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Systemic administration of GMP induces anxiolytic-like behavior in rats

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ABSTRACT

The glutamatergic system has received considerable attention over the last few years as potential target to develop anxiolytic drugs. Guanine based purines (GBPs) play an important neurmodulatory effect in the glutamatergic system. Several studies have shown the ability of the GBPs to reduce glutamatergic activity. In the present study, we investigated the anxiolytic effect of GBPs — by Guanosina Monophosphate (GMP) administration — in rodents. Adult male Wistar rats were pretreated with GMP (10, 25, 50, 100 and 150 mg/kg: i.p.); or saline (NaCl 0.9%; i.p.) (control); or, diazepam (2 mg/kg: i.p.) (positive control). One hour after the injection, the anxiety-related behaviors for each animal was evaluated in the light/dark, elevated plus-maze, and open field tasks. Additionally, purines concentration in the cerebrospinal fluid (CSF) was verified. The administration of 25 and 50 mg/kg GMP was able to promote anxiolytic-like behavior, in the light/dark and elevated plus-maze task, similar to diazepam effect. However, no changes in the open field task, or CSF purines concentration were found for either GMP or diazepam treated animals, when compared with saline group. Thus, this study suggests that acute administration of GMP was able to decrease the levels of anxiety in classical behavioral tasks.

1. Introduction

Anxiety disorders are common psychiatric diseases in medical practice. Anxiety is characterized by autonomic symptoms, such as, tachycardia, sweating, dyspnea, associated with feeling of fear, discomfort or psychological stress, causing great pain to the patients (Hoffman and Mathew, 2008).

The pathophysiological mechanisms underlying anxiety remain relatively obscure. However, different neurotransmitter seems to be involved. It is widely accepted that the GABAergic system plays an important role in this disorder (Nutt and Malizia, 2001). Benzodiazepines, which act by increasing the inhibitory GABAergic neurotransmission, have been established as a standard treatment for anxiety. However, their sedative-hypnotic effects, muscle relaxant properties, and memory impairing effects may limit their therapeutic application (Hoffman and Mathew, 2008). Therefore, the search for new effective pharmacological targets to treat anxiety with less adverse side effects has gained attention.

The glutamatergic system has received considerable attention over the last years as potential target to develop anxiolytic drugs (Bergink et al., 2004; Chojnacka-Wojcik et al., 2001; Cryan et al., 2003; Kapus et al., 2008; Palucha and Pilc, 2007). Glutamate, the main excitatory neurotransmitter in mammalian central nervous system (CNS), is essential for brain activity, modulates brain plasticity (such as, learning and memory), pain, and several brain responses to external stimuli (Izquierdo et al., 2006; Ozawa et al., 1998; Schmidt et al., 2008; Segovia et al., 2001). However, overstimulation of the glutamatergic system, caused by excess of extracellular glutamate levels (excitotoxicity), is implicated in various acute and chronic brain diseases, including neurodegenerative disorders, traumatic brain injury, cerebral ischemia, and seizures (Lipton and Rosenberg, 1994; Maragakis and Rothstein, 2006; Meldrum, 1994; Sheldon and Robinson, 2007). In addition, it is also widely accepted that the glutamatergic system is involved in the pathophysiology of some psychiatric disorders, such as anxiety and depression (Bergink et al., 2004; Cryan et al., 2003).

The glutamate acts on the ionotropic (ion channels — NMDA, AMPA and KA) and metabotropic (mGlu — coupled to G proteins) receptors (Maragakis and Rothstein, 2006; Ozawa et al., 1998; Sheldon and Robinson, 2007). NMDA receptors uncompetitive and competitive antagonists, as well as AMPA receptor non-competitive

Abbreviations: ADO, adenosine; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; BBB, blood brain barrier; CNS, central nervous system; CSF, cerebrospinal fluid; Dz, diazepam; GABA, γ-aminobutyric- acid; GBPs, guanine based purines; GDP, guanosine diphosphate; GMP, guanosine monophosphate; GTP, guanosine triphosphate; GUO, guanosine; HIPOX, hypoxanthine; HPLC, high-performance liquid chromatography; iGluR, ionotropic glutamate receptor; IMP, inosine monophosphate; INO, inosine; mGluR, glutamate metabotropic receptor; MK-801, dizocilpine; NaCl, sodium chloride; NMDA, N-methyl-D-aspartate; RA, risk assessment behavior; AU, uric acid; XANT, xanthine.

