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The activation of α_1-adrenoceptors is implicated in the antidepressant-like effect of creatine in the tail suspension test

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ABSTRACT

The antidepressant-like activity of creatine in the tail suspension test (TST) was demonstrated previously by our group. In this study we investigated the involvement of the noradrenergic system in the antidepressant-like effect of creatine in the mouse TST. In the first set of experiments, creatine administered by i.c.v. route (1 μg/site) decreased the immobility time in the TST, suggesting the central effect of this compound. The anti-immobility effect of peripheral administration of creatine (1 mg/kg, p.o.) was prevented by the pretreatment of mice with α-methyl-p-tyrosine (100 mg/kg, i.p., inhibitor of tyrosine hydroxylase), prazosin (1 mg/kg, i.p., α_1-adrenoceptor antagonist), but not by yohimbine (1 mg/kg, i.p., α_2-adrenoceptor antagonist). Creatine (0.01 mg/kg, subeffective dose) in combination with subeffective doses of amitriptyline (1 mg/kg, p.o., tricyclic antidepressant), imipramine (0.1 mg/kg, p.o., tricyclic antidepressant), reboxetine (2 mg/kg, p.o., selective noradrenaline reuptake inhibitor) or phenylephrine (0.4 μg/site, i.c.v., α_1-adrenoceptor agonist) reduced the immobility time in the TST as compared with either drug alone. These results indicate that the antidepressant-like effect of creatine is likely mediated by an activation of α_1-adrenoceptor and that creatine produces synergistic effects in the TST with antidepressants that modulate noradrenaline transporter, suggesting that an improvement in the response to the antidepressant therapy may occur when creatine is combined with these antidepressants. Furthermore, the synergistic effect of creatine (0.01 mg/kg, p.o.) and reboxetine (2 mg/kg, p.o.) combination was abolished by the α_1-adrenoceptor antagonist prazosin, indicating that the antidepressant-like effect of combined therapy is likely mediated by an activation of α_1-adrenoceptor.

1. Introduction

Depression is one of the most common illnesses characterized by a broad range of symptoms, including altered mood and cognitive functions, loss of interest or pleasure, feelings of guilt or low self-worth, disturbed sleep or appetite, low energy and recurrent thoughts of death or suicide. The antidepressants used for the treatment of this disorder act by increasing the availability of monoamines (serotonin, noradrenaline and dopamine) in the synaptic cleft by blocking their reuptake (tricyclics, selective serotonin reuptake inhibitors, selective noradrenaline reuptake inhibitors and dopamine reuptake inhibitors) or its metabolism (monoamine oxidase inhibitors) (Vetulani and Nalepa, 2000). Requisite brain regions that mediate these events are unknown, although Samson et al. (2011) reported that activation in the prefrontal cortex seems to play a crucial role in the treatment responses of patients with major depression.

Besides the monoaminergic hypothesis, impairment in the brain energetic metabolism has been implicated in the pathophysiology of depression. Several studies have demonstrated alterations in high-energy phosphate metabolism in subjects with depression (Kato et al., 1992; Volz et al., 1998). Indeed, a hypoactive prefrontal cerebral energy metabolism has been reported in unipolar and bipolar depression (Forester et al., 2009; Ketter et al., 2001). Of note, antidepressants treatment increased the energetic metabolism in the brain, since antidepressants treatment increased the citrate synthase activity in the mitochondrial brain (Hroudova and Fisar, 2010), creatine kinase activity in the cerebral cortex (Assis et al., 2009; Réus et al., 2012; Santos et al., 2009; Scaini et al., 2010) and brain levels of creatine-containing compounds (Papakostas, 2009; Silveri et al., 2003). Furthermore, electroconvulsive shock increased the cerebral glucose utilization in the hippocampus, suggesting that this intervention that is well-known to cause antidepressant effect, increases metabolic rates (Orzi et al., 1987). It is, therefore, feasible to suppose that agents that improve cellular bioenergetics may be useful in the treatment of depression.

In line with this, creatine (N-aminoiminomethyl-N-methylglycine), a guanidine compound that plays a pivotal role in brain energy homeostasis (Andres et al., 2008) and present a significant role in the pathophysiology

Abbreviations: AMPT, α-methyl-p-tyrosine; ANOVA, analysis of variance; DMSO, dimethylsulfoxide; FST, forced swimming test; TST, tail suspension test.

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and treatment of depression. Thus, an inverse correlation between Hamilton Depression Rating Scale scores and white matter creatine levels was shown (Dager et al., 2004). Furthermore, a single prolonged stress and forced swimming stress was reported to decrease creatine concentrations in the rat prefrontal cortex (Herring et al., 2008; Kim et al., 2010; Knox et al., 2010). Noteworthy, creatine supplementation has been proposed as a putative antidepressant in preclinical studies (Allen et al., 2010, 2012; Cunha et al., 2012) and clinical trials (Kondo et al., 2011, 2012; Roitman et al., 2007). However, the mechanisms underlying its antidepressant effects are not well established. Our group recently demonstrated that an acute administration of creatine to mice elicits an antidepressant-like effect in the tail suspension test (TST) dependent on the dopaminergic activation (Cunha et al., 2012). Furthermore, considering that noradrenaline terminals regulate extracellular dopamine concentrations in the prefrontal cortex (Yamamoto and Novotney, 1998), we hypothesized that the antidepressant-like effect of creatine also involves noradrenergic system modulation.

The noradrenergic system is involved in the regulation of many physiological and psychological processes, including the modulation of mood. The α-adrenergic receptors modulate noradrenaline release (Rump and Majewski, 1987), as well as dopamine release (Verheij and Cools, 2009) and are therefore potential targets for antidepressant drug development. Of note, a number of antidepressant drugs have been found to increase α_1-adrenoceptors function (Holsboer and Barden, 1996), since they are able to increase the density of α_1-adrenoceptors in the hippocampus and cerebral cortex of mice and rats (Deupree et al., 2007; Rehavi et al., 1980) and the α_1-adrenoceptor agonist affinity in the cerebral cortex (Klimek et al., 1991; Nowak and Przegaliński, 1988). Moreover, the α_1-adrenoceptor blockade in the central nervous system induces depression-related behavior in the TST (Stone and Quartermain, 1999). Indeed, the α_1- and α_2-adrenoceptors have been shown to underlie some of the antidepressant-like responses of drugs in behavioral models of depression (Danysz et al., 1986; Kitada et al., 1983; Masuda et al., 2001). Taking into account the established roles of α-adrenoceptors in mood disorders, we investigate the involvement of the α-adrenoceptor activation in the antidepressant-like effect of creatine. Furthermore, the possibility that creatine administration causes an enhancement of the anti-immobility action of noradrenaline reuptake inhibitors in the TST was also investigated.

2. Methods

2.1. Animals

Male Swiss mice (30–40 g) were obtained from the Central Biothery of Universidade Federal de Santa Catarina (Santa Catarina, Brazil). Animals were housed in groups of fourteen animals per plastic cage under controlled conditions of light (from 07:00 to 19:00 h) and temperature (21 ± 1 °C) were used. Mice were allowed free access to standard laboratory food and tap water, and to adapt to the laboratory environment for at least 1 week before the behavioral studies. Each experimental group consisted of 7–9 animals. Animals were randomly distributed into specified experimental groups. All manipulations were carried out between 14:00–17:00 h, with each animal used only once. All procedures in this study 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.

2.2. Drugs

The following drugs were used: creatine monohydrate, α-methyl-p-tyrosine (AMPT), prazosin hydrochloride, (R)-(−)-phenylephrine hydrochloride, yohimbine hydrochloride, imipramine hydrochloride (Sigma Chemical Co, USA), amitriptyline hydrochloride, reboxetine mesylate (Pfizer, Brazil). All drugs were dissolved in saline, except creatine, imipramine, amitriptyline and reboxetine which were dissolved in distilled water. All drugs were administered by intraperitoneal (i.p.) route, except creatine, imipramine, amitriptyline and reboxetine which were administered by oral route (p.o.) by gavage and creatine and phenylephrine that was administered by intracerebroventricular (i.c.v.) route. All drugs, except those administered centrally, were administered in a constant volume of 10 ml/kg body weight.

