ORIGINAL ARTICLE

# Signaling pathways underlying the antidepressant-like effect of inosine in mice

Filipe Marques Gonçalves[1] · Vivian Binder Neis[1] · Débora Kurrle Rieger[2] ·
Mark William Lopes[1] · Isabella A. Heinrich[3] · Ana Paula Costa[3] ·
Ana Lúcia S. Rodrigues[1,3] · Manuella P. Kaster[1] · Rodrigo Bainy Leal[1,3,4]

**Abstract** Inosine is a purine nucleoside formed by the breakdown of adenosine that elicits an antidepressant-like effect in mice through activation of adenosine $A_1$ and $A_{2A}$ receptors. However, the signaling pathways underlying this effect are largely unknown. To address this issue, the present study investigated the influence of extracellular-regulated protein kinase (ERK)1/2, $Ca^{2+}$/calmoduline-dependent protein kinase (CaMKII), protein kinase A (PKA), phosphoinositide 3-kinase (PI3K)/Akt, and glycogen synthase kinase 3beta (GSK-3β) modulation in the antiimmobility effect of inosine in the tail suspension test (TST) in mice. In addition, we attempted to verify if inosine treatment was capable of altering the immunocontent and phosphorylation of the transcription factor cyclic adenosine monophosphatate (cAMP) response-binding element protein (CREB) in mouse prefrontal cortex and hippocampus. Intracerebroventricular administration of U0126 (5 μg/mouse, MEK1/2 inhibitor), KN-62 (1 μg/mouse, CaMKII inhibitor), H-89 (1 μg/mouse, PKA inhibitor), and wortmannin (0.1 μg/mouse, PI3K inhibitor) prevented the antiimmobility effect of inosine (10 mg/kg, intraperitoneal (i.p.)) in the TST. Also, administration of a sub-effective dose of inosine (0.1 mg/kg, i.p.) in combination with a sub-effective dose of AR-A014418 (0.001 μg/mouse, GSK-3β inhibitor) induced a synergic antidepressant-like effect. None of the treatments altered locomotor activity of mice. Moreover, 24 h after a single administration of inosine (10 mg/kg, i.p.), CREB phosphorylation was increased in the hippocampus. Our findings provided new evidence that the antidepressant-like effect of inosine in the TST involves the activation of PKA, PI3K/Akt, ERK1/2, and CaMKII and the inhibition of GSK-3β. These results contribute to the comprehension of the mechanisms underlying the purinergic system modulation and indicate the intracellular signaling pathways involved in the antidepressant-like effect of inosine in a preclinical test of depression.

**Keywords** Inosine · Depression · Signaling pathways · Protein kinases · CREB

## Introduction

Major depressive disorder (MDD) is a serious public health problem, responsible for a profound negative impact on the quality life of patients and a socioeconomic burden [1–3]. Despite all the efforts towards elucidating the neurochemical alterations found in depressive patients, there is still a lack of understanding about the molecular basis behind this disorder. The current pharmacological therapy for MDD is not completely effective since two thirds of patients did not respond to the first prescribed drug and most antidepressants are associated with several side effects [4, 5].

Several clinical and preclinical studies have demonstrated the involvement of purinergic system in psychiatric disorders including MDD [6–8]. Accordingly, adenosine receptors are able to control cellular excitability and modulate several neurotransmission systems involved in behavioral, neurochemical, and

✉ Rodrigo Bainy Leal
rbleal@gmail.com

1 Biochemistry Graduate Program, Center of Biological Sciences, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil

2 Department of Nutrition, Center of Health Science, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil

3 Neuroscience Graduate Program, Center of Biological Sciences, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil

4 Department of Biochemistry, Universidade Federal de Santa Catarina, Florianópolis, SC 88040-900, Brazil

endocrine alterations associated with MDD, such as dopaminergic, serotoninergic, and glutamatergic systems [9, 10]. In addition, preclinical data have shown that modulation of adenosine receptors induces antidepressant-like effects in behavioral despair models in mice [11–14] and prevents depressive and anhedonic behavior of mice submitted to chronic unpredictable stress [6]. Also, an increase in adenosine $A_{2A}$ receptor expression in hippocampal synapses was found in a depression-prone mouse strain, and rats overexpressing adenosine $A_{2A}$ receptors exhibit a depressive, anhedonic, and anxious phenotype [15, 16].

Inosine is an endogenous purine nucleoside formed by cleavage of adenosine in a reaction catalyzed by adenosine deaminase (ADA) [17]. Even though few studies have focused on the physiological roles of inosine, there is a growing body of evidence suggesting that this nucleoside has significant biological effects. It has been suggested that inosine elicits neuroprotective effects in neurons and astrocytes subjected to hypoxia, glucose-oxygen deprivation, and oxidative damage [18, 19]; stimulates neurite outgrowth in vitro [20]; and induces axonal outgrowth in vivo and in vitro studies [21–25]. Moreover, systemic administration of inosine causes antinociceptive, antiallodynic, and antihyperalgesic effects in mice [26, 27]. Evidence from clinical studies has suggested antioxidant and protective properties of inosine in multiple sclerosis and Parkinson's disease patients [28–30]. Indeed, many of the behavioral and neuroprotective effects of inosine were associated with its ability to interact with different adenosine receptors. The antinociceptive effect of inosine involves adenosine $A_1$ receptor direct activation, suggesting that this nucleoside can act as adenosine $A_1$ receptor agonist [31]. Also, it was recently demonstrated that inosine can bind and activate $A_{2A}$ adenosine receptor [32]. Several works have shown that inosine has immunomodulatory effects through adenosine $A_3$ receptor activation [33–35].

It was previously reported by our group that intraperitoneal (i.p.) administration of inosine decreases the immobility time in behavioral despair models of depression by a mechanism dependent on the activation of adenosine $A_1$ and $A_{2A}$ receptors [36]. This antidepressant potential was further confirmed by Muto et al. [20], showing that chronic administration of inosine prevented the depressive-like effect in the forced swimming test (FST) and the decreased cellular proliferation in the dentate gyrus in mice subjected to chronic unpredictable stress. A single administration of inosine was also able to significantly increase extracellular-regulated protein kinase (ERK) phosphorylation and the expression of brain-derived neurotrophic factor (BDNF) in mouse hippocampus. Furthermore, the inosine-induced increase on BDNF mRNA was partially prevented by the pretreatment with adenosine $A_1$ receptor antagonist DPCPX, suggesting that adenosine $A_1$ receptor activation could be involved in the increase of BDNF expression induced by inosine in this model.

Activation of different kinases can influence cyclic adenosine monophosphatate (cAMP) response-binding element protein (CREB) by phosphorylation at Ser-133, resulting in expression of proteins related to cellular survival and neuroplasticity such as BDNF [37, 38]. It is widely recognized that changes in brain levels of BDNF are involved in the physiopathology of depression and represent a key target implicated in antidepressant responses. Notably, decreased CREB activity is frequently observed in preclinical models of depression and patients diagnosed with MDD [39, 40] and restored after antidepressant treatment [41]. Some of the kinases responsible for CREB activation are the protein kinase A (PKA), $Ca^{2+}$/calmoduline-dependent protein kinase (CaMKII), phosphoinositide 3-kinase (PI3K), glycogen synthase kinase 3 (GSK-3), and ERK1/2 [37, 38]. These enzymes are important regulators of receptor–effector cascades and are responsible for integrating signals between receptor activation and gene expression. In this context, activation of adenosine receptors may also modulate these signaling pathways [42, 43], prompting the hypothesis that these intracellular targets may underlie the beneficial effects of inosine in preclinical models of depression.

Taking into account that (i) inosine exerts an antidepressant-like effect in mice [36]; (ii) there is some evidence showing that inosine induces BDNF expression [20]; and (iii) the activation of CREB and modulation of MEK/ERK1,2, PKA, CaMKII, PI3K/Akt, and GSK-3β are important to the antidepressant responses [37, 38], this study was undertaken to investigate whether systemic administration of inosine is capable of increasing CREB phosphorylation and to evaluate the involvement of intracellular signaling pathways in the antidepressant-like effect of inosine in the tail suspension test (TST).