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antagonists can profoundly block anxiety-like behavior in rodents (Bergink et al., 2004; Chojnacka-Wojcik et al., 2001; Kapus et al., 2008; Kehne et al., 1991; Plaznik et al., 1994). In addition, selective antagonist of some subtypes of mGluR receptors can also display anxiolytic-like effects in rodents (Chojnacka-Wojcik et al., 2001; Palucha and Pilc, 2007; Spooren and Gasparini, 2004; Tatarczynska et al., 2001a,b). These studies indicate that anxiolytic-like effect can be achieved by blocking glutamatergic neurotransmission and that hyper stimulation of the glutamatergic transmission can be associated with anxiety related behavior. Thus, new drugs able to antagonize the glutamatergic neurotransmission could be effective to treat anxiety (Bergink et al., 2004; Palucha and Pilc, 2007; Spooren and Gasparini, 2004; Tatarczynska et al., 2001a,b).

Over the past 10 years, our group have shown that guanine-based purines (GBPs), such as, the nucleotides GTP, GDP, GMP and the nucleoside guanosine (GUO) exhibit important neuromodulatory function, including the extracellular antagonism of glutamatergic system (Schmidt et al., 2007). In vitro studies demonstrated that GBPs prevent cellular responses evoked by glutamate (Paz et al., 1994; Regner et al., 1998; Tasca et al., 1995), and stimulate glutamate uptake (Frizzo et al., 2002, 2003). In vivo studies had shown that GUO, administered either intraperitoneally (i.p.) or orally (p.o.), prevented seizures induced by the glutamatergic agents (quinolinic acid (QA), kainate and α-dendrotoxin) in rodents. These data suggest the neuroprotective role of GBPs in events caused by the glutamatergic excitotoxicity (Lara et al., 2001; Schmidt et al., 2000, 2007; Soares et al., 2004; Vinadé et al., 2003, 2005).

Behavioral experiments performed by our group demonstrated that GUO exerted an amnesic effect on inhibitory avoidance task in rats (Roesler et al., 2000; Saute et al., 2006; Vinadé et al., 2003, 2005). On the other hand, no obvious motor disturbance or sedative effects were observed after acute or chronic administration of GBPs, as evidenced with other glutamate antagonists such as MK-801 (Lara et al., 2001; Tort et al., 2004; Vinadé et al., 2003). In addition, GUO exhibited an anxiolytic effect in mice tested by the hole board task when administered ad libitum in the water for two weeks (Vinadé et al., 2003).

Considering the effects of GBPs on the glutamatergic system and the involvement of the glutamate in the pathophysiology of anxiety, the main objective of this study was to evaluate the effects of GMP on behavioral models of anxiety in rats.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (60–90 days old, weighing 250–350 g) were kept under a 12-hour light/dark cycle (light on at 7:00 AM) at constant temperature of 22 ± 1 °C. They were housed in plastic cages (5 per cage) with water and commercial food ad libidum. All behavioral procedures were conducted between 9:00 AM and 5:00 PM. All experimental procedures were performed in accordance with the Brazilian Society for Neuroscience and Behavior's recommendations for animal care.

The animals were handled in the same room during the 6 days preceding the experiment. At the day of behavioral task, the animals were acclimatized in the testing room for 1 h before the task.

Each animal was used once, because our previous observation that, 2 sequential different experiments with the same animal, promoted behavioral disturbances like anxiety-related behaviors (data not shown).

2.1.1. Chemicals

Guanosine monophosphate (GMP) was obtained from Sigma Chemicals (St. Louis, MO, USA). Diazepam was purchased from União Química Nacional S/A (Pouso Alegre, MG, Brazil). GMP was dissolved in saline 0.9% and diazepam was dissolved in saline solution containing 0.5% Tween 80. The anesthetic sodium thiopental was obtained from Cristália (Itapira, SP, Brazil).

