Guanosine and GMP increase the number of granular cerebellar neurons in culture: dependence on adenosine A_{2A} and ionotropic glutamate receptors

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Abstract

The guanine-based purines (GBPs) have essential extracellular functions such as modulation of glutamatergic transmission and trophic effects on neurons and astrocytes. We previously showed that GBPs, such as guanosine-5'-monophosphate (GMP) or guanosine (GUO), promote the reorganization of extracellular matrix proteins in astrocytes, and increase the number of neurons in a neuron-astrocyte co-culture protocol. To delineate the molecular basis underlying these effects, we isolated cerebellar neurons in culture and treated them with a conditioned medium derived from astrocytes previously exposed to GUO or GMP (GBPs-ACM) or, directly, with GUO or GMP. Agreeing with the previous studies, there was an increase in the number of β -tubulin III-positive neurons in both conditions, compared with controls. Interestingly, the increase in the number of neurons in the neuronal cultures treated directly with GUO or GMP was more prominent, suggesting a direct interaction of GBPs on cerebellar neurons. To investigate this issue, we assessed the role of adenosine and glutamate receptors and related intracellular signaling pathways after GUO or GMP treatment. We found an involvement of A_{2A} adenosine receptors, ionotropic glutamate N-methyl-D-aspartate (NMDA), and non-NMDA receptors in the increased number of cerebellar neurons. The signaling pathways extracellular-regulated kinase (ERK), calcium-calmodulin-dependent kinase-II (CaMKII), protein kinase C (PKC), phosphatidylinositol-3'-kinase (PI3-K), and protein kinase A (PKA) are also potentially involved with GMP and GUO effect. Such results suggest that GMP and GUO, and molecules released in GBPs-ACM promote the survival or maturation of primary cerebellar neurons or both via interaction with adenosine and glutamate receptors.

Keywords Guanosine · GMP · Astrocytic-conditioned medium · Cerebellar granule neurons · Adenosine receptors · Glutamate receptors

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Introduction

The guanine-based purines (GBPs) such as nucleotide guanosine-5'-monophosphate (GMP) or nucleoside guanosine (GUO) exert relevant extracellular effects as intercellular messengers, neuromodulators in the central nervous system (CNS), and trophic effects on astrocytes and neurons [1–3]. GBPs are released by astrocytes in basal conditions or when exposed to hypoxic or low glucose environment [4]. The nucleotides are hydrolyzed by ectonucleotidases, forming extracellular guanosine that may confer neuroprotective effects [5]. These purines modulate astrocyte function by increasing growth factor release [6] and promoting a modulatory effect on the glutamatergic system [7, 8]. The GBPs function in neurons is not clear yet. Effects like those observed in astrocyte cultures were found in hippocampal slices [3], where GBPs may act in both astrocytes and neurons.

Currently, there is no identified site of guanosine interaction in the brain. The first studies evaluating extracellular GBPs effects showed they could displace glutamate binding from their ionotropic or metabotropic receptors [9]. Evidence from previous studies using hippocampal slices suggests that guanosine and other GBPs may interact with glutamate transporters [10], or through signaling pathway activation [11]. There is also evidence of guanosine function via activation of calcium-activated potassium channels [12], and both adenosine A₁ and A_{2A} receptors (A₁R and A_{2A}R) modulation [8, 13].

In cell lineages such as PC12 cells, GTP, or GUO treatment resulted in increased growth factor levels, and neurite outgrowth and arborization [14, 15]. Moreover, GUO treatment prevented neuroblastoma cells from glutamate toxicity [16] and mitochondrial dysfunction [17].

Primary cerebellar neurons in culture are a useful tool to study granular neuron proliferation [18] and glutamatergic function and toxicity [19]. GUO treatment in cerebellar neurons enhances neurite extension and growth to rescue the cells subjected to hypoxic conditions, resulting in less cell death [20].

We previously showed that GMP or GUO modulate extracellular matrix proteins (ECM) such as laminin and fibronectin organization and promote an increase in the number of cerebellar neurons observed in a neuron-astrocyte co-culture model [21]. Then, it was shown that in neuron-astrocyte co-culture, ECM proteins and astrocytes participate in cell-cell contact to provide trophic support [22, 23], contributing to the increase in the number of cerebellar neurons.

To investigate the astrocyte effect in neuronal cultures exposed to GMP and GUO treatment, we performed experiments individually with astrocytic-conditioning medium previously treated with GMP or GUO. In another set of experiments, neurons were treated directly with GMP or GUO to assess their trophic effects. Additionally, we investigated the

involvement of adenosine receptors and ionotropic glutamate receptors (iGluRs) in the increase in the number of cerebellar neurons induced by GMP or GUO. We also checked for the participation of signaling pathways involved in cell survival such as extracellular-regulated kinase (ERK), calcium-calmodulin-dependent kinase-II (CaMKII), protein kinase C (PKC), phosphatidylinositol-3'-kinase (PI3-K), and protein kinase A (PKA).

