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ORIGINAL ARTICLE

Guanosine Promotes Proliferation in Neural Stem Cells from Hippocampus and Neurogenesis in Adult Mice

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Abstract

Neural stem cells can generate new neurons in the mouse adult brain in a complex multistep process called neurogenesis. Several factors regulate this process, including neurotransmitters, hormones, neurotrophic factors, pharmacological agents, and environmental factors. Purinergic signaling, mainly the adenosinergic system, takes part in neurogenesis, being involved in cell proliferation, migration, and differentiation. However, the role of the purine nucleoside guanosine in neurogenesis remains unclear. Here, we examined the effect of guanosine by using the neurosphere assay derived from neural stem cells of adult mice. We found that continuous treatment with guanosine increased the number of neurospheres, neural stem cell proliferation, and neuronal differentiation. The effect of guanosine to increase the number of neurospheres was reduced by removing adenosine from the culture medium. We next traced the neurogenic effect of guanosine in vivo. The intraperitoneal treatment of adult C57BL/6 mice with guanosine (8 mg/kg) for 26 days increased the number of dividing bromodeoxyuridine (BrdU)-positive cells and also increased neurogenesis, as identified by measuring doublecortin (DCX)-positive cells in the dentate gyrus (DG) of the hippocampus. Antidepressant-like behavior in adult mice accompanied the guanosine-induced neurogenesis in the DG. These results provide new evidence of a pro-neurogenic effect of guanosine on neural stem/progenitor cells, and it was associated in vivo with antidepressant-like effects.

Keywords Guanosine · Neural stem cells · Neurogenesis · Antidepressant · Hippocampus

Introduction

Adult neurogenesis is a form of neuroplasticity generating morphological and functional new neurons from neural stem/progenitor cells (NSCs). Studies on stem cell research have identified a physiological role of adult neurogenesis including learning, memory, and synaptic plasticity [1, 2], and it is implicated in the action of antidepressants [3]. The two major niches where adult NSCs reside are the subventricular zone (SVZ) along the lateral walls of the lateral ventricles and the subgranular zone (SGZ) of the hippocampus. Most adult NSCs are quiescent through inhibition of cell proliferation [4]. After activation, they divide, allowing them to be labeled with 5′-Bromo-2′-deoxyuridine (BrdU) and later give rise to the neuroblasts. The proliferating neuroblasts then exit the cell cycle to differentiate into newborn neurons expressing the microtubule-associated protein doublecortin (DCX). In the hippocampus, the neuroblasts from SGZ migrate and differentiate into dentate granule neurons in the inner granule cell layer (GCL) and later integrate into the existing circuitry [5, 6].

The neurogenic niche around NSCs is highly complex and surrounded by astrocytes, endothelial cells, and mature neurons to provide structural support and regulation of the NSCs [7]. Studies have identified a few modulators' signals [8] including signaling through purinergic receptors [9]. In the adult

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brain, there is evidence of the presence of membrane-bound ectonucleotidases, which hydrolyzes extracellular nucleotides tri- and/or diphosphates to modulate purinergic signaling [10]. Astrocytes are also a source of extracellular nucleotides to promote NSC proliferation in the adult hippocampus and in vitro [11].

Cell culture of NSCs from SGZ and the SVZ is achieved in the presence of epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2), to form free-floating clusters in suspension culture, designated neurospheres. The neural progenitor cells in the neurospheres produce and release adenosine-5′-triphosphate (ATP) locally resulting in the activation of purinergic receptors [12]. Moreover, the exogenous addition of ATP, ADP, and adenosine increase NSC proliferation [12, 13]. The expression of ectonucleotidases and the release of purine nucleotides may activate signaling via purine nucleosides.

Guanosine is a naturally occurring nucleoside with a vital neuromodulatory role in the extracellular environment of brain cells promoting neuroprotection and activation of signaling pathways and increasing the expression of new proteins [14]. Astrocytes release guanosine and other purines in physiological conditions where they may confer neuroprotective effects, as after brain injury [15–18]. Experimental studies have shown that exogenous guanosine treatment stimulates astrocyte proliferation associated with guanosine-induced adenosine release. Astrocytes treated with guanosine induce a peak of extracellular accumulation of adenosine and inosine, which may be directly released from astrocytes or derived from the extracellular breakdown of ATP [19, 20]. Guanosine treatment also affects SVZ NSC proliferation, through activation of the cAMP-CREB pathway, as demonstrated in NSC culture from postnatal rats [21].

Although guanosine does not have a clearly identified site of interaction in the brain, there is evidence that guanosine modulates the adenosine receptors (A_1R and $A_{2A}R$) to exert its neuroprotective effects [22]. In astrocytes, the protective effects of guanosine against ischemic-like situations are abolished by an A_1R antagonist and $A_{2A}R$ agonist [23]. Additionally, the blockade of $A_{2A}R$ inhibits the guanosine-induced increase in the number of cultured cerebellar neurons [24].

Systemic administration of guanosine (8 mg/kg, 2 to 8 weeks of treatment) triggers proliferation and remyelination after chronic spinal cord injury [25] and stimulates neurogenesis associated with improved motor skills [26]. Additionally, guanosine (5 mg/kg) promotes antidepressant-like effects following 21 days of treatment [27].

In this study, we examined the ability of guanosine to stimulate proliferation and differentiation in NSCs from adult mice. The current findings indicated that guanosine induced NSC activation, conversion into progenitor cells, and shifts in the differentiation toward a neuronal fate. In NSCs obtained from the DG, the proliferation effect was associated with the presence of extracellular adenosine. By tracing neurogenesis in vivo, we demonstrated that systemic administration of guanosine in adult mice increased hippocampal neurogenesis that was accompanied by antidepressant-like effects.

Materials and Methods

Animals

Experiments were conducted using adult C57BL/6 mice (male and female, 3 to 4 months) from our inbred colony, housed on a 12/12-h light/dark cycle, room temperature 21 ± 1 °C, with ad libitum access to food and water. The animals were treated, manipulated, and euthanized according to the "Principles of Laboratory Animal Care" (NIH 2011) and approved by the Committee on the Ethics of Animal Experiments of the Federal University of Santa Catarina (CEUA/UFSC 1454270417). All efforts were made to minimize the number of animals used and their suffering.

NSC Cell Culture and Immunocytochemistry

NSCs were isolated from adult mice (male and female, 3 to 4 months) and grown as floating spheres called neurospheres [28, 29]. For cytochemical analysis, neurospheres were seeded as adherent cells in traditional two-dimensional cultures. The DG of hippocampus and SVZ were microdissected and collected in cold Hank's buffered saline solution (HBSS) containing HEPES and dissociated in 0.05% Trypsin-EDTA (Gibco #25300062) for SVZ or a mix PDD (papain 2.5 U/ml Sigma #P4762, Dispase 1 U/ml Sigma #D4693, DNaseI 1 U/ml from Sigma #D5025) for DG. After dissociation, we added the trypsin inhibitor and centrifuged the sample at 300 g for 5 min. Cells were washed and resuspended in growth medium consisting of Neurobasal A (Gibco #21103-049), containing B27 (2%, Gibco #17504-044), GlutaMAX (1X, Gibco # 35050-038), Heparin (2 μg/ml, Sigma #H3149), Antibiotic-Antimycotic (Anti-Anti, 50 U/ml, Gibco #15240-062), fibroblast growth factor-2 (bFGF) (20 ng/ml, PrepoTech #100-18B), and epidermal growth factor (EGF) (20 ng/ml, PrepoTech #100-15). Cells were passaged at least twice before use to generate secondary neurospheres.