2.3. I.c.v. administration

I.c.v. administration was performed 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 Laursen and Belknap (1986), which was modified from the method of Haley and McCormick (1957). Briefly, the animals were anesthetized with ether and then gently restrained by hand for i.c.v. injections. The sterilization of the injection site was carried out using gauze embedded in 70% ethanol. Under light anesthesia (i.e. just that necessary for loss of the postural reflex), 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. A volume of 5 μl of sterile saline containing the drugs was injected directly into the lateral ventricle, at the following coordinates from bregma taken from the atlas of Franklin and Paxinos (1997): anterioposterior (AP) = −0.1 mm; mediolateral (ML) = 1 mm; and dorsoventral (DV) = −3 mm. Mice exhibited normal behavior within 1 min after injection. After completion of the experiments, all animals were decapitated and their brains were examined freshly.

It is possible to check the site of the i.c.v. injections by visual inspection of the dissected brain. This is a standard procedure commonly performed to determine the accuracy of the injection 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).

2.4. Experimental design

2.4.1. Effect of i.c.v. administration of creatine in the TST or open-field test

In a first set of experiments, mice were administered with creatine at doses of 0.01–1 μg/site, 15 min before the TST or open-field test in order to investigate its central effect.

2.4.2. Effect of administration of antidepressants in the TST or open-field test

Furthermore, mice were injected with the tricyclic antidepressants imipramine and amitriptyline and the selective noradrenaline reuptake inhibitor reboxetine (orally) at doses: 0.1–1, 1–10, and 2–20, respectively, 60 min before the TST or open-field test in order to establish effective and subeffective doses of these antidepressants for subsequent studies.

2.4.3. Effect of AMPT administration on the creatine-induced reduction in the immobility time in the TST and in the number of crossings in the open-field test

To test the hypothesis that the antidepressant-like effect of creatine is mediated through an interaction with the noradrenergic system, animals were pretreated with AMPT (100 mg/kg, an inhibitor of the enzyme tyrosine hydroxylase) 4 h before the administration of creatine (1 mg/kg, p.o.). This dose was previously established by our group to produce an antidepressant-like effect in the TST, without altering locomotor activity of mice (Cunha et al., 2012). As a positive control, α-methyl-p-tyrosine (100 mg/kg, i.p.) was administered 4 h before the treatment of mice with reboxetine (20 mg/kg, p.o.). A further 60 min elapsed between reboxetine administration and the behavioral tests (TST or open-field test) (Fig. 1A).

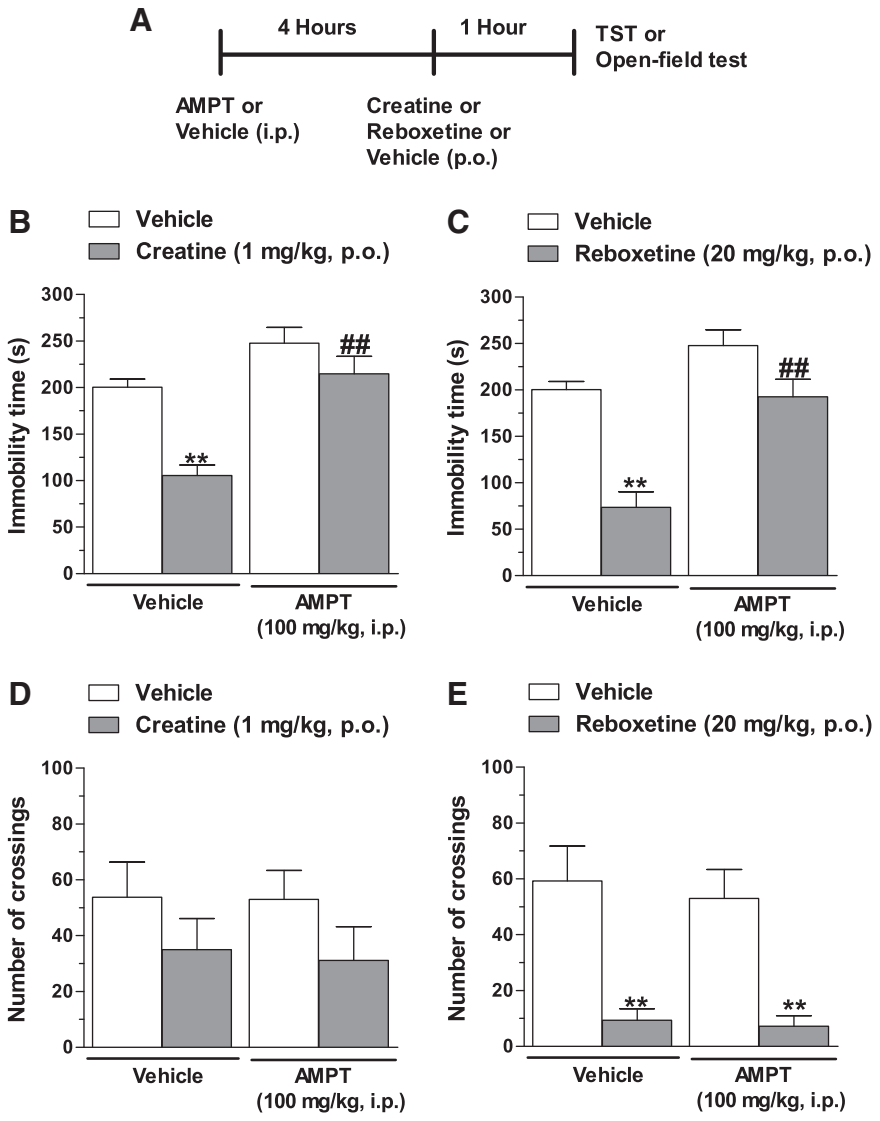

Fig. 1. Effect of creatine on AMPT-treated mice. Timeline of experimental protocol of administrations and behavioral tests (Panel A). Effect of pretreatment of mice with AMPT (100 mg/kg, i.p., a tyrosine hydroxylase inhibitor) and treatment with creatine (1 mg/kg, p.o.) or reboxetine (20 mg/kg, p.o.) on the immobility time in the TST (Panels B and C, respectively) and on the number of crossings in the open-field test (Panels D and E, respectively). Each column represents the mean + S.E.M. $^{**}P<0.01$ compared with the vehicle-treated control; $^{\#\#}P<0.01$ as compared with the same group pretreated with vehicle (two-way ANOVA followed by Newman–Keuls post-hoc test).

2.4.4. Effect of administration of α-adrenoceptors agonists in the TST or open-field test

Also, mice were injected by i.c.v. route with phenylephrine (an α_1-adrenoceptor agonist) at the dose range of 0.4–40 μg/site 15 min before the TST or open-field test in order to establish effective and subeffective doses of these α_1-adrenoceptor agonists for subsequent studies.

2.4.5. Effect of the pretreatment with prazosin on the antidepressant-like effect of creatine in the TST and in the number of crossings in the open-field test

In order to investigate the involvement of α_1-adrenoceptors in the anti-immobility effect of creatine in the TST, mice were pretreated with prazosin (1 mg/kg, i.p., an α_1-adrenoceptor antagonist), 30 min before the administration of creatine (1 mg/kg, p.o.). Animals were submitted to the TST 60 min later (Fig. 2A). As a positive control, mice were pretreated with vehicle (control group) or prazosin (1 mg/kg, i.p.) and 30 min later they received vehicle or phenylephrine (40 μg/site, i.c.v., an α_1-adrenoceptor agonist). After 15 min of the last treatment, the TST or open-field test was carried out (Fig. 2B).