Materials and methods

Reagents

All chemicals listed below were purchased from Sigma. GMP; GUO; adenosine receptor antagonists: A₁R antagonist, DPCPX (1,3-dipropyl-8-cyclopentylxanthine); A_{2A}R antagonist, ZM24138 [4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol]; non-selective adenosine receptor antagonist, 8-PT (8-phenyltheophylline); glutamate receptor antagonists: non-competitive *N*-methyl-D-aspartate (NMDA) antagonist, MK-801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-iminemaleate]; non-NMDAR [kainate/(α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) AMPA] antagonist, GAMS (c-d-glutamylaminomethylsulphonic acid); inhibitor of nucleoside transporter, dipyridamole; signaling pathway inhibitors: mitogen-activated protein kinase (MAPK) extracellular signal regulated kinase kinase (MEK1)-specific inhibitor, PD98059; phosphatidylinositol-3 kinase (PI3-K)-selective inhibitor, Wortmannin; Ca²⁺-calmodulin-dependent protein kinase-II (CaMKII) selective inhibitor, KN-62; protein kinase C (PKC) selective inhibitor, chelerythrine; and protein kinase A (PKA) selective inhibitor, H-89.

Primary culture of astrocytes and conditioned medium derived from astrocytes (ACM)

All experiments were performed in accordance with the "Principles of laboratory animal care" (NIH 2011) and were approved by the local Ethical Committee of Animal Research (CEUA/UFSC-PP955). Primary culture of astrocytes was prepared from cerebella of postnatal day 1 to 3 Wistar rats (male and female) as previously described [21]. Cells were plated at a density of 1.5×10^5 cells onto glass cover slips/24-well plate or plastic culture flasks, previously coated with poly-ornithine (10 μ g/mL, Sigma) in DMEM-F12 medium supplemented with 10% fetal bovine serum (FBS) (Gibco). Cell cultures were incubated at 37 °C in a humidified 5% CO₂, 95% air atmosphere. Cell culture medium was changed 24 h after plating and subsequently every third day until reaching confluence, which usually occurs after 7–10 days in vitro (DIV).

For the preparation of ACM, confluent cultures of astrocytes were carefully and extensively washed with serum-free DMEM-F-12 medium, as previously described (Trentin et al., 1995). The astrocyte cultures were treated with concentrations of 1 μ M, 100 μ M, or 1 mM of GMP or GUO diluted in serum-free DMEM-F12 for 24 hours. After this time, the medium was changed to a fresh DMEM-F12 medium and ACM was harvested after 24 h of conditioning and clarified by centrifugation at 1500 $\times$ g for 10 min to use immediately or stored in aliquots at -20°C .

From now on we will use the term, GMP-ACM (astrocytic-conditioned medium) referring to medium collected from astrocyte cultures previously exposed for 24 h to GMP; GUO-ACM, referring to medium collected from astrocyte cultures previously exposed for 24 h to GUO; and ACM referring to medium collected from astrocyte cultures treated only with serum-free DMEM-F12 medium.

Primary culture of cerebellar neurons

Primary neurons were prepared from cerebella of 7-day-old Wistar rats (P7) as previously described [24]. Briefly, neurons were freshly dissociated from cerebella, and plated at a density of 5×10^4 cells onto glass cover slips/24-well plates previously coated with poly-ornithine (10 μ g/mL), in Neurobasal medium supplemented with B27 (Neurobasal/B27, Invitrogen). Neuronal cultures were maintained at 37°C in a humidified 5% CO_2 , 95% air atmosphere.

Neuronal cell treatment

After 24 h, neurons were carefully washed with serum-free Neurobasal/B27 medium and then treated with GBPs-ACM or directly with GBPs. In the first set of experiments, neurons were treated with GMP-ACM or GUO-ACM (in the concentrations described above, 1 μ M, 100 μ M, or 1 mM of GMP or GUO or control ACM) and maintained in culture for 24 h before analyzing cellular viability and neuritogenesis. In another set of experiments, neurons were incubated at final concentrations of 1 μ M, 100 μ M, or 1 mM of GMP or GUO diluted in serum-free Neurobasal/B27 medium for 24 h. Control cells were maintained in serum-free Neurobasal/B27 medium.

For evaluation of adenosinergic or glutamatergic system or intracellular signaling pathways, neurons were exposed to specific antagonists or inhibitors 30 min prior to GMP or GUO (100 μ M) treatment. The final concentration for each substance was DCPX 100 nM, ZM241385 50 nM, 8-PT 20 nM, MK-801 1 μ M, GAMS 100 μ M, dipyrindamole 10 μ M, PD98059 50 μ M, Wortmannin 1 μ M, KN-62 10 μ M, chelerythrine 10 μ M, and H-89 5 μ M.