Secondary neurospheres were dissociated and resuspended in N2 growth medium with N2 supplement (Gibco #17502-408), DMEM/F12 (Gibco #12500-062), L-glutamine (2 mM, Sigma #G8540), antibiotic-antimycotic (50 U/ml, Gibco #15240-062), bFGF (20 ng/ml, PrepoTech #100-18B), and EGF (20 ng/ml, PrepoTech #100-15). Cells were seeded (5×10^4 cells/ml) into uncoated 24-well plates and cultured for 7 (SVZ) to 14 (DG) days in suspension to form

neurospheres in the N2 control medium. When cells measured between 50 and 200 mm, they were analyzed by microscopy.

Neurospheres were cultured under additional treatment with guanosine (GUO, 100 μM, Sigma #G6264). This guanosine concentration was previously shown as neuroprotective [22, 24, 30]. In some experiments, we also tested adenosine (ADO, 100 μM, Sigma #A4036), adenosine deaminase (ADA, 0.5 U/ml, Sigma #10102105001), and a mixture of ADA + GUO for their effects on neurospheres.

After imaging, neurospheres were fixed for 20 min with 4% paraformaldehyde in phosphate buffered saline (PBS). Whole neurospheres immunostaining was performed following free-floating immunohistochemistry protocol [31, 32]. Immunocytochemistry was performed by washing the cells in PBS and permeabilized 5 min with PBS/0.5% Triton X-100. The cells were then blocked in PBS/0.5% Triton X-100 containing 10% normal donkey serum (Sigma #D9663) for 1 h at room temperature. Next, the cells were incubated overnight at 4 °C in PBS/0.3% Triton X-100 containing 5% serum and the appropriate mixture of primary antibodies. Primary antibodies used were mouse anti-Nestin (1:500, Abcam #6142) for neural stem/progenitor cells, rabbit anti-Ki-67 (1:500, Millipore #9260) for cell proliferation, mouse anti-β-tubulin III (1:500, Sigma #T8578) for neurons, and rabbit anti-GFAP (1:600, Sigma #G9269) for astrocytes. After washing in PBS/0.1%, Triton X-100, for 5 min, cells were incubated 1 h with the secondary antibody diluted 1:2000, donkey anti-rabbit IgG labeled with Alexa Fluor 488 (Invitrogen #A212006) and donkey anti-mouse IgG Alexa Fluor 594 (Invitrogen #A11005). All samples were counterstained with Hoechst 33342 (5 μg/ml, Sigma #B2261), and whole neurospheres were mounted onto cover glass with ProLong Gold antifade reagent (Invitrogen #P10144) and viewed for triple immunofluorescence in a Confocal microscope DMI6000 B (Leica).

Cells plated in monolayer for proliferation assay were seeded with 1×10^5 cells/ml on poly-L-ornithine/laminin-coated glass coverslips in 24-well plates and incubated at 37 °C in 5% CO_2 in N2 control medium. For treatment experiments, 100 μM of guanosine was added to the in N2 medium. After cells reach confluency, cells were rinsed with PBS (pH 7.4) and fixed for 20 min with 4% paraformaldehyde in PBS for immunocytochemistry. Primary antibodies used were mouse anti-Nestin (1:500, Abcam #6142) for neural stem/progenitor cells and rabbit anti-Ki-67 (1:500, Millipore #9260) for cell proliferation. All samples were counterstained with Hoechst 33342 dye to label cell nuclei (5 μg/ml, Sigma #B2261) and mounted onto cover glass with ProLong Gold antifade reagent (Invitrogen #P10144).

To induce differentiation, single neurospheres with similar size were plated on poly-L-ornithine/laminin-coated glass coverslips in 24-well plates and incubated at 37 °C in 5% CO_2. Cells were allowed to grow for 24 h in the N2 growth medium, and the next day, we withdrew the growth factors, which were replaced by 1% fetal bovine serum (FBS, Gibco #10437010). For treatment experiments, 100 μM of guanosine was added to the medium. After 7 days, cells were rinsed with PBS (pH 7.4) and fixed for 20 min with 4% paraformaldehyde in PBS for immunocytochemistry. Primary antibodies used were mouse anti-β-tubulin III (1:500, Sigma #T8578) for neurons and rabbit anti-GFAP (1:600, Sigma #G9269) for astrocytes. All samples were counterstained with Hoechst 33342 dye to label cell nuclei (5 μg/ml, Sigma #B2261) and mounted onto cover glass with ProLong Gold antifade reagent (Invitrogen #P10144).

Imaging and Quantification of Neurospheres and Monolayer Cell Cultures

The micrographs were taken at × 100 total magnification using a microscope Axiovert 40 CFL (Zeiss) equipped with a digital camera Axio-Cam MRc (Zeiss). Five representative images were taken from randomly selected fields (ROI) for each well, and analysis was carried out using Zen software (Zeiss) by manually counting and measuring the diameters of each neurospheres using a circle measurement tool, which provides the diameter (μm). Neurospheres of diameter less than 50 μm were excluded from the count. The results are representative from triplicates of three or four separate experiments ($n = 3$–4). After micrographs were taken, neurospheres were centrifuged, treated with 0.05% Trypsin-EDTA at 37 °C for 5 min, washed, and mechanically dissociated. The cell number was counted using a hemocytometer.

For NSCs plated in monolayer, images were captured in a Confocal microscope DMI6000 B (Leica) at × 60 total magnification. The results are representative from duplicates of three separate experiments ($n = 3$). Five representative images were taken from randomly selected fields (ROI) from each coverslip/well, and analysis was carried out using Zen software (Zeiss) by manually counting. At least 100 cells per well were counted. Neuronal differentiation and proliferation were quantified as percentage of positive immunolabeled (β-tubulin-III+ or Ki-67+) cells over the total nuclei staining with Hoechst in each selected area. The fraction of positively stained cells is presented in the labeling index (the number of positively stained cells/total number of cells × 100).

Drug Treatments

A summary of the in vivo experimental procedure, including guanosine treatment, proliferation assays, and behavioral experiments, is presented in Fig. 5.

Guanosine was prepared fresh daily diluted in 0.9% saline. Adult mice (male, 3 to 4 months) were injected intraperitoneally once a day for 26 days either with 8 mg/kg of GUO or vehicle for control animals. Alternatively, to evaluate the

effect of an acute guanosine treatment on depressive-like behavior, animals received a single administration with 8 mg/kg of guanosine or vehicle 24 h before testing. This concentration of guanosine has been previously described as neurogenic [26]. Animals were euthanized the day after the last guanosine injection.

Proliferation studies in vivo were performed by BrdU assay (5′-Bromo-2′-deoxyuridine, Sigma #5002). The sterile solution of BrdU was prepared in 0.9% saline at a dilution of 10 mg/ml. For tracing the neurogenesis, adult mice were injected intraperitoneally with BrdU once a day for 5 days (50 mg/kg) during the second week of guanosine treatment (Fig. 5). Animals were euthanized 12 days after the last BrdU injection, at day 26.

