Creatine, similarly to ketamine, affords antidepressant-like effects in the tail suspension test via adenosine A₁ and A_{2A} receptor activation

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Received: 12 January 2015 / Accepted: 10 February 2015 / Published online: 22 February 2015
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Abstract The benefits of creatine supplementation have been reported in a broad range of central nervous systems diseases, including depression. A previous study from our group demonstrated that creatine produces an antidepressant-like effect in the tail suspension test (TST), a predictive model of antidepressant activity. Since depression is associated with a dysfunction of the adenosinergic system, we investigated the involvement of adenosine A₁ and A_{2A} receptors in the antidepressant-like effect of creatine in the TST. The anti-immobility effect of creatine (1 mg/kg, *po*) or ketamine (a fast-acting antidepressant, 1 mg/kg, *ip*) in the TST was prevented by pretreatment of mice with caffeine (3 mg/kg, *ip*, nonselective adenosine receptor antagonist), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (2 mg/kg, *ip*, selective adenosine A₁ receptor antagonist), and 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo-{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)-phenol (ZM241385) (1 mg/kg, *ip*, selective adenosine A_{2A} receptor antagonist). In addition, the combined administration of subeffective doses of creatine and adenosine (0.1 mg/kg, *ip*, nonselective adenosine receptor agonist) or inosine (0.1 mg/kg, *ip*, nucleoside formed by the breakdown of adenosine) reduced immobility time in the TST. Moreover, the administration of subeffective doses of creatine or ketamine combined with N-6-cyclohexyladenosine (CHA) (0.05 mg/kg, *ip*, selective adenosine A₁ receptor agonist), N-6-[2-(3,5-dimethoxyphenyl)-2-(methylphenyl)ethyl]adenosine (DPMA) (0.1 mg/kg, *ip*, selective adenosine A_{2A} receptor agonist), or dipyrindamole (0.1 µg/mouse, *icv*, adenosine transporter

inhibitor) produced a synergistic antidepressant-like effect in the TST. These results indicate that creatine, similarly to ketamine, exhibits antidepressant-like effect in the TST probably mediated by the activation of both adenosine A₁ and A_{2A} receptors, further reinforcing the potential of targeting the purinergic system to the management of mood disorders.

Keywords Adenosine · Antidepressant · Creatine · Depression · Tail suspension test

Abbreviations

CHA	N6-cyclohexyladenosine
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
DPMA	N6-[2-(3,5-dimethoxyphenyl)-2-(methylphenyl)ethyl]adenosine
TST	Tail suspension test
<i>icv</i>	Intracerebroventricular
<i>ip</i>	Intraperitoneal
<i>po</i>	Per os
ZM241385	4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo-{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)-phenol

Introduction

Major depression is a severe psychiatric disorder that affects about 16 % of the population during their lifetime, and it is predicted to become the second leading cause of disability by the year 2020 [1–6]. There is a lack of a clear understanding of the molecular basis responsible for this condition, and the efficacy of the therapeutic approaches is still far from optimal [3]. Since the delay to reach efficacy with current

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antidepressants is a major drawback, particularly for suicide risk patients, the development of fast-acting antidepressants represents a significant advance for the treatment of depression [7]. Interestingly, in recent preclinical and clinical reports ketamine emerges as a novel rapid-acting antidepressant agent [8–10]. However, the mechanisms of action implicated in the ketamine antidepressant-like effect are not well established.

Recently, it was also hypothesized that compounds that modulate the bioenergetics status of the cell might represent new pharmacologic approaches for psychiatric disorders [11]. In this context, a study conducted by Assis et al. [12] showed that the acute ketamine administration produced an antidepressant-like effect that was associated with increased creatine kinase activity in striatum, cerebral cortex, and cerebellum. The creatine kinase/phosphocreatine system is a rapid alternative source for ATP synthesis in the brain, controlling neural energy demands [13–15].

Both clinical and preclinical evidence have demonstrated that creatine (*N*-aminoiminomethyl-*N*-methylglycine, a substrate of creatine kinase enzyme) improves mood, possibly by restoring brain energy levels and homeostasis [16–22]. Of note, creatine supplementation has been reported to improve depressive symptoms in 1–2 weeks of treatment in depressive patients, faster than the currently available antidepressants, which require 4–5 weeks to produce their therapeutic effects [19, 21].

