



The antidepressant-like effect of guanosine involves the modulation of adenosine A₁ and A_{2A} receptors

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Abstract

Guanosine has been considered a promising candidate for antidepressant responses, but if this nucleoside could modulate adenosine A₁ (A₁R) and A_{2A} (A_{2A}R) receptors to exert antidepressant-like actions remains to be elucidated. This study investigated the role of A₁R and A_{2A}R in the antidepressant-like response of guanosine in the mouse tail suspension test and molecular interactions between guanosine and A₁R and A_{2A}R by docking analysis. The acute (60 min) administration of guanosine (0.05 mg/kg, p.o.) significantly decreased the immobility time in the tail suspension test, without affecting the locomotor performance in the open-field test, suggesting an antidepressant-like effect. This behavioral response was paralleled with increased A₁R and reduced A_{2A}R immunocontent in the hippocampus, but not in the prefrontal cortex, of mice. Guanosine-mediated antidepressant-like effect was not altered by the pretreatment with caffeine (3 mg/kg, i.p., a non-selective adenosine A₁R/A_{2A}R antagonist), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX — 2 mg/kg, i.p., a selective adenosine A₁R antagonist), or 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo-{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)-phenol (ZM241385 — 1 mg/kg, i.p., a selective adenosine A_{2A}R antagonist). However, the antidepressant-like response of guanosine was completely abolished by adenosine (0.5 mg/kg, i.p., a non-selective adenosine A₁R/A_{2A}R agonist), N-6-cyclohexyladenosine (CHA — 0.05 mg/kg, i.p., a selective adenosine A₁ receptor agonist), and N-6-[2-(3,5-dimethoxyphenyl)-2-(methylphenyl)ethyl]adenosine (DPMA — 0.1 mg/kg, i.p., a selective adenosine A_{2A} receptor agonist). Finally, docking analysis also indicated that guanosine might interact with A₁R and A_{2A}R at the adenosine binding site. Overall, this study reinforces the antidepressant-like effect of guanosine and unveils a previously unexplored modulation of the modulation of A₁R and A_{2A}R in its antidepressant-like effect.

Keywords A₁ receptors · A_{2A} receptors · Depression · Guanosine · Tail suspension test

Introduction

Major depressive disorder (MDD), a medical condition mainly characterized by depressed mood and anhedonia, is one of the most common and prevalent psychiatric illnesses, comprising the leading cause of disability and suicide worldwide [1]. Currently, the most prescribed antidepressant agents include monoaminergic system-targeted drugs, such as monoamine oxidase inhibitors and monoamine reuptake inhibitors [2]. Despite their widespread use, these drugs still present several challenges, particularly the treatment

non-responsiveness, a delayed onset effect, and undesirable adverse effects. Therefore, these limitations underscore a significant unmet need for novel antidepressants with distinct mechanisms from conventional medications [3, 4].

Recent studies have provided evidence that purinergic signaling may represent a promising target underlying the pathophysiology of MDD and antidepressant responses [5, 6]. Purinergic signaling is a complex and essential system that can shape a wide range of physiological functions, including synaptic transmission, neurotransmitter release, and neuromodulation [7]. In particular, adenosine is an adenine-based nucleoside that has been consistently reported to play a crucial role in the onset of MDD [8, 9] and antidepressant-like responses [10, 11]. More pertinently, the impact of adenosine on brain function is mainly dependent on the activity of adenosine inhibitory A₁ receptors (A₁R) and facilitatory A_{2A} receptors (A_{2A}R), which are widely distributed in the brain and are

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known to regulate mood [12] as well as to be implicated in antidepressant actions [6, 11, 13–15]. These findings indicate that adenosine-based purinergic signaling could represent a novel mechanism underpinning antidepressant responses.

Within this context, compelling studies have revealed surprisingly that the purine nucleoside guanosine may be a novel and promising target for antidepressant responses [16, 17]. Guanosine is an endogenous neuromodulator that has been shown to exert a plethora of activities in the central nervous system, such as neuroprotection, neuronal growth, differentiation, and survival [18–20]. Of special interest, patients with MDD have been reported to present reduced plasma levels of guanosine, suggesting that this nucleoside may be involved in the onset of MDD episodes [21, 22]. Accordingly, overwhelming evidence has demonstrated the ability of guanosine to produce antidepressant-like effects after an acute [23–27] or repeated [28–31] regimen of administration in rodents.

The key molecular targets for guanosine's antidepressant effect include (i) modulation of NMDA receptors [24], (ii) activation of alpha-amino-3-hydroxy-methyl-5-4-isoxazole propionic acid (AMPA) receptors [25], (iii) stimulation of mitogen-activated protein kinases (MAPKs)/extracellular signal-regulated kinase (ERK) pathway [26], and (iv) activation of the mechanistic target of rapamycin complex 1 (mTORC1) signaling pathway, brain-derived neurotrophic factor (BDNF) availability, and synaptic protein translation in the hippocampus and prefrontal cortex [16, 25]. Additionally, the antidepressant-like response elicited by guanosine was also associated with increased hippocampal neuronal differentiation [29] and the suppression of oxidative stress- and neuroinflammation-related pathways [26, 28].

Interestingly, recent studies have demonstrated that guanosine is effective in affording neuroprotection by modulating A_1R and $A_{2A}R$ [32–34]. However, the involvement of A_1R and $A_{2A}R$ in the behavioral responses displayed by guanosine is still controversial and remains to be fully understood [35–37]. More importantly, if guanosine could modulate A_1R and $A_{2A}R$ to exert antidepressant-like actions remains to be elucidated. Given this background, the present study aimed to investigate the role of A_1R and $A_{2A}R$ in the antidepressant-like response displayed by guanosine in the mouse tail suspension test. Additionally, we also sought to predict the binding affinity between guanosine and A_1R and $A_{2A}R$ using the molecular docking analysis.

Material and methods

Animals

Considering that the prevalence of depression is higher in women than in men [1], this study was performed using

192 adult female Swiss mice. The animals (30–40 g, 45–60 days of age) were maintained under controlled temperature (21 ± 1 °C) and humidity ($50 \pm 20\%$) with a 12-h:12-h light/dark cycle (lights on at 7:00 a.m.). Mice were housed in groups of 8 in a cage ($41 \times 34 \times 16$ cm) with free access to food and water. The protocols were approved by the Institutional Ethics Committee (numbers 00795 and 7,485,180,518) and carried out in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Pharmacological treatments and experimental design

The first experimental approach was designed to validate the antidepressant-like effect of guanosine in the tail suspension test, as previously demonstrated [24, 25], as well as to investigate if this behavioral response could be paralleled with alterations in the immunocontent of A_1R and $A_{2A}R$ in the hippocampus and prefrontal cortex. To address this experiment, mice ($n = 8/\text{group}$) received a single administration of vehicle or guanosine (0.05 mg/kg, p.o.), and 60 min after the treatments, they were subjected to the tail suspension test and open-field test (10 min apart). Guanosine, obtained from Sigma Chemical Co. (St. Louis, USA) and dissolved in distilled water, was freshly prepared and administered orally (p.o.) in a volume of 10 ml/kg body weight. All doses and time points of administration were chosen based on previous studies [24, 25]. After the behavioral tests, mice were immediately euthanized by decapitation and the hippocampus and prefrontal cortex were dissected to measure the immunocontent of adenosine A_1R and $A_{2A}R$ by Western Blotting.