Behavioral Analysis

All tests were carried out between 9:00 and 14:00 h. Behavioral analysis was performed in the last week of treatment in the following order: 22nd day, open field; 23th day, object location; 24th day, Y-maze; and 25th day, tail suspension test (Fig. 5). Another set of mice were used to test the acute effect of GUO on tail suspension test, 24 h after a single guanosine (8 mg/kg) administration. Mice were habituated for at least 1 h before the beginning of the tests to the test's room lighting and temperature conditions. On behavioral test days, animals were treated with guanosine in the afternoon.

Open Field

At the 22nd day of treatment, animals were placed in the center of a white-floor clear-wall acrylic box (40 cm × 40 cm × 40 cm) and allowed to explore it for 30 min freely. The arena was cleaned thoroughly with 20% ethanol in between trials to eliminate olfactory clues. The arena floor was divided into 16 equal quadrants by imaginary lines, which defined the center (four central quadrants) and periphery of the arena. Total distance traveled and time in the center of the arena were analyzed. The amount of time spent in the center of the arena is inversely proportional to anxiety-like behavior [33]. Animal exploration at the open field was monitored through a video camera positioned above the apparatuses, and the videos were later analyzed with the ANY Maze® video tracking (Stoelting Co., Wood Dale, IL, USA) by an experienced experimenter who was unaware of the experimental group of the animals tested.

Object Location Test

At the 23rd day of treatment, object location test was carried out in the same arena to which animals were habituated during the open-field test on the previous day and filmed. Cylindrical plastic objects were fixed to the arena using tape to prevent displacement due to animal exploratory activity during test sessions. In the training session, animals were placed at the center of the arena in the presence of two identical objects located at two arena corners, side by side. Animals could explore them freely for 5 min. The amount of time spent exploring each object was measured with a stopwatch by a trained experimenter blind to animal conditions. The arena was cleaned thoroughly with 20% ethanol in between trials to eliminate olfactory clues. After a 90-min interval, animals were again placed in the arena for a 5-min-long test session, when one object used during training was relocated to the opposite corner of the arena. The amount of time spent exploring each object (familiar vs. relocated object) was again measured. Results were expressed as a percentage of time exploring each object during the test session or as discrimination index (DI), ((DI = time exploring the relocated object − time exploring the familiar object)/(time exploring relocated + familiar) × 100). Animals that recognize the object in a familiar position as such (i.e., learned the task) explore the relocated object > 50% of the total time [34, 35].

Y-Maze Test

At the 24th day of treatment, animals were subjected to the Y-maze spontaneous (or continuous) alternation test. The Y-shaped arena contains three opaque gray arms (D, 30 cm × W, 10 cm × H, 16 cm) at 120° angles from each other. Mice were put at the center of the arena and allowed to explore it for 8 min freely. The arena was cleaned thoroughly with 20% ethanol in between trials to eliminate olfactory clues. An observer, blind to the animal conditions and in an adjacent room, counted the number of alternations between arms and the total number of arm entries. An alternation occurs when an animal enters the three different arms of the maze in three consecutive arm entries. Results are expressed as a percentage of alternations relating to total arm entries [36].

Tail Suspension Test

At the 25th day of treatment, mice were subjected to the tail suspension test. Another independent group of mice received a single administration of 8 mg/kg of guanosine or vehicle 24 h before testing. Visually and acoustically isolated mice were suspended by the tail using adhesive tape 50 cm above the floor, for 6 min. A tape was fixed 1 cm from the animal's tail top. Immobility time was defined as the amount of time during which the mice hung passively without moving [37]. Total immobility time was counted using a stopwatch by an experienced experimenter blinded to the animals' experimental group.

Tissue Processing and Immunohistochemistry

After guanosine or saline treatment and behavioral testing, 4 to 5 animals from each experimental group were randomly chosen for immunohistochemistry analysis. Mice were anesthetized with chloral hydrate and were perfusion fixed with 50 ml of 0.9% saline followed by 100 ml 4% paraformaldehyde (PFA). The brains were post fixed in 4% PFA and equilibrated in 30% sucrose. After, the brains were embedded in medium (Tissue-Tek OCT compound; VWR #25608-930) and serially sectioned (40 μm-thick) in the coronal plane throughout the dorsal-ventral extent of the hippocampus corresponding to the whole hippocampal extension according to the following coronal coordinates from bregma, − 0.94 to − 2.92 mm (http://www.mbl.org, coronal C57BL/6 J atlas). The sections were cut at − 22 °C with a cryostat (Leica) and stored at − 20 °C in cryoprotectant solution. The section was then processed for free-floating immunohistochemistry.

Immunohistochemical staining was performed as previously described [38]. Briefly, brain sections in a 1-in-5 series were collected from the cryoprotectant solution, washed three times for 5 min each in 0.1 M PBS, and then incubated in a blocking solution containing 10% normal donkey serum and 0.5% Triton X-100 in PBS for 1 h at room temperature.

BrdU immunostaining was followed according to manufacturer instructions. BrdU is a thymine nucleotide analog that is readily incorporated into the DNA of dividing cells during the S-phase of the cell cycle and is a commonly used marker for cellular proliferation and cell fate analysis using cell-specific markers [39]. Sections were incubated in HCL (1 N) for 10 min on ice to denature DNA, followed by HCl (2 N) for 10 min at room temperature before placing them in an incubator for 20 min at 37 °C. The sections were incubated with 0.1 M borate buffer (pH 8.5) for 12 min and rinsed three times for 5 min each with 0.01 M PBS (pH 7.4) with 0.1% Triton X-100 at room temperature. The sections were then incubated with 0.01 M PBS (pH 7.4)/ 0.5% Triton X-100/glycine (0.3 M) containing 10% normal donkey serum for 1 h prior to incubating for 2 days in − 4 °C in a solution containing 5% normal donkey serum, 0.3% Triton X-100, with the primary antibody rat anti-BrdU (1:200 Accurate Chem #MCA2060GA). For double labeling, rabbit anti-doublecortin (DCX) (1:500, Cell signaling #4604S) was added in the following day. Doublecortin is transiently expressed in proliferating progenitor cells and newly generated neuroblasts shortly after cell fate determination [40]. Negative controls were generated by replacing the primary antibodies with PBS. After rinsing three times for 10 min each in PBS/0.1% Triton X-100, the sections were incubated 1 h in fluorescent-coupled secondary antibodies diluted 1:2000 (Alexa Fluor Donkey anti-rat 647, Alexa Fluor donkey anti-rabbit 488, Life Technologies). After a final rinse in PBS for 10 min, sections were counterstained with Hoechst 33342 (5 μg/ml), mounted on slides (Superfrost Plus; Daigger), air-dried, and coverslipped with ProLong Gold antifade reagent (Invitrogen).