Although it is supposed that the mechanisms underlying creatine-induced mood modulation involve the enhancement in energy storage, a second line of evidence has demonstrated direct neuromodulatory effects for creatine in neurotransmitter systems and intracellular targets [22–24]. It was recently shown by our group that the antidepressant-like effect of creatine in preclinical models is dependent on the modulation of the monoaminergic and glutamatergic systems and several intracellular pathways involved in neuronal plasticity and cell survival [16–18, 22, 25].

Adenosine is a metabolic product of ATP, which is produced in an activity-dependent manner to play neuromodulatory and homeostatic roles in brain circuits [26, 27]. The adenosinergic system can affect the efficiency of synaptic transmission and neurotransmitter release through a combined activation of inhibitory adenosine A₁ receptors and facilitatory adenosine A_{2A} receptors in neuronal circuits [28, 29]. There is a growing body of evidence suggesting that these adenosine receptors could be promising therapeutic targets in a wide range of conditions, including neurodegenerative and psychiatric disorders such as major depression [30–34]. However, the interaction between antidepressant compounds and adenosine receptors in the central nervous system remains largely unexplored. The present study investigated the involvement of the adenosinergic system, especially adenosine A₁ and A_{2A} receptors in the antidepressant-like effect of creatine and ketamine in the tail suspension test (TST).

Materials and methods

Animals

Male Swiss mice (30–40 g, 8–12 weeks old) were used in the present study. Animals were housed in groups of 14 animals per cage under controlled conditions of light (from 07:00 to 19:00 h) and temperature (21±1 °C) with free access to standard laboratory food and tap water. Each experimental group consisted of 7–8 animals. Animals were randomly distributed into specified experimental groups. The manipulations were carried out between 13:00 and 16:00 h, with each animal used only once. All procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee of the Institution. All efforts were made to minimize animals suffering and to reduce the number of animals used in the experiments.

Drugs

The following drugs were used: creatine monohydrate and fluoxetine (Sigma Chemical Co., USA) were dissolved in distilled water, adenosine, inosine, N-6-cyclohexyladenosine (CHA), caffeine, and ketamine (Sigma Chemical Co., USA) were dissolved in saline, N-6-[2-(3,5-dimethoxyphenyl)-2-(methylphenyl)ethyl]adenosine (DPMA) (Sigma Chemical Co., USA), 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo-{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)-phenol (ZM241385), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), and dipyridamole (Tocris Cookson, USA) were dissolved in saline with 5 % dimethyl sulfoxide (DMSO). The drugs were administered by intraperitoneal (*ip*) route, except creatine, which was administered by oral route (*po*) by gavage and dipyridamole that was administered by intracerebroventricular (*icv*) route. All drugs, except dipyridamole, were administered in a constant volume of 10 mL/kg body weight.

Icv administration

Icv administration was performed by a “free-hand” technique using a microsyringe (25 µL, Hamilton) connected to a 26-gauge stainless steel needle that was inserted perpendicularly 2 mm deep through the skull according to the procedure originally described by [35] and adapted by [17]. Briefly, the animals were lightly anesthetized with ether (i.e., just that necessary for loss of the postural reflex) and then gently restrained by hand for *icv* injections. The sterilization of the injection site was carried out using gauze embedded in 70 % ethanol. Under light anesthesia, the needle was inserted unilaterally 1 mm to the midline point equidistant from each eye, at an equal distance between the eyes and the ears and perpendicular to the plane of the skull. Dipyridamole was injected directly into the

lateral ventricle, at the following coordinates from bregma taken from the atlas of Franklin and Paxinos (1997) [36]: anteroposterior=−0.1 mm; mediolateral=1 mm; and dorso-ventral=−3 mm. Mice exhibited normal behavior within 1 min after injection. After completion of the experiments, all animals were decapitated and their brains were freshly examined.

It is possible to check the site of the *icv* injections by visual inspection of the dissected brain. This is a standard procedure commonly performed to determine the accuracy of the technique. Results from mice presenting misplacement of the injection site or any sign of cerebral hemorrhage were excluded from the statistical analysis (overall less than 5 % of the total animals used).

Experimental design

In order to investigate the involvement of the adenosine A₁ and A_{2A} receptors in the antidepressant-like effect of creatine or fluoxetine (conventional antidepressant) in the TST, animals were pretreated with caffeine (3 mg/kg, *ip*, a nonselective adenosine receptor antagonist), DPCPX (2 mg/kg, *ip*, a selective adenosine A₁ receptor antagonist), ZM241385 (1 mg/kg, *ip*, a selective adenosine A_{2A} receptor antagonist), or with appropriate vehicle. After 30 min, the animals received creatine (1 mg/kg, *po*) or fluoxetine (10 mg/kg, *po*) or vehicle before being tested in the TST or open-field 60 min later. In a separate set of experiments, caffeine, DPCPX, ZM241385, or vehicle were administered 30 min before ketamine (a fast-acting antidepressant) or vehicle. After 30 min, the animals were submitted to TST or open-field test.

