Effects of 3 weeks GMP oral administration on glutamatergic parameters in mice neocortex

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Abstract Overstimulation of the glutamatergic system (excitotoxicity) is involved in various acute and chronic brain diseases. Several studies support the hypothesis that guanosine-5'-monophosphate (GMP) can modulate glutamatergic neurotransmission. The aim of this study was to evaluate the effects of chronically administered GMP on brain cortical glutamatergic parameters in mice. Additionally, we investigated the neuroprotective potential of the GMP treatment submitting cortical brain slices to oxygen and glucose deprivation (OGD). Moreover, measurements of the cerebrospinal fluid (CSF) purine levels were performed after the treatment. Mice received an oral administration of saline or GMP during 3 weeks. GMP significantly decreases the cortical brain glutamate binding

and uptake. Accordingly, GMP reduced the immunocontent of the glutamate receptors subunits, NR2A/B and GluR1 (NMDA and AMPA receptors, respectively) and glutamate transporters EAAC1 and GLT1. GMP treatment significantly reduced the immunocontent of PSD-95 while did not affect the content of Snap 25, GLAST and GFAP. Moreover, GMP treatment increased the resistance of neocortex to OGD insult. The chronic GMP administration increased the CSF levels of GMP and its metabolites. Altogether, these findings suggest a potential modulatory role of GMP on neocortex glutamatergic system by promoting functional and plastic changes associated to more resistance of mice neocortex against an in vitro excitotoxicity event.

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Abbreviations

GMP	Guanosine-5'-monophosphate
OGD	Oxygen and glucose deprivation
CSF	Cerebrospinal fluid
GFAP	Glial fibrillary acid protein
Snap 25	Synaptosomal-associated protein 25
NMDA	<i>N</i> -Methyl-D-aspartic
NR2A/B	Subunit 2A/B of <i>N</i> -methyl-D-aspartic receptor
AMPA	(2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid)
GluR1	Subunit 1 of AMPA receptor
PSD-95	Postsynaptic density protein 95
EAAC1	Excitatory amino acid carrier 1
GLAST	Glutamate/aspartate transporter

GLT-1	Glutamate transporter-1
GBPs	Guanine-based purines
HBSS	Hank's buffered salt solution
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
GTP	Guanosine-5'-triphosphate
GDP	Guanosine-5'-diphosphate
GUO	Guanosine
XAN	Xanthine
UA	Uric acid

Introduction

Glutamate is the main excitatory neurotransmitter in mammalian central nervous system. It acts via ionotropic (ligand-gated ion channel) or metabotropic (coupled to G proteins) receptors, modulating several essential brain processes such as learning and memory, pain, ontogeny, development, and aging [1–4].

However, overstimulation of the glutamatergic system, which occurs when glutamate levels in the synaptic cleft increase over the physiological range (excitotoxicity), is involved in various acute and chronic brain diseases including neurodegenerative diseases, traumatic brain injury, cerebral ischemia, and seizures [1, 2, 5, 6]. There are strong evidences pointing that glutamatergic excitotoxicity may be prevented by astrocytic glutamate uptake, a process responsible for maintaining the extracellular glutamate below toxic levels [7, 8]. In fact, modulators of glutamatergic system homeostasis have then been proposed as a potential therapeutic tool to attenuate excitotoxicity.

Several studies support the hypothesis that guanosine-5'-monophosphate (GMP) can modulate glutamatergic neurotransmission. It has been reported that GMP is able to inhibit the binding of glutamate and its analogs to brain membrane preparations [9–16], to prevent cell responses to glutamate [17–25], and to stimulate glutamate uptake by astrocytes [26].

The neuroprotective potential of GMP against excitotoxicity has also been reported both by in vitro as by in vivo experiments. Hippocampal slices submitted to oxygen and glucose deprivation (OGD) followed by reperfusion have their viability restored when GMP is added to the incubation medium [27–29]. Additionally, GMP prevented seizures and striatal neuronal damage promoted by quinolinic acid, a hyper stimulator of the glutamatergic system [30–35].

The aim of this study was to evaluate, for the first time, the effects of chronic (3 weeks ad libitum) orally administered GMP on brain cortical glutamatergic parameters in mice. We also investigated the neuroprotective potential of the GMP treatment by submitting brain cortical

slices to OGD. In addition, we aimed to measure the concentration of purines levels in the cerebrospinal fluid (CSF) after the administration.

Materials and methods

Reagents

GMP, *N*-methyl-D-glucamine, bovine serum albumin, protease and phosphatase inhibitor cocktail and antibodies against glial fibrillary acid protein (GFAP), Snap 25, and Actin were from Sigma Chemicals. Antibody against NR2 A/B (*N*-methyl-D-aspartic receptor subunit) was from Chemicon; antibodies against subunit 1 of 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid (AMPA) receptor (GluR1; AMPA receptor subunit) was from UpState; antibody against postsynaptic density protein 95 (PSD-95) was from Affinity BioReagents; antibody against excitatory amino acid carrier 1 (EAAC1) was from Alpha Diagnostic; antibodies against glutamate/aspartate transporter (GLAST; batch Ab#314) and glutamate transporter-1 (GLT-1; batch Ab#360) were from the same preparations as previously published [36]. The horseradish peroxidase-conjugated secondary antibody against rabbit and mouse, and the chemiluminescence (ECL) were from Amersham Pharmacia Biotech. X-ray films were from Kodak X-Omat, Rochester, NY, USA. L-[³H] glutamate (specific activity 30 Ci/mmol) was from Amersham International, UK. The anesthetic sodium thiopental was from Cristália (Itapira, SP, Brazil). All other chemicals were of analytical grade or higher.