Immunocytochemistry assay

The neuronal cerebellar cultures of each experimental setting were fixed in 4% paraformaldehyde for 30 min, permeabilized with cold 0.2% Triton X-100 for 15 min. Samples were blocked for 1 h at room temperature with blocking buffer containing 5% normal goat serum. Then, coverslips were washed three times with PBS (3 min) followed by incubation for 1 h with primary antibody against β -tubulin III (1:400, Millipore). Afterward, cells were washed again three times with PBS (5 min), followed by incubation at room temperature with a peroxidase-conjugated secondary antibody (1:200, Invitrogen). Negative controls were obtained by omitting primary antibodies; in all cases, no reactivity was observed. Neurons were imaged in phase-contrast by a system coupled to a Nikon microscope (Nikon Eclipse TE300). The number of neurons, neurites per neuron, and the neurite length were analyzed using the Software Sigma Scan Pro (Jandel Scientific, San Rafael, CA). Three independent experiments assayed in triplicates, at least 100 neurons from five randomly chosen fields (approximately 0.36 mm^2/field) per well, were analyzed. The data were stored, and morphometric analyses were carried out using the Microsoft Excel version 2003.

Hoechst 33342 staining

The cell number and nuclei morphology were determined with chromatin dye Hoechst 33342 staining. After treatment, cerebellar neurons were incubated with Hoechst 33342 (5 μ g/mL) in PBS for 30 min at 37°C and washed twice with PBS (pH 7.4). Images were captured under a fluorescence microscope $\times 60$ magnification (Nikon Eclipse TE300) in order to count cell number. To each treatment, Hoechst-labeled cells were calculated from five randomly chosen fields per well, from triplicates of three experiments ($n = 3$). For determination of apoptotic cells, the pyknotic nuclei were counted and calculated as a percentage of the total number of cells.

MTT assay

Cell viability was determined through MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay (Mosmann, 1983; Liu et al., 1997). The tetrazolium ring of MTT is cleaved by various dehydrogenases in active mitochondria and then precipitated as a blue formazan product. After the treatment, neurons were washed and incubated with MTT (0.5 mg/mL) for 20 min at 37°C . After the removal of the medium, the precipitated formazan was solubilized with dimethyl sulfoxide and viable cells were quantified spectrophotometrically at a wavelength of 550 nm.

Statistical analysis

For all experiments, values are means $\pm$ SEM (standard error of the mean) from at least three independent experiments carried out in triplicate. Statistical analyses were performed by using one-way or two-way analysis of variance (ANOVA) followed by Tukey's post hoc test with GraphPad and SPSS 10.0 software. Results were designated significant when the p value < 0.05 .

Results

The effect of GBPs-ACM, GMP, or GUO in the number of cerebellar neurons, nuclear morphology, and neuritogenesis

We previously reported that GMP or GUO treatment induces ECM protein reorganization and increase the number of neurons in a neuron-astrocyte co-culture [21]. In the present study, we used two different approaches to further investigate the trophic effects of GMP or GUO. (1) Cerebellar neurons were isolated and treated them with conditioned medium derived from astrocytes exposed to guanine-based purines (GBPs-ACM). Using this approach, we examined potential effects of soluble factors secreted by astrocytes, and excluded possible effects resulting from astrocyte juxtaposition to neurons in the mixed-cell cultures. (2) Cerebellar neurons were isolated and treated them directly with guanine-based purines, GMP or GUO.

There was an increase in the number of β -tubulin III-positive neurons when neurons were treated with 1 mM of GMP-ACM compared to control ACM ($p < 0.05$). The experiments in which GMP was applied directly in neurons resulted in a more significant increase in the number of β -tubulin III-positive neurons treated with 1 μ M ($p < 0.05$), 100 μ M ($p < 0.01$), or 1 mM ($p < 0.01$) of GMP compared to control (Fig. 1a, b). Moreover, the treatment with 100 μ M of GMP significantly ($p < 0.05$) increased the number of β -tubulin III-positive neurons when compared to the same concentrations of GMP-ACM.

Neurons treated with GUO-ACM had a significant increase in the number of β -tubulin III-positive neurons when treated with 100 mM ($p < 0.05$), or 1 mM ($p < 0.01$) of GUO-ACM compared to control ACM. The experiments in which GUO was applied directly in neurons showed a significant increase in the number of β -tubulin III-positive neurons treated with 1 μ M ($p < 0.001$), 100 μ M ($p < 0.001$), or 1 mM ($p < 0.01$) of GUO compared to control neurons (Fig. 1a, c). When compared to the same concentrations of GUO-ACM, direct GUO treatment with 1 μ M ($p < 0.01$) or 100 μ M ($p < 0.05$)

significantly increased the number of β -tubulin III-positive neurons. Representative images of Hoechst 33342 staining also confirmed the increase in the number of cells after GMP or GUO and GBPs-ACM treatment (Fig. 1d, panel).