In order to investigate a possible synergistic effect between creatine and adenosinergic agonists, animals were treated with a subeffective dose of creatine (0.01 mg/kg, *po*) or vehicle 30 min before administration of adenosine (0.1 mg/kg, *ip*), inosine (0.1 mg/kg, *ip*), CHA (0.05 mg/kg, *ip*, a selective adenosine A₁ receptor agonist), DPMA (0.1 mg/kg, *ip*, a selective adenosine A_{2A} receptor agonist), or vehicle. A further 30 min was allowed to elapse before the animals were tested in the TST or open-field test. In a separate set of experiments, animals were treated simultaneously with subeffective dose of ketamine (0.1 mg/kg, *ip*) or vehicle and CHA (0.05 mg/kg, *ip*), DPMA (0.1 mg/kg, *ip*) or vehicle.

In another set of experiments, mice were pretreated with creatine (0.01 mg/kg, *po*) 45 min before dipyridamole (0.1 µg/mouse, *icv*, an adenosine transporter inhibitor) administration, and 15 min later, the TST or open-field test was carried out. Alternatively, animals were pretreated with ketamine (0.1 mg/kg, *ip*) 15 min before dipyridamole (0.1 µg/mouse, *icv*) administration and 15 min later, the TST or open-field test was carried out. Control animals received appropriate vehicle.

The doses of drugs (adenosine receptor antagonists and agonists, adenosine, inosine, and dipyridamole) used were

selected on the basis of literature data and on previous results from our group [32, 37, 38]. The doses of antidepressant compounds (creatine, ketamine, and fluoxetine) used were selected based on previous results from our laboratory [16, 39, 40].

Behavioral tests

Tail suspension test (TST)

The tail suspension test (TST) has become one of the most widely used models for assessing antidepressant-like activity in mice. The test is based on the fact that animals subjected to the short-term inescapable stress of being suspended by their tail will develop an immobile posture. The total duration of immobility induced by tail suspension was measured according to the method described by [41]. 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. Immobility time was recorded during a 6-min period. Mice were considered immobile only when they hung passively and completely motionless [16, 39, 42].

Open-field test

To assess the possible effects of drugs on locomotor activity, mice were evaluated in the open-field paradigm as previously described [18, 42]. Mice were individually placed in a wooden box (40×60×50 cm) with the floor divided into 12 equal rectangles. The number of rectangles crossed by the animal with its four paws (crossing) was registered during a 6-min period. The number of crossings was considered as indicative of locomotor activity. The behavior was recorded by an observer blind to the drug treatment. The floor of the open-field apparatus was cleaned with 10 % ethanol between tests.

Statistical analysis

Comparisons between experimental and control groups were performed by two-way ANOVA followed by Tukey's HSD test when appropriate. A value of $p < 0.05$ was considered to be significant.

Results

Effect of pretreatment of mice with caffeine in the antidepressant-like effect of creatine, ketamine, and fluoxetine in the TST

In order to investigate the possible interaction between creatine and adenosine receptors in the TST, the animals were pretreated with caffeine (3 mg/kg, *ip*) and submitted to the TST. The results depicted in Fig. 1c show that by blocking

both A_1 and A_{2A} receptors, caffeine significantly prevented the decrease in immobility time elicited by creatine (1 mg/kg, *po*) in the TST. Two-way ANOVA revealed a significant effect of the caffeine treatment [$F(1,27)=8.64$, $p<0.01$], creatine treatment [$F(1,27)=6.71$, $p<0.05$], and caffeine $\times$ creatine treatment interaction [$F(1,27)=5.93$, $p<0.05$]. Furthermore, caffeine (3 mg/kg, *ip*) significantly prevented the decrease in the immobility time elicited by the fast-acting antidepressant ketamine (1 mg/kg, *ip*) in the TST (Fig. 1d). Two-way ANOVA revealed a significant effect of the ketamine treatment [$F(1,24)=6.29$, $p<0.05$] and caffeine $\times$ ketamine treatment interaction [$F(1,24)=5.05$, $p<0.05$].

The result depicted in Fig. 1e shows that the anti-immobility effect of fluoxetine (10 mg/kg, *po*) was not prevented by pretreatment of mice with caffeine (3 mg/kg, *ip*) in the TST. A two-way ANOVA showed significant differences for fluoxetine treatment [$F(1,28)=23.23$, $p<0.01$],

but not for caffeine treatment and caffeine $\times$ fluoxetine treatment interaction.