Animals

Male adult Swiss albino mice (3–4 months of age, 30–40 g) were kept on a 12 h light/dark cycle (light on at 7:00 am) at temperature of 22±1°C, housed in plastic cages (four per cage) with commercial food ad libitum. All procedures were carried out according to the Brazilian Society for Neuroscience and Behavior's recommendations for animal care and the US National Institutes of Health guide for the care and use of laboratory animals, designed to minimize the suffering and the number of animals used. All behavioral procedures were conducted between 3:00 and 6:00 PM.

Treatment

The mice received water (control group) or GMP solution (1.5 mg/ml) for 3 weeks ad libitum from the bottle water. The GMP dose and time used was based on a previous pilot study, which indicated that GMP administration for 3 weeks

with a lower (0.5 mg/mL) or a higher (3 mg/mL) dose, or only during 1 week, had none or the same effect of the dose here used, respectively, on glutamate uptake and on in vitro viability of neocortical slices against OGD (data not shown). The liquid consumption was monitored every 2 days.

Slice and tissue preparation

The GMP effects were evaluated on brain cortical preparations based on previous studies indicating that this brain structure is responsive to guanine-based purines (GBPs) [37, 38].

Briefly after the administration, the animals were decapitated, their brains were immediately removed and the cerebral cortices were dissected onto Petri dishes. Cortices from both brain hemispheres from each animal were used. The right one was microslided and the left one was wholly frozen at -20°C in 25 mM Hepes buffer (pH 7.4), containing 0.1% sodium dodecyl sulfate (SDS) and a protease and phosphatase inhibitor cocktail.

The cortical coronal slices (0.4 mm) from the right parietal area were obtained using a McIlwain tissue chopper as previously described [38]. The slices were used for glutamate uptake or OGD assays.

The intact left cortices were used up to 1 month after decapitation to isolate plasma membrane or to analyze the immunocontent of specific proteins.

The animals were randomly assigned to the experiments.

Glutamate uptake

Glutamate uptake was assessed as fully detailed elsewhere [38]. From each animal, six slices were used. The cortical slices were separated in two equal sets, placed into separated 24-well culture plates; one plate was maintained at 37°C and the other on ice for evaluation of total and Na^{+} -independent glutamate uptake, respectively, with Hank's buffered salt solution (HBSS) containing (in mM): 137 NaCl, 0.63 Na_2HPO_4 , 4.17 NaHCO_3 , 5.36 KCl, 0.44 KH_2PO_4 , 1.26 CaCl_2 , 0.41 MgSO_4 , 0.49 MgCl_2 , and 5.5 glucose (pH 7.2). For the total glutamate uptake, the slices were washed once with 1 mL of 37°C HBSS and then pre-incubated at 37°C for 15 min followed by the addition of 0.33 Ci/mL L- ^3H glutamate and 100 μM (final concentration) glutamate. Incubation was stopped after 7 min with two ice-cold washes of 1 mL HBSS, immediately followed by the addition of 0.5 N NaOH, which were then kept overnight. Na^{+} -independent uptake was measured using the same protocol described above, with differences in the temperature (4°C) and medium composition (*N*-methyl-D-glucamine instead of sodium chloride). Results (Na^{+} -dependent uptake) were considered as the difference between both uptakes. The incorporated

radioactivity was measured in a Wallac 1409 liquid scintillation counter.

[^3H] Glutamate binding to brain plasma membranes

Brain plasma membrane preparations Plasma membrane preparations were carried out as previously described [39]. The frozen isolated parietal cortices were thawed at room temperature and all subsequent isolation procedures were performed at 4°C . The cortices were homogenized in 0.32 M sucrose (10% w/v) and centrifuged twice at $1,000\times g$; both supernatants were joined and centrifuged at $11,000\times g$ for 20 min. The obtained pellet was osmotically lysed by resuspension in 5 mM Tris/HCl pH 7.4 for 30 min. This resuspended pellet was centrifuged at $60,000\times g$ for 120 min in a sucrose density gradient (49%, 28.5%, and 10%). The plasma membranes were collected from the interface between 49% and 28.5% sucrose layers which were then washed twice in Tris/HCl buffer at $18,000\times g$ for 15 min.