## Materials and methods

### Animals

Male Swiss mice (30–35 g) were provided by the Federal University of Santa Catarina (UFSC, Brazil) breeding colony. Animals were maintained at controlled temperature at 20–22 °C with free access to water and food under a 12/12-h light–dark cycle (lights on at 07:00 h). The cages were placed in the experimental room for 24 h before the tests for acclimatization, and all manipulations were performed between 9:00 and 17:00 h. The procedures in this study were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and all the experimental protocols were approved by the Institutional Ethics Committee (protocol number PP00772). All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

## Drugs and treatment

The following drugs were used: inosine, N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide (H-89), 4-[2-[(5-isoquinolinyl-sulfonyl) methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl) propyl] phenyl ester (KN-62), U0126, wortmannin, and AR-A014418. All drugs were obtained from Sigma-Aldrich Chemical Co., St. Louis, USA, and dissolved in saline (0.9% NaCl) with dimethyl sulfoxide (DMSO) at a final concentration of 1% DMSO and administered by intracerebroventricular (i.c.v.) route, except inosine that was dissolved in saline (0.9% NaCl) and administered by intraperitoneal route (i.p.). The drugs were freshly prepared before treatment and administered in a constant volume of 10-ml/kg body weight (i.p. route) or 5-μl/mouse (i.c.v. route). Control animals received the appropriate vehicle, and all the doses were based on previous studies [36, 44, 45].

The i.c.v. injections were performed as previously described [46, 47]. Briefly, a 0.4-mm-external-diameter hypodermic needle attached to a cannula, which was linked to a 25-μl Hamilton syringe, was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mice. A volume of 5 μl was then administered into the left lateral ventricle. The injection was given over 30 s, and the needle remained in place for another 30 s in order to avoid the reflux of the substances injected. The injection site was 1 mm to the left from the mid-point on a line drawn through to the anterior base of the ears. The asepsis of the injection site was carried out using gauze embedded in 70% ethanol. To ascertain that the drugs were administered exactly into the cerebral ventricle, the brains were carefully dissected and examined macroscopically after the test. Results from mice presenting misplacement of the injection site or any sign of cerebral hemorrhage were excluded from the statistical analysis (less than 5% of the total animals used).

## Experimental design

In order to verify if the activation of MEK/ERK 1/2, PKA, CaMKII, and PI3K/Akt are involved in the antidepressant-like effect of inosine, mice were treated with specific inhibitors of these enzymes 15 min before inosine (10 mg/kg, i.p.) administration. The following inhibitors were used: U0126 (5 μg/mouse, MEK1/2 inhibitor), KN-62 (1 μg/mouse, CaMKII inhibitor), H-89 (1 μg/mouse, PKA inhibitor), and wortmannin (0.1 μg/mouse, i.c.v., PI3K inhibitor). In order to investigate if the antidepressant-like effect of inosine is mediated by the inhibition of GSK-3β activity, mice were treated with a sub-effective dose of inosine (0.1 mg/kg, i.p.) or vehicle, and 15 min after, they were injected with a sub-effective dose of AR-A014418 (0.001 μg/mouse, i.c.v., selective GSK-3β inhibitor) or vehicle. In all experiments, the TST was performed 30 min after inosine administration.

In another set of experiments, animals were treated with inosine (10 mg/kg, i.p.) or vehicle, and 30 min or 24 h after treatment, they were killed by decapitation and had their hippocampi and prefrontal cortex dissected for neurochemical analyses.

## TST

The TST was performed according to the method previously described [48]. Mice were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. The immobility time was manually recorded during a 6-min session, and mice were considered immobile only when they hung passively and completely motionless [49–51].

## Open-field test

Ten minutes after the TST, in order to rule out non-specific motor effects that could influence the TST results, the locomotor activity was assessed in the open-field test (OFT) paradigm as previously described [52, 53]. The test was carried out in a temperature and light-controlled room. Animals were individually placed in a wooden box (40 × 60 × 50 cm) with the floor divided into 12 rectangles. The numbers of squares crossed with all paws (i.e., crossings) were counted in a 6-min session. The arena floor was cleaned with 10% ethanol between tests in order to hide animal clues.

## Western blotting

To quantify CREB phosphorylation and immunocontent, western blotting analysis was performed as previously described [47, 54, 55]. Animals were euthanized by decapitation; brains were excised from the skull; and hippocampi and prefrontal cortex were dissected into cold saline solution, placed in liquid nitrogen, and then stored at −80 °C until use. Briefly, samples were mechanically homogenized in 300 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, and Amresco protease inhibitor cocktail catalog number M222 (working concentration 0.5 mM AEBSF, 0.3 μM aprotinin, 10 μM bestatin, 10 μM E-64, 10 μM leupeptin, 50 μM EDTA). Lysates were centrifuged (10,000g for 10 min, at 4 °C) in order to eliminate cellular debris. The supernatants were diluted 1/1 (v/v) in 100 mM Tris (pH 6.8), 4 mM EDTA, and 8% SDS, followed by boiling for 5 min. Thereafter, samples were diluted in 40% glycerol, 100 mM Tris, and bromophenol blue (pH 6.8) in the ratio of 25:100 (v/v), and β-mercaptoethanol at a final concentration of 8% was added to each sample. Protein content was estimated by the method described by Peterson [56]. The samples (60 μg of total protein/track) were electrophoresed in 10% SDS-PAGE minigels and transferred to nitrocellulose

membranes using a semi-dry blotting apparatus (1.2 mA/cm$^2$; 1.5 h). To verify transfer efficiency process, membranes were stained with Ponceau stain. The membranes were blocked with 5% bovine serum albumin (BSA) in TBS (10 mM Tris, 150 mM NaCl, pH 7.5). The total and phosphorylated forms of CREB and β-actin were detected after overnight incubation with specific antibodies diluted in TBS-T containing 2% BSA. The primary rabbit antibodies were diluted 1:1000 for the phosphorylated (Ser133) and total forms of CREB (Cell Signaling) and 1:2000 for mouse anti-β-actin (Santa Cruz Biotechnology). Membranes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated antirabbit or antimouse antibody (1:5000, Millipore) for protein detection. The reactions were developed by chemiluminescence substrate (LumiGLO). After blocking and incubation steps, membranes were washed three times (5 min) with TBS-T (10 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5). The bands were quantified using the Scion Image® software. CREB phosphorylation was determined as a ratio of optical density (OD) of phosphorylated band/OD of total band, and the expression of CREB was determined as a ratio of OD CREB band/OD of β-actin band.

## Statistical analysis

Data are presented as mean ± standard error of mean (SEM). Differences among experimental groups were determined by two-way ANOVA followed by Newman-Keuls post hoc test when appropriate for behavioral tests or by Student's $t$ test for western blot experiments. A value of $p < 0.05$ was considered to be significant.

## Results

Figure 1a shows that pretreatment of mice with U0126 (5 μg/mouse, i.c.v., MEK 1/2 inhibitor) significantly prevented the decrease in immobility time caused by inosine in the TST. A two-way ANOVA revealed a main effect of inosine treatment [$F_{(1,24)} = 4.35, p < 0.05$], no main effect of U0126 treatment [$F_{(1,24)} = 3.19, p = 0.059$], and a significant interaction between inosine × U0126 treatment [$F_{(1,24)} = 11.36, p < 0.01$]. Figure 1b shows that neither administration of U0126 alone nor in combination with inosine was able to affect locomotor activity of mice in the OFT. The two-way ANOVA revealed no significant effect of inosine [$F_{(1,24)} = 0.26, p = 0.62$], U0126 treatment [$F_{(1,24)} = 0.0032, p = 0.95$], or inosine × U0126 treatment interaction [$F_{(1,24)} = 0.96, p = 0.34$].

The treatment of mice with KN-62 (1 μg/mouse, i.c.v., CaMKII inhibitor) was able to abolish the reduction of immobility time elicited by inosine in the TST, as shown in Fig. 2a. A two-way ANOVA revealed a main effect of inosine
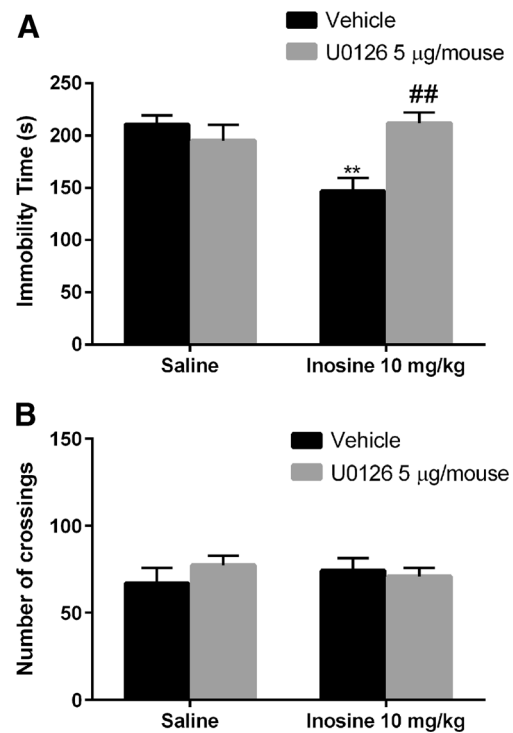


**Fig. 1** Involvement of MEK/ERK1/2 pathway in the antidepressant-like effect of inosine in the TST. Effect of treatment of mice with the MEK1/2 inhibitor U0126 (5 μg/mouse, i.c.v.) on the antidepressant-like effect of inosine (10 mg/kg, i.p.) in the TST (**a**) and locomotor activity in the OFT (**b**). Values are expressed as SEM of six to eight mice. **$p < 0.01$ compared with the vehicle-treated control group. ##$p < 0.01$ compared with inosine-treated group

treatment [$F_{(1,23)} = 8.38, p < 0.05$], no main effect of KN-62 treatment [$F_{(1,23)} = 2.17, p = 0.15$], but a significant interaction between inosine × KN-62 treatment [$F_{(1,23)} = 21.49, p < 0.001$]. The results depicted in Fig. 2b reveal that locomotor activity in the OFT was not affected by inosine treatment [$F_{(1,23)} = 0.38, p = 0.15$], KN-62 treatment [$F_{(1,23)} = 2.00, p = 0.17$], or by the interaction between inosine × KN-62 [$F_{(1,23)} = 1.61, p = 0.22$].

