDA receptor and PSD-95 in the prefrontal cortex in major depression. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 33, 70–75.

Garcia, L.S., Comim, C.M., Valvassori, S.S., Reus, G.Z., Stertz, L., Kapczinski, F., Gavioli, E.C., Quevedo, J., 2009. Ketamine treatment reverses behavioral and physiological alterations induced by chronic mild stress in rats. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 33, 450–455.

Gupta, D., Radhakrishnan, M., Kurhe, Y., Thangaraj, D., Prabhakar, V., Kanade, P., 2014. Antidepressant-like effects of a novel 5-HT3 receptor antagonist 6z in acute and chronic murine models of depression. Acta Pharmacol. Sin. 35, 1493–1503.

Isingrini, E., Camus, V., Le Guisquet, A.M., Pingaud, M., Devers, S., Belzung, C., 2010. Association between repeated unpredictable chronic mild stress (UCMS) procedures with a high fat diet: a model of fluoxetine resistance in mice. PLoS One 5, e10404.

Jacobson, L.H., Cryan, J.F., 2007. Feeling strained? Influence of genetic background on depression-related behavior in mice: a review. Behav. Genet. 37, 171–213.

Jernigan, C.S., Goswami, D.B., Austin, M.C., Iyo, A.H., Chandran, A., Stockmeier, C.A., Karolewicz, B., 2011. The mTOR signaling pathway in the prefrontal cortex is compromised in major depressive disorder. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 35, 1774–1779.

Kaster, M.P., Gadotti, V.M., Calixto, J.B., Santos, A.R., Rodrigues, A.L., 2012. Depressive-like behavior induced by tumor necrosis factor-alpha in mice. Neuropharmacology 62, 419–426.

Kaster, M.P., Machado, N.J., Silva, H.B., Nunes, A., Ardais, A.P., Santana, M., Baqi, Y., Muller, C.E., Rodrigues, A.L., Porciuncula, L.O., Chen, J.F., Tome, A.R., Agostinho, P., Canas, P.M., Cunha, R.A., 2015. Caffeine acts through neuronal adenosine A2A receptors to prevent mood and memory dysfunction triggered by chronic stress. Proc. Natl. Acad. Sci. U. S. A. 112, 7833–7838.

Kessler, R.C., Berglund, P., Demler, O., Jin, R., Koretz, D., Merikangas, K.R., Rush, A.J., Walters, E.E., Wang, P.S., National Comorbidity Survey R, 2003. The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R). JAMA 289, 3095–3105.

Krishnan, V., Nestler, E.J., 2008. The molecular neurobiology of depression. Nature 455, 894–902.

Kumar, B., Kuhad, A., Chopra, K., 2011. Neuropsychopharmacological effect of sesamol in unpredictable chronic mild stress model of depression: behavioral and biochemical evidences. Psychopharmacology 214, 819–828.

Li, N., Lee, B., Liu, R.J., Banasr, M., Dwyer, J.M., Iwata, M., Li, X.Y., Aghajanian, G., Duman, R.S., 2010. mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. Science 329, 959–964.

Li, N., Liu, R.J., Dwyer, J.M., Banasr, M., Lee, B., Son, H., Li, X.Y., Aghajanian, G., Duman, R.S., 2011. Glutamate N-methyl-D-aspartate receptor antagonists rapidly reverse behavioral and synaptic deficits caused by chronic stress exposure. Biol. Psychiatry 69, 754–761.

Lu, X.Y., Kim, C.S., Frazer, A., Zhang, W., 2006. Leptin: a potential novel antidepressant. Proc. Natl. Acad. Sci. U. S. A. 103, 1593–1598.

Machado, D.G., Cunha, M.P., Neis, V.B., Balen, G.O., Colla, A., Grando, J., Brocardo, P.S., Bettio, L.E., Capra, J.C., Rodrigues, A.L., 2012. Fluoxetine reverses depressive-like behaviors and increases hippocampal acetylcholinesterase activity induced by olfactory bulbectomy. Pharmacol. Biochem. Behav. 103, 220–229.

Manosso, L.M., Moretti, M., Rodrigues, A.L., 2013. Nutritional strategies for dealing with depression. Food Funct. 4, 1776–1793.

Manosso, L.M., Moretti, M., Ribeiro, C.M., Goncalves, F.M., Leal, R.B., Rodrigues, A.L., 2015. Antidepressant-like effect of zinc is dependent on signaling pathways implicated in BDNF modulation. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 59, 59–67.

Manosso, L.M., Moretti, M., Colla, A.R., Ribeiro, C.M., Dal-Cim, T., Tasca, C.I., Rodrigues, A.L., 2016. Involvement of glutamatergic neurotransmission in the antidepressant-like effect of zinc in the chronic unpredictable stress model of depression. J. Neural Transm. 123, 339–352.

Mineur, Y.S., Belzung, C., Crusio, W.E., 2006. Effects of unpredictable chronic mild stress on anxiety and depression-like behavior in mice. Behav. Brain Res. 175, 43–50.

Moretti, M., Freitas, A.E., Budni, J., Fernandes, S.C., Balen Gde, O., Rodrigues, A.L.S., 2011. Involvement of nitric oxide-cGMP pathway in the antidepressant-like effect of ascorbic acid in the tail suspension test. Behav. Brain Res. 225, 328–333.

Moretti, M., Freitas, A.E., Budni, J., Fernandes, S.C.P., Balen, G., Rodrigues, A.L.S., 2011b. Involvement of nitric oxide–cGMP pathway in the antidepressant-like effect of ascorbic acid in the tail suspension test. Behav. Brain Res. 225, 328–333.