2.1.2. Drugs administration

The animals were divided into the following groups: Saline (NaCl 0.9%) (control group); GMP 10, 25, 50, 100 and 150 mg/kg (GMP group); and diazepam 2 mg/kg (positive control group). The diazepam dose was chosen based on dose response curve performed prior the study (data not shown). The standard anxiolytic compound diazepam was employed as a positive anxiolytic control in all experiments. All groups received a 1 mL/kg intraperitoneally (i.p.) administration of the drugs 1 h before each behavioral task. To minimize the interference of the natural variability of the animals on the tasks, all plastic cages contained 1 animal that received saline, other that received diazepam and 3 animals that received different doses of GMP.

2.2. Behavioral tasks

2.2.1. Light/dark task

The light/dark task was performed as previously described (Crawley and Goodwin, 1980) with some modifications. The light/dark apparatus consisted of an acrylic rectangular box with two separated chambers. One chamber had black walls and floor, with size of 210 × 350 × 410 mm (height × length × width) and was not illuminated. The other side had white walls and floor, with size of 210 × 450 × 410 mm (height × length × width) and illuminated by a 100 W white lamp overhead. The two compartments were separated by a wall, which had a small opening (80 × 50 mm, height × length) at floor level. For each experiment, the animal was placed in the white chamber and allowed to explore the two-chamber area for 5 min. The following parameters were recorded by a trained and blinded-to-treatment observer: number of transitions between the two chambers, time spent in the light chamber, latency time to enter for the first time in dark chamber and the risk assessment behavior (RA, i.e., the number of times the animal in the dark compartment explored the light compartment). After each experiment, the apparatus was cleaned with alcohol 70° and dried before the next animal.

2.2.2. Elevated plus-maze task

The elevated plus-maze was performed as previously described (Pellow et al., 1986). The elevated plus-maze apparatus, entirely made of wood, consisted of two open arms (50 × 10 cm, length × width) and two enclosed arms (50 × 10 × 40 cm; length × width × height) separated by a central platform (5 × 5 cm; length × width) arranged so that the two identical arms of each type were opposite to each other. The height of the maze was 70 cm, and the experiments were conducted under dim red light in a quiet room. The animals were placed individually on the central platform of the plus-maze facing an open arm, and observed individually for 5 min by a trained and blinded-to-treatment observer. The number of transitions between the open and enclosed arms and of the total arm entries, and the time spent into open arms were recorded. After each session the apparatus was cleaned with alcohol 70° and dried before the next animal.

2.2.3. Open field task

Exploratory activity, locomotor activity and anxiety-like behavior of rats were evaluated during an open field session of 8 min (the first 3 min was considered as a measure of novelty exploratory activity and the last 5 min as locomotor activity). The test was performed by placing each individual animal without previously habituation in the center of a square arena (50 × 50 × 50 cm, length × width × height) with a black floor and walls. All sessions were recorded by a video-camera (positioned above and at ca. 90° to the square arena) connected to a monitor. Videotapes were blinded scored by a trained

observer using dedicated software (ANY-maze®). The videos were subsequently placed in randomized order in a separate ANY-maze protocol for a trained observer to score using a keyboard-based behavioral tracking system, blinded to the treatment group. The time spent in the inner area of the apparatus was considered as a anxiolytic-like behavior. After each session the apparatus was cleaned with alcohol 70° and dried before the next animal.

2.3. Cerebrospinal fluid (CSF) purines analysis

2.3.1. Cerebrospinal fluid (CSF) sampling

To evaluate whether changes in CSF purines profile could be correlated with the observed anxiolytic-like effect, we measured the CSF purines concentration on the GMP or diazepam treated animals. One hour after receiving GMP (50 and 150 mg/kg; i.p), saline (NaCl 0.9%; i.p.) or diazepam (2 mg/kg; i.p), the rats were anesthetized with sodium thiopental (40 mg/kg, 1 mL/kg, i.p.), and placed in a stereotaxic apparatus. The CSF was collected (40 to 80 mL) by direct puncture of the cisterna magna with an insulin syringe (27 gauge × 1/2-inch length). Individual samples with visible blood contamination were discarded. All samples were centrifuged at 10.000 *g* at 4 °C in an Eppendorf centrifuge for 10 min to obtain cell-free supernatants and then stored in single tubes at −70 °C.