Similarly, pretreatment of mice with H-89 (1 μg/mouse, i.c.v., PKA inhibitor) blocked the antiimmobility effect induced by inosine in the TST, as illustrated in Fig. 3a. A two-way ANOVA revealed no main effect of inosine treatment [$F_{(1,26)} = 1.59, p = 0.22$], a main effect of H-89 pretreatment [$F_{(1,26)} = 6.75, p < 0.05$], and a significant inosine treatment × H-89 treatment interaction [$F_{(1,26)} = 4.48, p < 0.05$]. Also, the two-way ANOVA revealed no significant effect of inosine treatment [$F_{(1,26)} = 1.27, p = 0.27$], H-89 treatment [$F_{(1,26)} = 0.75, p = 0.39$], and inosine × H-89 treatment interaction [$F_{(1,26)} = 0.43, p = 0.52$] in the number of crossings in the OFT, as shown in Fig. 3b.

Figure 4a shows the effect of wortmannin treatment (0.1 μg/mouse, i.c.v., PI3K inhibitor) on the reduction of immobility time elicited by inosine in the TST. The two-way ANOVA revealed a main effect of inosine treatment [$F$
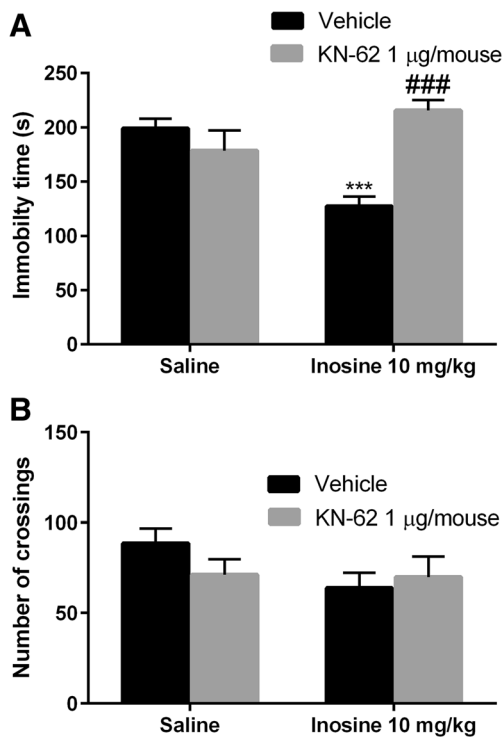
**Fig. 2** Involvement of CaMKII in the antidepressant-like effect of inosine in the TST. Effect of treatment of mice with the CaMKII inhibitor KN-62 (1 μg/mouse, i.c.v.) on the antidepressant-like effect of inosine (10 mg/kg, i.p.) in the TST (a) and locomotor activity in the OFT (b). Values are expressed as SEM of six to eight mice. ***$p < 0.001$ compared with the vehicle-treated control group. ###$p < 0.001$ compared with inosine-treated group

**Fig. 3** Involvement of PKA in the antidepressant-like effect of inosine in the TST. Effect of treatment of mice with the PKA inhibitor H-89 (1 μg/mouse, i.c.v.) on the inosine-induced (10 mg/kg, i.p.) antidepressant-like effect in the TST (a) and locomotor activity in the OFT (b). Values are expressed as SEM of seven to eight mice. *$p < 0.05$ compared with the vehicle-treated control group. #$p < 0.05$ compared with inosine-treated group

$(1,25) = 6.67$, $p < 0.05$], wortmannin treatment [$F (1,25) = 16.78$, $p < 0.001$], and inosine treatment × wortmannin treatment interaction [$F (1,25) = 7.49$, $p < 0.01$]. Post hoc analysis indicated that wortmannin administration abolished the antiimmobility effect of inosine in this test. As indicated in Fig. 4b, the number of crossings in the OFT was not altered by inosine and/or wortmannin (inosine treatment [$F (1,25) = 0.01$, $p = 0.89$], wortmannin treatment [$F (1,25) = 2.00$, $p = 0.16$], and inosine treatment × wortmannin treatment interaction [$F (1,25) = 2.25$, $p = 0.15$].

To test the hypothesis that GSK-3β is involved in the antidepressant-like effect of inosine in the TST, mice were treated with a combination of sub-effective doses of inosine (0.1 mg/kg, i.p.) and the selective GSK-3β inhibitor AR-A014418 (0.001 μg/mouse, i.c.v.). The two-way ANOVA revealed a main effect of inosine treatment [$F (1,24) = 5.74$, $p < 0.05$], AR-A014418 treatment [$F (1,24) = 6.74$, $p < 0.05$], and inosine × AR-A014418 interaction [$F (1,24) = 8.01$, $p < 0.01$]. Post hoc analysis indicated a significant reduction in the immobility time in the TST when sub-effective doses of inosine and AR-A014418 were combined, as shown in Fig. 5a. The results in Fig. 5b indicate that none of the treatments alone or in combination altered locomotor activity in the OFT
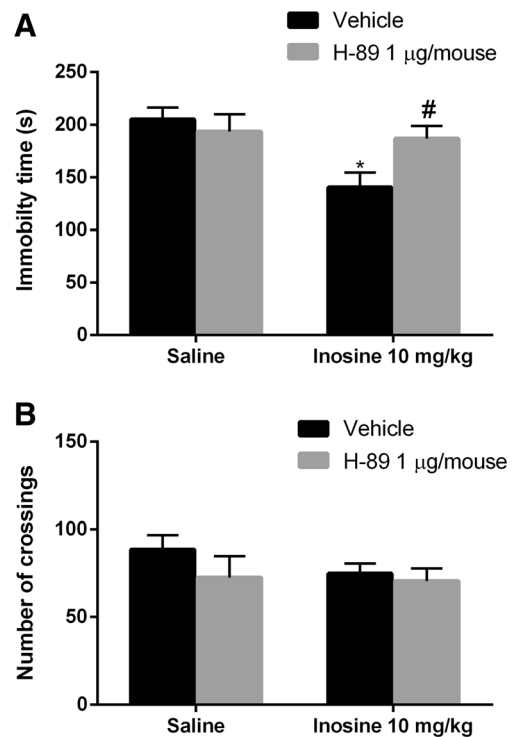
(inosine treatment [$F (1,24) = 0.48$, $p = 0.50$], AR-A014418 treatment [$F (1,24) = 0.22$, $p = 0.64$], and inosine × AR-A014418 treatment interaction [$F (1,24) = 2.44$, $p = 0.13$]).

