Journal of Psychiatric Research 115 (2019) 103–112

Contents lists available at ScienceDirect

Journal of Psychiatric Research

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

Augmentation effect of ketamine by guanosine in the novelty-suppressed feeding test is dependent on mTOR signaling pathway

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ARTICLE INFO

Keywords:
Augmentation strategy
Depression
Ketamine
Guanosine
Novelty-suppressed feeding

ABSTRACT

The ketamine's potential for the treatment of refractory depression and anxiety has been considered one the most important discoveries in the last years, however, repeated use of ketamine is limited due to its side/adverse effects. Therefore, the search for effective augmentation strategies that may reduce ketamine doses is welcome. Therefore, this study sought to augment the effect of ketamine by guanosine in the novelty-suppressed feeding (NSF) test, a behavioral paradigm able to detect depression/anxiety-related behavior. Acute administration of guanosine (0.05 mg/kg, p.o.), similar to ketamine (1 mg/kg, i.p.), produced a rapid behavioral response in mice submitted to NSF test. Moreover, the coadministration of sub-effective doses of guanosine (0.01 mg/kg, p.o.) and ketamine (0.1 mg/kg, i.p.) was effective in mice submitted to NSF test. Subsequently, the intracellular mechanism underpinning the augmentation effect of ketamine by guanosine was investigated. Our results suggest that augmentation response of ketamine by guanosine in the NSF test probably involves the activation of mTOR signaling, since the treatment with rapamycin (0.2 nmol/site, i.c.v., a selective mTOR inhibitor) completely abolished this effect. This augmentation strategy also increased mTOR phosphorylation (Ser^{2448}) in the hippocampus, reinforcing the role of mTOR in this augmentation response. However, no changes in the p70S6K, PSD-95, GluA1, and synapsin immunocontents were found in the hippocampus of ketamine plus guanosine-treated mice. Overall, results provide evidence that guanosine is able to augment the effect of ketamine in the NSF test via mTOR activation, a finding that might have therapeutic implications for the management of depression/anxiety.

1. Introduction

Major depressive disorder and anxiety are currently the most prevalent psychiatric disorders, becoming more and more common in modern society, which in turn causes a profound socioeconomic burden (Kessler et al., 2007; Papakostas and Ionescu, 2015; World Health Organization, 2017). Despite benzodiazepines have been used for over 50 years, monoamine reuptake inhibitors antidepressants are routinely prescribed for anxiety disorders (Griebel and Holmes, 2013). Unfortunately, although there are available antidepressant medications used in the therapy, the treatment has serious limitations related to the low response rate and time lag for the therapeutic effect, reducing the adhesion of the patients to treatments (Crisafulli et al., 2011; Papakostas and Ionescu, 2015). Taking into account these flaws, the search for novel antidepressants/anxiolytics treatments, especially those affording fast-acting responses with higher efficacy and fewer side effects are urgently needed.

Ketamine, an N-methyl-D-aspartate (NMDA) receptor antagonist, is often used as an anesthetic agent, although a large body of clinical evidence and experimental studies have demonstrated the rapid and robust antidepressant-like effect elicited by a single sub-anesthetic dose of ketamine (Berman et al., 2000; Li et al., 2010; Zarate et al., 2006; Zhou et al., 2014). Moreover, this fast-acting antidepressant effect of ketamine is observed in patients resistant to two or more typical antidepressants, even with suicidal ideation (DiazGranados et al., 2010; Price et al., 2009; Zarate et al., 2006). Likewise, compelling reports demonstrated that ketamine exerted anxiolytic effects (Fraga et al., 2018; Ionescu et al., 2015; Krystal et al., 1994). Remarkably, the fast-acting effect of ketamine seems to be unleashed by the release of brain-derived neurotrophic factor (BDNF) with subsequent activation of the mechanistic target of rapamycin protein (mTOR). Thereafter, mTOR signaling culminates in the phosphorylation of 70 kDa ribosomal

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https://doi.org/10.1016/j.jpsychires.2019.05.017
Received 20 February 2019; Received in revised form 29 April 2019; Accepted 15 May 2019

protein S6 kinase (p70S6K), facilitating the translation of synaptic proteins such as postsynaptic density protein-95 kDa (PSD-95), AMPA receptor subunits (GluA1) and synapsin, events that are associated with synaptogenesis and improved synaptic function (Li et al., 2010; 2011; R. Liu et al., 2016; Zhang et al., 2018; Zhou et al., 2014). Questions have been raised about the safety of prolonged use of ketamine, however, the repeated use of ketamine is reported to cause psychotomimetic or dissociative side effects and neurotoxicity thereby limiting its use (Behrens et al., 2007; Sleigh et al., 2014). Thus, considering these limitations, the investigation of molecules that might share similar mechanisms of action to ketamine or even attenuate its side effects, emerge as a promising therapeutic strategy.

In fact, the augmentation strategies, in which ongoing antidepressant drug treatment is combined with non-antidepressant agents have been postulated as useful for the management of depression (Otte et al., 2016). Typically, the goal of augmentation strategies is to obtain symptom remission more rapidly when such remission cannot be obtained by optimizing monotherapy. Previous papers reported that augmentation strategies produced significantly greater improvement than monotherapy (Cussotto et al., 2017; Han et al., 2014; Kale and Addepalli, 2014). Another possibility of augmentation strategy is the reduction of dosage of ongoing antidepressants, which in turn could decrease their adverse effects (Barowsky and Schwartz, 2006). Therefore, among the molecules that present a potential to augment the behavioral effects of ketamine, stands out guanosine.

Guanosine, a guanine-based purine, occurs naturally in the central nervous system being released from glial cells under physiological conditions and even more during pathological events, thereby suggesting that it could be an endogenous neuroprotective nucleoside (Ciccarelli et al., 2001, 1999). A vast number of studies have indicated the neuroprotective effect of guanosine in several models of disease (Dal-Cim et al., 2016; 2013; 2011; Lanznaster et al., 2017; Molz et al., 2011; Paniz et al., 2014; Petronilho et al., 2012; Quincozes-Santos et al., 2013), which may be related to its ability to reduce neuroinflammation, oxidative stress, and glutamatergic excitotoxicity (Bettio et al., 2016a, 2016b). Furthermore, guanosine has been postulated as an extracellular neuromodulator able to induce synthesis and liberation of neurotrophic factors, cell differentiation, and neuritogenesis (Bau et al., 2005; Bettio et al., 2016c; Guarnieri et al., 2009; Gysbers and Rathbone, 1996a, 1996b). Of note, guanosine is under active investigation as a potential mood modulator, because its action on glutamatergic transmission. Moreover, plasma levels of guanosine were reduced in patients with major depressive disorder, which reinforces the notion that this nucleoside plays a role in the pathophysiology of this disorder (Ali-Sisto et al., 2016).