Cell Counting

Brain sections were mounted on glass slides, and fluorescence signals were imaged using a Zeiss LSM710 confocal laser scanning microscope. Quantification of BrdU+ cells was determined by manual counting all fluorescent cells in one of every fifth coronal section of the granular and subgranular layer (SGZ) of the DG of the hippocampus. BrdU+ cells were counted using a 40× objective, the sum was multiplied by 5 to obtain an estimate of the total number of BrdU+ cells per animal and reported as mean ± SE. Small BrdU-labeled nuclei (presumed to be glial precursors) at the hilar border and linear (endothelial-like) immunostained forms were excluded from the analysis [41]. For double labeling, the percentage of BrdU-labeled cells that also expressed DCX+ (BrdU/DCX+) was determined by counting of labeled cells throughout the DG with a 60× oil immersion objective. Colocalization analysis was manually done by visual inspection of the size and shape of cells throughout a Z-stack (spanned between 3 and 5 μm in the Z-dimension). Cells were scored positive when the DCX+ labeling was unambiguously associated with a BrdU+ cells. Colocalization was identified by the appearance of a merged color. Only DCX+ immature neurons that had a minimal dendritic tree overlap with adjacent cells were included to avoid ambiguity [42].

Statistics

All data are expressed as mean ± SEM. The *n* value represents the number of independent experiments and/or the number of mice. Data were analyzed using either a one-sample Student's *t* test comparing the mean exploration percentage time for each object with the chance value of 50% or Student's *t* test comparing experimental groups. The analysis with one-way analysis of variance (ANOVA) was followed by Tukey's multiple comparisons test, and differences were considered significant at $p < 0.05$. All statistical analysis was conducted using GraphPad Prism software.

Results

Guanosine Stimulates Proliferation of Neural Stem Cells

All secondary neurospheres from SVZ abundantly expressed the Nestin and Ki-67 counterstained with Hoechst indicating that they were neural stem/progenitor cells (after 7 DIV, Fig. 1a). To ascertain the effects of guanosine (GUO) on NSC self-renewal and clonality, we cultured NSCs from

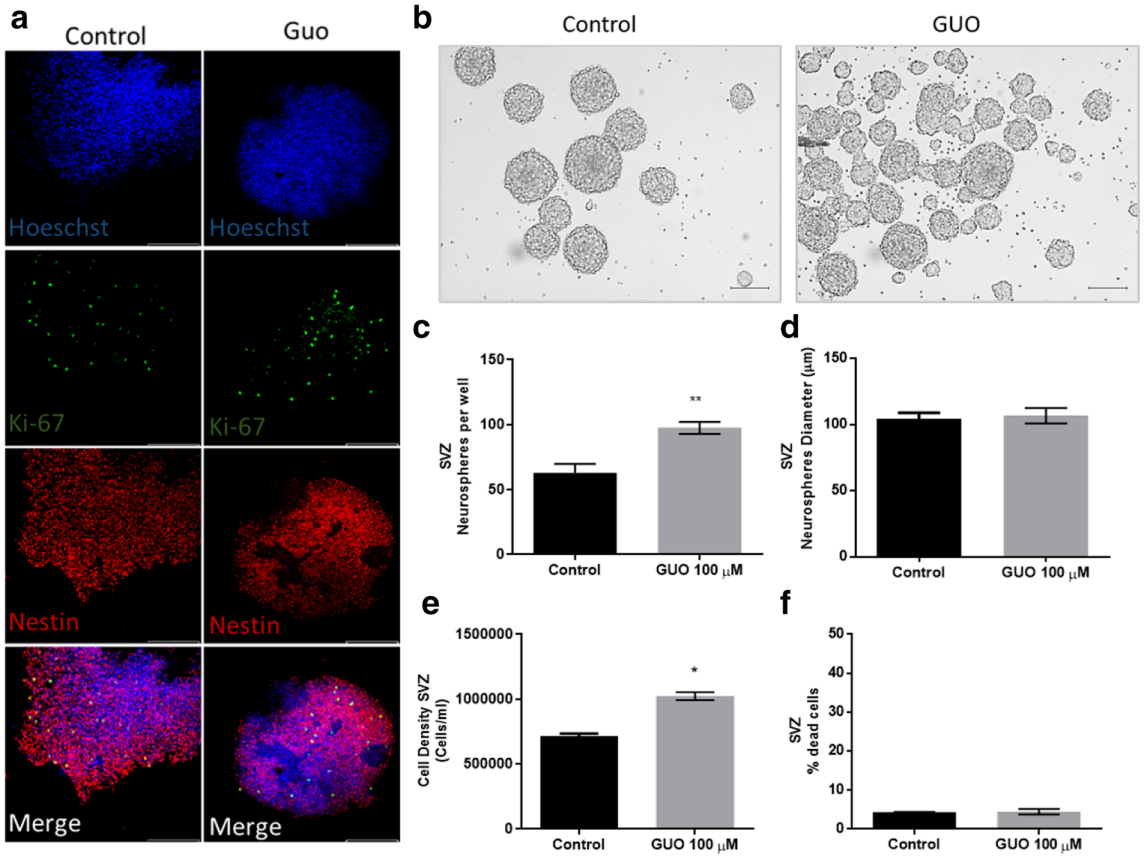

Fig. 1 **a** Representative images of NSCs from SVZ grown as neurospheres for 7 days, after immunocytochemistry with Hoechst (blue, staining nucleus), Ki-67 (green, staining proliferative cells), and Nestin (red, staining neuroprogenitor cells). Scale bar, 50 μm. **b** Representative images of NSCs from SVZ grown as neurospheres for 7 days. Scale bar, 100 μm. **c**, **d** Quantitative analysis of neurosphere per well and mean diameter of neurospheres formed after control or guanosine (GUO) treatment. **e** Quantitative analysis of cells after neurosphere dissociation with trypsin. **f** Percentage of cell death after neurosphere dissociation and counted in hemocytometer with trypan blue. Data represent mean ± SEM, * $p < 0.05$, ** $p < 0.01$ compared with the control group. $n = 5$–6/group. Two-tailed Mann-Whitney t test

SVZ for 7 days or NSCs from DG for 14 days in the N2 growth medium. For treatment experiments, 100 μM of guanosine was added to the N2 medium. Quantitative analysis revealed that the neurosphere-forming frequency in cultures treated with GUO was significantly increased in SVZ (control, 62.89 ± 6.973; GUO, 97.47 ± 4.501; $P = 0.004$; Fig. 1b–c) and DG (control, 80.33 ± 2.75; GUO, 111 ± 4.59; $P = 0.029$; Fig. 2a–b) as compared with the control. The number of NSCs obtained following dissociation was significantly increased with GUO treatment in SVZ (1.4-fold increase $P = 0.02$; Fig. 1d) and DG (1.2-fold increase $P = 0.03$; Fig. 2c). GUO did not alter mean neurosphere diameter in SVZ (control, 104.4 ± 4.476; 106.6 ± 5.907; Fig. 1e), DG (control, 113.2 ± 1.877; GUO, 102.5 ± 3.168; Fig. 2d), or cell death (Figs. 1f and 2e).

Previous studies have demonstrated that NSCs express purinergic adenosine (A_1, A_{2A}, and A_{2B}) [13] and ATP (some P2Y and P2X) receptors [43]. In this study, we used the same concentration of adenosine (ADO, 100 μM) to compare with GUO treatment in NSC cultures from DG. At this concentration, adenosine did not show significant differences in the number of neurospheres per well as compared with control. But when compared with GUO, there is a significant 1.4-fold decrease (GUO, 111 ± 4.59; ADO, 74.78 ± 16.61; $P < 0.05$, Fig. 2b). Adenosine treatment significantly reduced ($P < 0.0001$) cell density and neurospheres diameter (ADO, 59.12 ± 3.541) compared with control and GUO (Fig. 2 c and d), with a small significant increase ($P < 0.05$) in cell death as compared with GUO (Fig. 2e).