Finally, in order to evaluate if a single administration of inosine is able to induce changes in CREB phosphorylation and immunocontent, western blotting analyses were carried out in homogenates of prefrontal cortex and hippocampus of mice 30 min and 24 h after the i.p. administration of inosine. As demonstrated in Fig. 6a, b, inosine did not induce alterations in CREB phosphorylation or in CREB immunocontent in hippocampus and prefrontal cortex of mice 30 min after its administration (hippocampus P-CREB 30 min $t(12) = 1.28$, $p = 0.22$; hippocampus T-CREB 30 min $t(12) = 1.20$, $p = 0.25$; prefrontal cortex P-CREB 30 min $t(12) = 1.28$, $p = 0.77$; prefrontal cortex T-CREB 30 min $t(12) = 4.38$, $p = 0.10$). Conversely, as shown in Fig. 6c, 24 h after inosine administration, a significant increase in hippocampal CREB phosphorylation ($t(14) = 2.40$, $p < 0.05$), without alterations in CREB immunocontent ($t(14) = 1.46$, $p = 0.17$), was observed. The results in Fig. 6d indicate that no alterations were observed in CREB phosphorylation or immunocontent in the prefrontal cortex of animals 24 h after inosine administration (P-CREB $t(14) = 1.15$, $p = 0.24$; T-CREB $t(14) = 1.61$, $p = 0.55$).
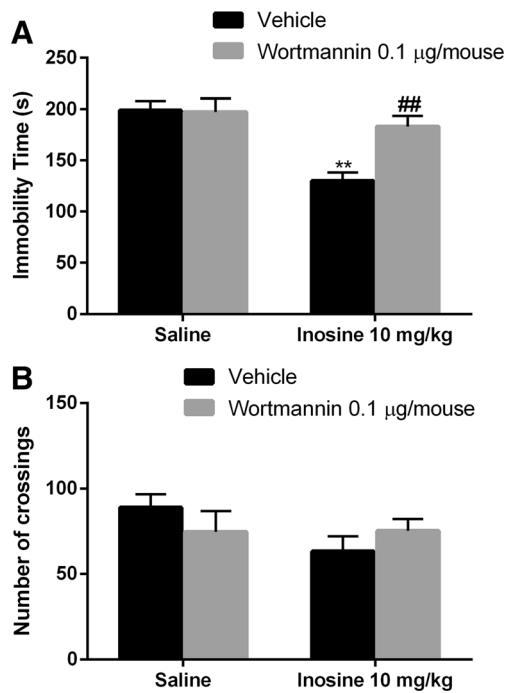
Fig. 4 Involvement of PI3K/Akt in the antidepressant-like effect of inosine in the TST. Effect of treatment of mice with the PI3K inhibitor wortmannin (0.1 μg/mouse, i.c.v.) on the inosine-induced (10 mg/kg, i.p.) antidepressant-like effect in the TST (a) and locomotor activity in the OFT (b). Values are expressed as mean + SEM of six to eight mice. **$p < 0.01$ compared with the vehicle-treated control group. ##$p < 0.01$ compared with inosine-treated group
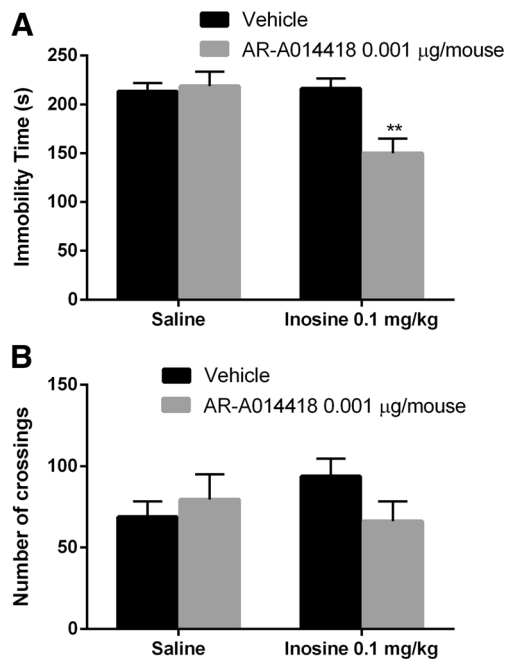


Fig. 5 Involvement of GSK-3β inhibition in the antidepressant-like effect of inosine in the TST. Effect of treatment of mice with the GSK-3β inhibitor AR-A014418 (0.001 mg/mouse, i.c.v.) in combination with a sub-effective dose of inosine (0.1 mg/kg, p.m.) in the TST (a) and locomotor activity in the OFT (b). Values are expressed as mean ± SEM of seven mice. **$p < 0.01$ compared with the vehicle-treated control group

## Discussion

In the present study, we demonstrated the involvement of several kinases in the antidepressant-like effect elicited by inosine in TST. The inhibition of PKA, CaMKII, MEK/ERK1/2, and PI3K was able to prevent the decrease of immobility time elicited by inosine in this behavioral despair model. Along this framework, inosine had a synergic effect with a GSK-3β inhibitor in the TST. Moreover, our study also showed that a single administration of inosine is sufficient to increase CREB phosphorylation in the hippocampus after 24 h. These results reinforce the role of intracellular pathways controlling neuroplasticity in the antidepressant-like effect of inosine.

We used the TST as a predictive test to evaluate antidepressant activity. Despite its limitations, including the fact that it is sensitive to acute antidepressant treatment and has mostly predictive validity, this behavioral despair test was designed to assess antidepressant or depressant activity of compounds and allows the study of their mechanism of action. In this paradigm, animals are placed in an acute, stressful, and inescapable situation and a decrease in the immobility time is an indication of antidepressant-like action. One of the main concerns of the TST is the occurrence of false-positive results due to alterations in the locomotor activity of animals [48]. For this reason we also performed the OFT and we observed that none of the treatments alone or in combination was able to alter locomotor activity of mice.

Inosine is widely distributed in the central nervous system (CNS), and among all nucleosides and their metabolites, this purine has the highest overall concentration in various brain regions, including hippocampus [57]. Brain levels of inosine are increased after its oral administration [20], raising the possibility that exogenous inosine might impact the CNS where it can act as a neuromodulator and activate different cellular targets including the adenosine receptors [29, 28]. However, we cannot rule out the involvement of different purine metabolites or even the involvement of adenosine itself in the behavioral effects observed. As indicated by clinical studies, inosine may also partially act via its breakdown product, uric acid [30, 29]. Lowered uric acid levels were found in depressive patients [58], a compound that exerts antioxidant and neuroprotective proprieties in different pathological conditions including Parkinson's disease [30, 29]. The antidepressant potential of inosine is in agreement with clinical data showing decreased levels of inosine in the serum of patients diagnosed with MDD [7]. Also, the activity of ADA, the enzyme involved in inosine formation, was decreased in MDD patients, and an inverse relationship was found between ADA activity and severity of the depressive symptoms [59]. Literature data have shown that activation of adenosine receptors is essential for many of the biological effects of inosine. Indeed, most of preclinical data suggest that its antidepressant,

**Fig. 6** Effect of inosine treatment (10 mg/kg, i.p.) on CREB immunocontent and phosphorylation (Ser133) in the hippocampus and prefrontal cortex of mice. **a**, **b** The representative western blotting and the quantification of phospho-CREB (P-CREB) and total immunocontent of CREB (T-CREB) in the hippocampus (**a**) and prefrontal cortex of mice (**b**) 30 min after inosine administration. **c**, **d** The representative western blotting and the quantification of P-CREB and T-CREB in hippocampus (**c**) and prefrontal cortex of mice (**d**) 24 h after inosine administration. Results were quantified as percent of control and expressed as mean ± SEM of seven to eight mice. *$p < 0.05$ compared with the vehicle-treated control group
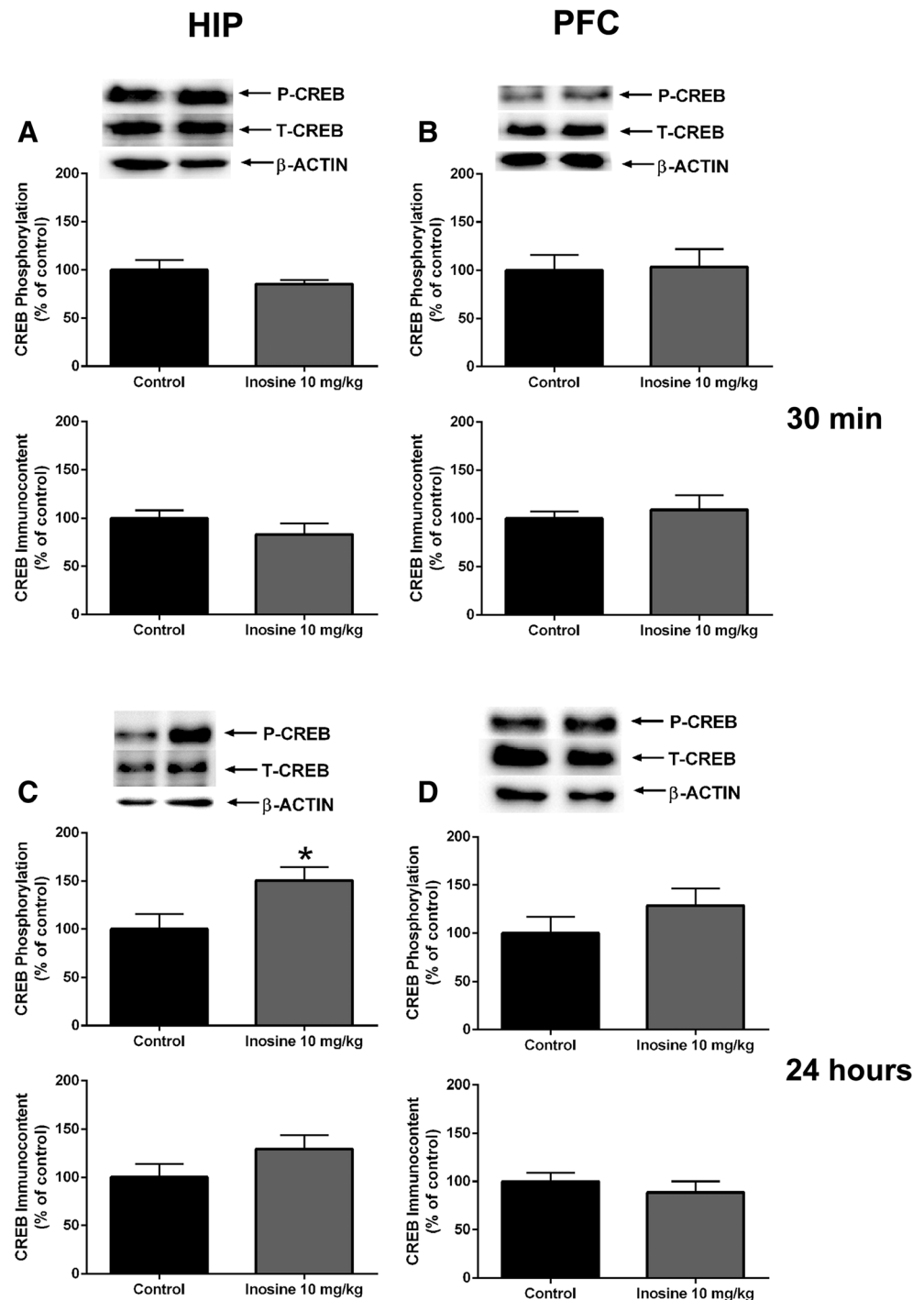
antinociceptive, and neuroprotective effects occur through activation of adenosine $A_1$ and $A_{2A}$ receptors [31, 36]. Moreover, inosine reduced ischemic brain injury in rats via adenosine $A_3$ receptor-dependent pathway [60]. Meanwhile, the activation of these receptors is able to modulate several signaling pathways related to neuroplasticity and cell survival such as MEK/ERK1/2, CaMKII, PKA, PI3K/Akt, and GSK-3β [42, 43].