Effects of the combined treatment of creatine, adenosine, or inosine in the TST

We further investigated the effect of the subeffective doses of adenosine (0.1 mg/kg, *ip*) or inosine (0.1 mg/kg, *ip*) in combination with creatine (0.01 mg/kg, *po*) in the immobility time in the TST. The results presented in Fig. 2b show that the administration of the adenosine produced a synergistic antidepressant-like effect when combined with a subeffective dose of creatine (0.01 mg/kg, *po*) in the TST. A two-way ANOVA revealed significant differences for adenosine treatment [$F(1,26)=21.46$, $p<0.01$], creatine treatment [$F(1,26)=13.58$, $p<0.01$], and adenosine $\times$ creatine treatment interaction [$F(1,26)=9.04$, $p<0.01$]. As presented in Fig. 2c, a

Fig. 1 Effect of pretreatment of mice with caffeine on the anti-immobility effect of creatine, ketamine, and fluoxetine in the TST. Timeline of experimental protocols of administrations and behavioral tests (**a** and **b**). Effect of the pretreatment with caffeine (3 mg/kg, *ip*, a nonselective adenosine receptor antagonist) on the anti-immobility effect of creatine (1 mg/kg, *po*), ketamine (1 mg/kg, *ip*), and fluoxetine

(10 mg/kg, *po*) in the TST (**c**, **d**, and **e**, respectively) and on ambulation in the open-field test (**f**, **g**, and **h**, respectively). Each column represents the mean $\pm$ S.E.M. ($n=7-8$). ** $p<0.01$ as compared with the vehicle-treated control. # $p<0.05$, ## $p<0.01$ as compared with the same group pretreated with vehicle. (two-way ANOVA followed by Tukey's HSD post hoc test)

Fig. 2 Synergistic antidepressant-like effect of combined administration of subeffective doses of creatine and adenosine or inosine in the TST. Timeline of experimental protocols of administrations and behavioral test (**a**). Effect of the treatment with subeffective doses of creatine (0.01 mg/kg, *po*) in combination with adenosine (0.1 mg/kg, *ip*, a nonselective adenosine receptor agonist) or inosine (0.1 mg/kg, *ip*, a purine formed during the breakdown of adenosine) on the immobility time in the TST (**b** and **c**, respectively) and on the number of crossings in the open-field test (**d** and **e**, respectively). Each column represents the mean $\pm$ S.E.M. $**p < 0.01$ as compared with the vehicle-treated control (two-way ANOVA followed by Tukey's HSD post hoc test)

similar result was obtained with the combined administration of subeffective doses of creatine and inosine. Two-way ANOVA revealed significant differences for inosine treatment [$F(1,26)=14.69$, $p < 0.01$], creatine treatment [$F(1,26)=10.25$, $p < 0.01$], and inosine $\times$ creatine treatment interaction [$F(1,26)=4.29$, $p < 0.05$].

Involvement of adenosine A₁ receptor on the antidepressant-like effect of creatine and ketamine in the TST

Further, we tried to unravel the adenosine receptor subtypes involved in the antidepressant-like effect of creatine in the FST. To this end, mice were pretreated with DPCPX (2 mg/kg, *ip*) or ZM241385 (1 mg/kg, *ip*). The results in Fig 3c show that the pretreatment of mice with the adenosine A₁ receptor antagonist DPCPX (2 mg/kg, *i.p*) prevented the antidepressant-like effect elicited by creatine. Two-way

ANOVA showed significant differences for DPCPX treatment [$F(1,28)=19.69$, $p < 0.01$], creatine treatment [$F(1,28)=9.01$, $p < 0.01$], and DPCPX $\times$ creatine treatment interaction [$F(1,28)=10.16$, $p < 0.01$]. Moreover, DPCPX (2 mg/kg, *ip*) significantly prevented the decrease in the immobility time elicited by fast-acting antidepressant ketamine (1 mg/kg, *ip*) in the TST (Fig. 3d). Two-way ANOVA revealed a significant effect of DPCPX treatment [$F(1,20)=6.99$, $p < 0.05$] and DPCPX $\times$ ketamine treatment interaction [$F(1,20)=6.11$, $p < 0.05$].