NSC proliferation is sensitive to differential adenosine concentration, and the addition of adenosine in lower concentrations (1 μM) has been shown to increase the number of neurospheres [44]. Next, we examined whether extracellular adenosine levels affect guanosine-induced proliferation. The neurospheres cultured from DG were pretreated with adenosine deaminase (ADA, 0.5 U/ml) to remove extracellular adenosine levels by converting it to inosine. We observed a significant 1.5-fold decrease in the neurosphere-forming frequency compared with the control group (DG control, 80.33 ± 2.75; ADA, 53.50 ± 6.245; $P < 0.05$) and a significant change in the effect of guanosine by a 1.4-fold decrease compared with GUO treatment alone (GUO, 111 ± 4.59; GUO +

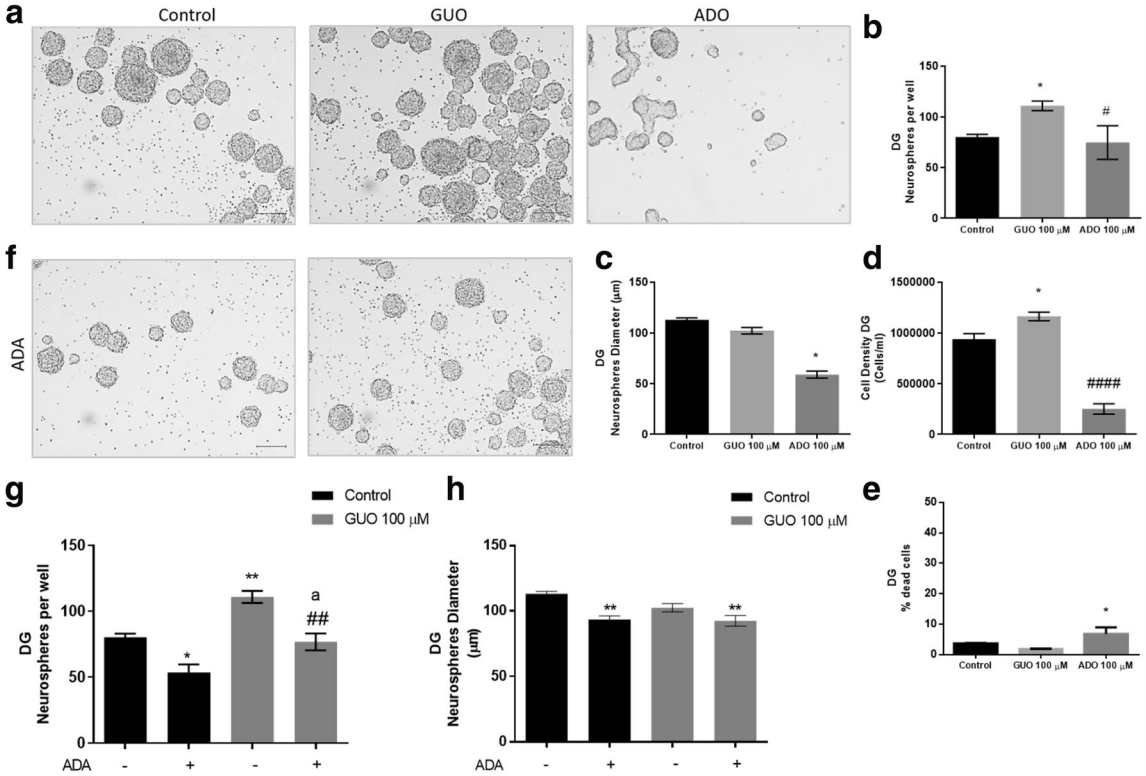

Fig. 2 **a** Representative images of NSCs from DG grown as neurospheres for 10–14 days. Scale bar, 100 µm. **b**, **c** Quantitative analysis of neurospheres per well and mean diameter of neurospheres formed after control, guanosine (GUO), or Adenosine (ADO) treatment. **d** Quantitative analysis of cells after neurosphere dissociation with trypsin. **e** Percentage of cell death after neurosphere dissociation and counted by hemocytometer with trypan blue. **f** Representative images of NSCs from DG after treatment with adenosine deaminase (ADA). Scale bar, 100 µm. **g**, **h** Quantitative analysis of neurospheres per well and mean diameter of neurospheres formed after control or guanosine (GUO) with the addition of ADA. Data represent mean ± SEM, * $p < 0.05$, ** $p < 0.01$ compared with control group; # $p < 0.05$, ## $p < 0.01$, #### $p < 0.0001$ compared with GUO treatment alone; [a] $p < 0.05$ compared with control-treated with ADA. $n = 3$–4/group; one-way ANOVA followed by Tukey's multiple comparisons test

ADA, 76.83 ± 6.405; $P < 0.01$; Fig. 2f–g). There was a significant decrease ($P < 0.01$) in the neurospheres' diameters following ADA treatment (Fig. 2h).

The neurosphere clusters contain, in addition to NSCs, neuronal and glial progenitors in different states of differentiation. To determine whether GUO affected the Nestin-positive cell division with less accompanying differentiation, NSCs from DG were grown as an adherent monolayer in appropriate medium and labeled with the proliferation marker Ki-67. As shown in Fig. 3a, the cells abundantly express the NSC marker Nestin, and the percentage of Nestin+ Ki-67+ is significantly increased ($P < 0.05$) when NSCs were cultured with GUO in the presence of growth factors (Fig. 3b).

Guanosine Stimulates NSC Differentiation

We then investigated the effect of GUO on NSC differentiation. NSCs isolated from DG are multipotent and can differentiate into both neurons and glia under differentiation conditions [29]. Neurospheres from DG were induced to differentiate after the withdrawal of growth factors in coated coverslips. After 1 week of differentiation, neurospheres typically give rise to mostly GFAP+ astrocyte and few β-tubulin-III+ neurons (Fig. 4a) [29, 45]. The GUO treatment resulted in a significant increase ($P < 0.05$) in the percentage of β-tubulin III+ neurons compared with the control group (Fig. 4 b and c).

Tracing Neurogenesis In Vivo

To investigate the effects of GUO treatment on adult neurogenesis in vivo, adult C57BL/6 mice received intraperitoneal GUO administration during 26 consecutive days, followed by BrdU administration to label dividing cells (Fig. 5). We observed a significant 1.3-fold increase in the number of BrdU-labeled cells in the SGZ of DG from mice treated with GUO compared with control (control, 408.8 ± 42.93; GUO, 542.0 ± 26.25; $P = 0.031$, Fig. 6).