Within this context, our research group has demonstrated that guanosine given systemically or centrally produces an antidepressant-like effect in two widely used predictive tests, namely the tail suspension test (TST) and forced swimming test (FST), through the modulation NMDA receptors and PI3K/mTOR pathway, as well as by decreasing hippocampal oxidative damage (Bettio et al., 2012, 2014). Moreover, the anxiolytic-like effect of guanosine was previously demonstrated (Almeida et al., 2017). Noteworthy, a single administration of a sub-effective dose of guanosine when combined with a sub-effective dose of ketamine affords an antidepressant-like effect, which suggests a potential augmentation effect (Bettio et al., 2012), a finding that deserves further investigation. Given this background, this study sought to investigate the effect of sub-effective dose of guanosine combined with a sub-effective dose of ketamine, a putative augmentation strategy, in the novelty-suppressed feeding (NSF) test. This is a behavioral paradigm initially described to study anxiolytic drugs, but that has been increasingly used to assess the efficacy of antidepressants agents, since this test detects depression-related behaviors (Blasco-Serra et al., 2017).

2. Material and methods

2.1. Animals

The behavioral experiments were conducted using male *Swiss* mice (30–40 g), maintained under a controlled temperature (20–22 °C) and humidity (50 ± 20%) with a 12:12 h light/dark cycle (lights on at 7:00 a.m.). Animals were maintained with free access to food and water, except when they were 24-h food deprived prior to the NSF test. The animals were caged in groups of 10 in a 41 × 34 × 16 cm cage and, the behavioral test was carried out between 9.00 a.m. and 04.00 p.m. The animals were used according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and the experiments were performed after approval of the protocol by the Institutional Ethics Committee. All efforts were done in order to minimize animal suffering and to reduce their number to the minimum necessary to demonstrate consistent effects in the experiments.

2.2. Drugs and treatments

The first set of experiments were designed to obtain the active and sub-effective doses of guanosine and ketamine in the NSF test. To conduct the treatment protocol, mice were randomly assigned to five experimental groups (n = 9–10/group): (1) control (vehicle); (2) guanosine (0.01 mg/kg, p.o.); (3) guanosine (0.05 mg/kg, p.o.); (4) ketamine (0.1 mg/kg, i.p.) and (5) ketamine (1 mg/kg, i.p.). Guanosine and ketamine, obtained from Sigma Chemical Co., St. Louis, U.S.A, were dissolved in distilled water and saline (0.9%), respectively. Guanosine was administered orally (p.o.) whereas ketamine was administered intraperitoneally (i.p.) in a volume of 10 ml/kg. Mice were submitted to the NSF test 60 min after receiving a single administration of guanosine treatment or 30 min after receiving ketamine treatment (Fig. 1A). All the drugs were freshly prepared before administration and the doses were chosen based on previous studies (Bettio et al., 2012; Ludka et al., 2013).

In the second set of experiments, to test the hypothesis of the augmentation effect of ketamine by guanosine in the NSF test (n = 9–10/group), mice were administered with sub-effective doses of guanosine (0.01 mg/kg, p.o.) and ketamine (0.1 mg/kg, i.p.), and were submitted to the behavioral test 60 min after receiving guanosine and 30 min after receiving ketamine treatment as shown in Fig. 1B.

In a third set of experiments, to investigate the role of mTOR pathway in the augmentation response of ketamine by guanosine (n = 9–10/group), rapamycin (a selective mTOR inhibitor) was administered 45 min after guanosine administration as indicated in Fig. 1C. The animals were subjected to behavioral test 15 min later. Rapamycin was dissolved in 100% DMSO and administered by intracerebroventricular route (i.c.v.) in a volume of 3 µl per mouse (0.2 nmol/site). 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 (Pazini et al., 2016). Briefly, a 0.4-mm external diameter hypodermic needle attached to a cannula linked to a 25-µl Hamilton syringe was inserted perpendicularly through the skull (no more than 2 mm into the brain of each mouse). Rapamycin was 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 midpoint on a line drawn through to the anterior base of the ears. Then, 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%).

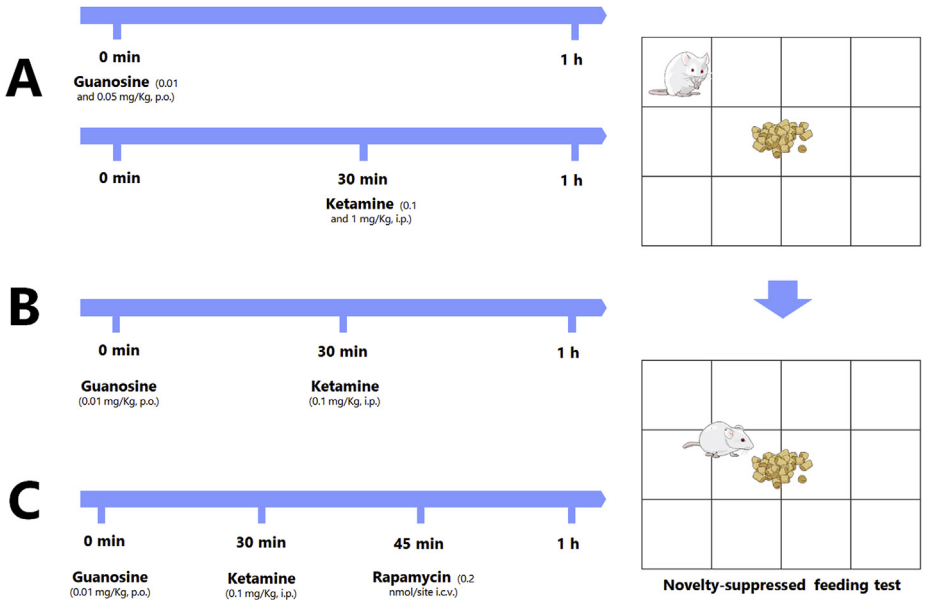

Fig. 1. Schedule of treatments and behavioral test. (A) Mice received guanosine (0.05 and 0.01 mg/kg, p.o.) or ketamine (1 and 0.1 mg/kg, i.p.) and 60 min or 30 after the treatments, respectively, they were submitted to the NSF test. (B) To test the hypothesis of the augmentation effect of ketamine by guanosine, mice received sub-effective doses of guanosine (0.01 mg/kg, p.o.) and ketamine (0.1 mg/kg, i.p.). Mice were submitted to the NSF test after the treatments. (C) To investigate the role of mTOR pathway in the augmentation response of ketamine by guanosine, mice received rapamycin (0.2 nmol/site, i.c.v., a selective mTOR inhibitor) 45 min after guanosine and 15 min after ketamine administration. The animals were subjected to NSF test 15 min later.