The second experimental approach was designed to evaluate if the antidepressant-like effect of guanosine is mediated by adenosine A_1R and $A_{2A}R$. In a first set of experiments to address this second hypothesis, mice were pretreated with vehicle, caffeine (3 mg/kg, i.p., a non-selective adenosine $A_1R/A_{2A}R$ antagonist), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX – 2 mg/kg, i.p., a selective adenosine A_1R antagonist), or 4-(2-[7-amino-2-{2-furyl}] {1,2,4} triazolo- {2,3-a} {1,3,5} triazin-5-yl-amino]ethyl)-phenol (ZM241385 – 1 mg/kg, i.p., a selective adenosine $A_{2A}R$ antagonist). After 30 min, mice received the administration of vehicle or guanosine (0.05 mg/kg, p.o.), and then, they were subjected to the tail suspension test and open-field test (10 min apart) 60 min later. All doses and time points of administration were chosen based on previous studies [10, 13].

In another set of experiments, mice were treated with vehicle or guanosine (0.05 mg/kg, p.o.), and following 30 min, they received the administration of adenosine (0.5 mg/kg, i.p., a non-selective adenosine $A_1R/A_{2A}R$

agonist), N-6-cyclohexyladenosine (CHA — 0.05 mg/kg, i.p., a selective adenosine A₁ receptor agonist), or N-6-[2-(3,5-dimethoxyphenyl)-2-(methylphenyl)ethyl]adenosine (DPMA — 0.1 mg/kg, i.p., a selective adenosine A_{2A} receptor agonist). After 30 min, mice were subjected to the tail suspension test and open-field test (10 min apart). All doses and time points of administration were chosen based on previous studies [10, 13].

A schedule of experimental approaches, pharmacological treatments, behavioral tests, and Western blotting analysis is provided in Fig. 1.

Tail suspension test

The total immobility time of mice suspended by the tail was measured as previously proposed [38]. Briefly, visually isolated mice were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time was recorded for a 6-min period by an experienced observer blind to the experimental groups. Mice

were considered immobile only when they hung passively and completely motionless. The immobility time in the tail suspension test was taken as indicative of antidepressant-like responses [39].

Open-field test

The locomotor activity of mice was assessed in the open field apparatus, which consists of a wooden box (40 × 60 cm × 50 cm) with the floor divided into 12 equal squares [40]. At the start of each trial, mice were placed in the left corner of the field and allowed to freely explore the arena. The number of crossings (squares crossed with all paws) was registered for 6 min [41, 42]. The arena floor was cleaned with 10% ethanol between tests.

Western blotting

The hippocampus and prefrontal cortex were quickly dissected and snap-frozen with liquid nitrogen before

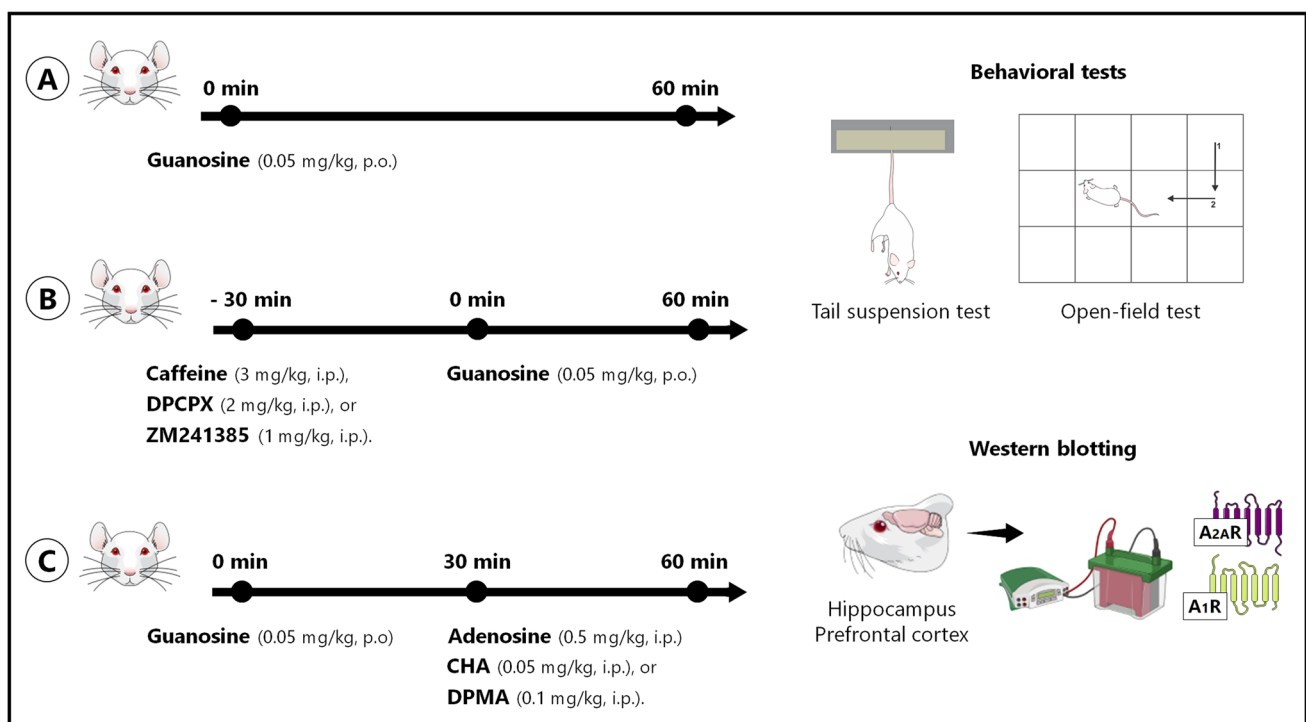


Fig. 1 Schedule of experimental approaches, pharmacological treatments, behavioral tests, and Western blotting analysis. In the first experimental approach, female Swiss mice were administered orally (p.o.) with vehicle or guanosine (0.05 mg/kg, p.o.), and 60 min after the treatments, they were subjected to the tail suspension test and open-field test (10 min apart). After the behavioral tests, mice were immediately euthanized by decapitation and the hippocampus and prefrontal cortex were dissected to measure the immunoccontent of adenosine A₁R and A_{2A}R by Western blotting (A). In the second experimental approach, mice were pretreated with vehicle, caffeine

(3 mg/kg, i.p.), DPCPX (2 mg/kg, i.p.), or ZM241385 (1 mg/kg, i.p.) and 30 min after the treatments, they received the administration of vehicle or guanosine (0.05 mg/kg, p.o.). Following 60 min of the treatments, mice were subjected to the tail suspension test and open-field test (B). In the third experimental approach, mice were treated with vehicle or guanosine (0.05 mg/kg, p.o.), and following 30 min, they received the administration of adenosine (0.5 mg/kg, i.p.), CHA (0.05 mg/kg, i.p.), or DPMA (0.1 mg/kg, i.p.). After 30 min of the treatments, mice were subjected to the tail suspension test and open-field test (C). Figure designed using images from Mind the Graph