Moreover, in order to determine the involvement of different adenosine receptor subtypes in the antidepressant-like effect of creatine and ketamine, mice were treated with subeffective doses of the selective adenosine A₁ receptor agonist CHA (0.05 mg/kg, *ip*) in combination with a subeffective dose of creatine (0.01 mg/kg, *po*) or ketamine (0.1 mg/kg, *ip*). The results presented in Fig. 4c show that the administration of CHA produced a synergistic antidepressant-like effect when combined with a subeffective dose of creatine

Fig. 3 Effect of pretreatment of mice with DPCPX on the anti-immobility effect of creatine and ketamine in the TST. Timeline of experimental protocols of administrations and behavioral tests (**a** and **b**). Effect of the pretreatment with DPCPX (2 mg/kg, *ip*, a selective adenosine A₁ receptor antagonist) on the anti-immobility effect of creatine (1 mg/kg, *po*) and ketamine (1 mg/kg, *ip*) in the TST (**c** and **d**, respectively) and on ambulation in the open-field test (**e** and **f**, respectively). Each column represents the mean + S.E.M. ($n=7-8$). ** $p<0.01$ as compared with the vehicle-treated control. # $p<0.05$, ## $p<0.01$ as compared with the same group pretreated with vehicle. (two-way ANOVA followed by Tukey's HSD post hoc test)

in the TST. Two-way ANOVA revealed significant differences for creatine treatment [$F(1,27)=14.53$, $p<0.01$], CHA treatment [$F(1,27)=14.62$, $p<0.01$], and creatine $\times$ CHA treatment interaction [$F(1,27)=4.88$, $p<0.05$]. In addition, CHA also produced a synergistic antidepressant-like effect when combined with a subeffective dose of ketamine (0.1 mg/kg, *ip*) in the TST (Fig. 4d). Two-way ANOVA revealed significant differences for ketamine treatment [$F(1,20)=22.85$, $p<0.01$], CHA treatment [$F(1,20)=23.46$, $p<0.01$], and ketamine $\times$ CHA treatment interaction [$F(1,20)=4.88$, $p<0.05$].

Adenosine A_{2A} receptors are implicated in the antidepressant-like effect of creatine and ketamine in the TST

The results depicted in Fig. 5c show that the pretreatment of animals with the selective A_{2A} receptor antagonist ZM241385 (1 mg/kg, *ip*) prevented the effect of creatine (1 mg/kg, *ip*) in the TST. Two-way ANOVA revealed main differences for creatine treatment [$F(1,27)=7.89$, $p<0.01$] and ZM241385 $\times$ creatine treatment interaction [$F(1,27)=12.33$, $p<0.01$], but not for ZM241385 treatment [$F(1,27)=3.40$, $p=0.07$].

Moreover, ZM241385 also significantly prevented the decrease in the immobility time elicited by ketamine (1 mg/kg, *ip*) in the TST (Fig. 5d). Two-way ANOVA revealed a significant effect of ketamine treatment [$F(1,20)=5.35$, $p<0.05$] and ZM241385 treatment $\times$ ketamine treatment interaction [$F(1,20)=4.48$, $p<0.05$]. Thus, the antidepressant-like effect of creatine, similar to ketamine, is dependent, at least in part, on both A₁ and A_{2A} receptor subtypes.

The Fig. 6c shows that the administration of DPMA (0.1 mg/kg, *ip*), a selective A_{2A} receptor agonist, potentiated the action of a subeffective dose of creatine (0.01 mg/kg, *po*) in the TST. Two-way ANOVA showed significant differences for creatine treatment [$F(1,31)=21.88$, $p<0.01$], DPMA treatment [$F(1,31)=14.94$, $p<0.01$], and creatine $\times$ DPMA treatment interaction [$F(1,31)=4.51$, $p<0.05$]. DPMA also produced a synergistic antidepressant-like effect when combined with a subeffective dose of ketamine (0.1 mg/kg, *ip*) in the TST (Fig. 6d). Two-way ANOVA revealed significant differences for the ketamine treatment [$F(1,27)=23.81$, $p<0.01$], DPMA treatment [$F(1,27)=22.75$, $p<0.01$], and ketamine $\times$ DPMA treatment interaction [$F(1,27)=3.36$, $p=0.05$].