2.3. Novelty-suppressed feeding (NSF) test

The NSF test was performed as proposed previously (Bodnoff et al., 1988; Dulawa and Hen, 2005). This test measures the latency of mice in approaching and eating food in a novel environment following an extended period (up to 24 h) of food deprivation. The latency to begin eating reflects how the animal copes with a behavioral conflict. Because the ability to solve conflicts is inversely related to anxiety and depression, and since NSF test assesses anhedonia in a situation where there is a conflict between food reward and novel open space, this test has been used for depression-related assessments (Dulawa and Hen, 2005; Powell et al., 2012). The mice were weighed, and all food was removed from their cages, although water continued to be provided with free access. Approximately 24 h after the removal of the food, mice were placed in an illuminated and soundproofed wooden box (40 × 60 cm and 50 cm height). A small piece of mouse chow was placed in the center of the box and each mouse was placed in the corner of the testing arena, and the time until the first feeding episode was recorded within 10 min. Immediately after the mouse began to eat the chow, the tested animal was placed alone in a cage with a weighed piece of chow for 5 min and, at the end of this period, the amount of food consumed was determined by weighing the piece of chow. After, all mice from a single cage had been tested, mice returned to the cage with free access to food and water (Blasco-Serra et al., 2017; Fukumoto and Chaki, 2015).

2.4. Western blotting

To quantify mTOR, p70S6K, PSD-95, GluA1 and synapsin immunocontents, Western blot analyses were performed as previously described (Pazini et al., 2016). Animals were euthanized by rapid decapitation immediately after the behavioral test and the hippocampus was quickly dissected and snap-frozen with liquid nitrogen prior to storage at −80 °C until use. Briefly, samples were mechanically homogenized in 400 µl of 50 mM TRIS pH 7.0, 1 mM EDTA, 100 mM NaF, 0.1 mM PMSF, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, Sigma Protease Inhibitor Cocktail (P2714). Lysates were centrifuged (10000 g for 10 min, at 4 °C) to eliminate cellular debris. The supernatants were diluted 1/1 (v/v) in 100 mM TRIS pH 6.8, 4 mM EDTA, 8% SDS, and boiled for 5 min. Thereafter, sample dilution (40% glycerol, 100 mM TRIS, bromophenol blue, pH 6.8) in the ratio 25:100 (v/v) and β-mercaptoethanol (final concentration 8%) were added to the samples. Protein content was quantified using bovine serum albumin as a standard (Peterson, 1977). The samples containing 60 µg protein/track were separated by SDS-PAGE (miniVE Vertical Electrophoresis System TM, GE Healthcare Life Sciences, Piscataway, NJ, USA) using 7–10% gel and the proteins were transferred to nitrocellulose membranes using a semi-dry blotting apparatus (1.2 mA/cm2; 1.5 h). To verify transfer efficiency process, membranes were stained with Ponceau (Leal et al., 2002) and subsequently, the membranes were blocked with 5% bovine serum albumin (BSA) in TBS (10 mM Tris, 150 mM NaCl, pH 7.5). The immunocontent of total and phosphorylated forms of mTOR (Ser^{2448}) and p70S6K (Thr^{389}), as well as PSD-95, GluA1, synapsin and β-actin (loading control) immunocontents were detected using specific antibodies (obtained from Cell Signaling Technology, Inc. and diluted by a factor 1:1000) incubated overnight diluted in TBS-T (10 mM Tris, 150 mM NaCl, 0.1% Tween-10, pH 7.5) containing 2.5% BSA. Subsequently, the membranes were incubated with anti-rabbit antibody horseradish peroxidase-conjugated secondary antibody (Cell Signaling, 1:2500) for 60 min, and the immunoreactive bands were developed using a chemiluminescence kit (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 (OD) of the bands was quantified using Image Lab Software® 4.1 (Bio-Rad Laboratories). The phosphorylation levels of mTOR and p70S6K were determined as a ratio of OD of the phosphorylated band over OD of the total band. The immunocontents of PSD-95, synapsin and GluA1 were determined as a ratio of OD of PSD-95, synapsin and GluA1 band over the OD of the β-actin band. Results are expressed as compared to control group 100%.

2.5. Statistical analysis

The results were expressed as means ± S.E.M. Differences among experimental groups were determined by one-way or two-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test, when appropriate. A value of $P < 0.05$ was considered to be significant.

3. Results

3.1. Effect of guanosine and ketamine in the NSF test

To determine the effective and sub-effective doses of guanosine and ketamine in the NSF test, two doses of these compounds were chosen.

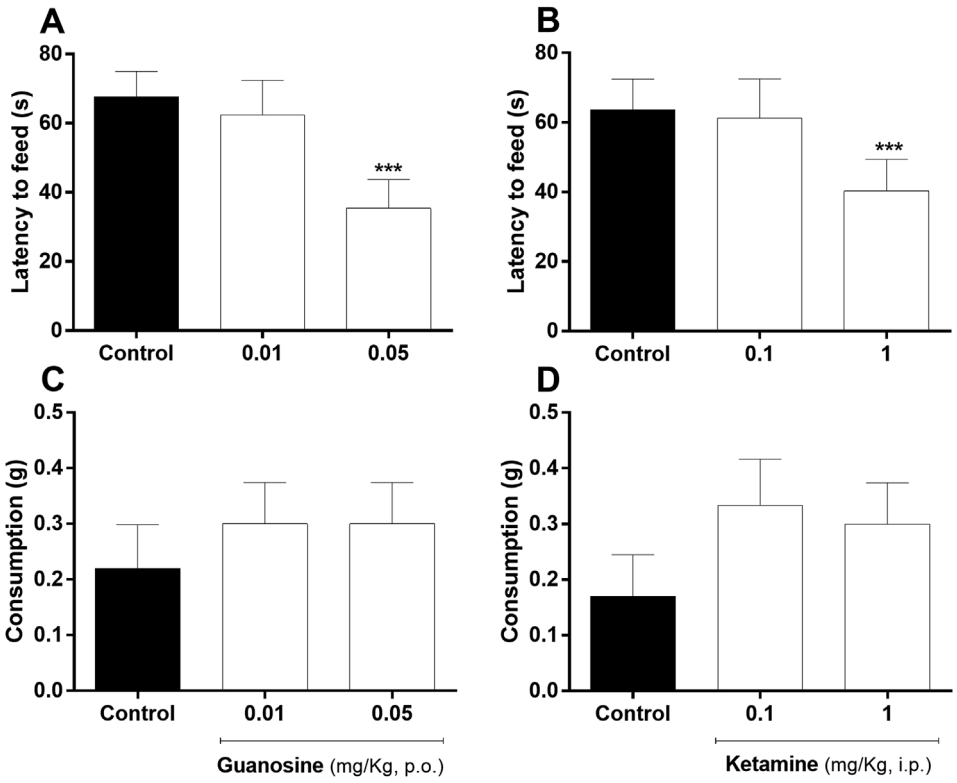

Fig. 2. Effect of a single administration of guanosine (A and C) or ketamine (B and D) on the latency to feed and food consumption in mice submitted to the NSF test. Guanosine (0.05 or 0.01 mg/kg, p.o.) and ketamine (1 or 0.1 mg/kg, i.p.) were administered 60 min and 30 prior to the test, respectively. Values are expressed as means ± S.E.M (n = 9–10). ***$P < 0.001$ as compared with the vehicle-treated control (one-way ANOVA followed by Newman-Keuls post hoc test).