[^3H] Glutamate binding protocol The binding assay of [^3H] glutamate was performed at 35°C in small polycarbonate tubes (total incubation volume 500 μL) containing 10 mM Tris/HCl pH 7.4 and 40 nM [^3H] glutamate [39]. Incubation was started by the addition of plasma membrane preparation (50 μg protein). After 30 min, tubes were centrifuged at $16,000\times g$ for 10 min at 4°C . The supernatant was discarded and the walls of the tubes and the surface of the pellets were quickly and carefully rinsed with cold distilled water. The pellets were solubilized with 0.3 mL of NaOH 0.1 M overnight. Bound radioactivity was measured by using a Wallac scintillation counter. Unspecific binding (10–20% of the total binding) was determined by adding 200 μM nonradioactive glutamate to the medium in a parallel assay. Specific binding was considered as the difference between total binding and unspecific binding. Experiments were performed in triplicate.

Western blotting proteins analysis

For Western blotting analysis, the frozen isolated parietal cortices were thawed at room temperature and then homogenized in 25 mM Hepes buffer (pH 7.4) containing 0.1% SDS and a protease and phosphatase inhibitors cocktail and normalized with a sample buffer (4% sodium dodecylsulfate, 2.1 mM EDTA, 50 mM Tris buffer (pH 7.4), and 5% β -mercaptoethanol). For each protein evaluated, a concentration curve (5–50 μg) was previously made in the same gel in order to determine the amount of protein to be used in the experiments; accordingly, samples (30 μg protein/well) with the exception for glutamate transporters (20 μg protein/well), were subjected to electrophoresis and

transferred to a nitrocellulose membrane. Membranes were processed as follows: (1) blocking with 5% bovine serum albumin for 2 h, (2) incubation with primary antibody overnight, (3) incubation with horseradish peroxidase conjugated secondary antibody for 2 h, (4) chemiluminescence (ECL) was detected using X-ray films. Protein loading was controlled by additionally staining blots with β -actin antibodies. The films were scanned and bands intensity was analyzed using ImageJ (developed at the US National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>).

Oxygen and glucose deprivation

Six slices were used from each animal. The cortical slices were separated in two equal sets (control and OGD group), placed into separated 24-well culture plates, and pre-incubated for 30 min at 37°C in the modified Krebs–Henseleit solution containing (in mM): 137 NaCl, 0.63 Na₂HPO₄, 4.17 NaHCO₃, 5.36 KCl, 0.44 KH₂PO₄, 1.26 CaCl₂, 0.41 MgSO₄, 0.49 MgCl₂, 25 HEPES buffer, and 5.5 glucose (pH 7.2). After preincubation, the medium in the control plate was replaced with a new modified Krebs–Henseleit solution and the plate was maintained at 37°C for 60 min. For ischemic conditions, the slices were exposed to a model of OGD, as previously described [40] with some modifications [41]. Briefly, after pre-incubation, OGD slices were washed twice with the modified Krebs–Henseleit glucose-free solution and incubated for 60 min (OGD period) at 37°C in a chamber saturated with nitrogen.

After the 60-min period, the media from both control and OGD slices were replaced by incubation solution (with glucose) and cellular viability was immediately verified. The period of 60 min of ischemic insult was chosen after a time curve of OGD evaluating cell viability by the 3(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (data not shown).

Analysis of cellular viability (MTT assay) Cellular viability assay was performed by colorimetric MTT method. After the 60-min period, both control and OGD slices were incubated with 0.5 mg/mL of MTT at 37°C for 20 min. The formazan product generated during the incubation was solubilized in dimethyl sulfoxide and measured at 560 and 630 nm. Only viable cells are able to reduce MTT.

CSF purine levels measurement

CSF sampling

For CSF purines concentration measurement, we used specific mice not used in the others experiments. Mice

were anesthetized with sodium thiopental (60 mg/kg, 10 ml/kg, i.p.) and placed in a stereotaxic apparatus, where the CSF was drawn (10–20 μ l per mouse), with the help of a magnifying glass, by direct puncture of the cisterna magna with an insulin syringe (27 gauge $\times$ 1/2 in length). All samples were centrifuged at 10,000 $\times$ g in an Eppendorf centrifuge for 10 min at 4°C and the supernatant was frozen at –70°C until analysis.

High-performance liquid chromatography procedure

After being thawed at room temperature, CSF aliquots were centrifuged at 1,000 $\times$ g for 5 min for obtaining cell-free supernatants. High-performance liquid chromatography (HPLC) was used for determination of purines concentration [42]. CSF concentrations of the following compounds were determined: guanosine-5'-triphosphate (GTP), guanosine-5'-diphosphate (GDP), GMP, guanosine (GUO), xanthine (XAN), and uric acid (UA). Analyses were performed with Shimadzu class-VP chromatography system consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Shimadzu SIL-10AF auto injector valve with 50 μ l loop and a 254-nm UV detector. Separations were achieved on a Supelco C18 250 $\times$ 4.6 mm, 5 μ m particle size columns. The mobile phase flowed at a rate of 1.2 mL/min and the column temperature was 24°C. Buffers composition remained unchanged (A: 150 mmol/l phosphate buffer, pH 6.0, containing 150 mmol/L KCl; B: buffer A with 15% acetonitrile). The gradient profile was modified to the following content of buffer B in the mobile phase: 0% at 0.00 min, 2% at 0.05 min, 7% at 2.45 min, 50% at 10.00 min, 100% at 11.00 min, and 0% at 12.40 min. Samples of 10 μ l were injected into the injection valve loop.