storage at $-80\text{ }^{\circ}\text{C}$ until use [43, 44]. Briefly, samples were mechanically homogenized in 50 mM TRIS pH 7.0, 1 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaF, 0.1 mM phenylmethylsulphonyl fluoride, 2 mM Na_3VO_4 , 1% Triton X-100, 10% glycerol, and Sigma Protease Inhibitor Cocktail (P2714). Lysates were centrifuged (10,000 g for 10 min, at $4\text{ }^{\circ}\text{C}$) to eliminate cellular debris. The supernatants were diluted 1/1 (v/v) in 100 mM TRIS pH 6.8, 4 mM EDTA, 8% SDS, and boiled for 5 min. Thereafter, sample dilution (40% glycerol, 100 mM TRIS, bromophenol blue, pH 6.8) in the ratio 25:100 (v/v) and β -mercaptoethanol (final concentration 8%) were added to the samples. Protein content was quantified using bovine serum albumin (BSA) as a standard [45]. The samples containing 60 μg protein/track were separated by SDS-PAGE (miniVE Vertical Electrophoresis System TM, GE Healthcare Life Sciences, Piscataway, NJ, USA) using 7–10% gel, and the proteins were transferred to nitrocellulose membranes using a semi-dry blotting apparatus (1.2 mA/cm²; 1.5 h). To verify the transfer efficiency, membranes were stained with Ponceau [46], and subsequently blocked with 5% BSA in TBS (10 mM Tris, 150 mM NaCl, pH 7.5). The immunocontent of adenosine A₁R (Santa Cruz Biotechnology®, CA, USA — 1:500 dilution) and A_{2A}R (Santa Cruz Biotechnology®, CA, USA — 1:500 dilution), as well as β -actin (loading control, Cell Signaling Technology — 1:1000 dilution), were detected using antibodies diluted in TBS-T (10 mM Tris, 150 mM NaCl, 0.1% Tween-10, pH 7.5) containing 2.5% BSA and incubated overnight. Subsequently, the membranes were incubated with anti-rabbit antibody horseradish peroxidase-conjugated secondary antibody (Cell Signaling, 1:2500) for 60 min, and the immunoreactive bands were developed using a chemiluminescence kit (Amersham ECL Select, Piscataway, USA). All blocking and incubation steps were followed by three washes (5 min) of the membranes with TBS-T [47–49]. The optical density (OD) of the bands was quantified using the Image Lab Software® 4.1 (Bio-Rad Laboratories). The immunocontent of A₁R and A_{2A}R was determined as the ratio of the respective protein band over the OD of the β -actin band.

Molecular docking

The molecular docking analysis was carried out to predict the binding affinity between guanosine and A₁R and A_{2A}R. The coupling energy of the guanosine at the adenosine (A₁R/A_{2A}R non-selective agonist) binding site to A₁R and A_{2A}R (crystallographic structure) was evaluated using the DockThor 2.0 software [50]. To compare the docking properties of guanosine, we also estimated the docking energies of adenosine to A₁R and to A_{2A}R. The protein

database (RSCB — Protein Data Bank) was used to obtain the structures of A₁R (PDB 6D9H code) and A_{2A}R (PDB 2YDO code). Moreover, the hydrogen-bond interaction patterns and hydrophobic contacts between guanosine and A₁R or A_{2A}R were performed using LigPlot + 2.2.5 graphical interface [51]. All binders underwent geometric optimization in the Chimera 1.14 software. The preparation of A₁R and A_{2A}R included the removal of water molecules, the addition of hydrogens, and the assignment of Gasteiger charges. Additionally, the poses of ligands were also observed using the Chimera 1.14.

Statistical analysis

The D'Agostino-Pearson test was used to assess data normality. Differences among experimental groups were determined by Student's *t*-test or two-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test, when appropriate. Values of $P < 0.05$ were considered significant. The results are expressed as means \pm standard error of the mean (S.E.M.).

Results

The antidepressant-like effect of guanosine is paralleled with alterations on hippocampal and prefrontocortical adenosine A₁R and A_{2A}R immunocontent

In the first experimental approach, we aimed to validate the antidepressant-like effect of guanosine in mice subjected to the tail suspension test (Fig. 2) and open-field test (Fig. 2). Statistical analysis showed that the acute administration of guanosine (0.05 mg/kg, p.o.) significantly reduced the immobility time when compared to the vehicle-treated group ($t_{(14)} = 3.52$, $P < 0.01$), suggesting an antidepressant-like effect. No significant alterations were observed in the number of crossings ($t_{(14)} = 0.26$, $P = 0.79$) in the open-field test. Fluoxetine, the positive control, also reduced the immobility time in the tail suspension test without altering the number of crossings in the open-field test (supplementary information).

In the next step, we investigated if the antidepressant-like effect exerted by guanosine could be associated with alterations in the immunocontent of A₁R (Fig. 3) and A_{2A}R (Fig. 4) in the hippocampus and prefrontal cortex. Statistical analysis showed that the acute administration of guanosine (0.05 mg/kg, p.o.) significantly augmented the immunocontent of A₁R in the hippocampus (Fig. 3 and C; $t_{(12)} = 2.41$, $P < 0.05$), but not in the prefrontal cortex (Fig. 3 and B; $t_{(12)} = 0.12$, $P = 0.89$). Additionally, a single administration of guanosine (0.05 mg/kg, p.o.)

Fig. 2 Guanosine (0.05 mg/kg, p.o.) exerts an antidepressant-like effect in mice subjected to the tail suspension test (A) and open-field test. Values are expressed as means \pm S.E.M. ($n=8$). $**P<0.01$ as compared with the vehicle-treated group (Student's *t*-test)

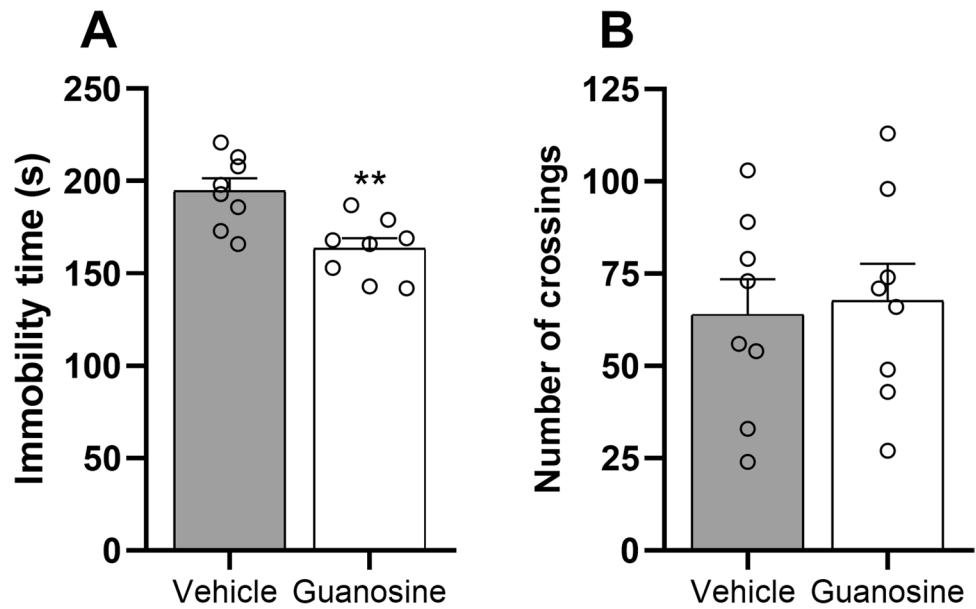
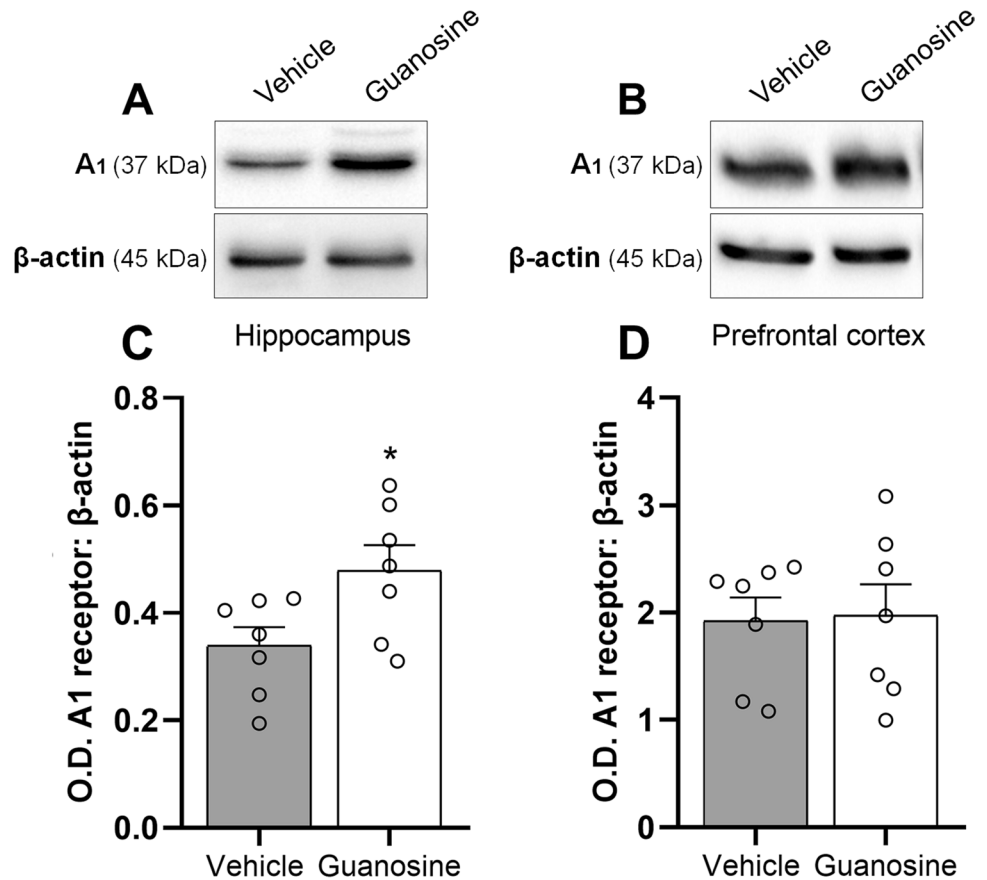