2.4.6. Interaction of creatine with α_1-adrenoceptor agonist phenylephrine in the TST and open-field test

Alternatively, animals were pretreated with a dose of creatine (0.01 mg/kg, p.o.) that was previously shown to be subeffective in the TST (Cunha et al., 2012) 45 min before the administration of subeffective doses of phenylephrine (0.4 μg/site, i.c.v.). After 15 min following the administrations, the TST or open-field test was carried out (Fig. 3A).

2.4.7. Effect of the pretreatment with yohimbine on the creatine-induced reduction in the immobility time in the TST and in the number of crossings in the open-field test

In an attempt to investigate the participation of α_2-adrenoceptors in the antidepressant-like effect of creatine in the TST, yohimbine (1 mg/kg, i.p., an α_2-adrenoceptor antagonist) or vehicle was administered 30 min before creatine (1 mg/kg, p.o.). The TST was carried out 60 min later (Fig. 4A).

2.4.8. Interaction of creatine with conventional antidepressants in the TST and open-field test

In order to investigate the potential synergistic effect of a subeffective dose of creatine (0.01 mg/kg) with subeffective doses

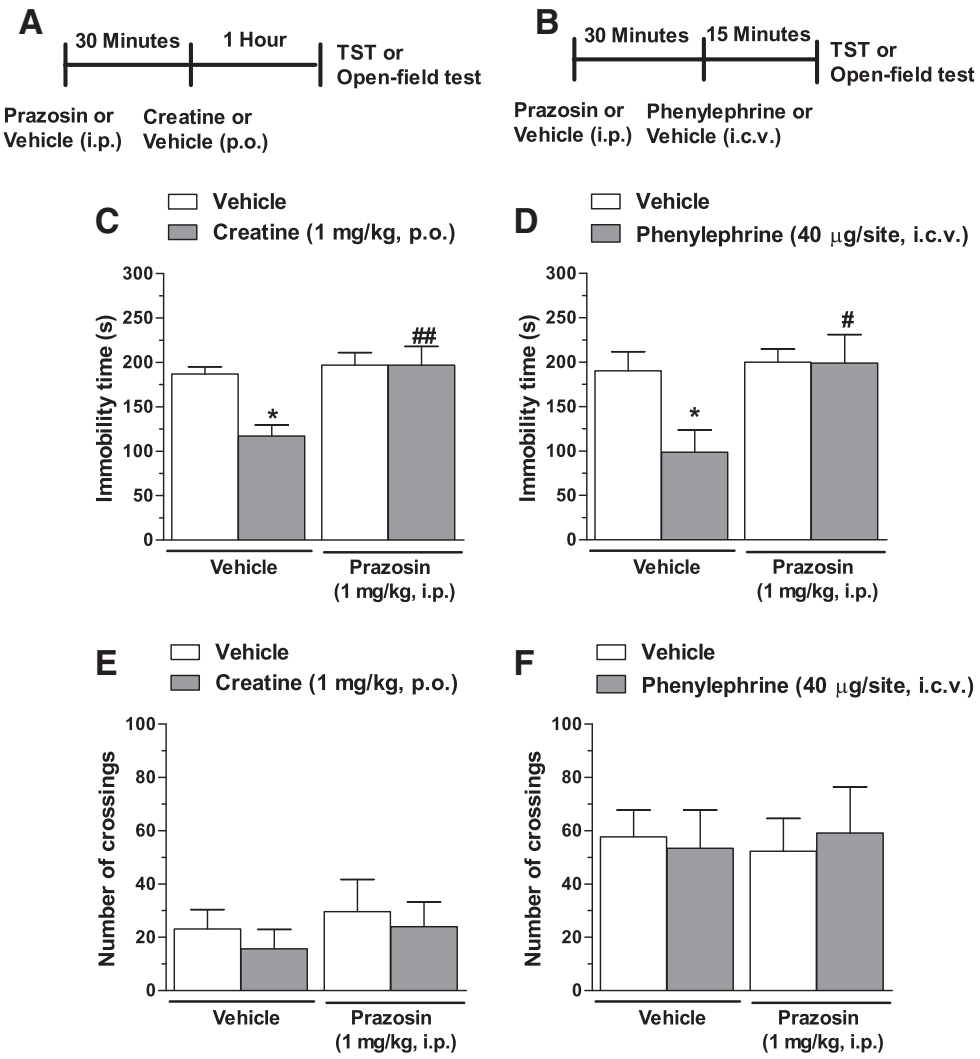

Fig. 2. Involvement of α_1-adrenoceptors activation on the antidepressant-like effect of creatine in the TST. Timeline of reversal protocol of the antidepressant-like effect of creatine or phenylephrine by prazosin (Panels A and B, respectively). Effect of pretreatment of mice with prazosin (1 mg/kg, i.p., an α_1-adrenoceptor antagonist) on the anti-immobility effect of creatine (1 mg/kg, p.o.) or phenylephrine (40 μg/site, i.c.v., an α_1-adrenoceptor agonist) in the TST (Panels C and D, respectively) and on the number of crossings in the open-field test (Panels E and F, respectively). Each column represents the mean + S.E.M. $^{*}P<0.05$ compared with the vehicle-treated control; $^{\#\#}P<0.01$ as compared with the same group pretreated with vehicle (two-way ANOVA followed by Newman–Keuls post-hoc test).

of antidepressants amitriptyline (1 mg/kg, p.o.), imipramine (0.1 mg/kg, p.o.) and reboxetine (2 mg/kg, p.o.) in the TST or open-field test, creatine or vehicle administration was followed by another immediate administration of the antidepressant drug or vehicle 60 min before the TST or open-field test (Fig. 5A).

2.4.9. Effect of prazosin administration on the antidepressant-like effect induced by the combination of sub-effective doses of creatine and reboxetine in the TST and in the number of crossings in the open-field test

In another experiment, mice were pretreated with prazosin (1 mg/kg, i.p.) or vehicle 30 min before the co-administration of creatine (0.01 mg/kg, p.o.) with reboxetine (2 mg/kg, p.o.). Animals were submitted to the TST 60 min later (Fig. 6A).

The doses of drugs (tyrosine hydroxylase inhibitor AMPT, noradrenergic receptor antagonists and agonists and amitriptyline, imipramine and reboxetine) used were selected on the basis of literature data and on previous results from our laboratory (Binfaré et al., 2009; Capra et al., 2010; Jesse et al., 2010; Kaster et al., 2007; Machado et al., 2007, 2009; O'Leary et al., 2007).

2.5. Behavioral tests

2.5.1. Tail suspension test (TST)

The 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 Steru et al. (1985). Briefly, mice were acoustically and visually isolated and 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. The immobility time was recorded by observers blind to the drug treatment (Binfaré et al., 2009; Cunha et al., 2008; Machado et al., 2009).

2.5.2. Open-field test

To assess the possible effects of drugs on locomotor activity, mice were evaluated in the open-field paradigm as previously described (Machado et al., 2009). Mice were individually placed in a wooden

