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Anxiolytic effect of chronic intake of supplemental magnesium chloride in rat

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ABSTRACT

Evidence suggest that magnesium dietary supplementation has several health benefits including lowering blood pressure, reducing insulin resistance, and improving symptoms of depression, anxiety, and migraine. Here, we aimed to study the effect of chronic magnesium supplementation on anxiety-like behavior in rats by supplementing with magnesium their drinking water for 30 days. Anxiety-like behavior was induced by subcutaneous injection of veratrin 30 min before performing elevated plus maze and open field tests to measure anxiety levels and locomotion, respectively. We quantify the concentration of magnesium in plasma and cerebrospinal fluid. We used diazepam to compare the efficacy of magnesium supplementation as an anxiolytic agent. Our results show that rats supplemented with magnesium had a statistically significant decrease in anxiety levels with not effects on locomotion and a statistically significant increase in concentration of magnesium in plasma and cerebrospinal fluid. However, the anxiolytic effect of magnesium supplementation washes-out in 12 days. We discuss the advantages of using supplemental magnesium as anxiolytic.

1. Introduction

Magnesium is an essential mineral that is required for several physiological functions and for regulation of biochemical and metabolic processes such as muscle contraction, blood pressure control and insulin metabolism [1–8]. In the nervous system, magnesium is important for nerve transmission and neuromuscular coordination [9]. Magnesium is as a voltage-dependent blocker of N-methyl-D-aspartate (NMDA) receptor, which plays a role in the influx of calcium in neurons. Magnesium blockade of NMDA receptor may have neuroprotective properties during glutamatergic excitatory signaling [10,11]. It is also involved in the modulation of GABAergic neurotransmission and adrenocorticotrophic hormone secretion, affecting several transduction pathways [12]. Magnesium deficiency has been associated with cardiovascular, metabolic, and respiratory diseases, as well as with neurological abnormalities such as stress, depression and anxiety [2,6,9,13–15].

Anxiety disorders are among the most prevalent mental disorders [16,17].These disorders are characterized by somatic, emotional, cognitive and behavioral symptoms that affect the daily life of an individual. Pathophysiology of anxiety disorders is multifactorial and may have different anatomical and / or molecular substrates. Therefore, treatments, known as anxiolytics, do not achieve complete remission of symptoms and their clinical use is limited due to the side effects that can occur [18]. Supplemental magnesium therapy may be effective for preventing some behavioral sequelae of depression and anxiety related to magnesium deficiency [19,20]. In rats, intravenous injection of Mg^{2+} after a traumatic brain injury improves post-traumatic depression and anxiety, and has been considered as a neuroprotective agent [21]. In mice, intraperitoneal injections of magnesium (20 and 30 mg / Kg) decrease anxiety-like behavior and enhance the effect of diazepam in behavioral tests such as the elevated plus [22]. Therefore, magnesium is considered an element with anxiolytic activity [23].

Current pharmacological treatments for anxiety disorders are aimed to induce a rapid anxiolytic effect. However, chronic treatment is often required to attenuate the symptoms of pathological anxiety [24]. In clinical practice, selective serotonin reuptake inhibitors (SSRIs) and benzodiazepines are the most used medications to treat symptoms of anxiety disorders. However, benzodiazepines are not recommended as the first line of treatment for generalized anxiety due to the dependence that they can cause [25–27]. Furthermore, dosimetry, route of

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administration and duration of treatment must be considered to prevent side effects such as fatigue, nausea, diarrhea, constipation, insomnia, drowsiness, sexual dysfunction, hypertension, cognitive problems, dry mouth, urine retention, dizziness, among others [28,29]