The analyses of pyknotic cells showed no significant changes in the number of chromatin condensation/apoptosis with Hoechst 33342 staining following GMP or GUO treatment and GBPs-ACM experiments (Fig. 1e, f). Also, there were also no significant changes in the cell viability (measured by MTT assay, Fig. 1g, h).

We next examined whether GBPs-ACM and GMP or GUO promote neuritogenesis after 24 h of treatment and there was no alteration in the number of neurites per neuron, distribution of neurite length, or in total neurite length when compared with control cultures (Tables 1 and 2).

GMP or GUO modulates glutamate and adenosine receptors to increase the number of cells in culture

Next, we evaluated the role of glutamate and adenosine receptors in the increase of neurons induced by GMP or GUO treatment. To address this issue, antagonists of glutamatergic and adenosinergic receptors (Figs. 2 and 3, respectively) were added to the neuronal cultures 30 min prior exposure to GMP or GUO (100 μ M) treatment for 24 h.

MK-801 is a high affinity and selective non-competitive antagonist of NMDA glutamate receptor [25]. GAMS is a weak selective and competitive antagonist of kainate/AMPA glutamatergic receptors [26]. The blockade of subtypes of iGluRs, NMDA, and kainate/AMPA with MK-801

Fig. 1 Effect of GBPs-ACM or GMP and GUO on number and survival of cerebellar granule cells. Cerebellar neurons were treated with 1 μ M, 100 μ M, or 1 mM of GBPs-ACM, or GMP or GUO for 24 h. **a** Representative phase-contrast microscopy images with β -tubulin III immunostaining from 100 μ M treatments with GMP or GUO. (Upper panel) ACM refers to neuronal cells treated with the conditioned medium collected from astrocyte cultures treated only with serum-free DMEM/F12. (Lower panel) Control refers to neuronal cells incubated with serum-free Neurobasal. **b, c** The number of neurons was obtained from images of β -tubulin III-positive neurons from triplicates of three independent experiments ($N = 3$). Two-way ANOVA showed * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared with their own control (striped bar), # $p < 0.05$ and ## $p < 0.01$ when compared GMP to GMP-ACM or GUO to GUO-ACM-treated group. **d** Representative images of Hoechst 33342 staining showing an increase in the number of cells after treatment with GBPs. The left panel shows the neurons treated with GBPs-ACM compared to their own control, the ACM. The right panel shows the neurons treated with GBPs compared to their own control. **e, f** Percentage of pyknotic nuclei obtained from Hoechst 33342 staining for determination of apoptotic cells. There were no statistically significant differences between the groups as determined by one-way ANOVA (**e** [$p = 0.0900$] and **f** [$p = 0.7421$]). **g, h** Neuronal viability compared to controls as determined by MTT assay. There were no statistically significant differences between the groups as determined by one-way ANOVA (**g** [$p = 0.9904$] and **h** [$p = 0.7163$]). Scale bar = 20 μ m

and GAMS, respectively, abrogates the GMP and GUO effect (Fig. 2).

Regarding adenosine receptors, only the inhibition of $A_{2A}R$ (with ZM241385, selective adenosine A_{2A} receptor antagonist) blocked the GMP and GUO effect (Fig. 3). No effect was observed with a non-selective (8-PT) antagonist of adenosine receptors, and a selective A_1R

antagonist (DPCPX). Besides, dipyrindamole, a potent nucleoside transporter inhibitor, did not alter GMP or GUO effects, indicating that both GMP and GUO had an extracellular effect of modulating these receptors. The glutamate and adenosine receptor antagonist treatment showed no effect when administered alone (panels of Figs. 2 and 3).

Fig. 1 (continued)

Signaling pathways involved in the effects of GMP or GUO

To investigate the signaling mechanism underlying the GMP and GUO effects on cell number, protein kinase inhibitors were applied to the cerebellar neuronal cultures 30 min before the GMP or GUO (100 μM) treatment of 24 h. Protein kinase inhibitors used in this study were PD98059 (a specific inhibitor of the activation of mitogen-activated protein kinase

kinase (MAPKK), 50 μM), Wortmannin (a potent and specific PI3-K inhibitor 1 μM), KN-62 (a selective inhibitor of rat brain CaMKII, 10 μM), chelerythrine (a selective antagonist of PKC, 10 μM), or H89 (a selective inhibitor of PKA, 5 μM).

The treatment with inhibitors blocked the increase in the number of neurons induced by GMP or GUO (Fig. 4). The inhibitor treatment showed no effect when administered alone (panel of Fig. 4), except H89, which showed no difference as compared to control and compared to GMP or GUO.