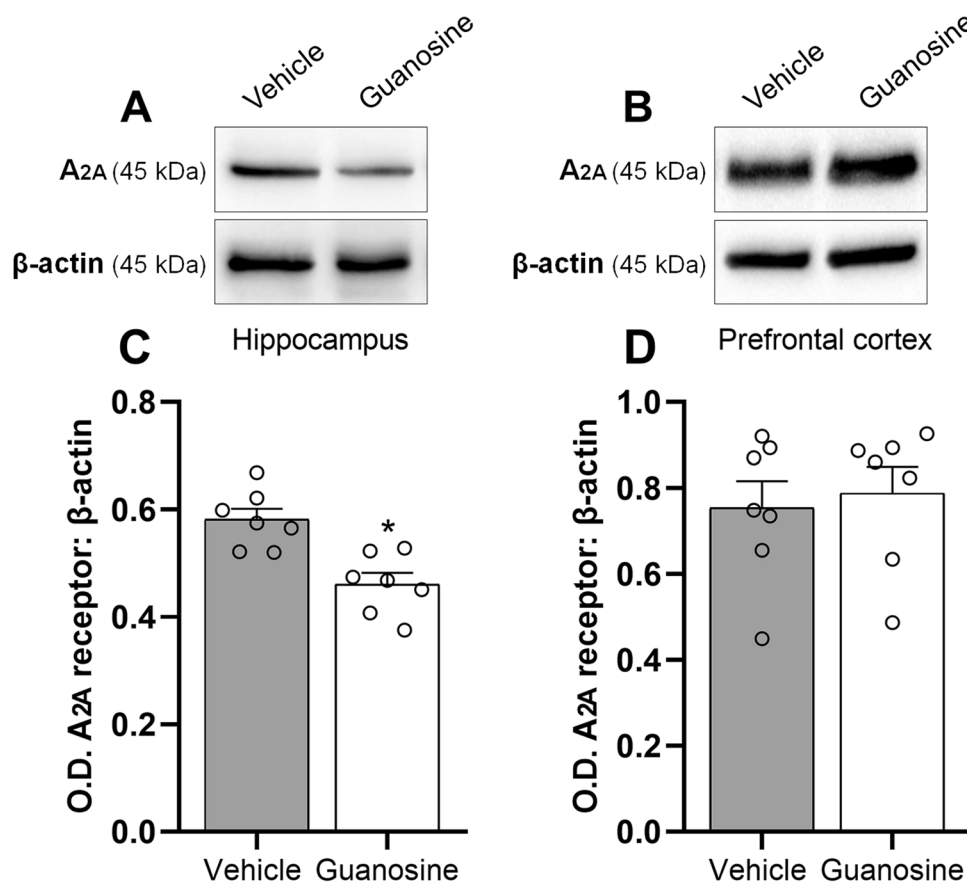
Fig. 3 Guanosine selectively increases A_1R immunocontent in the hippocampus of mice. Representative images of A_1R/β -actin and corresponding quantification in the hippocampus (A and C) and prefrontal cortex (B and D). Values are expressed as means (percentage of control) \pm S.E.M. ($n=7$). $*P<0.05$ as compared with the vehicle-treated group (Student's *t*-test)



significantly reduced the immunocontent of adenosine $A_{2A}R$ in the hippocampus (Fig. 4 and C; $t_{(12)}=4.10$, $P<0.01$), but not in the prefrontal cortex (Fig. 4 and 4; $t_{(12)}=0.38$, $P=0.70$). These results suggest that the

antidepressant-like response of guanosine may be paralleled with alterations in the immunocontent of adenosine receptors, particularly an increment of A_1R and a reduction of $A_{2A}R$ specifically in the hippocampus.

Fig. 4 Guanosine selectively reduces $A_{2A}R$ immunocontent in the hippocampus of mice. Representative images of $A_{2A}R/\beta$ -actin and corresponding quantification in the hippocampus (A and C) and prefrontal cortex (B and D). Values are expressed as means (percentage of control) \pm S.E.M. ($n=7$). * $P < 0.05$ as compared with the vehicle-treated group (Student's t -test)



The antidepressant-like effect of guanosine is associated with the modulation of A_1R and $A_{2A}R$

To reinforce the hypothesis that the antidepressant-like effect of guanosine may involve the modulation of A_1R and $A_{2A}R$, mice were pretreated with caffeine (3 mg/kg, i.p., a non-selective $A_1R/A_{2A}R$ antagonist), DPCPX (2 mg/kg, i.p., a selective A_1R antagonist), or ZM241385 (1 mg/kg, i.p., a selective $A_{2A}R$ antagonist). The results illustrated in Fig. 5 show the effect of the pretreatment with caffeine (Fig. 5 and 5), DPCPX (Fig. 5 and 5), or ZM241385 (Fig. 5 and 5) in the antidepressant-like effect of guanosine in the tail suspension test and open-field test. Two-way ANOVA revealed a significant main effect of guanosine [$F_{(1, 28)} = 17.95$, $P < 0.01$], with guanosine treated-mice presenting a significant reduction in the immobility time ($P < 0.01$) regardless of the administration with vehicle or caffeine. However, two-way ANOVA revealed no significant differences for caffeine [$F_{(1, 28)} = 2.23$, $P = 0.14$], and guanosine \times caffeine interaction [$F_{(1, 28)} = 0.13$, $P = 0.72$] in the immobility time (Fig. 5). No alterations were detected in the number of crossings (Fig. 5) in the open-field test (guanosine [$F_{(1, 28)} = 0.06$, $P = 0.76$], caffeine [$F_{(1, 28)} = 0.17$, $P = 0.67$], and guanosine \times caffeine interaction [$F_{(1, 28)} = 0.22$, $P = 0.63$]. Furthermore, two-way