As shown in Fig. 2A and B, the administration of guanosine (0.05 mg/kg) significantly decreased the latency to feed in the NSF test ($F_{(2, 26)} = 38.78$, $P < 0.001$), similar to the result obtained when mice were administered with 1 mg/kg ketamine ($F_{(2, 25)} = 16.56$, $P < 0.001$), which suggests a fast behavioral effect of these compounds. In order to rule out a possible interference of the treatments on food consumption, the amount of food consumed was determined for 5 min. As depicted in Fig. 2C and D, the treatments with guanosine or ketamine did not affect the consumption of food in the NSF test ($F_{(2, 26)} = 0.28$, $P > 0.05$ and $F_{(2, 25)} = 1.10$, $P > 0.05$, respectively), ruling out false-positive results.

In order to investigate the potential augmentation effect of ketamine by guanosine, sub-effective doses of these compounds were coadministered (Fig. 3A and B). Two-way ANOVA revealed significant differences for guanosine treatment [$F_{(1, 35)} = 20.69$, $P < 0.05$], ketamine treatment [$F_{(1, 35)} = 23.66$, $P < 0.05$] and guanosine treatment × ketamine treatment interaction [$F_{(1, 35)} = 14.03$, $P < 0.05$]. Post-hoc analysis showed that a single administration with a sub-effective dose of guanosine (0.01 mg/kg, p.o.) when combined with a sub-effective dose of ketamine (0.1 mg/kg, i.p.) significantly reduced the latency to feed ($P < 0.001$), indicating an augmentation effect of ketamine by guanosine in mice submitted to the NSF test, as depicted in Fig. 3A. Noteworthy, as illustrated in Fig. 3B, the coadministration with sub-effective doses of guanosine and ketamine did not significantly change the food consumption as compared to the control group. A two-way ANOVA revealed no significant effects for guanosine treatment [$F_{(1, 35)} = 0.265$, $P > 0.05$], ketamine treatment [$F_{(1, 35)} = 0.011$, $P > 0.05$] and guanosine treatment × ketamine treatment interaction [$F_{(1, 35)} = 3.072$, $P > 0.05$] in food consumption. These results indicate that the augmentation effect of ketamine by guanosine was not due to the food consumption behavior of mice.

3.2. Involvement of mTOR signaling pathway in the augmentation effect of ketamine by guanosine in the NSF test

In order to investigate the role of mTOR signaling pathway in the augmentation response of ketamine by guanosine, rapamycin (0.2 nmol/site, i.c.v., a selective mTOR inhibitor) was administered as indicated in Fig. 1C. Two-way ANOVA revealed significant differences for ketamine plus guanosine treatment [$F_{(1, 35)} = 9.37$, $P < 0.05$], rapamycin treatment [$F_{(1, 35)} = 4.57$, $P < 0.05$] and ketamine plus guanosine treatment × rapamycin treatment interaction [$F_{(1, 35)} = 6.38$, $P < 0.05$] in the latency to feed. The results depicted in Fig. 4A shows that coadministration of guanosine (0.01 mg/kg, p.o.) and ketamine (0.1 mg/kg, i.p.) significantly reduced the latency to feed ($P < 0.01$), but this result was completely abolished by rapamycin. Moreover, as illustrated in Fig. 4B, the coadministration of guanosine and ketamine did not significantly alter the consumption of food as compared to the control group. A two-way ANOVA revealed no significant effects for ketamine plus guanosine treatment [$F_{(1, 35)} = 0.129$, $P > 0.05$], rapamycin treatment [$F_{(1, 35)} = 0.046$, $P > 0.05$] and ketamine plus guanosine treatment × rapamycin treatment interaction [$F_{(1, 35)} = 0.069$, $P > 0.05$] in food consumption.

3.3. Effect of coadministration of guanosine and ketamine on mTOR, p70S6K, PSD-95, synapsin and GluA1 immunocontents in the hippocampus

Subsequently, we investigated whether the hippocampal phosphorylation of mTOR and p70S6K (a downstream target of mTOR) as well as the immunocontents of PSD-95, GluA1 and synapsin (proteins required for synaptic plasticity) were affected after a single or combined administration of sub-effective dose of guanosine (0.01 mg/kg, p.o.) and ketamine (0.1 mg/kg, i.p.). The results illustrated in Fig. 5A and B shows the hippocampal p-mTOR and total mTOR immunocontent of mice treated with guanosine and/or ketamine. A two-way ANOVA revealed no significant main effects for guanosine treatment [$F_{(1, 24)} = 3.163$, $P > 0.05$] and ketamine treatment [$F_{(1, 24)} = 3.405$, $P > 0.05$], but revealed a significant effect for guanosine treatment × ketamine treatment interaction on mTOR phosphorylation [$F_{(1, 24)} = 7.728$, $P < 0.05$]. Post-hoc analysis indicated that the coadministration with sub-effective doses of guanosine and ketamine was capable of increasing hippocampal mTOR phosphorylation (Ser^{2448}) as

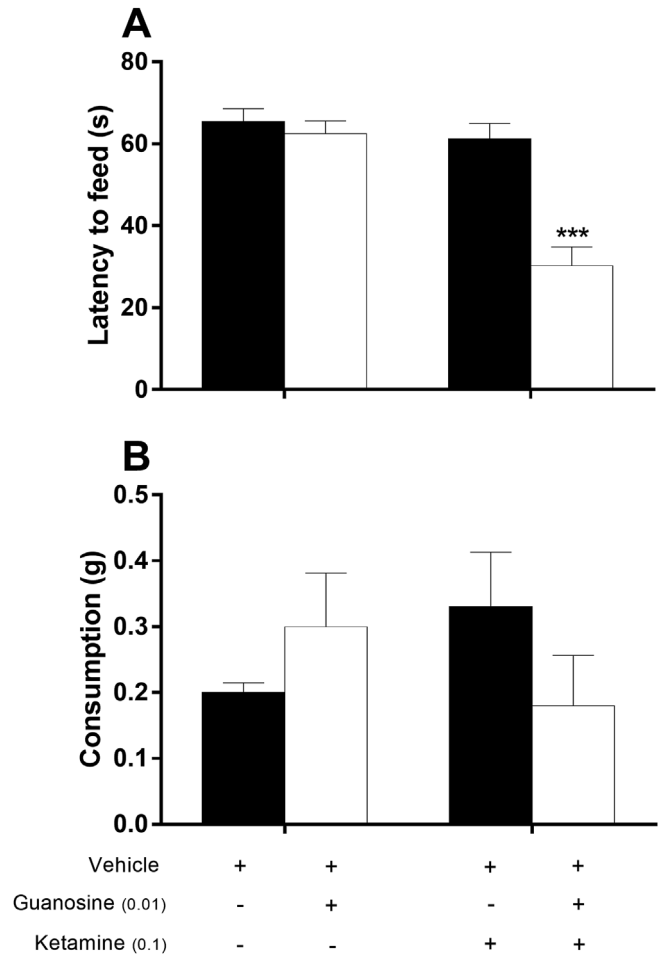

Fig. 3. Effect of a single administration with a sub-effective dose of guanosine or ketamine, as well as combined administration of guanosine plus ketamine on the latency to feed (A) and food consumption (B) in mice submitted to the NSF test. Guanosine (0.05 or 0.01 mg/kg, p.o.) and ketamine (1 or 0.1 mg/kg, i.p.) were administered 60 min and 30 prior to the test, respectively. Values are expressed as means ± S.E.M (n = 9–10). ***$P < 0.001$ as compared with the vehicle-treated control *(*two-way ANOVA followed by Newman-Keuls post hoc test).