Table 1 Effect of GBP-ACM on neuritogenesis. Cerebellar neurons were exposed to 100 μM of GBP-ACM for 24 h. Neurons were immunoassayed with β-tubulin III and length of neurites and the number of neurites per neuron was obtained using Sigma Scan Pro Software (Jandel Scientific). There were no statistically significant differences among the groups as determined by one-way ANOVA

ACM					
Neurite length distribution (μm)	Control	GMP 1 μM	100 μM	GUO 1 μM	100 μM
% neurons					
0–50	32.7 ± 14.0	45.3 ± 10.0	47.9 ± 1.0	53.7 ± 5.0	53.3 ± 4.0
50–100	38.4 ± 2.0	25.3 ± 0.3	35.9 ± 3.0	29.4 ± 4.0	31.9 ± 2.0
100–200	22.7 ± 6.0	25.3 ± 7.0	14.7 ± 0.5	16.6 ± 0.2	14.8 ± 6.0
> 200	6.3 ± 6.0	4.3 ± 3.0	1.7 ± 1.0	0.4 ± 0.4	0.0 ± 0.0
Total neurite length (μm)	71.1 ± 18.6	58.9 ± 10.2	51.5 ± 0.4	40.82 ± 5.9	41.0 ± 3.5
Neurites/neuron	Control	GMP 1 μM	100 μM	GUO 1 μM	100 μM
% neurons					
0	22.5 ± 2.0	27.0 ± 4.0	19.4 ± 2.0	28.6 ± 7.0	17.6 ± 4.0
1	70.6 ± 4.0	66.8 ± 5.0	67.8 ± 3.0	70.7 ± 9.0	68.8 ± 3.0
2	7.3 ± 0.3	8.4 ± 1.0	12.8 ± 0.9	7.4 ± 2.0	9.6 ± 1.0
3 and +	1.9 ± 0.7	1.7 ± 2.0	2.4 ± 2.0	0.5 ± 0.5	0.4 ± 0.4

Table 2 Effect of GMP or GUO on neuritogenesis. Cerebellar neurons were exposed to 100 μ M of GMP or GUO for 24 h. Neurons were immunoassayed with β -tubulin III and length of neurites and the number

of neurites per neuron was obtained using Sigma Scan Pro Software (Jandel Scientific). There were no statistically significant differences among the groups as determined by one-way ANOVA

Neurite length distribution (μ m)	Control	GMP			GUO		
		1 μ M	100 μ M	1 mM	1 μ M	100 μ M	1 mM
% neurons							
0–50	49.4 $\pm$ 14.0	70.9 $\pm$ 7.0	69.7 $\pm$ 13.0	80.3 $\pm$ 6.0	73.5 $\pm$ 10.0	71.0 $\pm$ 5.0	72.7 $\pm$ 10.0
50–100	39.61 $\pm$ 10.0	25.2 $\pm$ 6.0	19.6 $\pm$ 5.0	16.0 $\pm$ 4.0	21.6 $\pm$ 8.0	23.6 $\pm$ 3.0	22.1 $\pm$ 7.0
100–200	8.6 $\pm$ 3.0	4.0 $\pm$ 5.0	10.2 $\pm$ 7.0	3.7 $\pm$ 2.0	4.3 $\pm$ 2.0	4.3 $\pm$ 2.0	4.8 $\pm$ 3.0
> 200	2.1 $\pm$ 1.1	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Total neurite length (μ m)	34.5 $\pm$ 11.6	29.4 $\pm$ 5.1	24.5 $\pm$ 2.4	27.5 $\pm$ 8.1	22.4 $\pm$ 5.6	23.7 $\pm$ 6.7	24.4 $\pm$ 7.4
Neurites/neuron	Control	GMP			GUO		
		1 μ M	100 μ M	1 mM	1 μ M	100 μ M	1 mM
% neurons							
0	33.5 $\pm$ 6.0	28.0 $\pm$ 2.0	27.8 $\pm$ 2.0	25.7 $\pm$ 1.0	38.5 $\pm$ 0.3	39.8 $\pm$ 12.0	31.0 $\pm$ 2.0
1	57.8 $\pm$ 4.0	73.0 $\pm$ 8.0	67.4 $\pm$ 4.0	74.4 $\pm$ 9.0	59.7 $\pm$ 6.0	63.2 $\pm$ 11.0	71.0 $\pm$ 5.0
2	12.6 $\pm$ 8.0	4.6 $\pm$ 2.0	8.2 $\pm$ 4.0	7.4 $\pm$ 3.0	7.7 $\pm$ 3.0	4.6 $\pm$ 1.0	4.7 $\pm$ 2.0
3 and +	1.2 $\pm$ 6.0	3.8 $\pm$ 2.0	5.9 $\pm$ 4.0	1.0 $\pm$ 1.0	7.2 $\pm$ 5.0	5.6 $\pm$ 3.0	3.8 $\pm$ 4.0