Moretti, M., Budni, J., Ribeiro, C.M., Rodrigues, A.L.S., 2012a. Involvement of different types of potassium channels in the antidepressant-like effect of ascorbic acid in the mouse tail suspension test. Eur. J. Pharmacol. 687, 21–27.

Moretti, M., Colla, A., de Oliveira Balen, G., dos Santos, D.B., Budni, J., de Freitas, A.E., Farina, M., Rodrigues, A.L.S., 2012b. Ascorbic acid treatment, similarly to fluoxetine, reverses depressive-like behavior and brain oxidative damage induced by chronic unpredictable stress. J. Psychiatr. Res. 46, 331–340.

Moretti, M., Budni, J., Dos Santos, D.B., Antunes, A., Daufenbach, J.F., Manosso, L.M., Farina, M., Rodrigues, A.L.S., 2013. Protective effects of ascorbic acid on behavior and oxidative status of restraint-stressed mice. J. Mol. Neurosci. 49, 68–79.

Moretti, M., Budni, J., Freitas, A.E., Rosa, P.B., Rodrigues, A.L.S., 2014. Antidepressant-like effect of ascorbic acid is associated with the modulation of mammalian target of rapamycin pathway. J. Psychiatr. Res. 48, 16–24.

Moretti, M., Budni, J., Freitas, A.E., Neis, V.B., Ribeiro, C.M., de Oliveira Balen, G., Rieger, D.K., Leal, R.B., Rodrigues, A.L.S., 2015. TNF-alpha-induced depressive-like phenotype and p38(MAPK) activation are abolished by ascorbic acid treatment. Eur. Neuropsychopharmacol. 25, 902–912.

Moretti, M., Fraga, D.B., Rodrigues, A.L.S., 2017. Ascorbic acid to manage psychiatric disorders. CNS Drugs 31, 571–583.

Moretti, M., Ribeiro, C.M., Neis, V.B., Bettio, L.E.B., Rosa, P.B., Rodrigues, A.L.S., 2018. Evidence for the involvement of opioid system in the antidepressant-like effect of ascorbic acid. Naunyn Schmiedeberg's Arch. Pharmacol. 391, 169–176.

Moritz, B., Schwarzbold, M.L., Guarnieri, R., Diaz, A.P., AL, S.R., Dafre, A.L.S., 2017. Effects of ascorbic acid on anxiety state and affect in a non-clinical sample. Acta Neurobiol. Exp. 77, 362–372.

Neis, V.B., Bettio, L.E.B., Moretti, M., Rosa, P.B., Ribeiro, C.M., Freitas, A.E., Goncalves, F.M., Leal, R.B., Rodrigues, A.L.S., 2016. Acute agmatine administration, similar to ketamine, reverses depressive-like behavior induced by chronic unpredictable stress in mice. Pharmacol. Biochem. Behav. 150–151, 108–114.

Nemeroff, C.B., 2007. The burden of severe depression: a review of diagnostic challenges and treatment alternatives. J. Psychiatr. Res. 41, 189–206.

Olescowicz, G., Neis, V.B., Fraga, D.B., Rosa, P.B., Azevedo, D.P., Melleu, F.F., Brocardo, P.S., Gil-Mohapel, J., Rodrigues, A.L.S., 2018. Antidepressant and pro-neurogenic effects of agmatine in a mouse model of stress induced by chronic exposure to corticosterone. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 81, 395–407.

Peterson, G.L., 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. 83, 346–356.

Rice, M.E., 2000. Ascorbate regulation and its neuroprotective role in the brain. Trends Neurosci. 23, 209–216.

Rosa, P.B., Ribeiro, C.M., Bettio, L.E., Colla, A., Lieberknecht, V., Moretti, M., Rodrigues, A.L., 2014. Folic acid prevents depressive-like behavior induced by chronic corticosterone treatment in mice. Pharmacol. Biochem. Behav. 127 (1–6).

Rosa, P.B., Neis, V.B., Ribeiro, C.M., Moretti, M., Rodrigues, A.L., 2016. Antidepressant-like effects of ascorbic acid and ketamine involve modulation of GABAA and GABAB receptors. Pharmacol. Rep. 68, 996–1001.

Steru, L., Chermat, R., Thierry, B., Simon, P., 1985. The tail suspension test: a new method for screening antidepressants in mice. Psychopharmacology 85, 367–370.

Willner, P., Towell, A., Sampson, D., Sophokleous, S., Muscat, R., 1987. Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. Psychopharmacology 93, 358–364.

Wong, M.L., Licinio, J., 2001. Research and treatment approaches to depression. Nat. Rev. Neurosci. 2, 343–351.

Yoshimura, S., Sakamoto, S., Kudo, H., Sassa, S., Kumai, A., Okamoto, R., 2003. Sex-differences in adrenocortical responsiveness during development in rats. Steroids 68, 439–445.

Zarate Jr., C.A., Singh, J.B., Carlson, P.J., Brutsche, N.E., Ameli, R., Luckenbaugh, D.A., Charney, D.S., Manji, H.K., 2006. A randomized trial of an N-methyl-D-aspartate antagonist in treatment-resistant major depression. Arch. Gen. Psychiatry 63, 856–864.

Zhang, K., Xu, T., Yuan, Z., Wei, Z., Yamaki, V.N., Huang, M., Huganir, R.L., Cai, X., 2016. Essential roles of AMPA receptor GluA1 phosphorylation and presynaptic HCN channels in fast-acting antidepressant responses of ketamine. Sci. Signal. 9, ra123.

Zhang, K., Yamaki, V.N., Wei, Z., Zheng, Y., Cai, X., 2017. Differential regulation of GluA1 expression by ketamine and memantine. Behav. Brain Res. 316, 152–159.