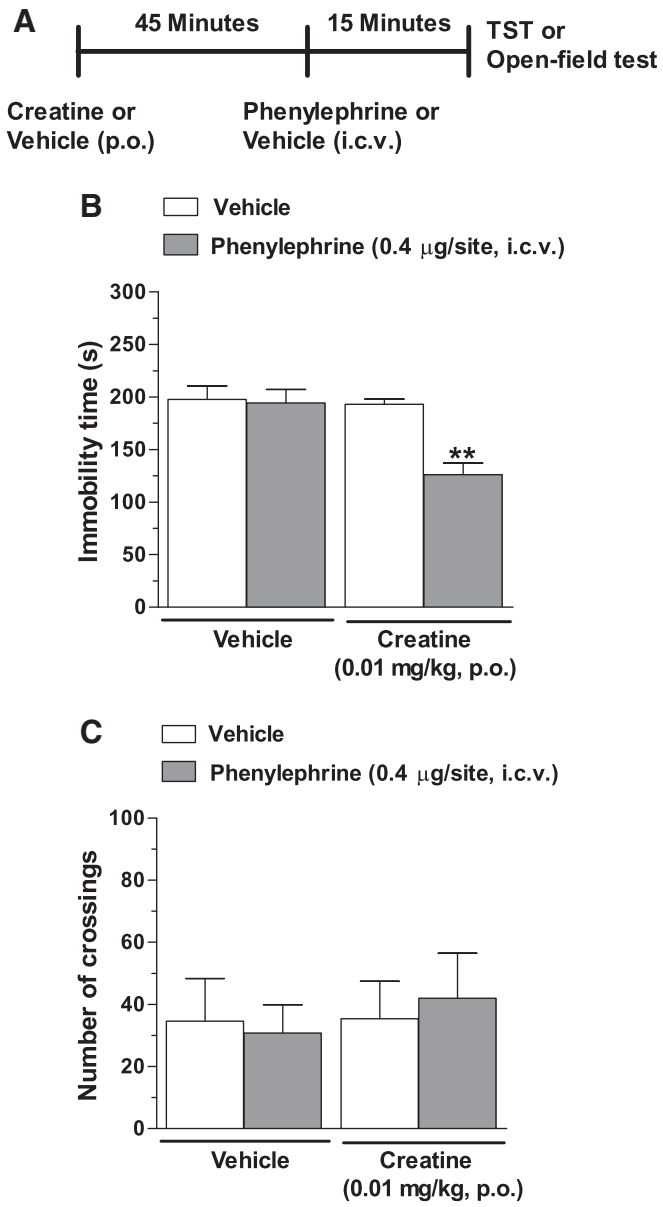

Fig. 3. Synergistic antidepressant-like effect of combination between subeffective doses of creatine and phenylephrine in the TST. Timeline of experimental protocol of administrations and behavioral tests (Panel A). Effect of the treatment with subeffective doses of creatine (0.01 mg/kg, p.o.) in combination with phenylephrine (0.4 μg/site, i.c.v., an α_1-adrenoceptor agonist) on the immobility time in the TST (Panel B) and on the number of crossings in the open-field test (Panel C). Each column represents the mean + S.E.M. $^{**}P<0.01$ compared with the vehicle-treated control (two-way ANOVA followed by Newman–Keuls post-hoc test).

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) and rising of the front paws (rearing) were registered during a period of 6 min. The number of crossings and rearings was considered as indicative of locomotor activity and exploratory behavior, respectively. The behavior was recorded by observers blind to the drug treatment. The floor of the open-field apparatus was cleaned with 10% ethanol between tests.

2.6. Statistical analysis

Comparisons between experimental and control groups were performed by one-way (dose–response curve of creatine or antidepressants or phenylephrine in the TST and open-field test in mice), two-way (experiments dealing with the involvement of the noradrenergic system in the effect of creatine in the TST or open-field test in mice) or three-way (experiments that aimed to analyze the involvement of the α_1-adrenoceptor in the effect of combined administration of creatine and reboxetine in the TST or open-field test) analysis of variance (ANOVA) followed by Newman–Keuls test when the F value was significant, as indicated on captions. A value of $P<0.05$ was considered to be significant.

3. Results

3.1. Effect of the acute intracerebroventricular treatment with creatine on the immobility time in the TST and on the number of crossings in the open-field test

Creatine administered at dose of 1 μg/site, but not at 0.01 and 0.1 μg/site, i.c.v., reduced the immobility time in TST in mice ($F(3,25)=5.16$; $P<0.01$; Table 1). Moreover, the one-way ANOVA revealed that the administration of creatine by i.c.v. route did not alter the number of crossings in the open-field test in mice ($P>0.05$).

3.2. Effect of acute treatment with amitriptyline, imipramine and reboxetine on the immobility time in the TST and on the number of crossings in the open-field test

The amitriptyline (1–10 mg/kg, a tricyclic antidepressant) administration by p.o. route at dose of 10 mg/kg reduced the immobility time in TST in mice ($F(2,19)=5.42$; $P<0.05$; Table 2). Imipramine (1 mg/kg, p.o., tricyclic antidepressant) reduced the immobility time in the TST ($F(2,24)=17.38$; $P<0.01$; Table 2). Moreover, reboxetine (selective noradrenaline reuptake inhibitor) administration by p.o. route at dose of 20 mg/kg reduced the immobility time in TST in mice ($F(2,21)=73.49$; $P<0.01$). Furthermore, amitriptyline administration at doses of 1–10 mg/kg, p.o., imipramine (0.1–1 mg/kg, p.o.) and reboxetine (2–20 mg/kg, p.o.) were tested in the open-field test in mice (Table 2). The one-way ANOVA revealed that the administration of amitriptyline or imipramine by p.o. route did not alter the number of crossings in the open-field test in mice (Table 2). Moreover, the one-way ANOVA revealed that the administration of reboxetine by p.o. route (2–20 mg/kg) decrease the number of crossings in the open-field test ($F(2,21)=4.92$; $P<0.05$).

3.3. Investigation of the pretreatment with AMPT on the effect of creatine and reboxetine in the TST and open-field test

The results depicted in Fig. 1B show that the anti-immobility effect of creatine (1 mg/kg, p.o.) was prevented by pretreatment of mice with the tyrosine hydroxylase inhibitor AMPT (100 mg/kg, i.p.) in the TST. A two-way ANOVA showed significant differences for AMPT treatment ($F(1,27)=29.17$; $P<0.01$), creatine treatment ($F(1,27)=19.44$; $P<0.01$), and AMPT × creatine treatment interaction ($F(1,27)=4.52$; $P<0.05$). The number of crossings in open-field test was not altered by AMPT and creatine treatments (Fig. 1D). A two-way ANOVA showed significant differences for creatine treatment ($F(1,29)=16.81$; $P<0.01$), but not for AMPT treatment and AMPT × creatine interaction.

The results depicted in Fig. 1C show that the anti-immobility effect of reboxetine (20 mg/kg, p.o.) was prevented by pretreatment of mice with the tyrosine hydroxylase inhibitor AMPT (100 mg/kg, i.p.) in the TST. A two-way ANOVA showed significant differences for AMPT treatment ($F(1,25)=28.83$; $P<0.01$), reboxetine treatment ($F(1,25)=34.55$; $P<0.01$), and AMPT × reboxetine treatment interaction ($F(1,25)=5.31$; $P<0.05$). Moreover, the effects of reboxetine and AMPT in the number of crossings in the open-field test are shown in Fig 1E. A two-way ANOVA showed significant differences for reboxetine treatment ($F(1,27)=25.28$; $P<0.01$), but not for AMPT treatment ($F(1,27)=0.20$; $P=0.63$) and AMPT × reboxetine treatment interaction ($F(1,27)=0.05$;