Protein determination

Protein concentration was determined by the method of Lowry [43] for glutamate uptake and by the Coomassie Blue method [44] for others parameters. Bovine serum albumin was used as standard.

Statistical analysis

The data obtained are expressed as means $\pm$ SEM of the mean. As the variances of the data were homogenous (analyzed by the Kolmogorov–Smirnov test), we used two-way analysis of variance (ANOVA) for analyzing the data from OGD experiment (factors were submission or not to OGD and treatment) followed by the Bonferroni post test. Unpaired Student's *t* test was used for analyzing the others results. *P* < 0.05 represents differences statistically significant. The number of animals used in each experiment is described in the figure legends.

Results

Effects of 3 weeks of oral GMP administration on functional and structural glutamatergic system parameters from neocortex

To investigate the GMP effects on glutamatergic system, we evaluated the glutamate binding to cortical brain plasma membranes and the glutamate uptake by slices from parietal cortex. GMP administration by 3 weeks caused a decrease in [^3H] glutamate binding (to $71.57 \pm 7.37\%$; Fig. 1a) and in [^3H] glutamate uptake (to $64.31 \pm 5.36\%$; Fig. 1b) compared to control animals ($P < 0.05$).

Since both parameters were decreased by GMP administration, the immunocontent of some specific proteins related to the glutamatergic neurotransmission was investigated: subunits of glutamatergic receptors (NMDA receptor subunit 2A/B of *N*-methyl-D-aspartic receptor (NR2A/B) and AMPA subunit GluR1), PSD-95 protein (protein implicated in connecting NMDA receptors to specific signal transduction pathways), glutamate transporter proteins (GLT-1 and GLAST—astrocytic, and EAAC1—neuronal), Snap 25 (protein involved in presynaptic vesicular fusion), and GFAP (astrocytic marker). In parietal cortex samples, oral GMP administration for 3 weeks reduced the immunocontent of NR2A/B (to $63 \pm 5\%$), GluR1 (to $69 \pm 9\%$) and PSD-95 (to $73 \pm 10\%$), compared to control animals (Fig. 2a–c, $P < 0.05$). The immunocontent levels of EAAC1 and GLT-1 were also reduced to $69 \pm 7\%$ and to $86 \pm 2\%$, respectively, compared to control (Fig. 3a, b, $P < 0.05$). The content of Snap 25 (Fig. 2d), GLAST, and GFAP (Fig. 3c, d) was not affected.

Effects of 3 weeks of oral GMP administration on cell viability (MTT) of cortical slices submitted to OGD

The two-way ANOVA analysis of variance was performed using the submission or not to OGD X treatment as factors and

the cell viability (by MTT assay) as the variable. The analysis showed an interaction between the factors [$F(1,30) = 11.40$, $P = 0.0039$]. As observed in Fig. 4, the basal viability of cortical slices was not affected by GMP administration. However, cellular injury caused by OGD was prevented by GMP orally administrated for 3 weeks ($P < 0.05$).

Effects of 3 weeks of oral GMP administration on CSF purines levels

GMP administration increased the CSF levels of GMP (to $120 \pm 4\%$), GUO (to $120 \pm 5\%$), XAN (to $150 \pm 8\%$), and UA (to $122 \pm 4\%$; Table 1); the GTP levels were not affected. The CSF GDP content was not reliably detected (data not shown).

Discussion

Three weeks ad libitum GMP administration (1.5 mg/ml) attenuated some brain cortical glutamatergic parameters: glutamate binding and uptake, and the immunocontent of several proteins related to the glutamate neurotransmission. In addition, GMP in vivo increased the resistance of neocortex to OGD (an ex vivo insult). Additionally, it was also demonstrated that GMP, GUO, XAN, and UA levels increased in CSF by oral GMP administration.

GMP effects on glutamate binding and on the levels of proteins related to glutamatergic signaling

Previous works have reported that in vitro GMP is able to inhibit the binding of glutamate and its analogs to brain membrane preparations [9–16]. In the present study, chronic (3 weeks) GMP administration caused a significant decrease of neocortex glutamate binding. Two hypotheses could be raised concerning these results:

Fig. 1 Effects of GMP oral administration for 3 weeks on glutamate binding to brain cortical plasma membranes (a) and on glutamate uptake by cortical slices (b). Data are expressed as mean $\pm$ SEM, $n = 12$ per group for both experiments. Statistical comparison between groups

was performed by unpaired Student's *t* test. Asterisk indicates a difference from control group at $P < 0.05$; 100% represents 16.3 ± 1.8 pmol/mg (a) and 0.40 ± 0.02 nmol/mg/min (b)

Fig. 2 Effects of GMP oral administration for 3 weeks on the cortical levels (evaluated by Western blotting) of glutamate ionotropic receptors subunits: **a** NMDA (NR2a/b); **b** AMPA (GluR1), and of glutamatergic synaptic proteins, **c** PSD-95; **d** Snap 25. Densitometric analysis of the bands corresponding to each protein and normalization to actin allowed comparison between the groups. Data are expressed as means $\pm$ SEM from three independent experiments, $n=6$ per group. Statistical comparison between groups was performed by unpaired Student's *t* test. Asterisk Indicates difference from control group at $P<0.05$. Representative Western blot images are shown under each figure. Protein loading of the gels was controlled by additionally staining blots with β -actin antibodies