Development of new pharmacological therapies for anxiety disorders represent a great unmet medical need [29]. A desirable pharmacological treatment must be effective with a minimum of side effects. Other characteristics as price, availability and ease of administration must be taken into consideration also. Evidence in humans suggest that magnesium supplementation has a beneficial effect on the subjective perception of anxiety [1]. In order to add new knowledge to the current evidence available from animal models, here we studied the effect of chronic magnesium supplementation on anxiety-like behavior in rats using a thorough experimental design. First, we controlled the baseline intake of magnesium by preparing potable water with concentration of ions similar to those present in tap water in the US. Second, we induced anxiety-like behavior by s.c. injection of veratrine [30]. Our rationale is that the anxiogenic effect of veratrine would emulate the acute phenomena occurring systemically during an anxiety attack, and this would allow us to assess objectively the anxiolytic effect of magnesium. Third, we used diazepam as positive control to compare quantitively the efficacy of magnesium supplementation as an anxiolytic agent. And last, by determining the concentrations of magnesium in plasma and cerebrospinal fluid, we established the time that takes for the anxiolitic effect to be washed-out. We propose that oral magnesium supplementation is an effective anxiolytic with a non-invasive administration, an affordable cost and without causing side effects.

2. Materials and methods

2.1. Ethical approval

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Universidad Veracruzana, accordingly to Official Mexican Standard NOM-062-ZOO-1999 (Technical Specifications for the Production, Care and Use of Laboratory Animals). In addition, we followed the U.S. National Institutes of Health (NIH) guidelines for animal care and handling.

2.2. Animals

Pregnant Wistar rats were housed in vivarium conditions under 12-h light/dark cycles (lights on at 08:00), temperature controlled (21 ± 1 °C) and humidity controlled, with food (Rismart, Mexico) and potable water provided *ad libitum*. We prepared the potable water to contain a similar concentration of ions present in tap water of Boston and New York, US (Azoulay et al., 2001) (in mg/L): 37.1 $CaCl_2 2H_2O$ (Fisher Scientific, NH, US), 11.5 $MgCl_2 6H_2O$ (Fermont, Mexico) and 43.8 NaCl (J.T. Baker, US). This formulation provides 1.4 mg/L of elemental magnesium. Offspring were weaned at P21 and males were housed in groups of 5 per cage with food and potable water *ad libitum*. In some groups, magnesium supplementation (see below) started at P90. We did not find statistically significant changes in body weight between rats that received magnesium supplementation (382 ± 2.27 g) and those with no supplementation (378 ± 3.9 g). Experimental manipulations and behavioral tests were conducted during the light period. Experiments were designed and performed with the goal of minimizing the number of animals used and their suffering.

2.3. Drugs

Anxiety was induced by s.c. injection of veratrine hydrochloride (Sigma-Aldrich, St. Louis, MO) dissolved in saline. Veratrine is a mixture of alkaloids including the voltage-gated sodium channel activator veratridine that has anxiogenic properties already tested in rats. At a dose of 0.6 mg/kg veratrine induces anxiety-like behaviors [30]. Diazepam (Valium, Roche) was used as a positive control anxiolytic drug and dissolved in physiological saline with 2% Tween® 80 (Sigma Aldrich, St. Louis, MO) which we also used as vehicle. Drugs were administered in a volume of 0.1 mL per 100 g of body weight and administered subcutaneously 30 min before the behavioral tests [30].

2.4. Experimental groups

We refer to potable water as that containing (in mg/L): 37.1 $CaCl_2$, 43.8 NaCl and 11.5 $MgCl_2$, and to supplemented water as that containing (in mg/L) 37.1 $CaCl_2$, 43.8 NaCl and 5 g/L $MgCl_2$. Thirty min before behavioral tests, rats received a s.c. injection of either vehicle or treatment.

Control (n = 9): drank potable water for 4 months. Received a s.c. injection of vehicle.

Magnesium supplementation (Mg^{2+}) (n = 10): drank potable water for 3 months and magnesium supplemented water for 1 month (dose ~50 mg/kg/day elemental Mg^{2+},~30 mL/day drinking water) [31] (Slutsky et al., 2010). Received a s.c. injection of vehicle.