2.3.2. High-performance liquid chromatography (HPLC) procedure

HPLC was performed with aliquots obtained from the CSF cell-free supernatants to measure purines levels. The measurement was done as described previously (Schmidt et al., 2009). The levels of the following purines were determined: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine (ADO), guanosine triphosphate (GTP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), guanosine (GUO), inosine monophosphate (IMP), inosine (INO), hypoxanthine (HIPOX), xanthine (XANT) and uric acid (AU). Analyses were performed with the 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 mL loop and a UV detector (Shimadzu, Kyoto, Japan). Separations were achieved on a Supelco 250 mm × 4.6 mm, 5 µm particle size column (Supelco, St Louis, MO, USA). The mobile phase flowed at a rate of 1.2 mL/min and the column temperature was 24 °C. Buffer composition remained unchanged (A: 150 mmol/L phosphate buffer, pH 6.0, containing 150 mmol/L potassium chloride; B: 15% acetonitrile in buffer A). 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 µl were injected into the injection valve loop. Absorbance was read at 254 nm. CSF concentrations of purines are expressed as mean ± SEM in micromoles.

2.4. Statistical analysis

Statistical analysis among groups were performed by one way ANOVA, plus the Tukey post hoc test if the variances of the data were homogenous, or Kruskal–Wallis analysis of variance followed by a Mann–Whitney *U* test when the variances of the data were not homogenous. All differences with $P<0.05$ were considered significant.

3. Results

3.1. Light/dark task

Animals treated with diazepam (2 mg/kg; i.p.) or GMP (25 and 50 mg/kg; i.p) exhibited a significant increase in the total exploration time on the light compartment of the apparatus (Fig. 1A, $P<0.05$). The number of transitions between the light and dark compartments was also increased in the diazepam treated animals and in the GMP group that received 50 mg/kg (Fig. 1B, $P<0.05$), when compared with saline group. No changes in the number of transitions were observed for animals receiving GMP 25 mg/kg. However, no changes in the number of RA, or the latency to enter in the dark chamber of the apparatus, was observed for all groups (data not shown).

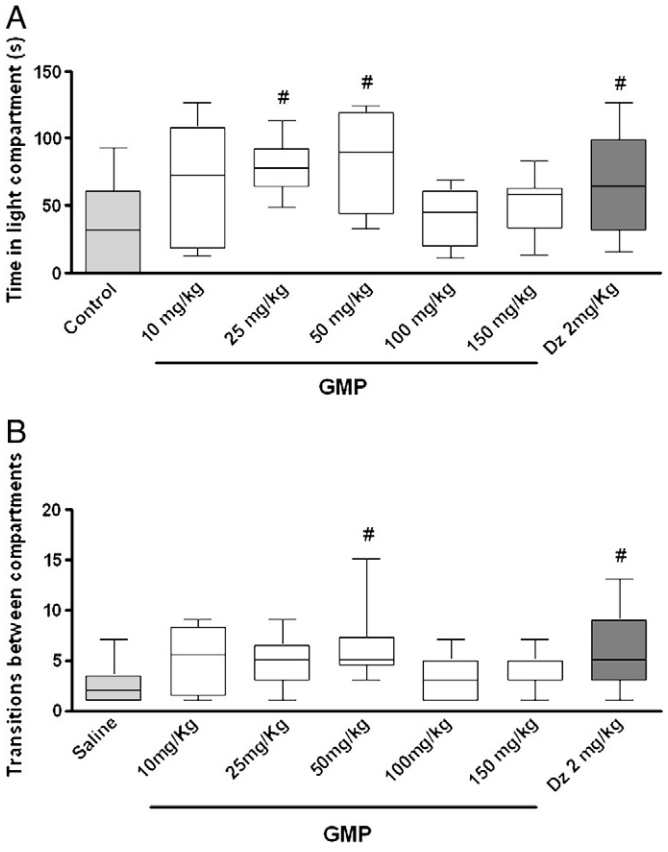

Fig. 1. Light/dark task. Effect of Dz or GMP on the total time spent in the light side (A) and on the total number of transitions (B). Data are reported as medians (interquartile ranges) analyzed by one-way ANOVA, $n = 10$ per group, # $P<0.05$ compared to saline. Dz: Diazepam; GMP: Guanosine Monophospate.