To examine neuronal differentiation and survival, we quantified BrdU+/DCX+ double-labeled neurons in the same animals. According to our timeline, DCX would label cells that are from a few days to about 2 weeks of age before mice euthanasia [46]. The ratios of BrdU+/DCX+ cells to BrdU+ cells significantly increase after GUO treatment compared

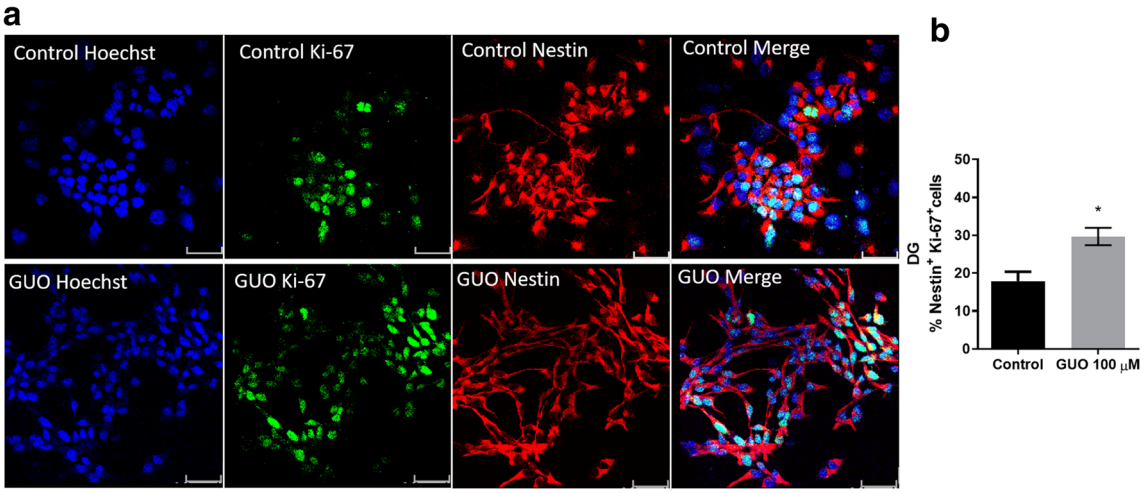

Fig. 3 **a** Representative images of NSCs from DG growing in an adherent monolayer in the presence of growth factors. Scale bar, 50 μm. Cells express the NSC marker Nestin (red), Ki67 (green), and Hoechst (blue, staining nucleus). **b** Percentage of Nestin+ Ki-67+ cells compared with total Hoechst cells. Data represent mean ± SEM * $p < 0.05$; $n = 3$/group; two-tailed Mann-Whitney t test

with control (control, 44.27% ± 9.430%; 79.32% ± 4.460; $P = 0.008$, Fig. 6 a and c).

Behavioral Analysis

Hippocampal adult neurogenesis is frequently associated with improved learning and memory and antidepressant-like behavior in various experimental settings [47, 48]. In the current study, we observed increased hippocampal neurogenesis after GUO treatment and therefore assessed the potential effects of GUO on memory performance and antidepressant-like behavior in adult mice.

First, the open-field test was used to access general health, exploratory behavior, and anxiety in mice after GUO treatment. There were no effects of GUO treatment on locomotor activity ($P > 0.05$, total distance, Fig. 7a) and anxiety-related behavior measured for 5 min ($P = 0.572$, time in the center, Fig. 7b) or 30 min (data not showed). Moreover, GUO treatment did not alter spatial learning and memory addressed on the object relocation test (Fig. 7c–e) or on the continuous alternation in the Y-maze test (Fig. 7f–g).

Importantly, a significantly decreased immobility time ($P > 0.05$) in the tail suspension test was found after GUO treatment for 26 days (Fig. 8a), indicative of an antidepressant-like profile. However, this effect was not observed following a single

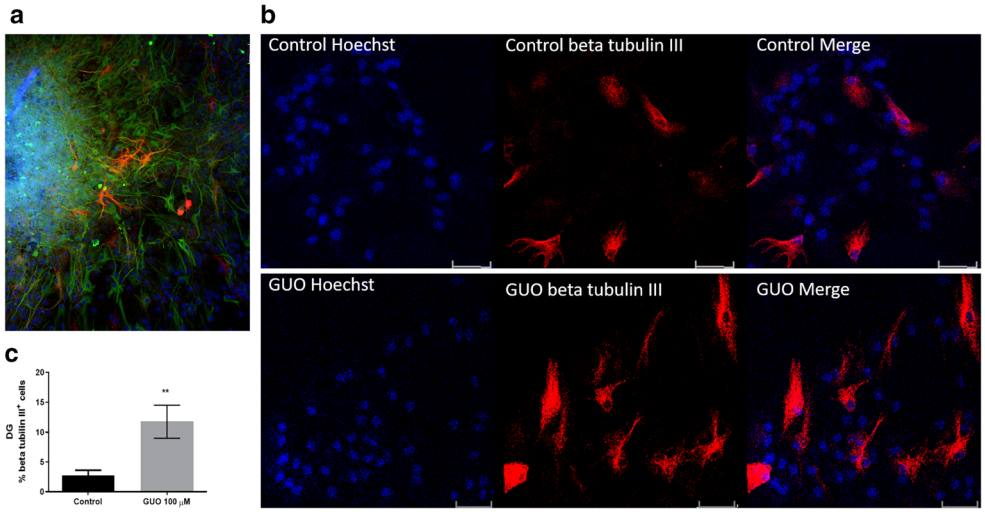

Fig. 4 **a** Representative images of NSCs from DG after 7 days of differentiation from neurospheres in a typical differentiation experiment, showing GFAP cells (green), beta-tubulin III cells (red), and Hoechst (blue, staining nucleus). **b** GUO treatment results in DG-NSCs differentiating into more neurons as demonstrated by immunostaining cells using the neuronal marker beta-tubulin III+ cells (red). Scale bar, 50 μm. **c** Quantitative analysis showing the percentage of positive beta-tubulin III cells compared with total Hoechst cells. Data represent mean ± SEM ** $p < 0.01$; $n = 3$/group; two-tailed Mann-Whitney t test

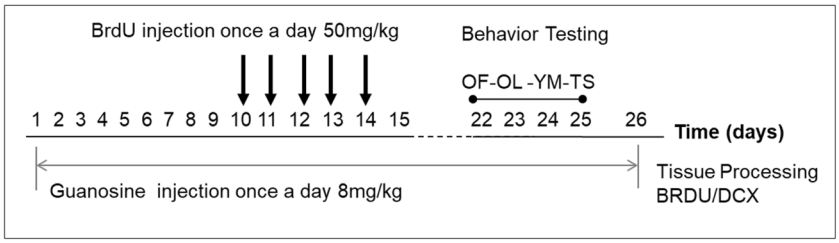

Fig. 5 Timeline for tracing neurogenesis in young adult mice treated with guanosine or control. Mice underwent behavioral analysis in the last week of treatment in the following order: 22nd day, open field (OF); 23th day, object Location (OL); 24th day, Y-maze (YM), and 25th day, tail suspension test (TS). After behavioral testing, the animals were euthanized, and immunohistochemistry for BrdU and doublecortin (DCX) was used to determine neurogenesis

GUO (8 mg/kg) administration 24 h before the tail suspension test (Fig. 8b).

Discussion

In the present study, we have explored the effects of GUO treatment on cell proliferation and the generation of new neurons in hippocampal NSCs in vitro. We also analyzed the effects of GUO on hippocampal neurogenesis in adult mice and the putative improvement on neurobehavioral performance.