Discussion

In the present study, we aimed to address the effect of GBPs and participation of astrocytic-conditioned medium on

maintenance and survival of cerebellar neurons in culture. The cerebellar cell culture is a versatile model to study neuronal proliferation, apoptosis, axonal, and dendrite development, among others [27]. Treatments with GBPs-ACM,

Fig. 2 Effect of glutamatergic antagonists in cerebellar granule neuronal cells treated with GMP or GUO. Cerebellar neurons were pre-incubated with glutamate receptors antagonists for 30 min and treated with 100 mM of GMP or GUO for an additional 24 h. Neurons were stained with Hoechst 33342. (Left) The average number of nuclei per field (five randomly chosen fields) from triplicates of three independent experiments

($N=3$). (Right) Representative images of Hoechst 33342 staining from neurons in the absence (a) or presence of GMP (b), GUO (f) treatment, and antagonists: MK-801 (NMDA receptor antagonist, 1 mM, c, d, e); GAMS (KA receptor antagonist, 100 mM, g, h, i). Two-way ANOVA showed *** $p < 0.001$ when compared with the control, # $p < 0.05$ and ## $p < 0.01$ compared to GMP or GUO-treated group. Scale bar = 20 μ m

Fig. 3 Effect of adenosine receptor antagonists in cerebellar granule neuronal cells treated with GMP or GUO. Cerebellar neurons were pre-incubated with adenosine receptor antagonists for 30 min and treated with 100 μ M of GMP or GUO for additional 24 h. Neurons were stained with Hoechst 33342. (Left) The average number of nuclei per field (five randomly chosen fields) from triplicates of three independent experiments ($N=3$). (Right) Representative images of Hoechst 33342 staining from

neurons in the absence (Control) or presence (GMP or GUO) of treatment and antagonists: 8-PT (a non-selective adenosine receptor antagonist, 20 nM); ZM (ZM241385, a selective A_{2A} R antagonist, 50 nM); DPCPX (a selective A_1 R antagonist, 100 nM); DIP (dipyridamole, an inhibitor of nucleoside transporter, 10 μ M). Two-way ANOVA showed ** $p < 0.01$ when compared with the control, ## $p < 0.01$ compared to GMP or GUO-treated group. Scale bar = 20 μ m

Fig. 4 Effect of signaling pathway inhibitors in cerebellar granule neuronal cells treated with GMP or GUO. Cerebellar neurons were pre-incubated with different protein kinase inhibitors for 30 min and then treated with 100 μ M of GMP or GUO for additional 24 h. Neurons were stained with Hoechst 33342. (Left) The average number of nuclei per field (five randomly chosen fields) from triplicates of three independent experiments ($N=3$). (Right) Representative images of Hoechst 33342

staining from neurons in the absence (Control) or presence (GMP or GUO) of treatment and inhibitors: PD (PD98059, a MEK/ERK inhibitor, 50 μ M); WORT (Wortmannin, a PI3K inhibitor, 1 μ M); KN (KN-62, a CaMKII inhibitor, 10 μ M); CHEL (chelerythrine, a PKC inhibitor, 10 μ M); H89 (a PKA inhibitor, 5 μ M). Two-way ANOVA showed *** $p < 0.001$ when compared with the control, ## $p < 0.01$ and #### $p < 0.001$ compared to GMP or GUO-treated group. Scale bar = 20 μ m

Fig. 5 Schematic diagram for proposed neurotrophic mechanism involved in the increase of cerebellar granule neurons number after GBP or GBP-ACM treatment. (1) The GBP-ACM may contain soluble factors which are important to neurons growth and development. (2) GMP or GUO may interact with A_{2A}R and/or iGluRs. GMP or GUO effects via A_{2A}R interaction and downstream signaling pathways (MEK/ERK, PKC, PKA) activation may modulate the release of trophic levels of glutamate, which is beneficial to neurons in cell culture. The blockade of iGluRs or A_{2A}R prevented the beneficial effect of GMP or GUO on increasing the number of neurons. Filled arrows represent the

results observed in this study. Traced arrows represent the hypothesis to GBPs interaction with A_{2A}R and/or iGluRs. ACM, astrocytic-conditioned medium; A_{2A}R, adenosine A_{2A} receptors; CaMKII, calcium-calmodulin dependent kinase-II; ERK, extracellular-regulated kinase; GBPs-ACM, guanine-based purines-astrocytic-conditioned medium; GMP, guanosine-5'-monophosphate; GPCR, G-protein-coupled receptors; GUO, Guanosine; iGluR, ionotropic glutamate receptors; PI3-K, phosphatidylinositol-3'-kinase; PKA, protein kinase A; PKC, protein kinase C

GMP, or GUO increase the number of cerebellar neurons in culture. However, no differences were found in the number of neurites *per* neuron and distribution of neurites after treatment with GMP, GUO, or GBPs-ACM. Additionally, we showed the involvement of NMDA and non-NMDA subtypes of iGluRs, and the adenosine A_{2A}R in the increase of cerebellar neurons induced by GMP or GUO. It was also possible to identify the involvement of the main signaling pathways involved in cell survival and proliferation underlying the effect of GMP and GUO treatment.