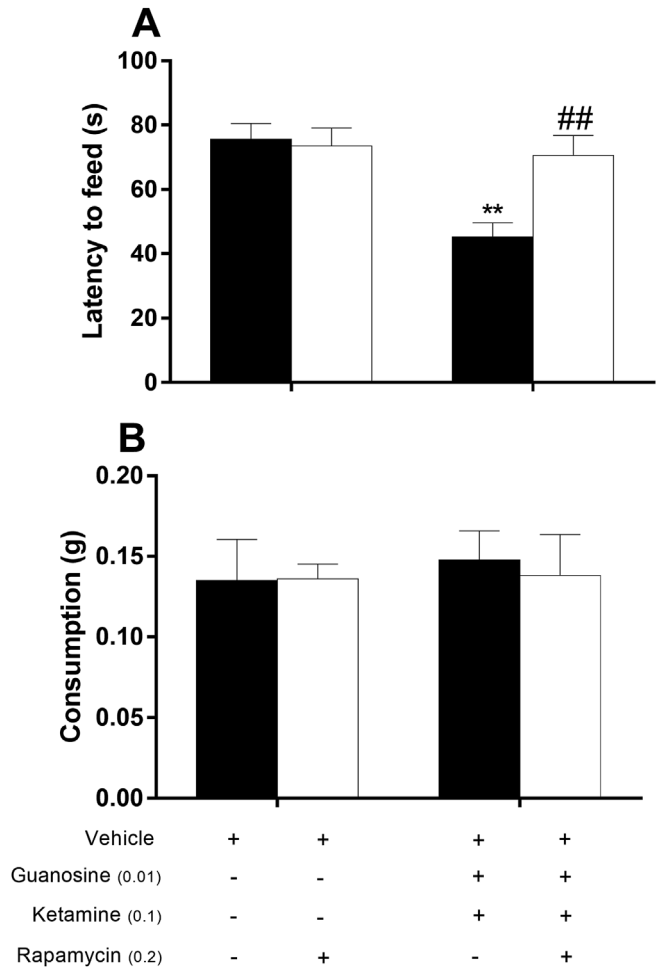

Fig. 4. Effect of rapamycin (an irreversible mTOR inhibitor, 0.2 nmol/site, i.c.v.) in the guanosine (0.01 mg/kg, p.o.) plus ketamine (0.1 mg/kg, i.p.)-induced reduction on the latency to feed (A) and food consumption (B) in mice submitted to the NSF test. Values are expressed as means ± S.E.M (n = 9–10). **$P < 0.01$ as compared with the vehicle-treated control; ##$P < 0.01$ as compared with the augmentation group (two-way ANOVA followed by Newman-Keuls post hoc test).

compared to the control group ($P < 0.05$). Regarding the total mTOR immunocontent, no significant effect was observed for guanosine [$F_{(1, 24)} = 0.431$, $P > 0.05$], ketamine [$F_{(1, 24)} = 0.229$, $P > 0.05$], and guanosine treatment × ketamine treatment interaction [$F_{(1, 24)} = 0.094$, $P > 0.05$].

Fig. 6 shows the effects of guanosine and ketamine, alone or in combination, on hippocampal p-p70S6K and total p70S6K immunocontent. As shown in Fig. 6A and B, guanosine and ketamine alone or in combination, did not cause any effect on hippocampal p-p70S6K (Thr^{389}) and total p70S6K immunocontent. A two-way ANOVA revealed no significant effects for guanosine treatment [$F_{(1, 24)} = 0.274$, $P > 0.05$], ketamine treatment [$F_{(1, 24)} = 0.089$, $P > 0.05$] and guanosine treatment × ketamine treatment interaction [$F_{(1, 24)} = 0.107$, $P > 0.05$] on p70S6K phosphorylation. Likewise, the two-way ANOVA revealed no significant effects for guanosine treatment [$F_{(1, 24)} = 0.026$, $P > 0.05$], ketamine treatment [$F_{(1, 24)} = 0.002$, $P > 0.05$] and guanosine treatment × ketamine treatment interaction [$F_{(1, 24)} = 0.009$, $P > 0.05$] on total p70S6K immunocontent.

The results depicted in Fig. 7 shows the influence of guanosine, ketamine, and guanosine plus ketamine treatments on the synaptic proteins PSD-95 (7A), GluA1 (7B) and synapsin (7C) immunocontents on the hippocampus of mice. The treatments with guanosine and ketamine, alone or in combination, were not able to alter the hippocampal immunocontent of PSD-95, GluA1, and synapsin. A two-way ANOVA revealed no significant effects for guanosine treatment [$F_{(1, 24)} = 0.041$, $P > 0.05$], ketamine treatment [$F_{(1, 24)} = 0.158$, $P > 0.05$] and guanosine treatment × ketamine treatment interaction [$F_{(1, 24)} = 0.714$, $P > 0.05$] on PSD-95 immunocontent. Similarly, the two-way ANOVA revealed no significant effects for guanosine treatment [$F_{(1, 24)} = 0.002$, $P > 0.05$], ketamine treatment [$F_{(1, 24)} = 0.468$, $P > 0.05$] and guanosine treatment × ketamine treatment interaction [$F_{(1, 24)} = 0.134$, $P > 0.05$] on GluA1 immunocontent. In addition, two-way ANOVA revealed no significant effects for guanosine treatment [$F_{(1, 24)} = 0.481$, $P > 0.05$], ketamine treatment [$F_{(1, 24)} = 0.015$, $P > 0.05$] and guanosine treatment × ketamine treatment interaction [$F_{(1, 24)} = 0.917$, $P > 0.05$] on synapsin immunocontent. Overall, none of the treatments in this time protocol was able to significantly change the phosphorylation of p70S6K and its downstream proteins required for synaptic plasticity, such as PSD-95, GluA1, and synapsin.