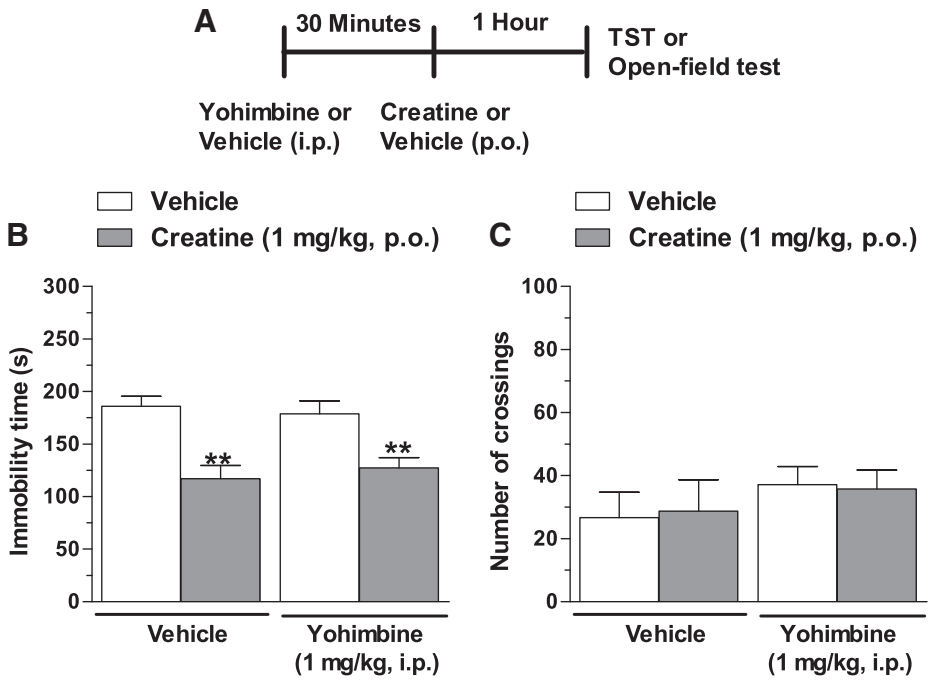

Fig. 4. α_2-adrenoceptors activation is not involved on the antidepressant-like effect of creatine in the TST. Timeline of reversal protocol of the antidepressant-like effect of creatine by yohimbine (Panel A). Effect of pretreatment of mice with yohimbine (1 mg/kg, i.p., an α_2-adrenoceptor antagonist) on the anti-immobility effect creatine (1 mg/kg, p.o.) in the TST (Panel B) and on the number of crossings in the open-field test (Panel C). Each column represents the mean + S.E.M. $^{**}P<0.01$, $^{*}P<0.05$ compared with the vehicle-treated control; $^{\#\#}P<0.01$ as compared with the same group pretreated with vehicle (two-way ANOVA followed by Newman–Keuls post-hoc test).

$P=0.66$). Reboxetine alone and in combination with AMPT, but not AMPT alone, reduced the locomotor activity of mice.

3.4. Effect of acute treatment with phenylephrine on the immobility time in the TST and on the number of crossings in the open-field test

Phenylephrine (an α_1-adrenoceptor agonist) administered by i.c.v. route at the dose of 40 μg/site, but not at the doses of 4 and 0.4 μg/site, reduced the immobility time in the TST (Table 2). The one-way ANOVA revealed a significant main effect of phenylephrine treatment ($F(3,28)=5.45$; $P<0.01$). Furthermore, phenylephrine administrations at doses of 0.4–40 μg/site, i.c.v. were tested in the open-field test in mice. The administration of phenylephrine did not alter the number of crossings in the open-field test (Table 2), as revealed by one-way ANOVA ($P>0.05$).

3.5. Investigation of the pretreatment with prazosin on the effect of creatine or phenylephrine in the TST and open-field test

The results presented in Fig. 2C show that the anti-immobility effect of creatine (1 mg/kg, p.o.) was prevented by pretreatment of mice with the α_1 adrenoceptor antagonist prazosin (1 mg/kg, i.p.) in the TST. A two-way ANOVA showed significant differences for prazosin treatment ($F(1,25)=9.87$; $P<0.01$), creatine treatment ($F(1,27)=5.97$; $P<0.05$) and prazosin × creatine treatment interaction ($F(1,25)=5.92$; $P<0.05$). The number of crossings in open-field test was not altered by the pretreatment with prazosin and creatine treatment ($P>0.05$, Fig. 2E).

Furthermore, the anti-immobility effect of phenylephrine (40 μg/site, i.c.v.) was prevented by pretreatment of mice with prazosin (1 mg/kg, i.p.) in the TST (Fig. 2D). A two-way ANOVA showed significant differences for prazosin treatment ($F(1,28)=30.83$; $P<0.01$) and prazosin × phenylephrine treatment interaction ($F(1,28)=6.16$; $P<0.05$), but not for phenylephrine treatment. Also, the number of crossings in open-field test was not altered by prazosin or phenylephrine alone or in combination ($P>0.05$, Fig. 2F).

3.6. Effect of the interaction of subeffective doses of creatine and phenylephrine on the immobility time in the TST and on the number of crossings in the open-field test

The α_1-adrenoceptor agonist phenylephrine, administered at a subeffective dose (0.4 μg/site, i.c.v.), exhibited a potent antidepressant-like effect when combined with a subeffective dose of creatine (0.01 mg/kg, p.o.) in the TST in mice as compared to the control or either drug alone (Fig. 3B). A two-way ANOVA showed significant differences for creatine treatment ($F(1,29)=25.65$; $P<0.01$), phenylephrine treatment ($F(1,29)=55.37$; $P<0.01$), and creatine × phenylephrine interaction ($F(1,29)=6.97$; $P<0.05$). Moreover, the number of crossings in open-field test was not altered by the administration of creatine and phenylephrine alone or in combination (Fig. 3C). A two-way ANOVA shows no difference for creatine treatment, phenylephrine treatment and creatine × phenylephrine interaction.

3.7. Effect of the pretreatment with yohimbine on the effect of creatine in the TST and open-field test

The result depicted in Fig. 4B shows that the anti-immobility effect of creatine (1 mg/kg, p.o.) was not prevented by pretreatment of mice with the α_2-adrenoceptor antagonist yohimbine (1 mg/kg, i.p.) in the TST. A two-way ANOVA showed significant differences for creatine treatment ($F(1,24)=26.26$; $P<0.01$), but not for yohimbine treatment and yohimbine × creatine treatment interaction. The number of crossings in open-field test was not altered by the pretreatment with yohimbine and creatine treatment ($P>0.05$, Fig. 4C).

3.8. Effect of the interaction of creatine with antidepressants on the immobility time in the TST and on the number of crossings in the open-field test

The tricyclic antidepressant amitriptyline, administered at a subeffective dose (1 mg/kg, p.o.), exhibited a potent antidepressant-like effect when combined with a subeffective dose of creatine (0.01 mg/kg,