This study showed the involvement of MEK/ERK1/2 pathway in the antidepressant-like effect of inosine considering that pretreatment with U0126, a selective inhibitor of MEK1/2 and consequently ERK1/2 activation [61], prevented the decrease of immobility time elicited by inosine in the TST. The MEK/ERK signaling pathway mediates the transduction of intracellular stimuli and gene expression, regulating a variety of cell activities such as cell proliferation, migration, and

differentiation [62]. Several studies have shown the involvement of this signaling pathway in MDD. Animal models of depression induced by chronic stress or corticosterone treatment are associated with decreased levels of ERK1/2 in prefrontal cortex and hippocampus of mice [63, 64]. Also, the inhibition of MEK/ERK cascade induces a depressive-like behavior and abolishes the effect of some antidepressants including desipramine, sertraline, and ketamine [65, 66]. Our results are consistent with literature data regarding the involvement of ERK activation in the neurobiological effects of inosine. In fact, oral administration of inosine induces an increase in ERK phosphorylation in the hippocampus of mice through both adenosine $A_1$ and $A_{2A}$ receptor activation. In addition, the neuroprotective activity of inosine in vitro is also dependent on ERK activation [20].

CaMKII is a prominent protein kinase largely distributed in the brain and concentrated in the postsynaptic densities and presynaptic terminals that control activity-dependent neuronal response [67, 68]. This enzyme has a critical role for regulation of synaptic plasticity, and modulation of CaMKII activity has been implicated in the pathophysiology of MDD and in antidepressant responses [69]. Noteworthy, it has been reported that activation of adenosine $A_1$ receptors may modulate $Ca^{2+}$ and increase CaMKII phosphorylation and activity [70, 43]. In the present study, we used KN-62, a selective CaMKII inhibitor, which has been extensively used as a tool to address the functional roles of this kinase [69, 71, 72]. Our results showed that KN-62 treatment fully abolished the decrease of immobility time induced by inosine in the TST. It is worthy to mention that KN-62 can also block P2 receptors, such P2X7 receptors [73]. However, these receptors are not activated by purines such as adenosine, and it is unlikely that inosine directly activates these receptors [74]. However, we cannot rule out the possibility of an indirect modulation of P2X7 receptors by inosine and further studies are necessary to investigate the role of P2 receptors in the antidepressant-like effect of inosine. Altogether, our findings suggest CaMKII activation as a key signaling element involved in the inosine antidepressant response.

We also demonstrated that H-89, a selective inhibitor of PKA activity, was able to prevent the antidepressant-like effect of inosine. Our results corroborate previous reports showing a role for PKA activity in the antidepressant response of compounds like memantine, creatine, and zinc chloride [75, 44, 76]. However, adenosine receptors are capable of modulating PKA activity in opposite ways. Adenosine $A_1$ receptors interact with Gi proteins that ultimately decrease the levels of cAMP, reducing PKA activity. On the other hand, the activation of adenosine $A_{2A}$ receptors is classically associated with a Gs-protein interaction, increased cAMP levels, and PKA activation [42]. Previous report indicates that the antidepressant-like effect of inosine may involve the activation of both $A_1$ and $A_{2A}$ receptors [36]. However, it is also important to highlight that these receptors are able to interact, and other non-classical transduction pathways might be operated under these conditions [77]. Therefore, additional studies will be required to explain the modulation of PKA activity by inosine and adenosine receptors.

It has been reported that adenosine receptors can activate PI3K/Akt [78], and in vitro studies have shown an increase in Akt phosphorylation elicited by inosine [79]. The activation of PI3K catalyzes the production of phosphatidylinositol-3,4,5-triphosphate and induces Akt activation, which is associated with several cellular processes, such as neurite outgrowth, cell survival, synaptic plasticity, and antidepressant responses [80, 38]. In this study, we show that wortmannin, a selective irreversible PI3K inhibitor, abolished the decrease of immobility time elicited by inosine in the TST, indicating the involvement of PI3K/Akt in its antidepressant-like effect. Once activated, one of the downstream targets of Akt is the enzyme GSK-3β. Akt can phosphorylate GSK-3β at Ser-9 residue leading to its inactivation. It was already demonstrated that GSK-3β is associated with the development of MDD, and GSK-3β inhibition is capable of potentiating the effect of antidepressants [81, 82]. This is in accordance with our results showing a synergistic antidepressant-like effect when sub-effective doses of inosine and AR-A014418, a selective GSK-3β inhibitor, were combined. Thus, our findings suggest that inosine, probably through activation of adenosine receptors, can trigger the activation of PI3K/Akt with the consequent inhibition of GSK-3β.

Overall, activation of MEK/ERK1,2, CaMKII, PKA, and PI3K/Akt can induce the phosphorylation of CREB at Ser-133, which is a committed step for activation of this transcription factor [83]. Moreover, inhibition of GSK-3β by Ser-9 phosphorylation, via PI3K/Akt, reduces the inhibitory effect of GSK-3β on CREB activity [84]. Our results showed increased CREB Ser-133 phosphorylation in the hippocampus 24 h after inosine administration. The hippocampus is one of the most prominent regions involved in mood regulation and largely sensitive to alterations in neuroplasticity and cell survival induced by stress and MDD. The phosphorylation of CREB can lead to transcription of several neurotrophic genes including BDNF [83]. Indeed, it was recently demonstrated that inosine administration enhanced BDNF expression and increased cell proliferation in the hippocampus of mice [20]. Moreover, a recent study concerning the antidepressant action of ketamine and its metabolite (2R,6R)-hydroxynorketamine (HNK) shows that immobility time in the FST was decreased at 1 and 24 h after treatment in mice. However, an increment of BDNF was observed in the hippocampus, but not in prefrontal cortex only 24 h after ketamine or HNK administration [85]. Therefore, the profile of CREB phosphorylation increment observed in response to inosine treatment (24 h), only in the hippocampus, is in agreement with that observed for BDNF expression in response to ketamine. Taken together, our

findings suggest that inosine can alter the complex intracellular signaling pathways that converge on CREB activation, an effect that might represent a promising strategy to manage depressive-related disorders. However, it remains to be answered if these initial effects elicited by a single inosine administration are capable of producing sustained antidepressant response or even neuroprotective effects in preclinical models associated with compromised neuroplasticity.