4. Discussion

To the best of our knowledge, this is the first study that reports the

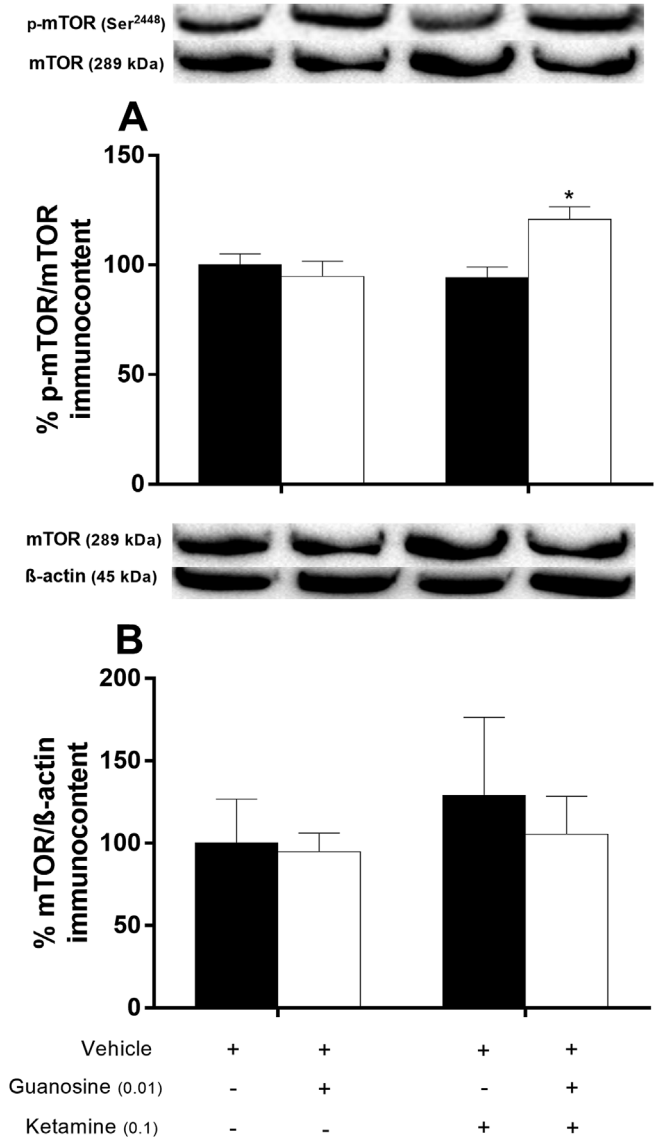

Fig. 5. Effect of treatment with sub-effective doses of guanosine (0.01 mg/kg, p.o.) and/or ketamine (0.1 mg/kg, i.p.) on p-mTOR (A) and total mTOR (B) immunocontent in the hippocampus of mice submitted to the NSF test. Values are expressed as means ± S.E.M (n = 6–7). *$P < 0.05$ as compared with the vehicle-treated control (two-way ANOVA followed by Newman-Keuls post hoc test).

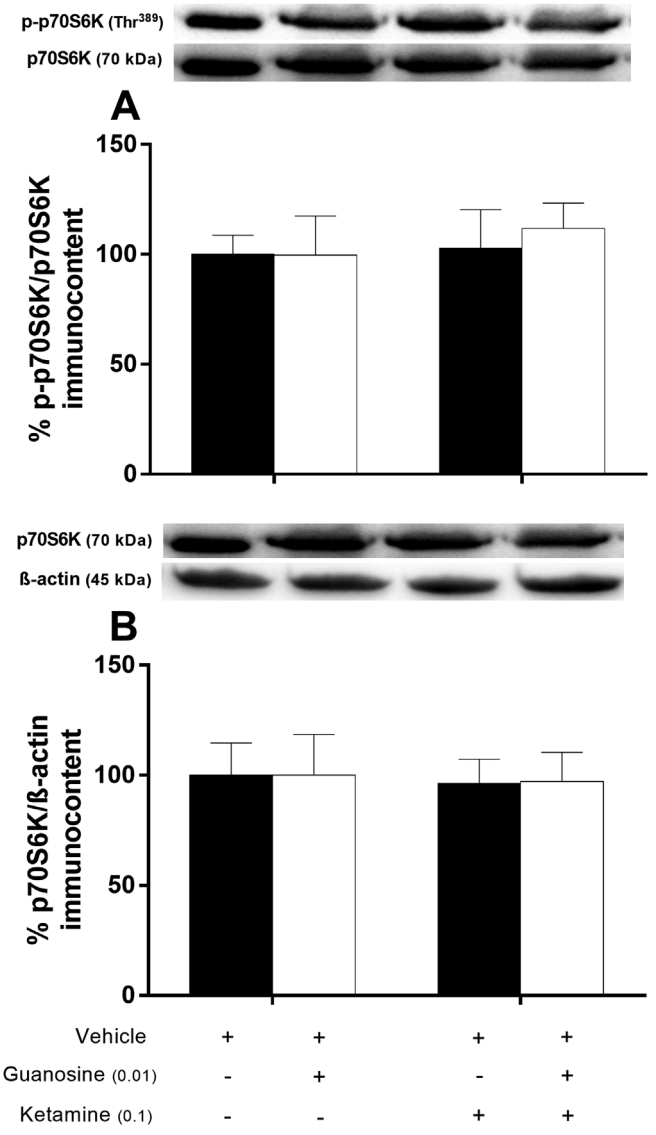

Fig. 6. Effect of treatment with sub-effective doses of guanosine (0.01 mg/kg, p.o.) and/or ketamine (0.1 mg/kg, i.p.) on p-p70S6K (A) and p70S6K (B) immunocontent in the hippocampus of mice submitted to the NSF test. Values are expressed as means ± S.E.M (n = 6–7) (two-way ANOVA followed by Newman-Keuls post hoc test).

augmentation effect of ketamine by guanosine and its intracellular mechanism in the hippocampus of mice. This rapid augmented effect of ketamine by guanosine in the NSF test is dependent on the mTOR-mediated signaling pathway, a key signaling underpinning the fast-acting antidepressant responses. The present study also provides a novel evidence regarding the fast-acting effect of guanosine *per se* in mice submitted to the NSF test, a behavioral paradigm capable of differentiating compounds with fast antidepressant effect, like ketamine, from conventional antidepressants.

Although antidepressant agents used in the therapy are effective in many patients, a large proportion of these individuals (approximately 50%) fail to achieve remission with first-line antidepressants and many remain treatment-resistant (Leary et al., 2015). Therefore, when first-line antidepressant treatment is unsuccessful, second-line strategies include dose optimization, switching to another antidepressant or combination with another antidepressant, and augmentation strategies (Barowsky and Schwartz, 2006). Given this scenario, augmentation strategies have been postulated for the management of depression, especially due to their ability to produce significantly greater improvement than monotherapy (Cussotto et al., 2017; Han et al., 2014; Kale and Addepalli, 2014; Otte et al., 2016). In addition, the augmentation strategies may allow the reduction of the antidepressant dose, which in turn could decrease adverse/side effects (Barowsky and Schwartz, 2006). Supporting the aforementioned reports and aiming at obtaining a more rapid remission of the depressive symptoms, the augmentation strategies should be considered.