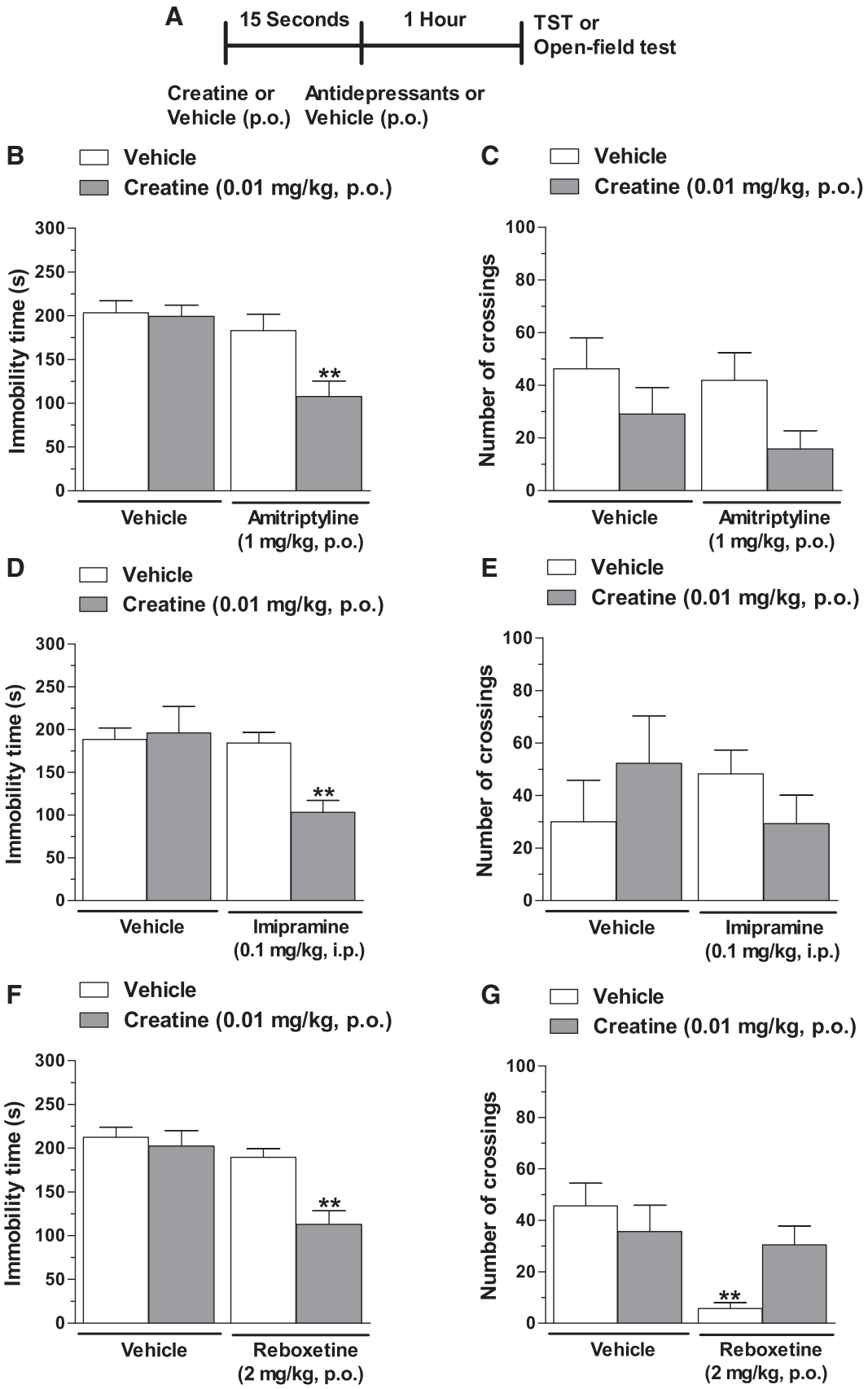

Fig. 5. Synergystic antidepressant-like effect of combined administration between subeffective doses of creatine and antidepressants in the TST. Timeline of experimental protocol of administrations and behavioral tests (Panel A). Effect of the treatment with subeffective doses of creatine (0.01 mg/kg, p.o.) in combination with amitriptyline (1 mg/kg, p.o.) or imipramine (0.1 mg/kg, p.o.) or reboxetine (2 mg/kg, p.o.) on the immobility time in the TST (Panels B, D and E, respectively) and on the number of crossings in the open-field test (Panels C, E, and F, respectively). Each column represents the mean + S.E.M. $^{**}P<0.01$ compared with the vehicle-treated control (two-way ANOVA followed by Newman–Keuls post-hoc test).

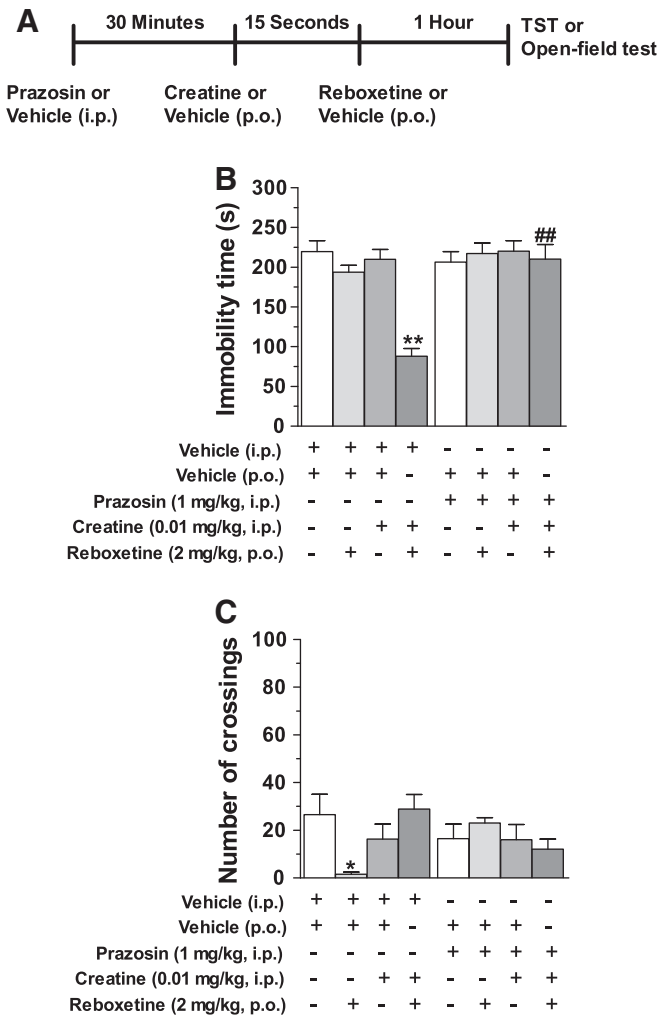

Fig. 6. Involvement of α_1-adrenoceptors activation on the antidepressant-like effect of combined administration of creatine and reboxetine in the TST. Timeline of experimental protocol of administrations and behavioral tests (Panel A). Effect of pretreatment of mice with prazosin (1 mg/kg, i.p., an α_1-adrenoceptor antagonist) on the anti-immobility effect of the co-treatment with subeffective doses of creatine (0.01 mg/kg, p.o.) in combination with reboxetine (2 mg/kg, p.o.) in the TST (Panel A) and on the number of crossings in the open-field test (Panels B). Each column represents the mean + S.E.M. $^{*}P<0.05$, $^{**}P<0.01$ compared with the vehicle-treated control; $^{\#\#}P<0.01$ as compared with the same group pretreated with vehicle (three-way ANOVA followed by Newman–Keuls post-hoc test).

p.o.) in the TST as compared to the control or either drug alone (Fig. 5B). A two-way ANOVA showed significant differences for amitriptyline treatment ($F(1,27)=12.35$; $P<0.01$), creatine treatment ($F(1,27)=6.25$; $P<0.05$), and amitriptyline × creatine interaction ($F(1,27)=4.95$; $P<0.05$). Moreover, the number of crossings in open-field test was not altered by the co-administration of creatine with amitriptyline (Fig. 5C),

Table 1
Effect of treatment with creatine (0.01–1 μg/site, i.c.v.) on the immobility time in the TST and in the number of crossings in the open-field test.

Compound	Dose	Immobility time (s)	Number of crossings
Vehicle	–	192.18 ± 20.06	63.2 ± 10.07
Creatine	0.01 μg/site, i.c.v.	209.83 ± 26.12	62.60 ± 15.60
Creatine	0.1 μg/site, i.c.v.	137.17 ± 16.57	72.60 ± 7.05
Creatine	1 μg/site, i.c.v.	92.83 ± 24.12*	70.05 ± 12.91

Results are expressed as mean ± S.E.M.
* $P<0.05$ compared with the vehicle-treated control (one-way ANOVA followed by Newman–Keuls post-hoc test).

Table 2
Effect of treatment with amitriptyline, imipramine, reboxetine and phenylephrine on the immobility time in the TST and on the number of crossings in the open-field test.

Compound	Dose	Immobility time (s)	Number of crossings
Vehicle	–	198.13 ± 9.88	37.00 ± 14.52
Amitriptyline	1 mg/kg, p.o.	164.71 ± 26.31	31.86 ± 9.49
Amitriptyline	10 mg/kg, p.o.	118.29 ± 13.22*	17.00 ± 3.64
Vehicle	–	221.78 ± 8.40	34.82 ± 10.68
Imipramine	0.1 mg/kg, p.o.	198.78 ± 11.24	48.29 ± 9.05
Imipramine	1 mg/kg, p.o.	147.56 ± 7.22**	42.13 ± 11.70
Vehicle	–	193.78 ± 6.96	47.22 ± 12.88
Reboxetine	2 mg/kg, p.o.	182.14 ± 11.43	13.00 ± 6.68*
Reboxetine	20 mg/kg, p.o.	42.88 ± 11.43**	11.75 ± 3.79*
Vehicle	–	229.00 ± 12.27	24.83 ± 9.58
Phenylephrine	0.4 μg/site, i.c.v.	186.38 ± 18.89	27.88 ± 8.52
Phenylephrine	4 μg/site, i.c.v.	157.57 ± 21.49	39.63 ± 7.14
Phenylephrine	40 μg/site, i.c.v.	134.14 ± 28.68*	22.29 ± 8.86

Results are expressed as mean ± S.E.M.
**P<0.01, *P<0.05 compared with the vehicle-treated control (one-way ANOVA followed by Newman–Keuls post-hoc test).

since only a main effect of creatine treatment was revealed by two-way ANOVA ($F(1,28)=4.52$; $P<0.05$).