Fig. 4 The antidepressant-like effect of administration of a subeffective dose of creatine or ketamine in combination with selective adenosine A₁ receptor agonist CHA in the TST. Timeline of experimental protocols of administrations and behavioral tests (**a** and **b**). Effect of CHA (0.05 mg/kg, *ip*, a selective adenosine A₁ receptor agonist) or DPMA (0.1 mg/kg, *ip*, a selective adenosine A_{2A} receptor agonist) treatment in combination with a subeffective dose of creatine (0.01 mg/kg, *po*) or ketamine (0.1 mg/kg, *ip*) on the immobility time in the TST (**c** and **d**, respectively) and on the ambulation in the open-field test (**e** and **f**, respectively). Each column represents the mean + S.E.M. ($n=7-8$). $**p<0.01$ as compared with the vehicle-treated group (two-way ANOVA followed by Tukey's HSD post hoc test)

Creatine or ketamine in combination with dipyrindamole produced a synergistic antidepressant-like effect in the TST

Another strategy used to investigate the involvement of the adenosinergic system in the effect of creatine in the TST was to enhance the extracellular adenosine levels by blocking the nucleoside transporter with dipyrindamole. As presented in Fig. 7c, the treatment of mice with the adenosine transporter inhibitor dipyrindamole (0.1 µg/mouse, *icv*) was able to potentiate the effect of a subeffective dose of creatine (0.01 mg/kg, *po*). Two-way ANOVA showed significant differences for creatine treatment [$F(1,30)=13.79$, $p<0.01$], dipyrindamole treatment [$F(1,30)=18.12$; $p<0.01$], and creatine × dipyrindamole treatment interaction [$F(1,30)=4.23$, $p<0.05$]. Also, dipyrindamole was able to potentiate the effect of a subeffective dose of ketamine (0.1 mg/kg, *ip*) (Fig. 7d). Two-way ANOVA showed significant differences for ketamine treatment [$F(1,39)=4.5$, $p<0.01$], dipyrindamole treatment [$F(1,39)=10.61$; $p<0.01$], and ketamine × dipyrindamole treatment interaction [$F(1,39)=4.61$, $p<0.01$].

It is important to mention that creatine, ketamine, or fluoxetine alone or in combination with the adenosine system modulators did not cause any alterations in the ambulatory behavior in the open-field test [$p>0.05$].

Discussion

Five decades of antidepressant research focused on the monoaminergic system and wrestled with the fact that monoaminergic antidepressants require several weeks to produce their full therapeutic effects. Therefore, there is a clear and urgent need for rapid-acting antidepressants with robust efficacy. Ketamine is clinically used as a dissociative anesthetic [43] and, also, for the acute treatment of depressive patients refractory to currently available treatments. For this reason, it is a prototype for a new generation of antidepressants that rapidly alleviate depressive symptoms [9, 44, 45]. In the present study, we confirmed the robust anti-immobility effect of acute administration of subanesthetic doses of ketamine in the TST.

Although ketamine was generally well tolerated, mild to moderate transient adverse effects were observed during infusion, as perceptual disturbances, dissociation, euphoria, dysphoria, and/or anxiety [46]. Furthermore, physical adverse effects include nausea, dizziness, and minimal increases in blood pressure and heart rate [46]. Thus, pharmacological compounds capable of mimicking the fast antidepressant effects of ketamine but without causing side effects might represent new therapeutic targets in the treatment of depression.

Currently, it is hypothesized that compounds that modulate the bioenergetics status of the cell might be good candidates for the development of new antidepressant strategies [11].

Fig. 5 Effect of pretreatment of mice with ZM241385 on the anti-immobility effect of creatine and ketamine in the TST. Timeline of experimental protocols of administrations and behavioral tests (**a** and **b**). Effect of the pretreatment with ZM241385 (1 mg/kg, *ip*, a selective adenosine A_{2A} receptor antagonist) on the anti-immobility effect of creatine (1 mg/kg, *po*) and ketamine (1 mg/kg, *ip*) in the TST (**c** and **d**, respectively) and on ambulation in the open-field test (**e** and **f**, respectively). Each column represents the mean + S.E.M. ($n=7-8$). ** $p<0.01$ as compared with the vehicle-treated control. ## $p<0.01$ as compared with the same group pretreated with vehicle (two-way ANOVA followed by Tukey's HSD post hoc test)

Creatine, a well-known energy enhancer compound, has shown promise as a safe, effective, and tolerable adjunct to medication for the treatment of depression [18–20]. The present study extends the literature data by showing that creatine also elicits antidepressant-like effect in the TST through an interaction with the neuromodulatory system operated by adenosine and its receptors. This effect of creatine is promising since many efforts have been devoted to find new therapeutics aiming at multiple cellular targets to treat complex disorders like major depression.