The use of GBPs-ACM in the cultured cerebellar neurons in the present study has allowed the evaluation of astrocyte-released factors rather than changes due to cell-cell contact for providing the trophic support. The ACM contains soluble and insoluble factors that have some regulatory effects on neurons [28]. In the present study, there was an increase in the number of cerebellar neurons exposed to ACM-GMP and ACM-GUO compared to only ACM treatment. Guanine-based purine treatment on astrocytes may trigger the production of soluble factors, membrane-bound proteins, and peptides that promote neuronal survival in the ACM-treated cells. For instance, there is evidence from PC-12 cells, demonstrating that GTP and GUO activate synthesis and release of neurotrophic factors, as FGF-2 and NGF [14]. Our data suggest that GMP and GUO improve astrocytic-conditioned medium to support the survival of the neurons, although the identity of the factors has remained elusive. Ongoing studies on proteomic analysis of

astrocyte-secreted proteins after guanosine treatment will help to elucidate some specific proteins that may mediate these functions.

Interestingly, direct treatment of neurons with GMP or GUO promoted an increased trophic effect when compared to GBPs-ACM treatment. One caveat of this study is that neuronal cells from astrocytic-conditioned medium received DMEM-F12 and the neurons directly treated with GBPs received Neurobasal medium, which optimizes the survival of primary rat neurons in culture [29]. It may result in less pyknotic cells as shown in Fig. 1e, f). The experiments also raised the hypothesis these purines could have molecular targets in neuronal cells. Still, there is still no identification of a selective site to GBPs in the brain. However, it has been postulated that GBPs effects are mediated through G protein-dependent signaling pathways since GUO effects on cell viability in hippocampal slices are prevented by Pertussis toxin-mediated inhibition of Gi/Go-proteins [8]. Some studies showed the presence of a single high-affinity binding site for [³H]GUO in rat brain membranes that did not involve receptors for adenosine [30, 31]. Moreover, a G protein-coupled receptor to GUO had been proposed [32], although it was not fully characterized or cloned.

Regarding a putative neuritogenic effect of GBPs, in the cerebellar neuronal cultures treated for 24 h with GBPs-ACM or directly with GMP or GUO, no effect was observed in any analyzed parameter (neurite length or distribution, and the

number of neurites per neuron). Similarly, in our previous study with cerebellar neuron-astrocyte co-cultures, no neurotogenic effect was observed [21]. However, critical studies showed GBPs (per se, or in association with trophic factors) induced neurite arborization outgrowth in PC12 cells [14, 33, 34], suggesting this effect would be more prone to happen in a more undifferentiated cell.

In this study, inhibition of iGluRs (NMDA and kainate/AMPA receptors) prevented the effect of GUO and GMP on the number of cerebellar neurons. Most of glutamate receptor subunits and subtypes are expressed in primary neuronal cell cultures similarly occurring in the developing rat brain, and low levels of extracellular glutamate exert trophic effects on neurons [35]. Some GBPs can inhibit glutamate binding at purified neuronal postsynaptic membrane preparations that are strong evidence of GBPs effect on glutamatergic receptors [36]. Moreover, GMP inhibits [³H]kainate binding in lysed membrane preparations from chick cerebellum [37]. Therefore, these studies suggest that GBPs may strengthen the inhibition of glutamatergic receptor together with iGluRs antagonists, MK-801, and GAMS, resulting in the blockade of effect of glutamatergic receptors in the cells [38]. However, the effects observed here may not be only a simple glutamate receptor blockade mechanism, as we and others previously demonstrated that GBP might also modulate adenosine receptor-induced signaling [17, 39].

Because of the molecular similarity between GUO and adenosine, some studies have investigated the potential effect of GBPs on adenosine receptors. Previous evidence from our laboratory indicated that GUO may act through both adenosine A₁R and A_{2A}R interaction [8, 13]. Considering the possibility of formation of A₁R-A_{2A}R oligomers and their role in regulating glutamatergic transmission, it is also suggested that GUO may interact with A₁R-A_{2A}R oligomers [40, 41]. Apparently when exerting neuroprotection, both A₁R and A_{2A}R are required to trigger GUO action in the human neuroblastoma cell line (SH-SY5Y) subjected to mitochondrial oxidative stress [17]. Conversely, in hippocampal slices subjected to oxygen-glucose deprivation, the A₁R, but not A_{2A}R, has shown to mediate the neuroprotective effect of GUO [8]. A similar effect occurs in cerebellar neurons exposed to hypoxia where A₁R mediates neuroprotection [42].