ANOVA revealed a significant main effect of guanosine [$F_{(1, 28)} = 13.44$, $P < 0.01$], with guanosine treated-mice presenting a significant reduction in the immobility time ($P < 0.01$) regardless of the administration with vehicle or DPCPX. However, two-way ANOVA revealed no significant differences for DPCPX [$F_{(1, 28)} = 0.18$, $P = 0.67$], and guanosine \times DPCPX interaction [$F_{(1, 28)} = 0.25$, $P = 0.61$] in the immobility time (Fig. 5). No alterations were detected in the number of crossings (Fig. 5) in the open-field test (guanosine [$F_{(1, 28)} = 0.10$, $P = 0.74$], DPCPX [$F_{(1, 28)} = 0.03$, $P = 0.85$], and guanosine \times DPCPX interaction [$F_{(1, 28)} = 0.01$, $P = 0.89$]. Likewise, two-way ANOVA revealed a significant main effect of guanosine [$F_{(1, 28)} = 36.60$, $P < 0.01$], with guanosine treated-mice presenting a significant reduction in the immobility time ($P < 0.01$) regardless of the administration with vehicle or ZM241385. However, two-way ANOVA revealed no significant differences for ZM241385 [$F_{(1, 28)} = 0.03$, $P = 0.85$], and guanosine \times ZM241385 interaction [$F_{(1, 28)} = 0.08$, $P = 0.77$] in the immobility time (Fig. 5). No alterations were detected in the number of crossings (Fig. 5) in the open-field test (guanosine [$F_{(1, 28)} = 0.04$, $P = 0.83$], ZM241385 [$F_{(1, 28)} = 0.09$, $P = 0.75$], and guanosine \times ZM241385 interaction [$F_{(1, 28)} = 0.08$, $P = 0.77$]. These results suggest that the acute antidepressant-like effect displayed by guanosine

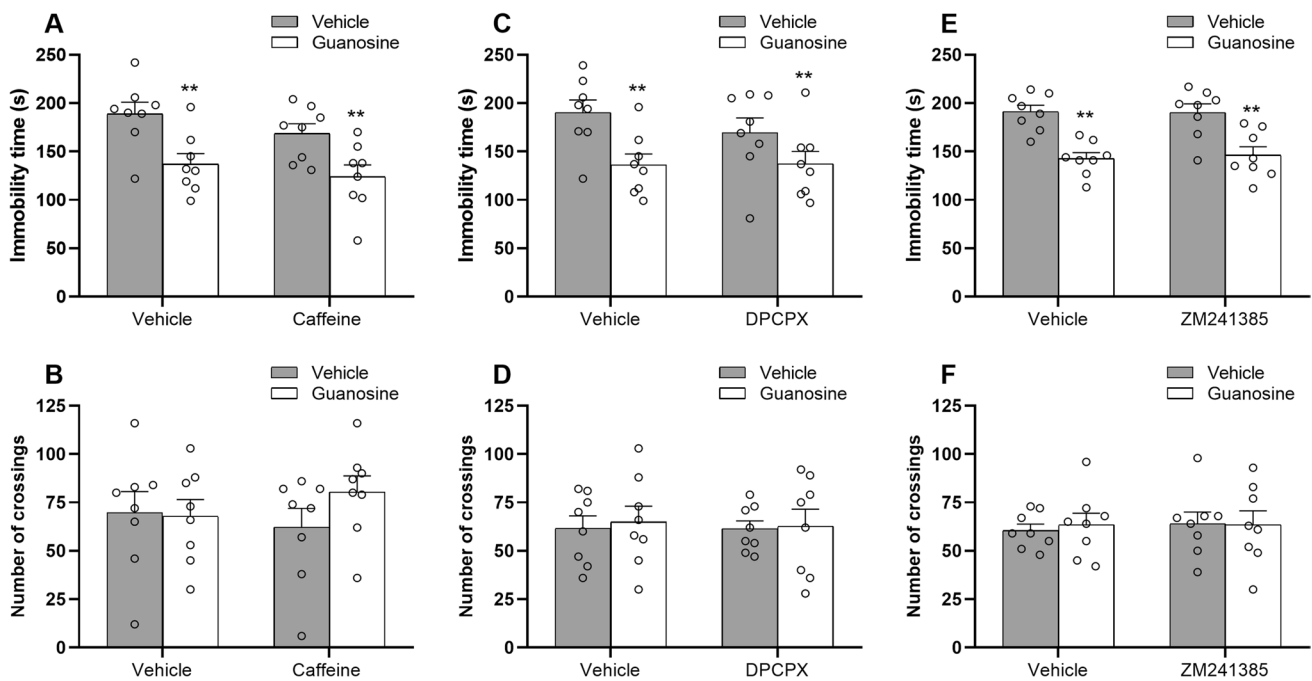


Fig. 5 The effects of caffeine (3 mg/kg, i.p., **A** and **B**), DPCPX (2 mg/kg, i.p., **C** and **D**), or ZM241385 (1 mg/kg, i.p., **E** and **F**) in the antidepressant-like effect of guanosine in mice subjected to the tail suspension test and open-field test. Values are expressed as

means \pm S.E.M. ($n=8$) followed by two-way ANOVA. * $P<0.05$ and ** $P<0.01$ as compared with the vehicle-treated group (i.e., significant main effect of guanosine)

in the tail suspension test may not involve, at least partially, the activation of adenosine A_1R and $A_{2A}R$.

Subsequently, we assessed the influence of the administration of adenosine (0.5 mg/kg, i.p., a non-selective $A_1R/A_{2A}R$ agonist), CHA (0.05 mg/kg, i.p., a selective adenosine A_1 receptor agonist), or DPMA (0.1 mg/kg, i.p., a selective adenosine A_{2A} receptor agonist) in the antidepressant-like responses elicited by guanosine. The results illustrated in Fig. 6 show the effect of the pretreatment with adenosine (Fig. 6 and 6), CHA (Fig. 6 and 6), or DPMA (Fig. 6 and 6) in the antidepressant-like effect of guanosine in the tail suspension test and open-field test. Two-way ANOVA revealed significant differences for guanosine [$F_{(1, 28)}=9.85$, $P<0.01$], adenosine [$F_{(1, 28)}=8.88$, $P<0.01$], and guanosine \times adenosine interaction [$F_{(1, 28)}=13.67$, $P<0.01$] in the immobility time (Fig. 6). Post hoc analysis indicated that guanosine administration significantly reduced the immobility time ($P<0.01$), but this anti-immobility effect was significantly abolished by adenosine ($P<0.01$). No alterations were detected in the number of crossings (Fig. 6) in the open-field test (guanosine [$F_{(1, 28)}=0.02$, $P=0.93$], adenosine [$F_{(1, 28)}=0.10$, $P=0.75$], and guanosine \times adenosine interaction [$F_{(1, 28)}=0.01$, $P=0.97$]. Furthermore, two-way ANOVA revealed significant differences for guanosine [$F_{(1, 28)}=18.27$, $P<0.01$], CHA [$F_{(1, 28)}=14.98$, $P<0.01$], and guanosine \times CHA interaction [$F_{(1, 28)}=16.28$, $P<0.01$] in the immobility time (Fig. 6). Post hoc analysis indicated