In conclusion, the present study provides combined pharmacological and biochemical evidence that indicates the participation of MEK/ERK1/2, CaMKII, PKA, and PI3K/Akt activation and GSK-3β inhibition in the antidepressant-like effect of inosine in a behavioral despair model of depression in mice. Furthermore, this study also identified that a single administration of inosine was able to increase hippocampal CREB phosphorylation in mice. It was already demonstrated that inosine has a highly safety profile and is devoid of significant side effects, even after prolonged treatment [29, 28]. These results suggest that inosine is able to target signaling pathways related to cell survival, neuroprotection, and neuroplasticity, further reinforcing the ability of the purinergic system to favorably impact mood.

**Compliance with ethical standards**

**Conflicts of interest** Filipe Marques Gonçalves declares that he has no conflict of interest.

Vivian Binder Neis declares that she has no conflict of interest.
Débora Kurrle Rieger declares that she has no conflict of interest.
Mark William Lopes declares that he has no conflict of interest.
Isabela A. Heinrich declares that she has no conflict of interest.
Ana Paula Costa declares that she has no conflict of interest.
Ana Lúcia S. Rodrigues declares that she has no conflict of interest.
Manuella P. Kaster declares that she has no conflict of interest.
Rodrigo Bainy Leal declares that he has no conflict of interest.

**Ethical approval** The procedures in this study were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and all the experimental protocols were approved by the Institutional Ethics Committee (protocol number PP00772). All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

# References

1. Kaster MP, Moretti M, Cunha MP, Rodrigues AL (2016) Novel approaches for the management of depressive disorders. Eur J Pharmacol 771:236–240. doi:10.1016/j.ejphar.2015.12.029
2. Krishnan V, Nestler EJ (2008) The molecular neurobiology of depression. Nature 455(7215):894–902. doi:10.1038/nature07455
3. Papakostas GI, Ionescu DF (2016) Towards new mechanisms: an update on therapeutics for treatment-resistant major depressive disorder. Mol Psychiatry 20(10):1142–1150. doi:10.1038/mp.2015.92
4. Nemeroff CB, Owens MJ (2002) Treatment of mood disorders. Nat Neurosci 5(Suppl):1068–1070. doi:10.1038/nn943
5. Ruhe HG, van Rooijen G, Spijker J, Peeters FP, Schene AH (2011) Staging methods for treatment resistant depression. A systematic review. J Affect Disord 137(1–3):35–45. doi:10.1016/j.jad.2011.02.020
6. Kaster MP, Machado NJ, Silva HB, Nunes A, Ardais AP, Santana M, Baqi Y, Muller CE, Rodrigues AL, Porciuncula LO, Chen JF, Tome AR, Agostinho P, Canas PM, Cunha RA (2015) Caffeine acts through neuronal adenosine $A_{2A}$ receptors to prevent mood and memory dysfunction triggered by chronic stress. Proc Natl Acad Sci U S A 112(25):7833–7838. doi:10.1073/pnas.1423088112
7. Ali-Sisto T, Tolmunen T, Toffol E, Viinamaki H, Mantyselka P, Valkonen-Korhonen M, Honkalampi K, Ruusunen A, Velagapudi V, Lehto SM (2016) Purine metabolism is dysregulated in patients with major depressive disorder. Psychoneuroendocrinology 70:25–32. doi:10.1016/j.psyneuen.2016.04.017
8. Ortiz R, Ulrich H, Zarate CA Jr, Machado-Vieira R (2014) Purinergic system dysfunction in mood disorders: a key target for developing improved therapeutics. Prog Neuro-Psychopharmacol Biol Psychiatry 57:117–131. doi:10.1016/j.pnpbp.2014.10.016
9. Scaccianoce S, Navarra D, Di Sciullo A, Angelucci L, Endroczi E (1989) Adenosine and pituitary-adrenocortical axis activity in the rat. Neuroendocrinology 50(4):464–468
10. Okada M, Nutt DJ, Murakami T, Zhu G, Kamata A, Kawata Y, Kaneko S (2001) Adenosine receptor subtypes modulate two major functional pathways for hippocampal serotonin release. J Neurosci 21(2):628–640
11. Kaster MP, Rosa AO, Rosso MM, Goulart EC, Santos AR, Rodrigues AL (2004) Adenosine administration produces an antidepressant-like effect in mice: evidence for the involvement of $A_1$ and $A_{2A}$ receptors. Neurosci Lett 355(1–2):21–24
12. El Yacoubi M, Costentin J, Vaugeois JM (2003) Adenosine $A_{2A}$ receptors and depression. Neurology 61(11 Suppl 6):S82–S87
13. Minor TR, Rowe M, Cullen PK, Furst S (2008) Enhancing brain adenosine signaling with the nucleoside transport blocker NBTI (S-(4-nitrobenzyl)-6-theoinosine) mimics the effects of inescapable shock on later shuttle-escape performance in rats. Behav Neurosci 122(6):1236–1247. doi:10.1037/a0013143
14. Yamada K, Kobayashi M, Shiozaki S, Ohta T, Mori A, Jenner P, Kanda T (2014) Antidepressant activity of the adenosine $A_{2A}$ receptor antagonist, istradefylline (KW-6002) on learned helplessness in rats. Psychopharmacology 231(14):2839–2849. doi:10.1007/s00213-014-3454-0
15. Coelho JE, Alves P, Canas PM, Valadas JS, Shmidt T, Batalha VL, Ferreira DG, Ribeiro JA, Bader M, Cunha RA, do Couto FS, Lopes LV (2014) Overexpression of adenosine $A_{2A}$ receptors in rats: effects on depression, locomotion, and anxiety. Front Psychiatry 5:67. doi:10.3389/fpsyt.2014.00067
16. Machado NJ, Simoes AP, Silva HB, Ardais AP, Kaster MP, Garcao P, Rodrigues DI, Pochmann D, Santos AI, Araujo IM, Porciuncula LO, Tome AR, Kofalvi A, Vaugeois JM, Agostinho P, El Yacoubi M, Cunha RA, Gomes CA (2016) Caffeine reverts memory but not mood impairment in a depression-prone mouse strain with up-regulated adenosine $A_{2A}$ receptor in hippocampal glutamate synapses. Mol Neurobiol. doi:10.1007/s12035-016-9774-9

17. Barankiewicz J, Cohen A (1985) Purine nucleotide metabolism in resident and activated rat macrophages in vitro. Eur J Immunol 15(6):627–631. doi:10.1002/eji.1830150618

18. Haun SE, Segeleon JE, Trapp VL, Clotz MA, Horrocks LA (1996) Inosine mediates the protective effect of adenosine in rat astrocyte cultures subjected to combined glucose-oxygen deprivation. J Neurochem 67(5):2051–2059

19. Cipriani S, Bakshi R, Schwarzschild MA (2014) Protection by inosine in a cellular model of Parkinson's disease. Neuroscience 274:242–249. doi:10.1016/j.neuroscience.2014.05.038

20. Muto J, Lee H, Uwaya A, Park J, Nakajima S, Nagata K, Ohno M, Ohsawa I, Mikami T (2014) Oral administration of inosine produces antidepressant-like effects in mice. Sci Rep 4:4199. doi:10.1038/srep04199

21. Wu MM, You SW, Hou B, Jiao XY, Li YY, Ju G (2003) Effects of inosine on axonal regeneration of axotomized retinal ganglion cells in adult rats. Neurosci Lett 341(1):84–86

22. Zurn AD, Do KQ (1988) Purine metabolite inosine is an adrenergic neurotrophic substance for cultured chicken sympathetic neurons. Proc Natl Acad Sci U S A 85(21):8301–8305

23. Benowitz LI, Goldberg DE, Irwin N (2002) Inosine stimulates axon growth in vitro and in the adult CNS. Prog Brain Res 137:389–399

24. Benowitz LI, Jing Y, Tabibiazar R, Jo SA, Petrausch B, Stuermer CA, Rosenberg PA, Irwin N (1998) Axon outgrowth is regulated by an intracellular purine-sensitive mechanism in retinal ganglion cells. J Biol Chem 273(45):29626–29634

25. Chen P, Goldberg DE, Kolb B, Lanser M, Benowitz LI (2002) Inosine induces axonal rewiring and improves behavioral outcome after stroke. Proc Natl Acad Sci U S A 99(13):9031–9036. doi:10.1073/pnas.132076299

26. Nascimento FP, Figueredo SM, Marcon R, Martins DF, Macedo SJ Jr, Lima DA, Almeida RC, Ostroski RM, Rodrigues AL, Santos AR (2010) Inosine reduces pain-related behavior in mice: involvement of adenosine $A_1$ and $A_{2A}$ receptor subtypes and protein kinase C pathways. J Pharmacol Exp Ther 334(2):590–598. doi:10.1124/jpet.110.166058

27. Macedo-Junior SJ, Nascimento FP, Luiz-Cerutti M, Santos AR (2012) Role of pertussis toxin-sensitive G-protein, $K^+$ channels, and voltage-gated $Ca^{2+}$ channels in the antinociceptive effect of inosine. Purinergic Signal 9(1):51–58. doi:10.1007/s11302-012-9327-2