The current results revealed that GUO treatment increased the proliferation of neural stem and progenitor cells, as evidenced by increased sphere-forming frequency in the neurosphere cultures from SVZ and DG, and the Ki-67 labeling to proliferating cells in the adherent monolayer culture. However, the diameter of the neurospheres, which is often used to assess mitogenic potential, was not affected. Therefore, taken together, these results indicate that GUO treatment is more involved in the increase of self-renewal capacity to form tertiary neurospheres. To the best of our knowledge, this is the first evidence showing that guanosine promotes the proliferation of adult NSCs in vitro. Further studies are necessary to fully address the GUO cell-intrinsic mechanisms to control cell cycle and maintenance of multipotency.

Our in vitro data suggest that extracellular adenosine is needed to increase the number of neurospheres, and guanosine alone cannot reverse the effects of inhibition of neurosphere-forming frequency due to ADA pretreatment. This observation suggests that some partial effects of adenosine together with guanosine are needed to induce cell proliferation. Then, as observed here, it is possible that low adenosine levels may be beneficial to this proliferative effect of guanosine. It was suggested that GUO might increase extracellular adenosine disposition in vascular and endothelial cell cultures [49], an effect that was not confirmed in neural cells, and the mechanism is not yet understood. Therefore, it is not clear whether GUO effects depend on increasing adenosine levels or may be due to a direct modulation on adenosine receptors.

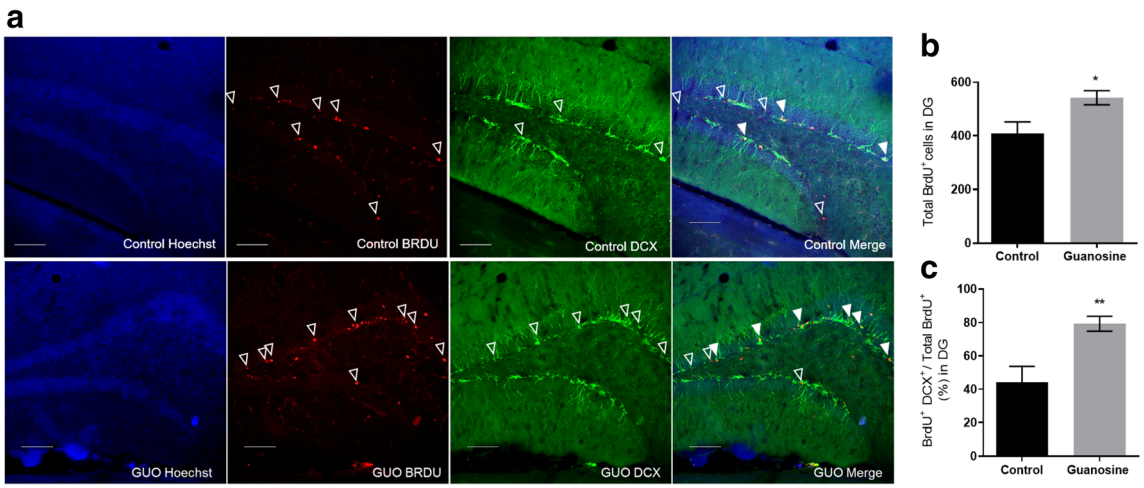

Fig. 6 **a** Representative photomicrographs of DG of hippocampus showing BrdU-positive and DCX-expressing neurons along with Hoechst33342 staining. Scale bar 100 μm. The open arrowheads indicate BrdU+ or GFP+ cells only, whereas the closed arrowheads indicate the colocalization in selected cells. The images were obtained by confocal microscopy. **b**, **c** Quantitative analysis of BrdU-positive cells and percentage of colocalization of BrdU+ and DCX+ in the SGZ of DG. BrdU+ and/or DCX-positive cells were manually counted in each section, regardless of the size or shape. Data represent mean ± SEM, * $p < 0.05$, $n = 4$–5/group; one and two-tailed Mann-Whitney T test, respectively

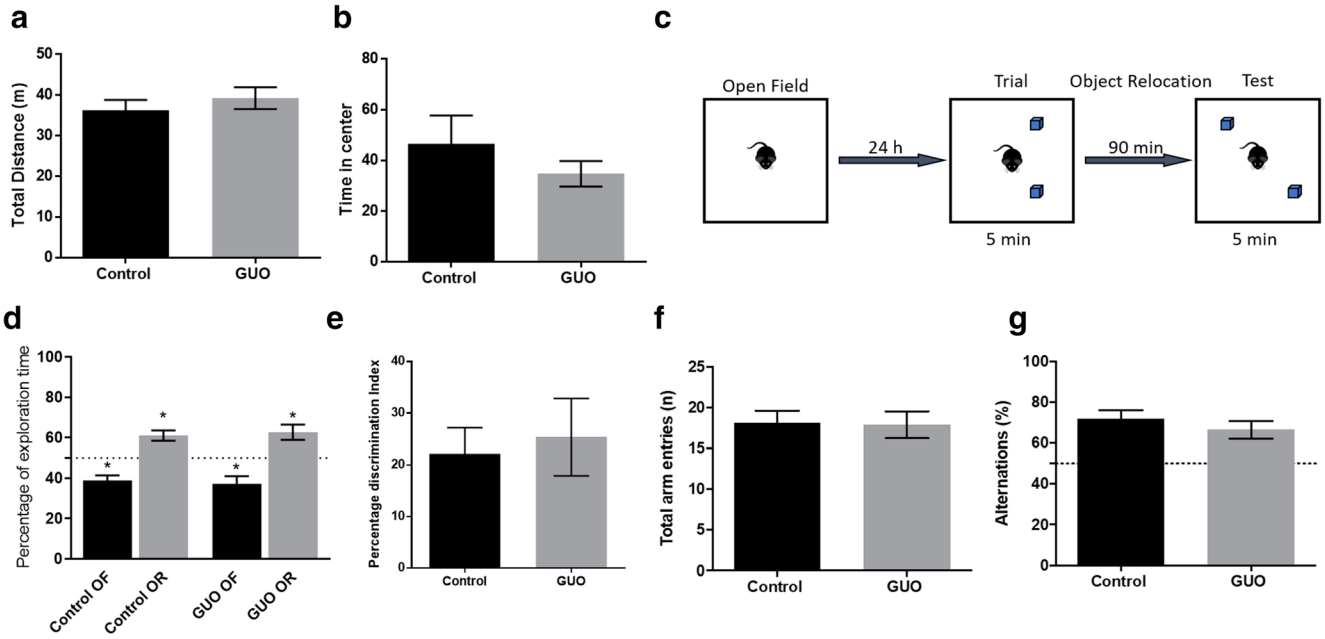

Fig. 7 **a** Quantitative analysis of general health of mice in the open-field arena, locomotion was measured for 30 min, **b** and time in the center was measured for 5 min using ANY-maze software. Data represent mean ± SEM, $n = 6–8$/group. No statistical significance, two-tailed Mann-Whitney T test. **c** Schematic overview showing object location trial and test. **d**, **e** Quantitative analysis from the object location test. Data represent mean ± SEM, $n = 13$/group. * $p < 0.05$, one-sample T test compared with chance level (50%) and Mann-Whitney T test, respectively. The discrimination index (DI) is calculated by ((time exploring the relocated object − time exploring the familiar object) / (time exploring relocated + familiar) × 100). Mice with data showing a large negative DI (≤ − 10) was excluded. OF, familiar object; OR, object relocation. **f**, **g** Quantitative analysis from continuous alternation in the Y-maze test and percentage of alternation compared with total arm exploration. Data represent mean ± SEM, $n = 6–8$/group. No statistical significance, two-tailed Mann-Whitney T test