Animals treated with the lowest (10 mg/kg), and the highest (100 and 150 mg/kg) doses of GMP did not exhibit alterations of the analyzed parameters when compared with controls (data not shown).

3.2. Elevated plus-maze task

Animals treated with diazepam (2 mg/kg; i.p.) or GMP (25 or 50 mg/kg; i.p) presented a significant increase in the time spent in open arms, compared with the saline group (Fig. 2, $P<0.05$).

On the other hand, the administration of diazepam or GMP was not able to change the number of entries in open, closed, and total arms, when compared with the saline group (data not shown).These data suggest that diazepam and GMP had no effect on the locomotor activity.

No alterations were observed in animals that received GMP 10 mg/kg, 100 and 150 mg/kg (data not shown).

3.3. Open Field task

I.p. administration of diazepam (2 mg/kg) or GMP (50 and 150 mg/kg) did not modify the total distance traveled by the animals in the 8 min of the open field session (Fig. 3A). There were no

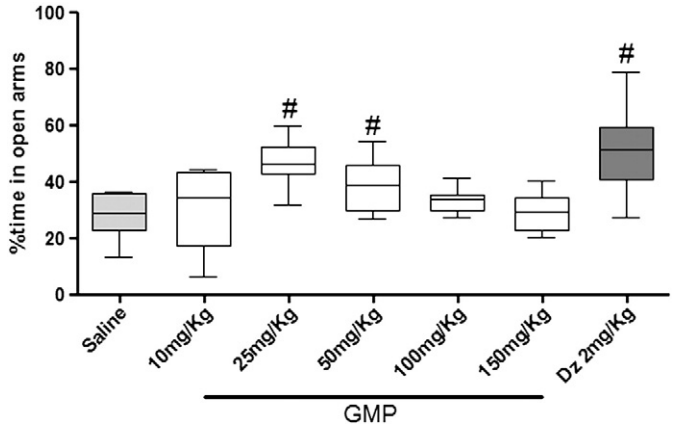

Fig. 2. Elevated plus-maze task. Effect of Dz or GMP on the time spent in open arms. Data reported as medians (interquartile ranges) analyzed by Kruskal–Wallis, followed by Mann–Whitney test, $n = 10$ per group, # $P<0.05$ compared to saline. Dz: Diazepam; GMP: Guanosine Monophospate.

alterations on the exploratory activity in the first 3 min (Fig. 3B) and on the locomotor activity in the last 5 min among groups (Fig. 3C).

No difference was observed in the time spent in the inner area of the open field for all groups (data not shown).

3.4. Cerebrospinal fluid (CSF) analysis

The analysis of the CSF 1 h after the i.p. administration of diazepam (2 mg/kg) or GMP (50 and 150 mg/kg) shows no alteration on the purines concentrations when compared with control group (data not shown).

4. Discussion

In the present study, we investigated the anxiety-like behavior in rats by the light/dark and elevated plus-maze tasks which are the two widely used protocols to predict anxiolytic-like or anxiogenic-like activity in rodents. The light/dark task is based on the innate aversion of rodents to brightly illuminated areas and on their spontaneous exploratory behavior in response to mild stressors, as novel environment and light. The elevated plus-maze is based on the natural aversion of rodents for open and elevated areas, as well as on their natural spontaneous exploratory activity in novel environments (Bourin and Hascoët, 2003; Prut and Belzung, 2003). We also evaluated the anxiety-like behavior in the open field task by measuring the time that the animal remain in the center of the open field arena which could indicate an anxiolytic-behavior effect, since it is well known that rodents prefer to walk close to the walls, a behavior called thigmotaxis.