28. Markowitz CE, Spitsin S, Zimmerman V, Jacobs D, Udupa JK, Hooper DC, Koprowski H (2009) The treatment of multiple sclerosis with inosine. J Altern Complement Med 15(6):619–625. doi:10.1089/acm.2008.0513

29. Schwarzschild MA, Ascherio A, Beal MF, Cudkowicz ME, Curhan GC, Hare JM, Hooper DC, Kieburtz KD, Macklin EA, Oakes D, Rudolph A, Shoulson I, Tennis MK, Espay AJ, Gartner M, Hung A, Bwala G, Lenehan R, Encarnacion E, Ainslie M, Castillo R, Togasaki D, Barles G, Friedman JH, Niles L, Carter JH, Murray M, Goetz CG, Jaglin J, Ahmed A, Russell DS, Cotto C, Goudreau JL, Russell D, Parashos SA, Ede P, Saint-Hilaire MH, Thomas CA, James R, Stacy MA, Johnson J, Gauger L, Antonelle de Marcaida J, Thurlow S, Isaacson SH, Carvajal L, Rao J, Cook M, Hope-Porche C, McClurg L, Grasso DL, Logan R, Orme C, Ross T, Brocht AF, Constantinescu R, Sharma S, Venuto C, Weber J, Eaton K (2013) Inosine to increase serum and cerebrospinal fluid urate in Parkinson disease: a randomized clinical trial. JAMA Neurol 71(2):141–150. doi:10.1001/jamaneurol.2013.5528

30. Bhattacharyya S, Bakshi R, Logan R, Ascherio A, Macklin EA, Schwarzschild MA (2016) Oral inosine persistently elevates plasma antioxidant capacity in Parkinson's disease. Mov Disord 31(3):417–421. doi:10.1002/mds.26483

31. Nascimento FP, Macedo-Junior SJ, Pamplona FA, Luiz-Cerutti M, Cordova MM, Constantino L, Tasca CI, Dutra RC, Calixto JB, Reid A, Sawynok J, Santos AR (2014)

Adenosine $A_1$ receptor-dependent antinociception induced by inosine in mice: pharmacological, genetic and biochemical aspects. Mol Neurobiol. doi:10.1007/s12035-014-8815-5

32. Welihinda AA, Kaur M, Greene K, Zhai Y, Amento EP (2016) The adenosine metabolite inosine is a functional agonist of the adenosine $A_{2A}$ receptor with a unique signaling bias. Cell Signal 28(6):552–560. doi:10.1016/j.cellsig.2016.02.010

33. Hasko G, Sitkovsky MV, Szabo C (2004) Immunomodulatory and neuroprotective effects of inosine. Trends Pharmacol Sci 25(3):152–157. doi:10.1016/j.tips.2004.01.006

34. Jin X, Shepherd RK, Duling BR, Linden J (1997) Inosine binds to $A_3$ adenosine receptors and stimulates mast cell degranulation. J Clin Invest 100(11):2849–2857. doi:10.1172/JCI119833

35. Gomez G, Sitkovsky MV (2003) Differential requirement for $A_{2a}$ and $A_3$ adenosine receptors for the protective effect of inosine in vivo. Blood 102(13):4472–4478. doi:10.1182/blood-2002-11-3624

36. Kaster MP, Budni J, Gazal M, Cunha MP, Santos AR, Rodrigues AL (2013) The antidepressant-like effect of inosine in the FST is associated with both adenosine $A_1$ and $A_{2A}$ receptors. Purinergic Signal 9(3):481–486. doi:10.1007/s11302-013-9361-8

37. D'Sa C, Duman RS (2002) Antidepressants and neuroplasticity. Bipolar Disord 4(3):183–194

38. Marsden WN (2012) Synaptic plasticity in depression: molecular, cellular and functional correlates. Prog Neuro-Psychopharmacol Biol Psychiatry 43:168–184. doi:10.1016/j.pnpbp.2012.12.012

39. Duric V, Banasr M, Stockmeier CA, Simen AA, Newton SS, Overholser JC, Jurjus GJ, Dieter L, Duman RS (2012) Altered expression of synapse and glutamate related genes in post-mortem hippocampus of depressed subjects. Int J Neuropsychopharmacol 16(1):69–82. doi:10.1017/S1461145712000016

40. Yuan P, Zhou R, Wang Y, Li X, Li J, Chen G, Guitart X, Manji HK (2009) Altered levels of extracellular signal-regulated kinase signaling proteins in postmortem frontal cortex of individuals with mood disorders and schizophrenia. J Affect Disord 124(1–2):164–169. doi:10.1016/j.jad.2009.10.017

41. Reus GZ, Stringari RB, Ribeiro KF, Ferraro AK, Vitto MF, Cesconetto P, Souza CT, Quevedo J (2011) Ketamine plus imipramine treatment induces antidepressant-like behavior and increases CREB and BDNF protein levels and PKA and PKC phosphorylation in rat brain. Behav Brain Res 221(1):166–171. doi:10.1016/j.bbr.2011.02.024

42. Jacobson KA, Gao ZG (2006) Adenosine receptors as therapeutic targets. Nat Rev Drug Discov 5(3):247–264. doi:10.1038/nrd1983

43. Liu AM, Wong YH (2004) G16-mediated activation of nuclear factor kappaB by the adenosine $A_1$ receptor involves c-Src, protein kinase C, and ERK signaling. J Biol Chem 279(51):53196–53204. doi:10.1074/jbc.M410196200

44. Manosso LM, Moretti M, Ribeiro CM, Goncalves FM, Leal RB, Rodrigues AL (2015) Antidepressant-like effect of zinc is dependent on signaling pathways implicated in BDNF modulation. Prog Neuro-Psychopharmacol Biol Psychiatry 59:59–67. doi:10.1016/j.pnpbp.2015.01.008

45. Zeni AL, Zomkowski AD, Maraschin M, Rodrigues AL, Tasca CI (2012) Involvement of PKA, CaMKII, PKC, MAPK/ERK and PI3K in the acute antidepressant-like effect of ferulic acid in the tail suspension test. Pharmacol Biochem Behav 103(2):181–186. doi:10.1016/j.pbb.2012.08.020

46. Kaster MP, Gadotti VM, Calixto JB, Santos AR, Rodrigues AL (2011) Depressive-like behavior induced by tumor necrosis factor-alpha in mice. Neuropharmacology 62(1):419–426. doi:10.1016/j.neuropharm.2011.08.018

47. Goncalves FM, Freitas AE, Peres TV, Rieger DK, Ben J, Maestri M, Costa AP, Tramontina AC, Goncalves CA, Rodrigues AL, Nagano CS, Teixeira EH, Nascimento KS, Cavada BS, Leal RB (2013) Vatairea macrocarpa lectin (VML) induces depressive-like behavior and expression of neuroinflammatory markers in mice. Neurochem Res 38(11):2375–2384. doi:10.1007/s11064-013-1150-9

48. Steru L, Chermat R, Thierry B, Simon P (1985) The tail suspension test: a new method for screening antidepressants in mice. Psychopharmacology 85(3):367–370

49. Cunha MP, Pazini FL, Rosa JM, Ramos-Hryb AB, Oliveira A, Kaster MP, Rodrigues AL (2015) Creatine, similarly to ketamine, affords antidepressant-like effects in the tail suspension test via adenosine $A_1$ and $A_{2A}$ receptor activation. Purinergic Signal. doi:10.1007/s11302-015-9446-7

50. Neis VB, Moretti M, Manosso LM, Lopes MW, Leal RB, Rodrigues AL (2015) Agmatine enhances antidepressant potency of MK-801 and conventional antidepressants in mice. Pharmacol Biochem Behav 130:9–14. doi:10.1016/j.pbb.2014.12.009

51. Moretti M, Budni J, Freitas AE, Rosa PB, Rodrigues AL (2013) Antidepressant-like effect of ascorbic acid is associated with the modulation of mammalian target of rapamycin pathway. J Psychiatr Res 48(1):16–24. doi:10.1016/j.jpsychires.2013.10.014

52. Rodrigues AL, Rocha JB, Mello CF, Souza DO (1996) Effect of perinatal lead exposure on rat behaviour in open-field and two-way avoidance tasks. Pharmacol Toxicol 79(3):150–156

53. Neis VB, Moretti M, Bettio LE, Ribeiro CM, Rosa PB, Goncalves FM, Lopes MW, Leal RB, Rodrigues AL (2016) Agmatine produces antidepressant-like effects by activating AMPA receptors and mTOR signaling. Eur Neuropsychopharmacol 26(6):959–971. doi:10.1016/j.euroneuro.2016.03.009