Furthermore, the tricyclic antidepressant imipramine administered at a subeffective dose (0.1 mg/kg, p.o.), exhibited antidepressant-like effect when combined with a subeffective dose of creatine (0.01 mg/kg, p.o.) in the TST as compared to control or either drug alone (Fig. 5D). A two-way ANOVA showed significant differences for imipramine treatment ($F(1,22)=4.89$; $P<0.05$), creatine treatment ($F(1,22)=8.50$; $P<0.01$), and imipramine × creatine interaction ($F(1,22)=7.06$; $P<0.05$). Moreover, the number of crossings in open-field test was not altered by the co-administration of creatine with imipramine (Fig. 5E).

Moreover, reboxetine administered at a subeffective dose (2 mg/kg, p.o.), exhibited antidepressant-like effect when combined with a subeffective dose of creatine (0.01 mg/kg, p.o.) in the TST as compared with the control group or either drug alone (Fig. 5F). A two-way ANOVA showed significant differences for reboxetine treatment ($F(1,26)=19.85$; $P<0.01$), creatine treatment ($F(1,26)=11.68$; $P<0.01$) and reboxetine × creatine interaction ($F(1,26)=6.95$; $P<0.05$). Moreover, reboxetine alone, but not in combination with creatine, reduced the locomotor activity of mice (Fig. 5G). A two-way ANOVA showed difference for reboxetine treatment ($F(1,27)=7.80$; $P<0.01$) and reboxetine × creatine interaction ($F(1,27)=4.65$; $P<0.05$), but not for creatine treatment.

3.9. Effect of pretreatment with prazosin on anti-immobility effect induced by the combined administration of creatine and reboxetine in the TST

The result depicted in Fig. 6B shows that the anti-immobility effect produced by the combined administration of creatine (0.01 mg/kg, p.o.) and reboxetine (2 mg/kg, p.o.) in the TST was prevented by pretreatment of mice with the α1-adrenoceptor antagonist prazosin (1 mg/kg, i.p.). A three-way ANOVA showed significant differences for reboxetine treatment ($F(1,51)=8.63$; $P<0.01$), creatine treatment ($F(1,51)=15.86$; $P<0.01$), prazosin treatment ($F(1,51)=14.94$; $P<0.01$), reboxetine × creatine interaction ($F(1,51)=9.97$; $P<0.01$), reboxetine × prazosin interaction ($F(1,51)=10.94$; $P<0.01$), creatine × prazosin interaction ($F(1.51)=16.19$; $P<0.01$), prazosin × creatine × reboxetine interaction ($F(1,51)=4.20$; $P<0.05$).

The number of crossings in open-field test was altered by treatment with reboxetine alone, but not with creatine, prazosin or combined administrations (Fig. 6C). A three-way ANOVA showed significant differences for prazosin × creatine × reboxetine interaction ($F(1,55)=9.05$; $P<0.01$), but not for creatine treatment, reboxetine treatment, prazosin

treatment, reboxetine × prazosin interaction, creatine × prazosin interaction and reboxetine × creatine treatment interaction.

4. Discussion

The results presented herein indicate that creatine produces antidepressant-like effect in TST dependent at least in part on the α_1-adrenoceptor activation. Interestingly, we also show that creatine presents adjunctive effect when associated with amitriptyline, imipramine and reboxetine, antidepressants that inhibit noradrenaline transporter. These findings contribute to the understanding of the mechanism underlying the antidepressant-like effect of creatine and are in line with the reported antidepressant effect of creatine shown in clinical (Amital et al., 2006; Kondo et al., 2011, 2012; Roitman et al., 2007) and preclinical studies (Allen et al., 2010, 2012; Cunha et al., 2012), further reinforcing the notion that creatine exerts an important role in the modulation of depression.

The antidepressant-like effect of creatine (p.o.) was observed 60 min after its administration by oral route. It is in line with the fact that systemic administration of creatine in rodents elicits several central effects such as antidepressant (Cunha et al., 2012), anticonvulsant (Magni et al., 2007; Royes et al., 2006) and spatial learning enhancement (Oliveira et al., 2008; Souza et al., 2012). Moreover, a single systemic administration of creatine was reported to increase striatal creatine and phosphocreatine content and creatine kinase in the brain of rats (Rambo et al., in press; Royes et al., 2006). In line with this, we show that creatine administered i.c.v. exerts antidepressant-like effect in the TST.

Creatine levels have been shown to be disrupted in the hippocampus of adult patients undergoing a first affective episode (Blasi et al., 2004) and to be significantly lower in euthymic adult male bipolar patients (Deicken et al., 2003). In addition, agents with reported antidepressant activity cause changes in brain levels of creatine-containing compounds, as acetyl-L-carnitine that increased phosphocreatine brain levels in geriatric depressed patients (Pettegrew et al., 2002) and in healthy mice (Smeland et al., 2012). Accordingly, two open-labeled studies found that creatine can significantly improve depressive mood (Amital et al., 2006; Roitman et al., 2007). Furthermore, eight weeks of creatine augmentation decrease the depression score in adolescents with antidepressant-resistant depression (Kondo et al., 2011). Moreover, creatine produced antidepressant-like effect in TST and forced swimming test (FST), two predictive tests of antidepressant properties, in rodents (Allen et al., 2010, 2012; Cunha et al, 2012).

The TST is widely used to assess the antidepressant properties of new drugs, as it is sensitive to all major classes of antidepressant drugs, including tricyclics, selective noradrenaline reuptake inhibitors and monoamine oxidase inhibitors (Steru et al., 1985) and is an important tool to study neurobiological mechanisms involved in antidepressant responses. The basic concept of this test called "searching–waiting strategy" can be explained by alteration between two kinds of behaviors, agitation and immobility, when mice were placed in aversive situations (Steru et al., 1985). The immobility in the TST represents a failure of persistence in escape-directed behavior and effective antidepressant treatments decrease immobility time in the TST (Cryan et al., 2005; Steru et al., 1985). "False" positive effects in this test do occur with stimulants interventions due to the generalized increase in motor activity, which is controlled for by use of an additional test for ambulation. Amount of ambulation has usually been scored spatially by the number of subdivisions entered in the open-field test. The reduction in the immobility time elicited by creatine in the TST cannot be attributable to a psychostimulant action of this compound. This conclusion derives from the fact that in our study creatine administered either by p.o. and i.c.v. routes at doses that produced a significant decrease in the immobility time (1 mg/kg, p.o. and 1 μg/site, i.c.v.) in the TST did not alter the locomotor activity in open-field test, as compared to control animals.