1. Chronic GMP administration could increase the levels of guanine purines in neocortex; this hypothesis is strengthened by the results of Table 1 indicating that GMP administration increased GMP CSF levels;
2. The content of neuronal postsynaptic proteins, such as the ionotropic glutamate receptors subunits, NR2ab and GluR1 (NMDA and AMPA receptors, respectively) was decreased by chronic GMP administration. Accordingly, the level of PSD-95, a protein that is highly enriched in the post synaptic density, implicated in connecting NMDA receptors to specific signal transduction pathways [45] was also decreased. Additionally, although there is no evidence in the literature that GMP can interact with metabotropic glutamate receptors, we cannot rule out the possibility that GMP may also affect metabotropic glutamate receptors. This question remains to be further explored.

Moreover, the levels of Snap 25, a protein essential in promoting presynaptic vesicular fusion, [46] were not affected by GMP administration. These results could imply that GMP effects on glutamate binding mainly affected sites located at neuronal pos synaptic terminals.

GMP effects on glutamate uptake and glutamate transporters proteins

Surprisingly when comparing to previous results from our and other groups, GMP chronic administration decreased the cortical glutamate uptake. Accordingly, the immunocontent of the glutamate transporters EAAC1 and GLT-1 (but not GLAST) were also decreased by GMP administration. Regarding GBPs, this is the first study to describe their chronic effects decreasing the glutamate uptake activity, which was unexpected since it has been shown that in vitro GMP (apparently through conversion to GUO), is able to enhance the glutamate uptake by cultured astrocytes and by brain cortical slices from rats [26, 47, 48]. These data concerning the reduced glutamate uptake observed in the present work could be looked at as inconsistent. However, this is the first study that investigated the effects of a chronic in vivo administration of GMP on glutamate uptake by cortical slices of adult mice (which also affected other glutamatergic parameters), thus pointing that the GMP effects on glutamate uptake may depend on the experimental models (in vivo or in vitro), the time and administration route used, and other parameters simultaneously affected. Accordingly, GMP

Fig. 3 Effects of GMP oral administration for 3 weeks on the cortical levels (evaluated by Western blotting) of glutamate transporters subunits: **a** EAAC1, **b** GLT-1, **c** GLAST, and of astrocytic protein: H-GFAP. Densitometric analysis of the bands corresponding to each protein and normalization to actin allowed comparison between the groups. Data are expressed as means $\pm$ SEM from three independent experiments, $n=6$ per group. Statistical comparison between groups was performed by unpaired Student's *t* test. Asterisk Indicates difference from control group at $P<0.05$. Representative Western blot images are shown under each figure. Protein loading of the gels was controlled by additionally staining blots with β -actin antibodies

chronically administered decreased both glutamate transporters and receptors content. These results raise the question what the primary effect of GMP: if one is the primary, the other is more likely a compensatory effect. Knowledge about

the time course of GMP events are still lacking and remain to be further investigated.

The reduction in glutamate uptake activity appears at first sight to correlate with the reduction of both EAAC1 and GLT-1 immunoreactivity. However, EAAC1 contributes little to the total uptake and most of the glutamate uptake observed in hippocampal slices is due to GLT-1 [6, 49, 50] in glutamatergic nerve terminals in spite of the fact that

Fig. 4 Effects of GMP oral administration for 3 weeks on cellular viability (MTT assay) of cortical slices submitted to ex vivo oxygen glucose deprivation-OGD. Data are expressed as means $\pm$ SEM, $n=12$ animals per group. Statistical comparison among groups was performed by two-way ANOVA followed by the Bonferroni post test. Asterisk indicates difference from all others groups at $P<0.05$

Table 1 Effects of GMP oral administration for 3 weeks on cerebrospinal fluid (CSF) purines concentration

Treatment	Control	GMP
CSF purine concentration (μ M)		
GTP	141 $\pm$ 0.05	1.61 $\pm$ 0.06
GMP	0.84 $\pm$ 0.02	1.01 $\pm$ 0.03 ^a
GUO	0.39 $\pm$ 0.02	0.47 $\pm$ 0.02 ^a
XAN	2.20 $\pm$ 0.04	3.30 $\pm$ 0.18 ^a
UA	5.25 $\pm$ 0.32	6.43 $\pm$ 0.22

Data are expressed as means $\pm$ SEM, $n=6$ per group. Statistical comparison between the groups was performed with unpaired Student's *t* test

^a Indicates a difference from control group ($P<0.05$)

80% of GLT-1 is in astroglia [51]. This raises the question if the reduced uptake is due to a selective effect on nerve terminal GLT-1 rather than total GLT-1, which is an additional aspect to be investigated with more details.

GMP neuroprotective effect against OGD

In the present study, in vivo GMP administration was neuroprotective against OGD damage through mechanisms not yet identified, which are under investigation by our group. However, it could be speculated that the neuroprotective effect of GMP here observed may be related to its effects on the glutamatergic system.