Induced anxiety (IA) (n = 9): drank potable water for 4 months. Received a s.c. injection of veratrine (0.6 mg/kg).

Diazepam (DZP) (n = 5): drank potable water for 4 months. Received a s.c. injection of the diazepam (1.0 mg/kg).

Magnesium supplementation + Induced Anxiety (Mg^{2+} + IA) (n = 9): drank potable water for 3 months and magnesium supplemented water for 1 month. Received a s.c. injection of veratrine (0.6 mg/kg).

Diazepam + Induced Anxiety (DZP + IA) (n = 9): drank potable water for 4 months. Received a s.c. injection of diazepam (1.0 mg/kg) followed by a s.c. injection of veratrine (0.6 mg/kg).

12 days of magnesium supplementation + Induced Anxiety (P102 Mg^{2+} + IA) (n = 9): drank potable water for 3 months, and supplemented water for 12 days. Received a s.c. injection of veratrine (0.6 mg/kg).

P102 + IA: drank potable water for 102 days. Received a s.c. injection of veratrine (0.6 mg/kg).

12-day washout period + Induced Anxiety (P132 Mg^{2+} + IA) (n = 9): drank potable water for 3 months, supplemented water for 1 month followed by potable water for 12 days. Received a s.c. injection of veratrine (0.6 mg/kg).

P132 + IA: drank potable water for 132 days. Received a s.c. injection of veratrine (0.6 mg/kg).

2.5. Behavioral procedures

We used the elevated plus maze test to measure anxiety. Number of entries to open arms and time spent in open arms were considered as anxiety-like behaviors [32]. We assessed locomotion in animals by quantifying the total number of entries in open and closed arms [33]. Exploratory behavior and locomotion were also measured with open field test.

Elevated plus maze (EPM) test. A rat EPM consisted of two open (50

× 10 cm) and two closed (50 × 10 × 40 cm) arms, extending from a central platform (10 × 10 cm). The maze was approximately 50 cm above the floor. Rats were individually placed at the center of the maze, facing an open arm and allowed to freely explore the maze for 5 min. The time spent on the open arms and the number of entries made into each arm was recorded using a camera (Logitech HD720p). Arm entry were defined as entry of all four paws into an arm. The number of entries and time spent in open arms were used as a measure of anxiety.

Open Field Test. Rats performed the open field test so we could measure exploration and locomotion behaviors. The open field box consisted of a square transparent Plexiglas box (60 × 60 × 40 cm height), with an outlined center area (30 × 30 cm). Rats were individually placed at the center of the arena and allowed to freely explore for 15 min. Total distance traveled was recorded and taken as a measure of locomotion. Time spent grooming, licking and scratching was quantified offline.

Video recordings were acquired using the Logitech webcam software with a 1280 × 720 pixels image resolution at 24 frames per second, in Windows media video format RGB color space. The webcam was placed perpendicularly over to EPM or the open field arena, on a custom polyvinyl chloride (PVC) pipe support. A dedicated room for behavioral tests was illuminated with fluorescent light. Analysis of each video recording was performed manually by two independent experts.

2.6. Measurement of plasma and CSF magnesium concentration [Mg^{2+}]

In order to confirm that the anxiolytic effect of chronic magnesium supplementation was due actually to the magnesium ingested, in all groups we measured the [Mg^{2+}] in plasma in P90, i.e., baseline, and after behavioral tests. Total [Mg^{2+}] in blood plasma and CSF were determined by Xilidyl blue method. Plasma was isolated by centrifugation 30 min after collection of blood from the lateral vein of the tail of the rat. CSF was isolated from the magna cistern. Ten microliters of plasma or CSF was added to 1 mL of the commercially available reagent (BioSystems, Reagents & Instruments COD11797) and the absorbance of the solution was read at 520 nm in a spectrophotometer (Jenway 6405 UV/Vis).