that the anti-immobility effect of guanosine in the immobility time ($P<0.01$) was significantly occluded by CHA administration ($P<0.01$). No alterations were observed in the number of crossings (Fig. 6) in the open-field test (guanosine [$F_{(1, 28)}=0.10$, $P=0.74$], CHA [$F_{(1, 28)}=0.01$, $P=0.95$], and guanosine \times CHA interaction [$F_{(1, 28)}=0.14$, $P=0.70$]. Likewise, two-way ANOVA revealed significant main effects for guanosine [$F_{(1, 28)}=17.83$, $P<0.01$], DPMA [$F_{(1, 28)}=12.94$, $P<0.01$], and guanosine \times DPMA interaction [$F_{(1, 28)}=19.73$, $P<0.01$] in the immobility time (Fig. 6). Post hoc analysis indicated that the ability of guanosine in reducing the immobility time ($P<0.01$) was significantly counteracted by DPMA administration ($P<0.01$). No alterations were detected in the open-field test (Fig. 6) in the open-field test (guanosine [$F_{(1, 28)}=0.01$, $P=0.98$], DPMA [$F_{(1, 28)}=0.28$, $P=0.59$], and guanosine \times DPMA interaction [$F_{(1, 28)}=0.14$, $P=0.71$]. These results suggest that the antidepressant-like effect exerted by guanosine in the tail suspension test may involve the modulation of adenosine $A_1R/A_{2A}R$.

Guanosine might interact with A_1R and $A_{2A}R$ at the adenosine binding site

In the first in silico approach, we aimed to predict the binding affinity between guanosine and A_1R as well as the guanosine- A_1R interactions (Fig. 7 and 7). The prediction

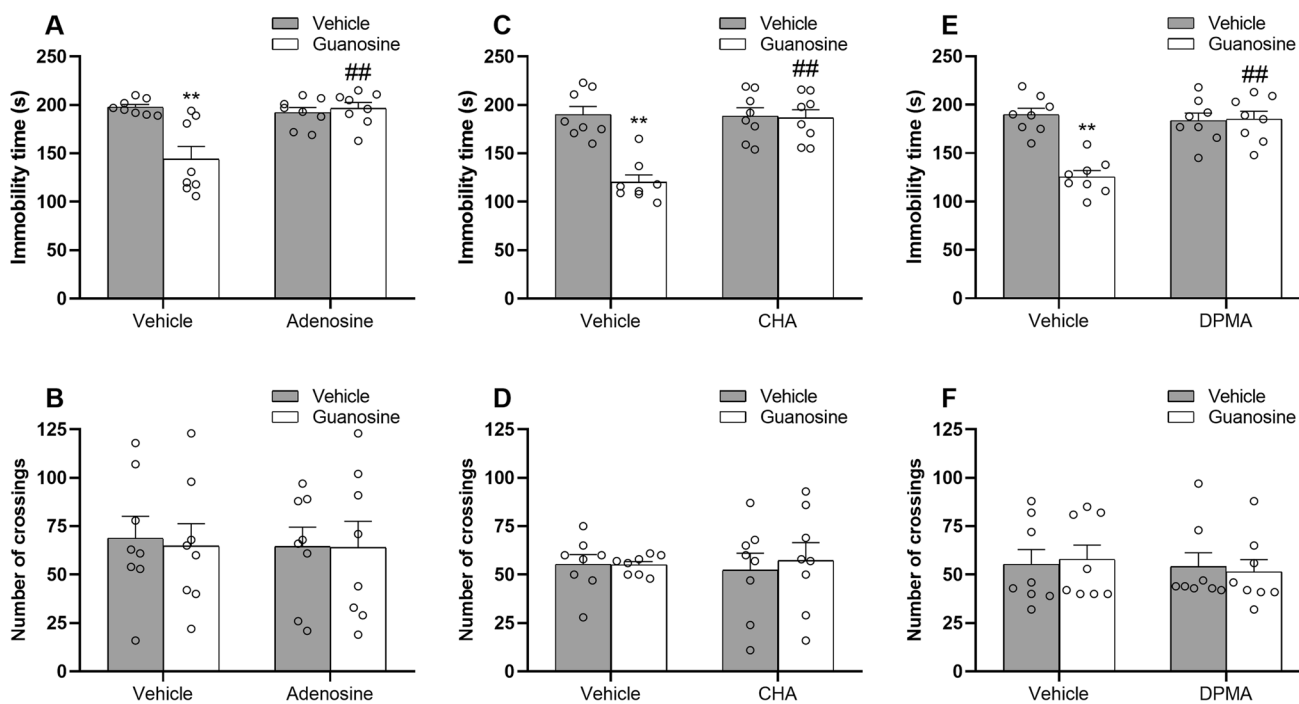


Fig. 6 The effects of adenosine (0.5 mg/kg, i.p., **A** and **B**), CHA (0.05 mg/kg, i.p., **C** and **D**), or DPMA (0.1 mg/kg, i.p., **E** and **F**) in the antidepressant-like effect of guanosine in mice subjected to the tail suspension test and open-field test. Values are expressed as

means \pm S.E.M. ($n=8$). ** $P < 0.01$ compared with the vehicle-treated group; ### $P < 0.01$ compared with the guanosine-treated group (two-way ANOVA followed by Newman-Keuls post hoc test)

of the guanosine binding affinity to A_1R was -8140 kcal/mol, according to the DockThor 2.0 software. Moreover, LigPlot+ analysis revealed 5 hydrogen-bond interactions (amino acid residues Asn254, Glu172, Glu170, and Ala66) (Fig. 7) and 28 hydrophobic contacts (amino acid residues Asn254, Glu172, Glu170, Ala66, Met180, Leu250, Phe171, Ile274, and Ile69) (Fig. 7) between guanosine and A_1R . In the second in silico approach, we aimed to predict the binding affinity between guanosine and $A_{2A}R$ as well as the guanosine– $A_{2A}R$ interactions (Fig. 7 and 7). The coupling energy obtained between guanosine and $A_{2A}R$ was $-8,069$ kcal/mol. Additionally, guanosine– $A_{2A}R$ interactions using the LigPlot+ software revealed 5 hydrogen-bond interactions (amino acid residues Asn253, Glu169, Phe168, and Ile66) (Fig. 7) and 39 hydrophobic contacts (amino acid residues Phe168, Ile66, Glu169, Leu249, Ile274, Leu167, and Met270) (Fig. 7). This prediction of $A_1R/A_{2A}R$ –guanosine binding data suggests that guanosine might interact with A_1R and $A_{2A}R$ at the adenosine binding site. Additionally, the prediction of the adenosine binding affinity to A_1R was -8291 kcal/mol while to $A_{2A}R$ was -7998 kcal/mol. Interestingly, most of the hydrogen-bond interactions and hydrophobic contacts observed herein with $A_1R/A_{2A}R$ –guanosine binding prediction were shown to be shared between adenosine and A_1R (hydrogen-bond interactions at amino acid residues Asn254

and Glu172; hydrophobic contacts at amino acid residues Asn254, Glu172, Met180, Leu250, Phe171, and Ile274) or $A_{2A}R$ (hydrogen-bond interactions at amino acid residues Asn253 and Glu169; hydrophobic contacts at amino acid residues Phe168, Ile66, Glu169, Leu249, Ile274, Leu167, and Met270).