In the 1960s, based on over a decade of accumulating data, a consensus was forming that catecholamines, specifically noradrenaline, play an important, possibly primary, role in the pathophysiology and subsequent treatment of mood disorders (Brunello et al., 2002; Bunney and Davis, 1965). Furthermore, antidepressant treatments with selective noradrenaline reuptake inhibitors and with serotonergic-specific agents cause decreased noradrenaline turnover (DeBellis et al., 1993; Javaid et al., 1983; Potter et al., 1985). Recent studies have reported the implication of noradrenaline as one of the important targets in the action of antidepressant compounds (Cardoso et al., 2009; Kaster et al., 2007; Machado et al., 2007, 2009). Furthermore, the treatment with acetyl-L-carnitine, compound endowed with antidepressant-like properties in the FST, increases the phosphocreatine and the phosphocreatine/creatine ratio associated with increases in the levels of noradrenaline in the cerebral cortex of mice (Di Cesare et al., 2011; Pulvirenti et al., 1990; Smeland et al., 2012). In the present work, the pretreatment of mice with AMPT was able to prevent the anti-immobility effect of creatine in the TST, an effect similar to the observed with the selective noradrenaline reuptake inhibitor reboxetine. These results suggest that the effect of creatine in the TST may be dependent on the bioavailability of noradrenaline in the synaptic cleft. The noradrenaline depletion paradigm has been successfully used to investigate the mechanism of action of antidepressants in preclinical and clinical studies (Kaster et al., 2007; Machado et al., 2008; Miller et al., 1996a,b). Studies reported the ability of selective inhibitor of the enzyme tyrosine hydroxylase AMPT to reduce dopamine and noradrenaline levels (57% and 53%, respectively) in mice, without affecting the levels of serotonin (Mayorga et al., 2001). Furthermore, AMPT generated a relapse of depression in depressed patients currently treated with a noradrenaline reuptake inhibitor, but not with patients treated with selective serotonin reuptake inhibitors (Bremner et al., 2003; Miller et al., 1996a,b). In addition, the acute administration of AMPT temporarily reversed the antidepressant response to desipramine, mazindol and mirtazapine in patients (Delgado et al., 1993, 2002; Miller et al., 1996a,b). Moreover, our group reported that the administration of AMPT at the same dose employed in the present study reversed the antidepressant-like action of antidepressant compounds, such as lamotrigine and rutin, in the FST and TST in mice, respectively (Kaster et al., 2007; Machado et al., 2008). We cannot rule out the possibility that the effect of AMPT to abolish the antidepressant-like property of creatine is due, at least in part, to a decrease in dopamine levels, since a previous study from our group demonstrated that the antidepressant-like effect of creatine could be mediated by activation of the dopaminergic system (Cunha et al., 2012).

Basic studies on the effects of antidepressants on brain α_1-adrenoceptor indirectly support the notion of an impaired function of α_1-adrenoceptor in depression. Many authors have reported that treatment with tricyclic antidepressants increases either density (Maj et al., 1985; Vetulani et al., 1984), agonist affinity (Maj et al., 2000; Menkes et al., 1983), electrophysiological response (Menkes et al., 1980), or behavioral response mediated by α_1-adrenoceptors (Maj et al., 2000; Menkes et al., 1983). The present study suggests that the antidepressant-like effect of creatine in the TST is dependent, at least in part, on an activation of α_1-adrenoceptor, since the pretreatment of mice with prazosin (α_1-adrenoceptor antagonist) significantly prevented the antidepressant-like effect evoked by creatine in the TST. In line with this, prazosin prevented the antidepressant-like effect of antidepressant compounds in the TST (Binfaré et al., 2009; Capra et al., 2010; Machado et al., 2009). Besides that, the antidepressant-like action of desipramine in the FST was prevented by the pretreatment of mice with prazosin (Danysz et al., 1986). Moreover, the α_1-adrenoceptor agonist phenylephrine produced an anti-immobility effect in the FST (Kitada et al., 1983), in agreement with the present study that shows an antidepressant-like effect produced by phenylephrine in the TST, which was prevented by the pretreatment of mice with prazosin. In addition, the results presented herein indicate that creatine administered at a subeffective dose exerts a synergistic anti-immobility effect in the

TST when associated with a subeffective dose of phenylephrine, reinforcing the assumption that creatine exerts an antidepressant-like effect by directly or indirectly activating α_1-adrenoceptors.

Our results also suggest that the effect of creatine in the TST is not dependent on an interaction with the α_2-adrenoceptor, considering that the pretreatment of mice with yohimbine (α_2-adrenoceptor antagonist) did not prevent the antidepressant-like effect evoked by creatine in the TST. Interestingly, the antidepressant-like action of some compounds is not blocked by yohimbine (Machado et al., 2009; Yi et al., 2011).

Taking into account that: (i) the adrenergic receptors may be physiologically activated by dopamine (Zhang et al., 2004), (ii) the α_1-adrenoceptor is a afferent system of the dopaminergic neurons (Lipinski et al., 1987), (iii) noradrenaline reuptake inhibitors can increase the extracellular concentrations of dopamine in the prefrontal cortex (Carboni et al., 1990; Tanda et al., 1994), (iv) the antidepressant-like effect of creatine in TST involves dopaminergic activation (Cunha et al., 2012), we cannot rule out the possibility that some of the actions of creatine in the TST may be mediated by an interaction between dopaminergic and noradrenergic systems.

The noradrenaline transporters located in the plasma membrane of noradrenergic neurons are responsible for the rapid reuptake of neuronally released noradrenaline into presynaptic terminals. It is therefore critically involved in the termination of noradrenergic neurotransmission regulating noradrenaline-mediated behavioral and physiological effects, including mood regulation (Bönisch and Brüss, 2006). The noradrenaline transporters are a target for antidepressants such as amitriptyline, imipramine and reboxetine which are clinically used for the treatment of depression (Haenisch and Bönisch, 2011). Imipramine and amitriptyline are dual serotonin and noradrenaline reuptake inhibitors (Carrodi and Fuxe, 1968; Richelson and Pfenning, 1984), whereas reboxetine is a selective noradrenaline reuptake inhibitor used in the treatment of depression (Hajós et al., 2004; Kasper et al., 2000). In the present study, amitriptyline, imipramine and reboxetine were effective in reducing the immobility time in the TST. TST is widely used to assess the antidepressant properties of new antidepressant interventions, as it is sensitive to all major classes of antidepressant drugs, including tricyclics and selective noradrenaline reuptake inhibitors (Caldarone et al., 2004; Cunha et al., 2008; O'Leary et al., 2007; Steru et al., 1985). Interestingly, in the present study, reboxetine decreased the ambulation of the animals in the open-field test. Thus, the antidepressant-like effect of reboxetine cannot be attributable to a psychostimulant action of this compound, since reboxetine administered by p.o. route at a dose that produced a significant decrease in the immobility time in the TST (20 mg/kg, p.o.) decreased the locomotor activity in open-field test.

The synergistic antidepressant-like effects of creatine with the tricyclics amitriptyline and imipramine, and selective noradrenaline reuptake inhibitor reboxetine were shown herein. The effect of the combined administration of reboxetine and creatine in the TST is similar to the reported synergistic effect elicited by the combined administration of reboxetine and metyrapone in the FST in rats (Rogóz, 2009). Moreover, lithium augmentation (the best validated augmentation strategy) should also be considered as a treatment strategy in case of nonresponse to specific noradrenergic antidepressant reboxetine (Khazaal et al., 2005). Furthermore, creatine blocked the reduction in the number of crossings induced by reboxetine administration in the open-field test. Noteworthy, we showed that the enhancement of the anti-immobility action of reboxetine by creatine in the TST was abolished by prazosin, further implicating the α_1-adrenoceptor activation in the effect of creatine in the TST.

We should take into account that the TST is a validated tool to screen acute antidepressant-like effects of certain compounds, but it does not measure depression (Cryan et al., 2005). Therefore, further studies are necessary to investigate if the synergistic antidepressant-like effect obtained with sub-effective doses of creatine and α_1-adrenoceptor agonists in the TST translate to animal models of depression or the clinical syndrome. Furthermore, further studies are needed to address if chronic treatment with creatine replicate the data obtained with creatine administered acutely.

5. Conclusions

In conclusion, our results provide convincing evidence that antidepressant-like effect of creatine in TST is dependent on an activation of α_1-adrenoceptors. A synergistic antidepressant-like effect was observed when subeffective doses of creatine and antidepressants that modulate the noradrenaline transporter were administered to mice, suggesting that creatine may be investigated in the future as an augmentation agent for the treatment of depression associated with noradrenergic dysfunctions. Finally, we indicate that combined administration of creatine with the selective noradrenaline transporter reboxetine at subeffective doses produced antidepressant-like effect in TST mediated by α_1-adrenoceptor activation. Collectively, the present findings indicate that creatine might constitute a potential and relevant alternative or adjunctive therapy for the management of depression. However, the results obtained with the TST should be considered with caution, and further studies with animal models of depression and clinical paradigms are welcome.

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