54. Peres TV, Eyng H, Lopes SC, Colle D, Goncalves FM, Venske DK, Lopes MW, Ben J, Bornhorst J, Schwerdtle T, Aschner M, Farina M, Prediger RD, Leal RB (2015) Developmental exposure to manganese induces lasting motor and cognitive impairment in rats. Neurotoxicology 50:28–37. doi:10.1016/j.neuro.2015.07.005

55. Lopes MW, Lopes SC, Costa AP, Goncalves FM, Rieger DK, Peres TV, Eyng H, Prediger RD, Diaz AP, Nunes JC, Walz R, Leal RB (2015) Region-specific alterations of AMPA receptor phosphorylation and signaling pathways in the pilocarpine model of epilepsy. Neurochem Int 87:22–33. doi:10.1016/j.neuint.2015.05.003

56. Peterson GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83(2):346–356

57. Kovacs Z, Dobolyi A, Juhasz G, Kekesi KA (2009) Nucleoside map of the human central nervous system. Neurochem Res 35(3):452–464. doi:10.1007/s11064-009-0080-z

58. Wen S, Cheng M, Wang H, Yue J, Wang H, Li G, Zheng L, Zhong Z, Peng F (2012) Serum uric acid levels and the clinical characteristics of depression. Clin Biochem 45(1–2):49–53. doi:10.1016/j.clinbiochem.2011.10.010

59. Elgun S, Keskinege A, Kumbasar H (1999) Dipeptidyl peptidase IV and adenosine deaminase activity. Decrease in depression. Psychoneuroendocrinology 24(8):823–832

60. Shen H, Chen GJ, Harvey BK, Bickford PC, Wang Y (2005) Inosine reduces ischemic brain injury in rats. Stroke 36(3):654–659. doi:10.1161/01.STR.0000155747.15679.04

61. Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem 273(29):18623–18632

62. Cheng P, Alberts I, Li X (2013) The role of ERK1/2 in the regulation of proliferation and differentiation of astrocytes in developing brain. Int J Dev Neurosci 31(8):783–789. doi:10.1016/j.ijdevneu.2013.09.008

63. Gourley SL, Wu FJ, Kiraly DD, Ploski JE, Kedves AT, Duman RS, Taylor JR (2008) Regionally specific regulation of ERK MAP kinase in a model of antidepressant-sensitive chronic depression. Biol Psychiatry 63(4):353–359. doi:10.1016/j.biopsych.2007.07.016

64. Liu D, Wang Z, Gao Z, Xie K, Zhang Q, Jiang H, Pang Q (2014) Effects of curcumin on learning and memory deficits, BDNF, and ERK protein expression in rats exposed to chronic unpredictable stress. Behav Brain Res 271:116–121. doi:10.1016/j.bbr.2014.05.068

65. Reus GZ, Vieira FG, Abelaira HM, Michels M, Tomaz DB, dos Santos MA, Carlessi AS, Neotti MV, Matias BI, Luz JR, Dal-Pizzol F, Quevedo J (2014) MAPK signaling correlates with the antidepressant effects of ketamine. J Psychiatr Res 55:15–21. doi:10.1016/j.jpsychires.2014.04.010

66. Duman CH, Schlesinger L, Kodama M, Russell DS, Duman RS (2007) A role for MAP kinase signaling in behavioral models of depression and antidepressant treatment. Biol Psychiatry 61(5):661–670. doi:10.1016/j.biopsych.2006.05.047

67. Celano E, Tiraboschi E, Consogno E, D'Urso G, Mbakop MP, Gennarelli M, de Bartolomeis A, Racagni G, Popoli M (2003) Selective regulation of presynaptic calcium/calmodulin-dependent protein kinase II by psychotropic drugs. Biol Psychiatry 53(5):442–449

68. Shonesy BC, Jalan-Sakrikar N, Cavener VS, Colbran RJ (2014) CaMKII: a molecular substrate for synaptic plasticity and memory. Prog Mol Biol Transl Sci 122:61–87. doi:10.1016/B978-0-12-420170-5.00003-9

69. Robison AJ (2014) Emerging role of CaMKII in neuropsychiatric disease. Trends Neurosci 37(11):653–662. doi:10.1016/j.tins.2014.07.001

70. Ethier MF, Madison JM (2006) Adenosine $A_1$ receptors mediate mobilization of calcium in human bronchial smooth muscle cells. Am J Respir Cell Mol Biol 35(4):496–502. doi:10.1165/rcmb.2005-0290OC

71. Tiraboschi E, Giambelli R, D'Urso G, Galietta A, Barbon A, de Bartolomeis A, Gennarelli M, Barlati S, Racagni G, Popoli M (2004) Antidepressants activate CaMKII in neuron cell body by Thr286 phosphorylation. Neuroreport 15(15):2393–2396

72. Du J, Szabo ST, Gray NA, Manji HK (2004) Focus on CaMKII: a molecular switch in the pathophysiology and treatment of mood and anxiety disorders. Int J Neuropsychopharmacol 7(3):243–248. doi:10.1017/S1461145704004432

73. Sanchez-Nogueiro J, Marin-Garcia P, Bustillo D, Olivos-Ore LA, Miras-Portugal MT, Artalejo AR (2014) Subcellular distribution and early signalling events of P2X7 receptors from mouse cerebellar granule neurons. Eur J Pharmacol 744:190–202. doi:10.1016/j.ejphar.2014.10.036

74. North RA, Jarvis MF (2013) P2X receptors as drug targets. Mol Pharmacol 83(4):759–769. doi:10.1124/mol.112.083758

75. Almeida RC, Souza DG, Soletti RC, Lopez MG, Rodrigues AL, Gabilan NH (2006) Involvement of PKA, MAPK/ERK and CaMKII, but not PKC in the acute antidepressant-like effect of memantine in mice. Neurosci Lett 395(2):93–97. doi:10.1016/j.neulet.2005.10.057

76. Cunha MP, Budni J, Pazini FL, Oliveira A, Rosa JM, Lopes MW, Leal RB, Rodrigues AL (2014) Involvement of PKA, PKC, CAMK-II and MEK1/2 in the acute antidepressant-like effect of creatine in mice. Pharmacol Rep 66(4):653–659. doi:10.1016/j.pharep.2014.03.004

77. Sheth S, Brito R, Mukherjea D, Rybak LP, Ramkumar V (2014) Adenosine receptors: expression, function and regulation. Int J Mol Sci 15(2):2024–2052. doi:10.3390/ijms15022024

78. Nayak GH, Prentice HM, Milton SL (2010) Neuroprotective signaling pathways are modulated by adenosine in the anoxia tolerant turtle. J Cereb Blood Flow Metab 31(2):467–475. doi:10.1038/jcbfm.2010.109

79. Gao Z, Li BS, Day YJ, Linden J (2001) $A_3$ adenosine receptor activation triggers phosphorylation of protein kinase B and protects rat basophilic leukemia 2H3 mast cells from apoptosis. Mol Pharmacol 59(1):76–82

80. Brazil DP, Hemmings BA (2001) Ten years of protein kinase B signalling: a hard Akt to follow. Trends Biochem Sci 26(11):657–664

81. Beaulieu JM, Gainetdinov RR, Caron MG (2009) Akt/GSK3 signaling in the action of psychotropic drugs. Annu Rev Pharmacol Toxicol 49:327–347. doi:10.1146/annurev.pharmtox.011008.145634

82. Rosa AO, Kaster MP, Binfare RW, Morales S, Martin-Aparicio E, Navarro-Rico ML, Martinez A, Medina M, Garcia AG, Lopez MG, Rodrigues AL (2008) Antidepressant-like effect of the novel thiadiazolidinone NP031115 in mice. Prog Neuro-Psychopharmacol Biol Psychiatry 32(6):1549–1556. doi:10.1016/j.pnpbp.2008.05.020

83. Shaywitz AJ, Greenberg ME (1999) CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu Rev Biochem 68:821–861. doi:10.1146/annurev. biochem.68.1.821

84. Li X, Jope RS (2010) Is glycogen synthase kinase-3 a central modulator in mood regulation? Neuropsychopharmacology 35(11): 2143–2154. doi:10.1038/npp.2010.105

85. Zanos P, Moaddel R, Morris PJ, Georgiou P, Fischell J, Elmer GI, Alkondon M, Yuan P, Pribut HJ, Singh NS, Dossou KS, Fang Y, Huang XP, Mayo CL, Wainer IW, Albuquerque EX, Thompson SM, Thomas CJ, Zarate CA Jr, Gould TD (2016) NMDAR inhibition-independent antidepressant actions of ketamine metabolites. Nature 533(7604):481–486. doi:10.1038/nature17998