The observation of an anxiolytic-like behavior in rodents is complex. The extent to which an anxiolytic compound can facilitate exploratory activity depends on the baseline behavior of the control group. Differences between the type and severity of external stressors might account for the variable results reported by different works. In the present study, we used a positive control group (classical anxiolytic drug — diazepam) in all behavioral tasks to establish which parameter(s) could indicate an anxiolytic effect compared with control group. We choose diazepam as a positive control because benzodiazepines are used to treat a broad spectrum of anxiety disorders. For the implementation of our experimental protocol, we changed the time of administration and the anxiolytic doses of diazepam described previously (Bourin and Hascoët, 2003; Prut and Belzung, 2003). We observed that the administration of lower doses of diazepam did not produce any effect on the behavior for all animals, while higher doses were sedative. These emphasize the difficulties to implement a reliable positive control of anxiolytic-like effects. According the dose response curve performed prior the study; the best suitable dose of diazepam to achieve an anxiolytic-like effect was 2 mg/kg, i.p., 60 min before the behavioral task.

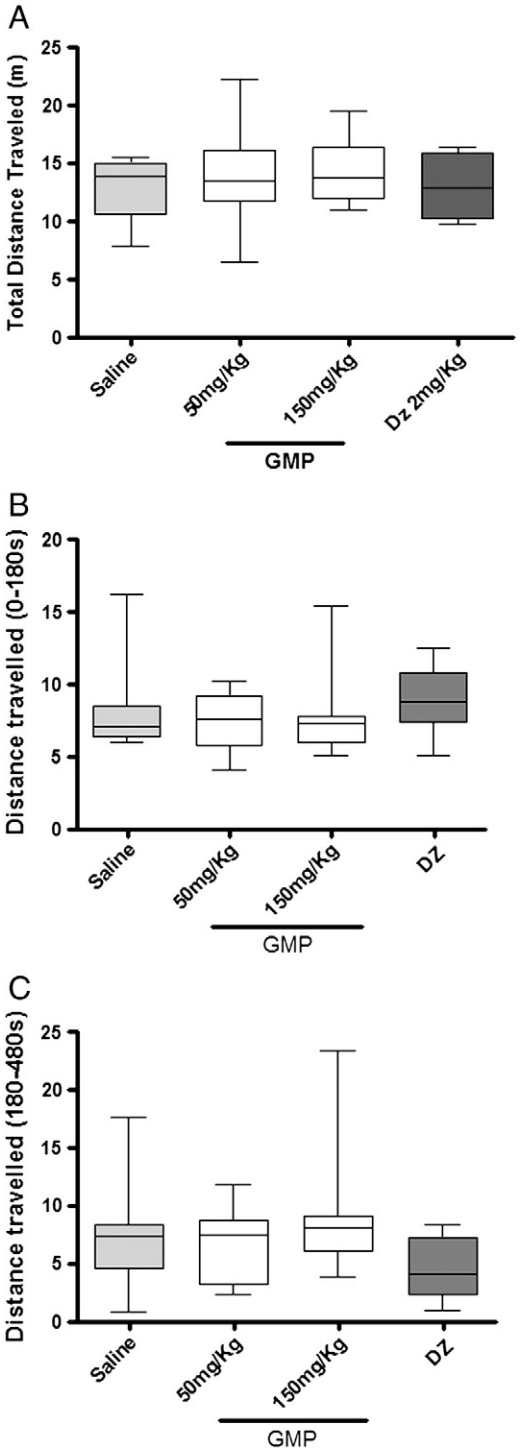

Fig. 3. Open field task. Effect of Dz or GMP on the total distance traveled in 8 min (A), in the exploratory activity in the first 3 min (B), and the locomotor activity in the last 5 min (C). Data reported as medians (interquartile ranges), analyzed by one-way ANOVA, $n = 7–9$ animals per group. No significant differences were observed among the groups. Dz: Diazepam, GMP: Guanosine Monophospate.