Glutamatergic excitotoxicity plays a pivotal role in the cellular damage during OGD. In brain preparations, OGD increases the extracellular glutamate concentration from a low micromolar to a low millimolar range [52, 53] mainly through the reverse transport activity [54, 55].

Several works have reported that in vitro GMP promotes neuroprotective effects in hippocampal slices submitted to OGD [27–29, 37]. These in vitro GMP neuroprotective effects is suggested to involve a direct interaction with ionotropic glutamate receptors [28, 29] and/or an increasing glutamate uptake activity [37]. However, the mechanisms involved with the neuroprotective effect here observed by in vivo chronic GMP administration seems to be different from those promoted by GMP in vitro. It could be speculated that the in vivo GMP chronic administration, by reducing the reverse transport activity (decreasing the EAAC1 and GLT-1 transporters level) and the levels of main excitotoxic target (decreasing the levels of glutamatergic receptors and the downstream signaling pathway), simultaneously contributed to a reduction of the extracellular glutamate levels and to a low responsiveness of neural cells to the excitotoxic stimuli.

GMP effects on CSF purines levels

In a previous study, we reported that acute GMP administration increases GUO but not GMP CSF level [34]. In contrast, here GMP chronic administration increased GMP as well as GUO and their metabolites CSF levels. These conflicting results are difficult to interpret as, to our knowledge, there is no work in the literature addressing GMP CSF transport across the blood–brain barrier. Of note, the significant production of GMP metabolites cannot be excluded from playing a role in the modulatory effects of GMP.

Conclusion

To our knowledge, this is the first study showing the effects of chronically oral GMP administration on brain

cortical glutamatergic parameters. The results present here demonstrated the potential modulatory role of GMP on neocortex glutamatergic system by promoting functional and plastic changes associated to more resistance of mice neocortex against an in vitro excitotoxicity event although its mechanism of action remains unclear. We are continuing to investigate the glutamatergic modulatory effects of GMP and the mechanisms underlying these effects.

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References

1. Danbolt NC (2001) Glutamate uptake. *Prog Neurobiol* 65(1):1–105
2. Beart PM, O'Shea RD (2007) Transporters for L-glutamate: an update on their molecular pharmacology and pathological involvement. *Br J Pharmacol* 150(1):5–17. doi:10.1038/sj.bjp.0706949
3. Izquierdo I, Bevilacqua LR, Rossato JI, Bonini JS, Medina JH, Cammarota M (2006) Different molecular cascades in different sites of the brain control memory consolidation. *Trends Neurosci* 29(9):496–505. doi:10.1016/j.tins.2006.07.005
4. Segovia G, Porras A, Del Arco A, Mora F (2001) Glutamatergic neurotransmission in aging: a critical perspective. *Mech Ageing Dev* 122(1):1–29
5. Maragakis NJ, Rothstein JD (2006) Mechanisms of disease: astrocytes in neurodegenerative disease. *Nat Clin Pract Neurol* 2(12):679–689. doi:10.1038/ncpneuro0355
6. Sheldon AL, Robinson MB (2007) The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention. *Neurochem Int* 51(6–7):333–355. doi:10.1016/j.neuint.2007.03.012
7. Chen Y, Swanson RA (2003) Astrocytes and brain injury. *J Cereb Blood Flow Metab* 23(2):137–149
8. Belanger M, Magistretti PJ (2009) The role of astroglia in neuroprotection. *Dialogues Clin Neurosci* 11(3):281–295
9. Hood WF, Thomas JW, Compton RP, Monahan JB (1990) Guanine nucleotide modulation of [3H]TCP binding to the NMDA receptor complex. *Eur J Pharmacol* 188(1):43–49
10. Baron BM, Dudley MW, McCarty DR, Miller FP, Reynolds IJ, Schmidt CJ (1989) Guanine nucleotides are competitive inhibitors of N-methyl-D-aspartate at its receptor site both in vitro and in vivo. *J Pharmacol Exp Ther* 250(1):162–169
11. Mendieta J, Gago F, Ramirez G (2005) Binding of 5'-GMP to the GluR2 AMPA receptor: insight from targeted molecular dynamics simulations. *Biochemistry* 44(44):14470–14476. doi:10.1021/bi051084x
12. Butcher SP, Roberts PJ, Collins JF (1986) Purine nucleotides inhibit the binding of DL-[3H] 2-amino-4-phosphonobutyrate (DL-[3H] APB) to L-glutamate-sensitive sites on rat brain membranes. *Biochem Pharmacol* 35(6):991–994