The creatine kinase/phosphocreatine system is a rapidly available alternative source for ATP synthesis in brain. This system plays an important role especially in cells with high energy demands like neurons, being an important target in the control of neurodegenerative states associated with several disorders [13–15]. In this context, creatine administered both systemic and centrally produces antidepressant-like effects in behavioral despair models like the TST [16–18]. In addition to the preclinical data, clinical trials have also demonstrated that creatine supplementation produces antidepressant effect and that creatine could be a new approach in the treatment of depression [21, 19, 20]. However, the mechanisms underlying

the homeostatic versus neuromodulatory roles of creatine in the brain still need to be explored. In this regard, our group recently demonstrated that the antidepressant-like effect of creatine in the TST is associated with the dopamine D₁ and D₂ receptors [16], α_1 -adrenoceptors [17], post-synaptic 5-HT_{1A} receptors [18] activation, NMDA receptor inhibition [22], as well as activation of intracellular kinases [25]. Since adenosinergic system, especially the inhibitory adenosine A₁ and the facilitatory adenosine A_{2A} receptors, are able to control several neurotransmission systems, such as dopaminergic [47], glutamatergic [48, 49], and serotonergic system [50], we investigated the involvement of this system in the antidepressant-like effect of creatine in the TST.

The adenosinergic system is also involved in the control of energy load and neuronal activity [51]. However, mainly by interacting with the high affinity A₁ and A_{2A} receptors, adenosine is also capable of modulating emotional behavior and is involved in several psychiatric condition including depression [32, 52] and anxiety [53, 54]. Taking into account the key role of adenosinergic system in mood regulation, the first set of experiments evaluated the influence of pretreating mice with the nonselective adenosine A₁ and A_{2A} receptor antagonist

Fig. 6 The antidepressant-like effect of combined administration of subeffective doses of creatine or ketamine and DPMA in the TST. Timeline of experimental protocols of administrations and behavioral tests (**a** and **b**). Effect of DPMA (0.1 mg/kg, *ip*, a selective adenosine A_{2A} receptor agonist) treatment in combination with a subeffective dose of creatine (0.01 mg/kg, *po*) or ketamine (0.1 mg/kg, *ip*) on the immobility time in the TST (**c** and **d**, respectively) and on the ambulation in the open-field test (**e** and **f**, respectively). Each column represents the mean + S.E.M. ($n=7-8$). ** $p<0.01$ as compared with the vehicle-treated group (two-way ANOVA followed by Tukey's HSD post hoc test)

caffeine in the effect of creatine, ketamine, and the conventional antidepressant fluoxetine (selective serotonin reuptake inhibitor) in the TST. Our results show, to our knowledge for the first time, that the antidepressant-like effects of creatine and ketamine, but not fluoxetine, were abolished by caffeine, suggesting that an activation of adenosine A₁ and/or A_{2A} receptors is involved in anti-immobility effects of these compounds in the TST. Interestingly, the selective adenosine A₁ receptor antagonist DPCPX and the selective adenosine A_{2A} receptor antagonist ZM241385 did not prevent the anti-immobility effect of fluoxetine in the forced swimming test [55]. Considering that caffeine has similar *in vitro* affinities for A₁ and A_{2A} receptors [56], to further address this issue, in the next set of experiments, the participation of either adenosine A₁ or A_{2A} receptor was investigated through the use of selective antagonists and agonists of these receptors.

The present results demonstrated that the activation of adenosine A₁ receptors is implicated, at least in part, in the antidepressant-like effect of creatine and ketamine in the TST, considering that the selective adenosine A₁ receptor antagonist DPCPX was able to prevent their effects in the TST. In addition, reinforcing our assumption, the treatment of mice with the selective adenosine A₁ receptor agonist CHA combined with a subeffective dose of creatine was able to cause a synergistic effect in the TST. In line with our findings, it has

been reported that adenosine A₁ receptors are implicated in the mechanism of action of some antidepressant compounds and approaches used to treat depression. A study showed that the anticonvulsant effect of tianeptine, an atypical antidepressant, is mediated by the activation of adenosine A₁ receptors [57]. In addition, the activation of adenosine A₁ receptors is required for the antidepressant effect of sleep deprivation [58]. In preclinical models, CCPA, a selective adenosine A₁ receptor agonist, reduced immobility time in the forced swimming test, an effect that is sustained for 36 h following its administration [58]. Moreover, adenosine A₁ receptor agonist CHA induces antidepressant-like profile in rats [59]. In line with these results, studies from our group also reported that DPCPX prevented the antidepressant-like effect of adenosine, inosine, and zinc chloride [32, 37, 38, 60].