In the present study, only the blockade of A_{2A}R interferes with the effect of GMP and GUO to increase the number of neurons in normal conditions. A_{2A}R are G protein-coupled receptors (GPCR) expressed in the brain and cell culture of cerebellar neurons [42]. A_{2A}R appears to affect neurotransmitter release, as the A_{2A}R agonist, CGS21680, increases glutamate release through PKA activation in cultured cells from medulla oblongata [43]. There is also evidence of A_{2A}R activation stimulating neurotrophic factors such as BDNF expression and secretion which is associated with cell survival, neurite outgrowth, and regeneration in cultured neurons [44].

We also observed that both GMP and GUO effects are associated with signaling pathways, increasing the hypothesis that these effects are mediated through the binding to unknown GPCRs or adenosine receptors. Here, it was found the direct effect of GMP and GUO on cell number is inhibited by signaling pathway inhibitor treatment such as MEK/ERK, PI3K, CaMKII, and PKC. The Ras-ERK and PI3K are signaling pathways coordinated by extracellular and intracellular cues to control cell survival, proliferation, motility, and metabolism [45]. Our results have been confirmed by previous studies using cell lines, showing that GBPs can elicit second messenger action. The inhibition of MEK/ERK, PI3K, and PKC pathway abolishes GUO-promoting cell survival, in the human neuroblastoma cell line [17], in C6 glioma cells [46], and microglia cell cultures [47]. Similar results of GUO suggesting activation of calcium-dependent K⁺ channel, promoting PKC, MEK, PI3-K, or PKA activation, were found in more complex models such as hippocampal slices [12, 48], and in vivo effects [49, 50]. Still, it is necessary to understand how these signaling pathways interact with each other to induce an increase in neuronal number. Considering these pathway organization, it is possible to speculate PI3K and MEK/ERK may be upstream of CaMKII, PKC, and PKA that could modulate trophic factors related to neuronal maintenance in culture and survival. Thus, most of the protective effects of guanosine depend on PI3K modulation [3].

Results of the blockade of iGluRs, A_{2A}R, and signaling pathways together with previous results of adenosine receptor modulation of glutamate release suggest that GMP and/or GUO interaction with A_{2A}R may be responsible for releasing trophic levels of glutamate that activates iGluR and maintain beneficial effects to neurons in culture. This hypothesis is presented in Fig. 5.

In conclusion, our data indicate that GBPs-ACM or direct treatment with GMP or GUO increases the number of cerebellar neurons in culture, thereby promoting neuronal maintenance without altering neuronal morphology. Inhibition of NMDA or AMPA/kainate receptors, A_{2A}R, and signaling pathways such as PI3K, MAPK/ERK, CaMKII, PKC, and PKA blocked the positive effects of GBPs treatment in neurons. This evidence suggests GBPs may control cell number and maintenance by modulation of iGluRs, and A_{2A}R, and triggering survival signaling pathways.

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Compliance with ethical standards

The procedures used in this study complied with the guidelines on animal care of the UFSC Ethics Committee on the Use of Animals (CEUA), which follow the Principles of laboratory animal care from NIH (2011).

Conflicts of interest Helena Decker declares that she has no conflict of interest.

Tetsade C. B. Piermartiri declares that she has no conflict of interest.
Cláudia B. Nedel declares that she has no conflict of interest.
Luciana F. Romão declares that she has no conflict of interest.
Sheila S. Francisco declares that she has no conflict of interest.
Tharine Dal-Cim declares that she has no conflict of interest.
Carina R. Boeck declares that she has no conflict of interest.
Vivaldo Moura-Neto declares that he has no conflict of interest.
Carla I. Tasca declares that she has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Abbreviations *ACM*, Astrocytic-conditioned medium; *A₁R*, Adenosine A₁ receptors; *A_{2A}R*, Adenosine A_{2A} receptors; *AMPA*, α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; *CaMKII*, Calcium-calmodulin dependent kinase-II; *Chel*, Chelerythrine; *DIP*, Dipyrindamole; *DPCPX*, 1,3-Dipropyl-8-cyclopentylxanthine; *ECM*, Extracellular matrix proteins; *ERK*, Extracellular-regulated kinase; *GAMS*, c-d-Glutamylaminomethylsulphonic acid; *GBPs-ACM*, Guanine-based purines-astrocytic-conditioned medium; *GMP*, Guanosine-5'-monophosphate; *GUO*, Guanosine; *iGluRs*, Ionotropic glutamate receptors; *MK-801*, (+)-5-Methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-iminemaleate; *MTT*, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; *NMDA*, N-methyl-D-aspartate; *8-PT*, 8-Phenyltheophylline; *PI3-K*, Phosphatidylinositol-3'-kinase; *PKA*, Protein kinase A; *PKC*, Protein kinase C; *Wort*, Wortmannin; *ZM24138*, 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol

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