13. Monahan JB, Hood WF, Michel J, Compton RP (1988) Effects of guanine nucleotides on *N*-methyl-D-aspartate receptor-ligand interactions. *Mol Pharmacol* 34(2):111–116
14. Paas Y, Devillers-Thiery A, Changeux JP, Medevielle F, Teichberg VI (1996) Identification of an extracellular motif involved in the binding of guanine nucleotides by a glutamate receptor. *EMBO J* 15(7):1548–1556
15. Sharif NA, Roberts PJ (1981) Regulation of cerebellar L-[3H] glutamate binding: influence of guanine nucleotides and Na⁺ ions. *Biochem Pharmacol* 30(21):3019–3022
16. Souza DO, Ramirez G (1991) Effects of guanine nucleotides on kainic acid binding and on adenylate cyclase in chick optic tectum and cerebellum. *J Mol Neurosci* 3(1):39–45
17. Tasca CI, Wofchuk ST, Souza DO, Ramirez G, Rodnight R (1995) Guanine nucleotides inhibit the stimulation of GFAP phosphorylation by glutamate. *Neuroreport* 6(2):249–252
18. Regner A, Ramirez G, Bello-Klein A, Souza D (1998) Effects of guanine nucleotides on glutamate-induced chemiluminescence in rat hippocampal slices submitted to hypoxia. *Neurochem Res* 23(4):519–524
19. Burgos JS, Aleu J, Barat A, Solsona C, Marsal J, Ramirez G (2003) Kainate-triggered currents in *Xenopus* oocytes injected with chick retinal membrane fragments: effect of guanine nucleotides. *Invest Ophthalmol Vis Sci* 44(7):3124–3129
20. Aleu J, Barat A, Burgos JS, Solsona C, Marsal J, Ramirez G (1999) Guanine nucleotides, including GMP, antagonize kainate responses in *Xenopus* oocytes injected with chick cerebellar membranes. *J Neurochem* 72(5):2170–2176
21. Burgos JS, Barat A, Ramirez G (2000) Guanine nucleotides block agonist-driven 45Ca²⁺ influx in chick embryo retinal explants. *Neuroreport* 11(10):2303–2305
22. Burgos JS, Barat A, Souza DO, Ramirez G (1998) Guanine nucleotides protect against kainate toxicity in an ex vivo chick retinal preparation. *FEBS Lett* 430(3):176–180
23. Paz MM, Ramos M, Ramirez G, Souza D (1994) Differential effects of guanine nucleotides on kainic acid binding and on adenylate cyclase activity in chick optic tectum. *FEBS Lett* 355(2):205–208
24. Tasca CI, Cardoso LF, Martini LH, Ramirez G, Souza DO (1998) Guanine nucleotides inhibit cAMP accumulation induced by metabotropic glutamate receptor activation. *Neurochem Res* 23(2):183–188
25. Tasca CI, Cardoso LF, Souza DO (1999) Effects of guanine nucleotides on adenosine and glutamate modulation of cAMP levels in optic tectum slices from chicks. *Neurochem Int* 34(3):213–220
26. Frizzo ME, Antunes Soares FA, Dall'Onder LP, Lara DR, Swanson RA, Souza DO (2003) Extracellular conversion of guanine-based purines to guanosine specifically enhances astrocyte glutamate uptake. *Brain Res* 972(1–2):84–89
27. Oleskovicz SP, Martins WC, Leal RB, Tasca CI (2008) Mechanism of guanosine-induced neuroprotection in rat hippocampal slices submitted to oxygen-glucose deprivation. *Neurochem Int* 52(3):411–418. doi:10.1016/j.neuint.2007.07.017
28. Oliveira IJ, Molz S, Souza DO, Tasca CI (2002) Neuroprotective effect of GMP in hippocampal slices submitted to an in vitro model of ischemia. *Cell Mol Neurobiol* 22(3):335–344
29. Molz S, Decker H, Oliveira IJ, Souza DO, Tasca CI (2005) Neurotoxicity induced by glutamate in glucose-deprived rat hippocampal slices is prevented by GMP. *Neurochem Res* 30(1):83–89
30. Schmidt AP, Souza DO (2010) The role of the guanine-based purinergic system in seizures and epilepsy. *The Open Neurosci Journal* 4:102–113. doi:10.2174/1874082001004010113
31. Malcon C, Achaval M, Komlos F, Partata W, Sauressig M, Ramirez G, Souza DO (1997) GMP protects against quinolinic acid-induced loss of NADPH-diaphorase-positive cells in the rat striatum. *Neurosci Lett* 225(3):145–148
32. Schmidt AP, Avila TT, Souza DO (2005) Intracerebroventricular guanine-based purines protect against seizures induced by quinolinic acid in mice. *Neurochem Res* 30(1):69–73
33. Schmidt AP, Lara DR, de Faria MJ, da Silveira PA, Onofre Souza D (2000) Guanosine and GMP prevent seizures induced by quinolinic acid in mice. *Brain Res* 864(1):40–43
34. Soares FA, Schmidt AP, Farina M, Frizzo ME, Tavares RG, Portela LV, Lara DR, Souza DO (2004) Anticonvulsant effect of GMP depends on its conversion to guanosine. *Brain Res* 1005(1–2):182–186. doi:10.1016/j.brainres.2004.01.053
35. Schmidt AP, Lara DR, Souza DO (2007) Proposal of a guanine-based purinergic system in the mammalian central nervous system. *Pharmacol Ther* 116(3):401–416. doi:10.1016/j.pharmthera.2007.07.004
36. Holmseth S, Scott HA, Real K, Lehre KP, Leergaard TB, Bjaalie JG, Danbolt NC (2009) The concentrations and distributions of three C-terminal variants of the GLT1 (EAAT2; slc1a2) glutamate transporter protein in rat brain tissue suggest differential regulation. *Neuroscience* 162(4):1055–1071
37. Frizzo ME, Lara DR, Prokopiuk Ade S, Vargas CR, Salbego CG, Wajner M, Souza DO (2002) Guanosine enhances glutamate uptake in brain cortical slices at normal and excitotoxic conditions. *Cell Mol Neurobiol* 22(3):353–363
38. Thomazi AP, Godinho GF, Rodrigues JM, Schwalm FD, Frizzo ME, Moriguchi E, Souza DO, Wofchuk ST (2004) Ontogenetic profile of glutamate uptake in brain structures slices from rats: sensitivity to guanosine. *Mech Ageing Dev* 125(7):475–481. doi:10.1016/j.mad.2004.04.005
39. Martini LH, Jung F, Soares FA, Rotta LN, Vendite DA, Frizzo ME, Yunes RA, Calixto JB, Wofchuk S, Souza DO (2007) Naturally occurring compounds affect glutamatergic neurotransmission in rat brain. *Neurochem Res* 32(11):1950–1956. doi:10.1007/s11064-007-9393-y
40. Strasser U, Fischer G (1995) Quantitative measurement of neuronal degeneration in organotypic hippocampal cultures after combined oxygen/glucose deprivation. *J Neurosci Methods* 57(2):177–186
41. Moreira JD, Knorr L, Thomazi AP, Simao F, Battu C, Osés JP, Gottfried C, Wofchuk S, Salbego C, Souza DO, Perry ML, Vinade L (2009) Dietary omega-3 fatty acids attenuate cellular damage after a hippocampal ischemic insult in adult rats. *J Nutr Biochem*. doi:10.1016/j.jnutbio.2009.01.013
42. Schmidt AP, Paniz L, Schallenger C, Bohmer AE, Wofchuk ST, Elisabethsky E, Portela LV, Souza DO (2009) Guanosine prevents thermal hyperalgesia in a rat model of peripheral mononeuropathy. *J Pain*. doi:10.1016/j.jpain.2009.06.010
43. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193(1):265–275
44. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
45. Gardoni F (2008) MAGUK proteins: new targets for pharmacological intervention in the glutamatergic synapse. *Eur J Pharmacol* 585(1):147–152. doi:10.1016/j.ejphar.2008.01.048
46. Bragina L, Candiracci C, Barbaresi P, Giovedi S, Benfenati F, Conti F (2007) Heterogeneity of glutamatergic and GABAergic release machinery in cerebral cortex. *Neuroscience* 146(4):1829–1840. doi:10.1016/j.neuroscience.2007.02.060
47. Frizzo ME, Lara DR, Dahm KC, Prokopiuk AS, Swanson RA, Souza DO (2001) Activation of glutamate uptake by guanosine in primary astrocyte cultures. *Neuroreport* 12(4):879–881
48. Frizzo ME, Schwalm FD, Frizzo JK, Soares FA, Souza DO (2005) Guanosine enhances glutamate transport capacity in brain cortical