Discussion

Several lines of evidence have demonstrated that disturbances in plasma levels of guanosine, an endogenous guanine-based purine, are associated with depressive symptoms in clinical studies [21, 22] as well as the ability of this nucleoside to produce antidepressant-like actions in preclinical reports [24–27]. In the present research, we corroborated these findings by showing that acute administration of guanosine (0.05 mg/kg, p.o.) significantly decreased the immobility time in the tail suspension test compared to vehicle-treated mice without altering the number of crossings in the open-field test. These results indicate that the anti-immobility effect displayed by this nucleoside in the tail suspension test was not affected by locomotor activity, as previously demonstrated [24–27]. Moreover, a study by Lara et al. [52] that administered guanosine at higher doses (2 and 7.5 mg/kg) to male Swiss mice reported no alteration

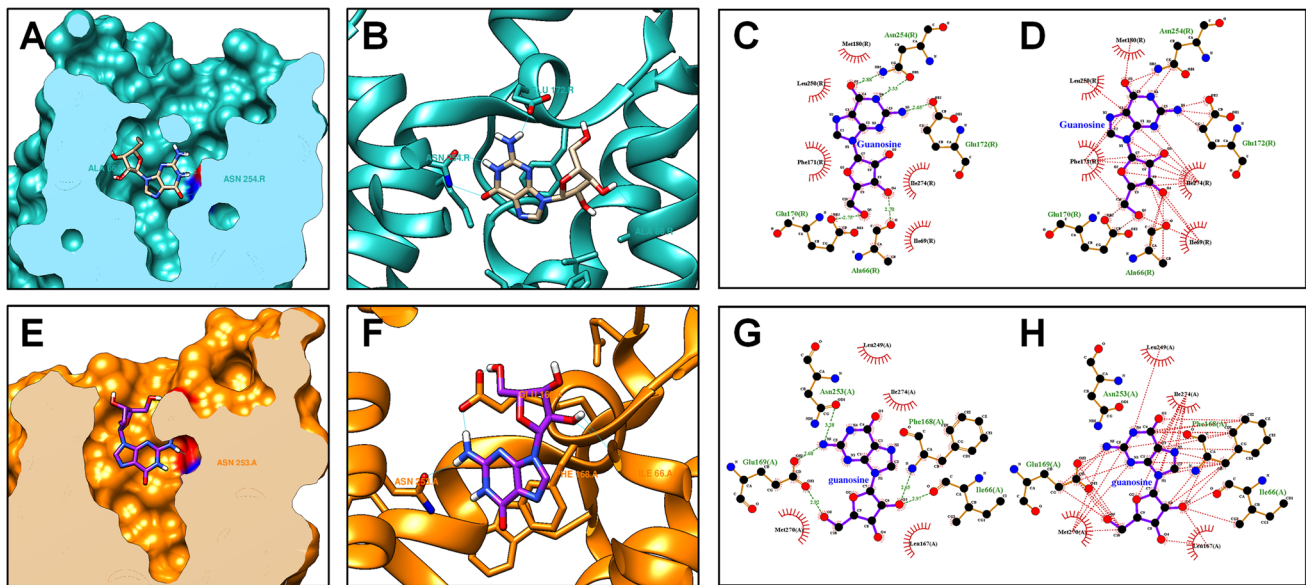


Fig. 7 Molecular interactions between guanosine and A_1R or $A_{2A}R$. The putative binding site of guanosine to A_1R (A). Structure of the A_1R complexed with guanosine (B). Representative pose of guanosine complexed with A_1R , in which guanosine– $A_{2A}R$ hydrogen bond are represented as green dashed lines (C). Representative pose of guanosine complexed with A_1R , in which guanosine– A_1R hydrophobic contacts are represented as red dashed lines (D). The putative

binding site of guanosine to $A_{2A}R$ (E). Structure of the $A_{2A}R$ complexed with guanosine (F). Representative pose of guanosine complexed with $A_{2A}R$, in which guanosine– $A_{2A}R$ hydrogen bonds are represented as green dashed lines (G). Representative pose of guanosine complexed with $A_{2A}R$, in which guanosine– A_1R hydrophobic contacts are represented as red dashed lines (H)

in the rotarod performance in guanosine-treated mice. Also, in agreement, a study by Schmidt et al. [53] showed that guanosine administered by i.t. route did not alter sensorimotor coordination of male Swiss mice in the rotarod and caused no significant alteration in episodes of rearing, crossing, grooming, and fecal boluses. Although the reduction of immobility time caused by drugs in the tail suspension test is not a definitive conclusion that this drug has an antidepressant effect, it is interesting to mention that guanosine has been consistently shown to elicit antidepressant-like responses in naïve mice and rodents subjected to models that induce depressive-like behavior [16, 17, 23–26, 54].

To further unravel the molecular basis associated with the antidepressant-like effect of guanosine, we next assessed the role of the modulation of A_1R and $A_{2A}R$ levels in the hippocampus and prefrontal cortex, two brain regions afflicted in MDD [55], in mice that exhibited a behavioral response to guanosine in the tail suspension test. Compelling studies have demonstrated that adenosine inhibitory A_1R and facilitatory $A_{2A}R$ may underlie the onset of depressive symptoms and antidepressant responses [12, 56]. Moreover, the overexpression of $A_{2A}R$ in forebrain neurons is paralleled with depression-like behavior [57] and chronic unpredictable stress increases $A_{2A}R$ immunocontent in the hippocampus [11]. Conversely, A_1R overexpression in forebrain neurons is associated with antidepressant-like effects and A_1R knockout mice displayed depressive-like phenotypes [58].

Furthermore, $A_{2A}R$ antagonists have been shown to evoke antidepressant-like effects in rodents subjected to the forced swim test and tail suspension test [59, 60], while increased A_1R function elicits antidepressant-like responses in these same behavioral paradigms [58, 61]. Here, to the best of our knowledge, we showed for the first time that acute administration of guanosine significantly increased A_1R and reduced $A_{2A}R$ immunocontent in the hippocampus, but not in the prefrontal cortex, of mice. Therefore, one may speculate that the ability of guanosine in inducing a hippocampal region-specific modulation of A_1R and $A_{2A}R$ immunocontent could contribute to the antidepressant-like effect of this nucleoside in the tail suspension test, although further studies are necessary to ascertain this hypothesis.