Ketamine has been reported to exhibit a rapid and long-lasting antidepressant effect in preclinical (Li et al., 2011; 2010; Zhou et al., 2014) and clinical studies (Berman et al., 2000; Zarate et al., 2006). However, the repeated use of ketamine might cause adverse effects, thereby limiting its usage (Behrens et al., 2007; Sleigh et al., 2014). Therefore, molecules that might share similar mechanisms of action to ketamine or even attenuate its adverse effects, arise as a promising therapeutic strategy. Guanosine is a guanine-based nucleoside recognized as an endogenous neuroprotective component (Ciccarelli et al., 1999, 2001). Intensive investigations regarding the cellular and

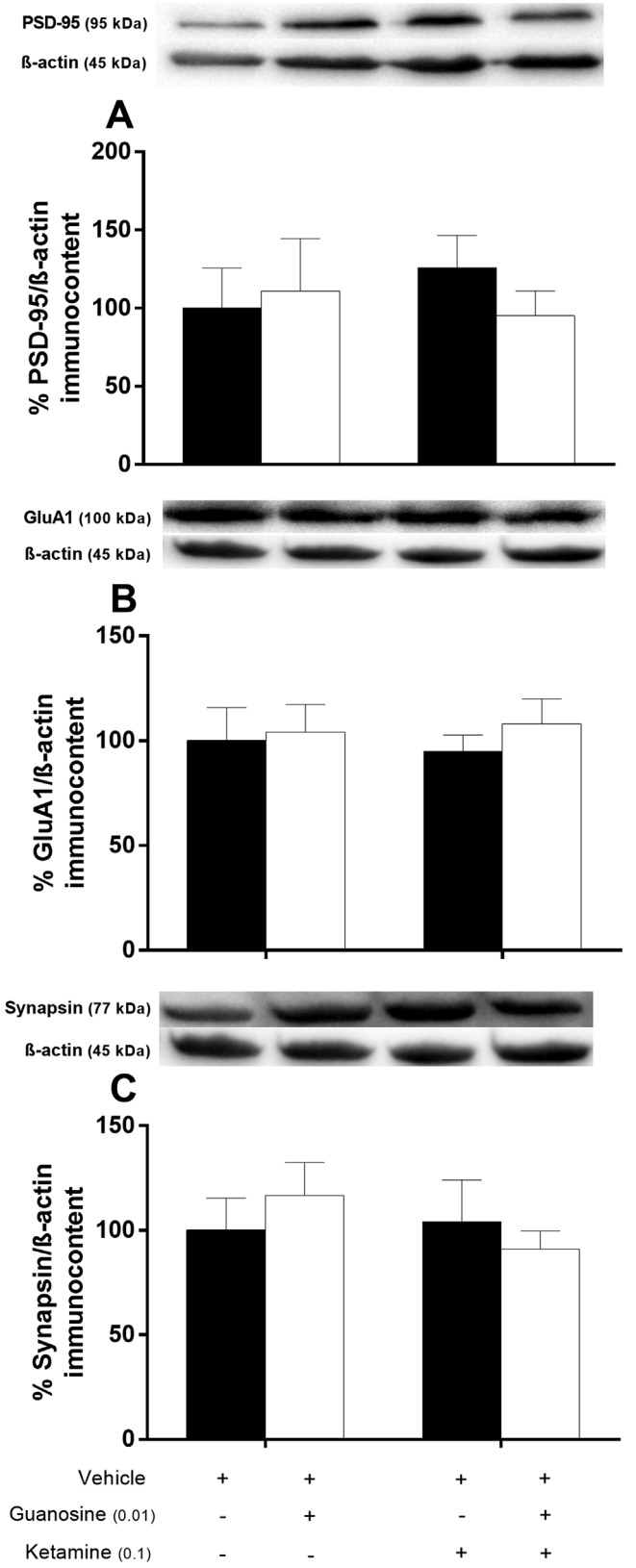

Fig. 7. Effect of treatment with sub-effective doses of guanosine (0.01 mg/kg, p.o.) and/or ketamine (0.1 mg/kg, i.p.) on PSD-95 (A), GluA1 (B) and synapsin (C) immunocontent in the hippocampus of mice submitted to the NSF test. Values are expressed as means ± S.E.M (n = 6–7) (two-way ANOVA followed by Newman-Keuls post hoc test).

molecular mechanisms of action of guanosine have demonstrated that its ability to mitigate the neuroinflammation, oxidative stress, and glutamatergic excitotoxicity may underlie its neuroprotective effects (Dal-Cim et al., 2016; 2013; 2011; Molz et al., 2011; Petronilho et al., 2012; Thomaz et al., 2016). Noteworthy, guanosine has been reported to present an antidepressant-like response (Bettio et al., 2012, 2014), and this effect seems to be unleashed by the modulation of NMDA receptors and mTOR signaling pathway, similarly to ketamine.

In the present study, we demonstrated that acute administration of guanosine (0.05 mg/kg, p.o.), similar to ketamine (1 mg/kg, i.p.), produced a rapid behavioral response in mice submitted to NSF test. The ability of guanosine to cause antidepressant-like effect at very low doses was a result previously obtained by our group in the TST (Bettio et al., 2012). In the FST, the active doses of guanosine are 0.5–5 mg/kg, p.o., whereas in the TST, the dose range that elicited antidepressant-effects are 0.05–0.5 mg/kg (Bettio et al., 2012). In addition, a chronic administration of guanosine for 21 days at the dose of 5 mg/kg also caused an antidepressant-like effect in the TST. Interestingly, in the present study we observe that a very low dose of guanosine (0.05 mg/kg, p.o.) was effective in the NSF test, a behavioral paradigm able to detect depression/anxiety-related behavior. Plasmatic levels of guanosine were reported to increase in a dose- and time-dependent manner following its systemic administration by intraperitoneal or oral route (Giuliani et al., 2012; Jiang et al., 2008; Schmidt et al., 2010; Vinadé et al., 2005). Regarding ketamine, its bioavailability is low (Gao et al., 2016), thereby this drug is generally administered by intravenous route in clinical studies (Berman et al., 2000; Zarate et al., 2006; DiazGranados et al., 2010) and by intraperitoneal route in preclinical studies (Li et al., 2010; Ludka et al., 2013; Pazini et al., 2016).

Despite the availability a considerable amount of literature data about fast-acting antidepressants in last years, few behavioral tests are capable of differentiating molecules with fast antidepressant effect, like ketamine, from conventional antidepressants. Behavioral tests like the TST and the FST have been classically used to track antidepressant drugs, especially monoamine-based drugs (Cryan et al., 2005). However, despite the excellent efficiency and effectiveness of these tests, they are not suitable for detecting fast-acting antidepressants. For this reason, animal models of chronic depression induced by stress, such as chronic treatment with corticosterone (Camargo et al., 2018; Pazini et al., 2016) and unpredictable mild chronic stress (Moretti et al., 2012; Neis et al., 2016) have been used to check compounds with rapid onset antidepressant response, since these models are sensitive to a single administration of rapid antidepressant agents. Although the excellent responsiveness of these models, they need extensive protocols to evaluate the fast-acting antidepressant effects. Therefore, reliable and simple tests with the ability to assess rapid effects of antidepressants are welcome.