- slices. *Cell Mol Neurobiol* 25(5):913–921. doi:[10.1007/s10571-005-4939-5](https://doi.org/10.1007/s10571-005-4939-5)
49. Eulenburg V, Gomeza J (2010) Neurotransmitter transporters expressed in glial cells as regulators of synapse function. *Brain Res Rev* 63(1–2):103–112
50. Haugeto O, Ullensvang K, Levy LM, Chaudhry FA, Honoré T, Nielsen M, Lehre KP, Danbolt NC (1996) Brain glutamate transporter proteins form homomultimers. *J Biol Chem* 271(44):27715–27722
51. Furness DN, Dehnes Y, Akhtar AQ, Rossi DJ, Hamann M, Grutle NJ, Gundersen V, Holmseth S, Lehre KP, Ullensvang K, Wojewodzic M, Zhou Y, Attwell D, Danbolt NC (2008) A quantitative assessment of glutamate uptake into hippocampal synaptic terminals and astrocytes: new insights into a neuronal role for excitatory amino acid transporter 2 (EAAT2). *Neuroscience* 157(1):80–94. doi:[10.1016/j.neuroscience.2008.08.043](https://doi.org/10.1016/j.neuroscience.2008.08.043)
52. Choi DW, Rothman SM (1990) The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. *Annu Rev Neurosci* 13:171–182. doi:[10.1146/annurev.ne.13.030190.001131](https://doi.org/10.1146/annurev.ne.13.030190.001131)
53. Nishizawa Y (2001) Glutamate release and neuronal damage in ischemia. *Life Sci* 69(4):369–381
54. Hamann M, Rossi DJ, Marie H, Attwell D (2002) Knocking out the glial glutamate transporter GLT-1 reduces glutamate uptake but does not affect hippocampal glutamate dynamics in early simulated ischaemia. *Eur J Neurosci* 15(2):308–314
55. Rossi DJ, Oshima T, Attwell D (2000) Glutamate release in severe brain ischaemia is mainly by reversed uptake. *Nature* 403(6767):316–321. doi:[10.1038/35002090](https://doi.org/10.1038/35002090)