To obtain deeper insights into the role of A_1R and $A_{2A}R$ in the antidepressant-like effect displayed by guanosine, we next investigated the impact of antagonists and agonists of A_1R and $A_{2A}R$ in the guanosine-mediated anti-immobility effect in the tail suspension test. Our results unveiled that the ability of guanosine in reducing the immobility time in this test was not altered by the pretreatment with a non-selective $A_1R/A_{2A}R$ antagonist, caffeine, or A_1R – $A_{2A}R$ -related selective antagonists, DPCPX and ZM241385, respectively. Interestingly, in another set of experiments, we observed that the anti-immobility response exerted by guanosine in mice that underwent the tail suspension test was completely occluded by adenosine (a non-selective $A_1R/A_{2A}R$ agonist) as well

as CHA (a selective adenosine A_1R agonist) and DPMA (a selective adenosine $A_{2A}R$ agonist). Importantly, these agonists were effective in blocking the guanosine-mediated antidepressant-like responses at doses that did not alter the immobility time in the tail suspension test *per se*, as previously demonstrated [10, 13]. At the same time, neither guanosine nor $A_1R/A_{2A}R$ agonists and antagonists, at given doses, modified basal locomotor activity. Considering that the behavioral response elicited by guanosine in the tail suspension test was blocked by A_1R and $A_{2A}R$ agonists, but not by A_1R and $A_{2A}R$ antagonists, these results suggest that a modulation of adenosine receptors may be related to the ability of guanosine to reduce the immobility time in the tail suspension test [37]. In line with our results, a recent study demonstrated that the ability of guanosine in producing anxiolytic-related responses involves the modulation of A_1R and $A_{2A}R$ [37]. Importantly, the authors have shown that the anxiolytic-like effect induced by guanosine in rats subjected to the elevated plus-maze test was blocked by $A_1R/A_{2A}R$ non-selective agonist (adenosine) and A_1R and $A_{2A}R$ selective agonists (CPA, CCPA, and CGS21680, respectively), but not by A_1R and $A_{2A}R$ antagonists (caffeine, DPCPX, and ZM241385), which resembles our findings. One possibility that has been raised to explain these results is that a functional competition between guanosine and adenosine or A_1R - $A_{2A}R$ selective agonists [37] may occur, or even an A_1 - A_{2A} receptor-receptor interaction, which have been shown to contribute to the guanosine-mediated responses [62]. Interestingly, a study by Kaster et al. [10] showed that both the intraperitoneal administration of adenosine and the intracerebroventricular (i.c.v.) administration of this nucleoside reduced the immobility time in the forced swimming test in mice, suggesting a centrally mediated modulation of adenosine receptors in the antidepressant-like responses in mice subjected to the forced swimming test following i.c.v. adenosine administration.

Providing additional support for our results, mounting evidence has indicated a functional crosstalk between guanosine and A_1R and $A_{2A}R$ agonists or antagonists [32–37]. Guanosine's ability to prevent oxygen/glucose deprivation-induced reduced hippocampal slices viability and glutamate uptake was abolished by CGS21680 ($A_{2A}R$ selective agonist), but not by ZM241385 ($A_{2A}R$ selective antagonist). The pretreatment of hippocampal slices with A_1R antagonist, DPCPX, occluded the effects of guanosine against oxygen/glucose deprivation-induced reduced slices viability but did not affect glutamate uptake [32]. Moreover, guanosine has also been shown to prevent reactive oxygen species production in hippocampal slices subjected to oxygen/glucose deprivation by modulating A_1R and $A_{2A}R$, as these responses were effectively abrogated by A_1R antagonist, DPCPX, and $A_{2A}R$ agonist, CGS21680 [33]. Guanosine-mediated anti-tremor and antioxidant effects in reserpinized mice

were shown to be A_1R -dependent and $A_{2A}R$ -independent, as these effects were abolished by A_1R antagonist DPCPX, but still present in $A_{2A}R$ -knockout mice [35]. Furthermore, guanosine-induced protective effects against oxidative burst and mitochondrial dysfunction induced by 6-OHDA in striatal slices were precluded by A_1R antagonist DPCPX and $A_{2A}R$ agonist CGS21680, but not by A_1R agonist CCPA [34]. Additionally, the neuroprotective effect displayed by guanosine against traumatic brain injury in rats was abrogated by A_1R antagonist DPCPX, but unaltered by the $A_{2A}R$ antagonist SCH58261 [63].

Our *in silico* results also uncovered that guanosine might interact with A_1R and $A_{2A}R$ at the adenosine binding site, reinforcing that A_1R and $A_{2A}R$ as likely targets for guanosine-mediated antidepressant-like responses. Of note, docking computational prediction of adenosine receptors–guanosine coupling affinities revealed a strong binding affinity between guanosine and A_1R or $A_{2A}R$, a response comparable to the binding affinity of adenosine to A_1R or $A_{2A}R$. Further analysis revealed critical hydrogen-bond interactions and hydrophobic contacts between guanosine and A_1R or $A_{2A}R$. Interestingly, most of the hydrogen-bond interactions and hydrophobic contacts observed herein with $A_1R/A_{2A}R$ –guanosine binding prediction were shown to be shared between adenosine and A_1R or $A_{2A}R$ (A_1R — PDB 6D9H code; $A_{2A}R$ — PDB 2YDO code), further reinforcing our experiments. These *in silico* results are in line with our *in vivo* data by showing that guanosine could act as a modulator or even an agonist of A_1R or $A_{2A}R$. However, we cannot rule out the possibility that guanosine interacts with putative guanosine receptors that may share some features with adenosine receptors, as has been suggested in previous studies. Although these receptors have not been fully characterized, specific receptor binding sites for [3H]guanosine in rat brain membranes have been reported in the last few years [64, 65]. Of special interest, [3H]guanosine binding sites in hippocampal membranes may be displaced by $A_1R/A_{2A}R$ non-selective agonist (adenosine) and A_1R or $A_{2A}R$ selective agonists (CPA and CGS21680, respectively) [37].

Taken together, our study reinforces the notion that guanosine is effective in exerting antidepressant-like responses and unveils a previously unexplored role of the modulation of A_1R and $A_{2A}R$ in the antidepressant-like effect of this nucleoside. Although our experiments did not fully elucidate if guanosine binds and acts through $A_1R/A_{2A}R$, we provided novel evidence that the anti-immobility effect of guanosine in the tail suspension test is paralleled with the hippocampal region-specific modulation of A_1R and $A_{2A}R$ levels. In addition, we suggest that guanosine may evoke the modulation of adenosine A_1R and $A_{2A}R$ to produce antidepressant-like responses. Our bioinformatics data also indicated that guanosine might interact with A_1R and $A_{2A}R$ at the adenosine binding site. Considering

that modulation of A₁R and A_{2A}R has been shown to evoke antidepressant-like effects in rodents [58–61] and caffeine (A₁R/A_{2A}R non-selective antagonist) is protective against MDD in humans [66, 67] and depressive-like behavior in rodents [11], here, we added new pieces of evidence indicating that the modulation of these receptors may comprise an important mechanism by which guanosine exerts its behavioral effects in the tail suspension test. However, it is essential to bear in mind that the tail suspension test is not a model of depression and that the results obtained in this test should be taken with care from the translational point of view. However, this test has been well recognized as a useful tool to elucidate some critical molecular targets implicated in antidepressant-like responses [38, 39]. Specifically, in the present study, our behavioral data associated with docking analysis help to characterize the possible existence of guanosine binding sites in adenosine receptors. Importantly, the docking properties of guanosine and adenosine are similar regarding the coupling energy. The results reported herein extend the assumption that guanosine may be further investigated as a useful approach for the management of MDD [16]. Importantly, future studies are welcome to characterize the role of A₁R and A_{2A}R in the antidepressant-like effect of guanosine using further molecular techniques, and animal models that induce depressive-like behavior.

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Author contribution Ana Lúcia S. Rodrigues designed the study and wrote the protocol. Anderson Camargo, Luis E. B. Bettio, Priscila B. Rosa, and Julia M. Rosa administered the drugs and performed the behavioral tests. Anderson Camargo performed Western blotting analysis. Glorister A. Altê carried out the bioinformatics studies. Anderson Camargo and Ana Lúcia S. Rodrigues contributed to undertake the statistical analysis and wrote the first draft of the manuscript, as well as approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval All procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the Institutional Ethics Committee.

Informed consent.
Not applicable.

Conflict of interest The authors declare no competing interests.

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