Previous studies confirmed the expression of adenosine receptor mRNA and protein in young and adult neurospheres, where adenosine treatment presented a concentration-dependent effect [50]. In secondary neurospheres cultured from adult mice, the lower concentration of adenosine (1 μM) may be beneficial to the neurosphere-forming frequency, where at high concentrations (10–100 μM), adenosine leads to an opposite effect [44, 50, 51], as observed in our study, although it was also observed that adenosine concentration of 10 and 30 μM induced proliferation of NSCs [13]. In that study, activation of adenosine A_1 receptor induces the proliferation of NSCs, and the blockade of MEK/ERK and Akt inhibits such effect. This same signaling pathway is often present in the protective action of GUO in several experimental models of neurodegeneration [14]. We previously showed that GUO neuroprotective effect depends on adenosine receptor modulation [22, 23]. Additionally, these intracellular pathways are involved in the effect of GUO promoting the survival and maturation of primary cerebellar neurons in culture [24]. However, the exact mechanisms and molecular targets involved in the GUO effect to induce NSC proliferation remain to be elucidated.

In our in vivo study, GUO treatment induced an increase in the percentage of cells expressing β-tubulin III+ neurons

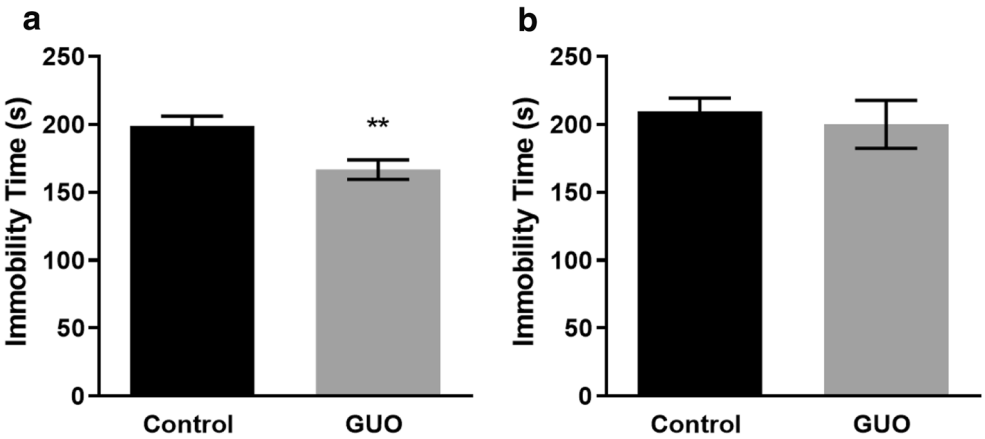

Fig. 8 **a** Quantitative analysis of antidepressant-like effect of guanosine treatment from mice subjected to the tail suspension test after 26 days of guanosine (GUO, 8 mg/kg) treatment and **b** after 24 h of a single GUO (8 mg/kg) administration. Data represent mean ± SEM, $n = 6–8$/group. ** $p < 0{,}01$, two-tailed Mann-Whitney T test

suggesting that GUO can promote a pro-neuronal fate choice on cell differentiation. By contrast, Benito-Muñoz et al. (2016) reported that the same concentration of adenosine inhibits neuronal differentiation in adult neurosphere culture through activation of A_1 receptors resulting in gliogenesis [51].

To further support the functional role of GUO, we analyzed the effect of GUO treatment in the neurogenic niche of adult C57BL/6 mice in vivo. In this study, we identified GUO as a neurogenic factor, increasing BrdU+ and BrdU/DCX+ cells in the SGZ of DG in the adult hippocampus. The last administration of BrdU was 12 days before fixation, suggesting a survival effect of BrdU+/DCX+ cells favoring increased net hippocampal neurogenesis resulting from guanosine treatment.

A recent related study in Swiss mice evaluated the effect of GUO on hippocampal cell proliferation by immunohistochemistry against the proliferating cell nuclear antigen (PCNA) and Ki-67 [27]. However, no significant differences between vehicle and GUO-treated mice (5 mg/kg/day, p.o., for 21 days) were observed with regard to the number of proliferating cells in the entire hippocampal DG. This absence of results may be due to the drawback of using Ki-67 for proliferation as it reflects only a snapshot of the amount of proliferation at the time of tissue collection [52]. On the other hand, our study using repeated administration of BrdU allows for labeling all dividing cells and further investigating the fate of BRDU+ NSCs in the brain.

A presumptive role of new neurons in hippocampal functions such as learning and memory or depression-related behaviors is one of the major hypotheses on adult neurogenesis. Our data, together with Bettio and collaborators [27], provide support to the association of neurogenesis and antidepressant effects following chronic GUO treatment. Interestingly, a single GUO administration did not alter the immobility time of mice in the tail suspension test when assessed after 24 h, indicating the relevance of the GUO neurogenic action in its antidepressant effect. Moreover, previous in vivo studies also showed that guanosine-induced neurogenesis is accompanied by an improved functional outcome in rodent models of Parkinsonism [26] and stroke [53]. Although the experimental conditions differ from ours, taken together, these data support the effect of GUO in neurogenesis and behavior.

While in this study we observed a neurogenesis increase throughout the dorsal-ventral extent of the hippocampus, it is worthwhile to notice that cells in the hippocampus present a functional heterogeneity within the structure. Studies targeting different regions on the hippocampus revealed that the dorsal DG controls spatial learning, memory, exploration, and navigation, as well as visuospatial processing, whereas the ventral DG regulates hormone release, reward processing, anxiety, depression, and executive function [54]. These findings correlate with Bettio (2016) reports showing that guanosine treatment resulted in a significant increase in the number of NeuroD+ cells localized to the ventral hippocampal DG [27]. We did not observe differences in cognitive tasks assessed in the object location or the Y-maze tests; however, there was little margin left to detect a possible improvement because all experimental groups performed well in the task. Future experiments with disruption of neurogenesis may determine the impact of GUO in cognitive function.

In summary, we showed that GUO treatment promotes cell proliferation and the generation of new neurons in hippocampal NSCs in vitro. Moreover, in adult mice, guanosine promotes hippocampal neurogenesis and an antidepressant-like effect, without altering parameters related to learning, memory, or anxiety-like behaviors. Although guanosine still does not have a clear molecular target identified, its neuroprotective effects are widely described [55]. Additionally, while guanosine may modulate extracellular adenosine levels to enhance neurogenesis in adults, it is a much safer drug without the undesirable peripheral side effects of adenosine [49]. Furthermore, the data presented here suggest that molecules that increase neurogenesis in adult-born neurons may be beneficial to treat depression.

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Compliance with Ethical Standards

Experiments followed the "Principles of Laboratory Animal Care" (NIH 2011) and were approved by the Committee on the Ethics of Animal Experiments of the Federal University of Santa Catarina (CEUA/UFSC 1454270417).

Conflict of Interest The authors declare that they have no conflict of interest.

Disclosures The financial support agencies had no further role in study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

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