One test with this capability is the NSF test, which is sensitive only to the chronic, but not acute administration of conventional antidepressants (Dulawa and Hen, 2005; Powell et al., 2012), such as selective serotonin reuptake inhibitors (Dulawa et al., 2004; Santarelli et al., 2003), tricyclic antidepressants (Bodnoff et al., 1988) and atypical antidepressants (Merali et al., 2003), which mirrors the effects of antidepressant treatment in human patients. Of note, this test is sensitive to a single administration of ketamine, which makes it able to track fast antidepressant agents (Brachman et al., 2016; Fukumoto et al., 2014; Iijima et al., 2012; Li et al., 2011; Wu et al., 2017). In the present study, the reduction in the latency to feed observed in this test seems to be unrelated to an increase of appetite since the amount of food consumed was not significantly affected by any treatment. These results reinforce the assumption that guanosine is effective to elicit rapid behavioral responses related to anxiety and depression, similarly to ketamine. Of note, compelling reports demonstrated that ketamine and guanosine exerted antidepressant and anxiolytic effects (Almeida et al., 2017; Fraga et al., 2018; Ionescu et al., 2015; Krystal et al., 1994).

Noteworthy, reinforcing the notion that guanosine could share the

mechanism of action of ketamine, a previous study reported that a single administration of a sub-effective dose of guanosine combined with a sub-effective dose of ketamine produced an antidepressant-like effect in the TST (Bettio et al., 2012). In keeping with these premises, in this study we purposed to augment the effect of ketamine in the NSF test by guanosine. This approach may be useful considering that chronic administration with ketamine is limited due to its adverse effects. Therefore, one may suppose that this augmentation strategy may reduce the dose of ketamine and consequently its adverse effects. Remarkably, a single administration with a sub-effective dose of guanosine (0.01 mg/kg) when combined with a sub-effective dose of ketamine (0.1 mg/kg) was effective in mice submitted to NSF test, which suggests a rapid and augmented effect. Some hypothesis could be raised to try to explain this above-mentioned augmented effect. A recent groundbreaking study showed that ketamine affects purine and pyrimidine metabolism and neurotransmission in brain (McGowan et al., 2018). Of note, a single administration of ketamine may increase the levels of guanosine diphosphate and guanosine triphosphate in the hippocampus and prefrontal cortex of stressed mice. This result suggests a possible relationship between ketamine and guanosine metabolism, an event that may underlie, at least in part, the augmentation effect of ketamine by guanosine. Another possibility, not excluding the former hypothesis, is that ketamine and guanosine have overlapped mechanism of action. Therefore, we decided to investigate the intracellular mechanism underpinning the augmentation effect of ketamine by guanosine in the NSF test. Particularly, we investigated whether the rapid and augmented effect of ketamine by guanosine in this test could be dependent on mTOR-mediated signaling pathway, that has been reported to be crucial for fast-acting antidepressant effects (Li et al., 2010; W.X. Liu et al., 2016; Voleti et al., 2013).

mTOR is a 289 kDa evolutionarily conserved serine/threonine protein kinase that may be activated by phosphorylation in response to environmental signals, such as growth factors, mitogens and stress (Abelaira et al., 2014; Réus et al., 2015). The mTOR phosphorylation at Ser^{2448} is an indicative of its activation, and upon activation, this protein exerts an essential role in the regulation of protein synthesis, thereby it is involved in neuronal processes as axonal sprouting and dendritic spine growth (Bockaert and Marin, 2015; Laplante and Sabatini, 2012; Li et al., 2010). In fact, Li et al. (2010) found for the first time that the activation of mTOR in the prefrontal cortex of rats underlies the fast antidepressant-like of ketamine. Indeed, dysfunction in mTOR signaling has been implicated in depression (Abelaira et al., 2014; Pazini et al., 2016; Réus et al., 2015). Robust deficits in the mTOR signaling in the prefrontal cortex of subjects diagnosed with major depressive disorder was reported (Jernigan et al., 2011). As shown herein, our results suggest that augmentation response of ketamine by guanosine in the NSF test probably involves the activation of mTOR pathway, since the treatment with rapamycin (a selective mTOR inhibitor) completely abolished this effect. This behavioral response was paralleled by increased mTOR phosphorylation at Ser^{2448} in the hippocampus of mice coadministered with ketamine and guanosine, which in turn reinforces the role of mTOR in the augmentation response of ketamine by guanosine.

Activated mTOR phosphorylates the p70S6K (Thr^{389}) and also phosphorylates and inactivates the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) facilitating translation initiation (Fingar et al., 2004). Among the proteins that have been functionally linked to activation of mTOR and p70S6K, stand out PSD-95, GluA1 and synapsin, which are required for the formation, maturation, and function of new synapses (Li et al., 2011, 2010). Mounting evidence indicates that phosphorylation of p70S6K and consequently the upregulation of PSD-95, GluA1 and synapsin underlie the fast-acting antidepressant response (Li et al., 2010; Pazini et al., 2016; Zhou et al., 2014). Interestingly, in prefrontal cortex of postmortem tissue of depressed subjects, reductions in the expression of p70S6K (Jernigan et al., 2011), PSD95 (Feyissa et al., 2009), GluA1 (Rafalo-Ulinska et al., 2016) and synapsin (Kang et al., 2012) were reported, reinforcing the notion that these proteins are crucial targets implicated in depressive disorders.

Considering this background, we investigated whether the increased levels of these synaptic proteins are associated with the augmented effect of ketamine by guanosine in the NSF test. However, this study did not find an increase in the phosphorylation of p70S6K (Thr^{389}) and on the immunocontents of PSD-95, GluA1, and synapsin in the hippocampus, although we cannot rule out the possibility that a significant effect may occur in another brain structure. Overwhelming pieces of evidence have indicated that prefrontal cortex is involved in the fast antidepressant responses (Li et al., 2011; 2010; Voleti et al., 2013; Zhou et al., 2014).

Collectively, our results significantly extend literature data by indicating that guanosine presents a fast-acting effect in the NSF test, a behavioral paradigm useful to assess the efficacy of fast antidepressants, similarly to ketamine. Interestingly, we provide clear evidence that guanosine is able to augment the effect of ketamine in this test by a mechanism dependent on mTOR signaling. This finding may indicate a pharmacodynamic interaction between ketamine and guanosine and/or that these drugs share common mechanisms. Finally, one may suppose that this augmentation strategy could help in the treatment of depression/anxiety, particularly by reducing the dose of ketamine, consequently diminishing undesirable side effects.

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 is no personal financial holding 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 Anderson Camargo designed the study and wrote the protocol. Anderson Camargo, Francis L. Pazini, Julia M. Rosa, Ingrid A. V. Wolin, Morgana Moretti, Priscila B. Rosa, and Vivian B. Neis administered the drugs and performed the behavioral test. Anderson Camargo and Ana Lúcia S. Rodrigues undertook the statistical analysis and wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Acknowledgments

This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, #449436/2014-4, #310113/2017-2, #150082/2018-5), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, and Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina. Ana L. S. Rodrigues is recipient of CNPq Research Productivity Fellowship. The authors thank the FAPESC scholarship and Laboratório Multiusuário de Estudos em Biologia (LAMEB) by support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpsychires.2019.05.017.

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