The main finding of the present work was that GMP at the dose of 50 mg/kg was able to consistently reproduce the anxiolytic effects of diazepam. On the other hand, in the open field task, neither diazepam nor GMP produced anxiolytic-like behavior. Therefore, predictive

value of this task appears to have limitations to test the anxiolytic effect of these drugs. Additionally, no locomotor activity alteration was observed after GMP or diazepam administration.

Interesting, GMP at intermediate doses (25 and 50 mg/kg) was able to decrease the anxiety levels in the classical behavioral tasks. Since, to our knowledge, no studies about the pharmacokinetics of i.p. injection of GMP were performed, it is difficult to understand why the GMP induces anxiolytic-like behaviors in a bell-shape dose response. Accordingly, more studies focusing the pharmacokinetics of GMP administration may help to clarify the dose-response effects observed in the present work.

The original reason that lead us to investigate whether GMP could induce anxiolytic-like behaviors was based on the fact that GMP can directly, but weakly, antagonizes iGluR (Baron et al., 1989; Mendieta et al., 2005; Paas et al., 1996; Porciúncula et al., 2002; Souza and Ramirez, 1991), and, that the administration of iGluR antagonists can induce anxiolytic-like behaviors in rodents (Bergink et al., 2004; Chojnacka-Wojcik et al., 2001; Kapus et al., 2008; Kehne et al., 1991; Plaznik et al., 1994). Considering that the behavioral changes observed 60 min after GMP administration were not accompanied by the increase in CSF GMP levels, we cannot confirm that the mechanism by which GMP induced anxiolytic-like behavior involved iGluR antagonism.

In addition, as changes in CSF GMP levels were not found, we cannot confirm that GMP crosses the blood brain barrier (BBB). However, organic anion transporter proteins, responsible to transport nucleotides analogs across BBB, were already described (Takeda et al., 2002; Strazielle and Ghersi-Egea, 2005). Thus these proteins could also transport GMP across the BBB. Moreover GMP seems to have a neuromodulatory role on CNS. This was attributed to the neuroprotective and behavioral effects after the intracerebroventricular, intrahippocampal and intrastriatal administration of GMP in rodents (Malcon et al., 1997; Rubin et al., 1996; Saute et al., 2006; Schmidt et al., 2005; Soares et al., 2004).

A previous study from our group reported that GMP (7.5 mg/kg; i.p.) increases CSF GUO levels 30 min after the administration, without altering GMP or adenosine CSF levels (Soares et al., 2004). Therefore, we cannot rule out the possibility that the CSF GBPs level varied before 60 min after GMP administration, causing the modulation of the glutamatergic system, which might be involved with the anxiolytic-related behavior observed.

GMP is metabolized both, systemically and in the CNS (Saute et al., 2006), suggesting that the metabolites of GMP may also be responsible for the anxiolytic-like effects observed. In agreement with this hypothesis, some effects of i.p. injection of GMP, such as, the anticonvulsant effect against QA-induced seizure (Soares et al., 2004) and the amnesic effect (Saute et al., 2006), are dependent on its conversion to GUO. In addition, GMP, through its conversion to GUO, increases glutamate uptake activity in astrocytes (Frizzo et al., 2003) which is the main mechanism to terminate glutamate physiological activity and avoid the glutamatergic excitotoxicity (Danbolt, 2001). As anxiety per se might ultimately arise from a shift towards neuronal hyper excitability (Bergink et al., 2004; Chojnacka-Wojcik et al., 2001; Cryan et al., 2003; Kapus et al., 2008; Palucha and Pilc, 2007), higher glutamate uptake ability might promote anxiolytic-like effect.

Additionally, there is a lack of studies focusing on the interaction between GBPs with other neurotransmitter systems besides glutamate. So, since the mechanisms underlying anxiety involves other neurotransmitters, the exactly mechanism by which GMP exert the anxiolytic effects needs more investigation.

To our knowledge, this is the first study showing anxiolytic-like effects after systemic administration of GMP in classical behavioral tasks. As GMP is an endogenous compound, apparently well tolerated with minor toxicity, it could eventually be developed as a new drug for anxiety treatment.

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