The involvement of adenosine A_{2A} receptors on depression and in the antidepressant action is not well established. Although literature data, through the genetic (adenosine A_{2A} receptor knockout mice) or pharmacological (A_{2A} receptor antagonists) approaches, have shown that adenosine A_{2A} receptors inhibition produces antidepressant-like effect in the forced swimming test and TST [31, 61, 62], other studies from our group have also demonstrated that activation of adenosine A_{2A} receptors is implicated in the antidepressant-like effect of adenosine, inosine, and zinc in the same tests [32, 37, 38].

Fig. 7 Synergistic antidepressant-like effect of combined administration of subeffective doses of creatine or ketamine and dipyridamole in the TST. Timeline of experimental protocols of administrations and behavioral tests (**a** and **b**). Effect of the treatment with subeffective doses of creatine (0.01 mg/kg, *po*) or ketamine (1 mg/kg, *ip*) in combination with dipyridamole (0.1 µg/mouse, *icv*, an adenosine transporter inhibitor) on the immobility time in the TST (**c** and **d**, respectively) and on the number of crossings in the open-field test (**e** and **f**, respectively). Each column represents the mean±S.E.M. ** $p<0.01$ as compared with the vehicle-treated control (two-way ANOVA followed by Tukey's HSD post hoc test)

This discrepancy might be due to differences in the animal model and/or procedures employed and the doses of adenosine A_{2A} receptor antagonists. Although at this stage the relation between adenosine A_{2A} receptors and depression is still circumstantial, it seems evident that adenosine A_{2A} receptors are able to modulate mood states, an effect that deserves further investigation. In the present study, we also show that the antidepressant-like effect of creatine and ketamine are dependent on adenosine A_{2A} receptor modulation, since the pretreatment of mice with caffeine or ZM241385, an adenosine A_{2A} receptor antagonist, prevented the antidepressant-like effect of these compounds in the TST. In addition, literature data show that creatine produced anti-inflammatory effects in endothelial cells that were suppressed by ZM241385 [63]. Similar to our results, the anti-inflammatory effect of ketamine was also blocked by ZM241385 treatment [64]. In addition, the pretreatment of mice with the selective adenosine A_{2A} receptor agonist DPMA was able to cause a synergistic effect with subeffective doses of creatine or ketamine in the TST, reinforcing the importance of adenosine A_{2A} receptor activation for the antidepressant-like effect of these compounds. One possibility to account for the involvement of adenosine A_{2A} receptors in the antidepressant-like effect of creatine and

ketamine is that these effects could be indirectly mediated by the inhibition of NMDA receptors. It is well known that adenosine A_{2A} receptors inhibit NMDA currents in rat striatal neurons [49].

Our results also show that subeffective doses of adenosine or inosine were able to potentiate the effect of creatine in the TST. In addition, adenosine administration was also able to potentiate the effect of ketamine, as well as of the other NMDA receptor antagonists MK-801 and zinc, in the forced swimming test [52]. These results further reinforce the importance of the adenosinergic system in the antidepressant-like effect of creatine and ketamine.

The present study also shows that the treatment of mice with a subeffective dose of dipyridamole was effective to augment the effect of creatine and ketamine in the TST. Dipyridamole is known to inhibit adenosine uptake, increasing the bioavailability of adenosine in the synaptic cleft [65] and was reported to potentiate the effect of zinc chloride in the forced swimming test [38]. Our results suggest that the increase of adenosine levels in the synaptic cleft plays a critical role in the effect of creatine and ketamine in the TST. Somewhat in line with these results, the antidepressant effect of citalopram was proposed to be related to the parallel increase

of adenosine and serotonin levels in plasma of depressed patients [66].

It is important to note that all the behavioral responses in the TST elicited by creatine or ketamine alone or in combination with adenosinergic modulators cannot be attributed to any unspecific locomotor effect, since ambulation in the open-field test was not altered by any treatment.

We demonstrated herein that creatine, an energy enhancer compound endowed with pleiotropic effects that include its ability to produce antidepressant-like effects through a modulation of monoaminergic and glutamatergic systems [16–18, 22] and intracellular signaling pathways implicated in cellular survival and neuroplasticity [25], produced an anti-immobility effect in the TST by activating both adenosine A₁ and A_{2A} receptors. Interestingly, a similar mechanism of action was observed in animals treated with ketamine. This property of creatine is especially relevant taking into account that compounds able to hit multiple targets implicated in complex disorders might represent promising pharmacological approaches.

Acknowledgments This study was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), #308723/2013-9, Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES), NENASC project (PRONEX program CNPq/FAPESC).

Conflict of interest The authors declare no conflict of interest.

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