2.7. Experimental design

Experiment 1. In order to test the sole effect of chronic 30-day magnesium supplementation, s.c. injection of veratrine (0.6 mg/kg) or s.c. injection of the diazepam (1.0 mg/kg) on baseline anxiety, we quantified the number of entries in open arms in the EPM in rats and compared them with rats that received no treatment. Control rats and those that received veratrine (dubbed IA) or diazepam (DZP) drank potable water for 4 months. The rational of this set of experiments was to verify the anxiolytic effect of magnesium supplementation and diazepam, and the anxiogenic effect of veratrine. Groups: Control, Mg^{2+}, IA, DZP.

Experiment 2. In order to test the anxiolytic effect of chronic 30-day magnesium supplementation or of the drug diazepam, we tested in EPM and open field rats with induced anxiety that either received or not chronic magnesium supplementation or diazepam. Groups: IA, DZP + IA, Mg^{2+} + IA

Experiment 3. We questioned how many days of supplementation are necessary to reach plasmatic [Mg^{2+}] comparable to that seen after 30 days of supplementation? In a group of rats that drank potable water we measured the baseline plasmatic [Mg^{2+}] in P90. Then, we separated rats in two groups: control group (n = 5) and Mg^{2+} supplemented group (n = 9). We obtained blood every three days and determined in which day plasmatic [Mg^{2+}] reached values similar to those seen after 30 days of supplementation. In day 13, we induced anxiety by injecting veratrine to rats from both groups and we quantified the number of entries and time spent in open arms, and the total number of entries to open and close arms in the EPM test. Groups: P102 + IA, P102 Mg^{2+} + IA

Experiment 4. We explored how long the anxiolytic effect of 30-day magnesium supplementation lasted after discontinuing supplementation. In a set of rats that received 30-day magnesium supplementation Mg^{2+}, in day 31 we either provided potable water (n = 5) or supplemented water (n = 9) and measured plasmatic [Mg^{2+}] every three days. In order to measure whether the anxiolytic effect seen was in fact due to the increase in plasmatic [Mg^{2+}], we induced anxiety by injecting veratrine to rats in both groups when plasmatic [Mg^{2+}] reached values similar to baseline. Groups: P132 + IA, P132 Mg^{2+} + IA.

2.8. Data collection and analysis

Statistical analysis was performed with Prisma 8 software (La Jolla, California). All data are expressed as mean ± SEM. Normal distribution was tested using the Shapiro-Wilk test. If data met conditions for normality, the comparisons were performed using Student's *t*-test or repeated measures one- way analysis of variance (ANOVA) follow by Dunnett's post hoc tests. If data were not normally distributed, then Kruskal-Wallis, or Friedman's test nonparametric with Dunn's post hoc test and Mann Whitney test were used. Significance was set at $p < 0.05$. In Table 1 we provide a summary of statistical tests used in figures that show statistically significant differences.

3. Results

3.1. Experiment 1. Baseline anxiety

We tested the effect of chronic magnesium supplementation, veratrine or diazepam on baseline anxiety by quantifying the number of total entries to open and closed arms in the EPM (Fig. 1). We found a statistically significant decrease in the number of entries to open arms in the

Table 1
Statistical tests.

Figure	Data structure	Type test	p value	*post-hoc*
1A	Non-normal	Kruskal-Wallis	0.014	Dunn's
1B	Non-normal	Kruskal-Wallis	0.0083	Dunn's
2A	Non-normal	Kruskal-Wallis	0.0015	Dunn's
2B	Non-normal	Kruskal-Wallis	0.0006	Dunn's
3A	Non-normal	Kruskal-Wallis	0.0087	Dunn's
3B	Non-normal	Kruskal-Wallis	0.042	Dunn's
4	Non-normal	Friedman's test	<0.0001	Dunn's
6	Normal	RM one-way ANOVA F (5, 40) = 113.0	<0.0001	Dunnett's

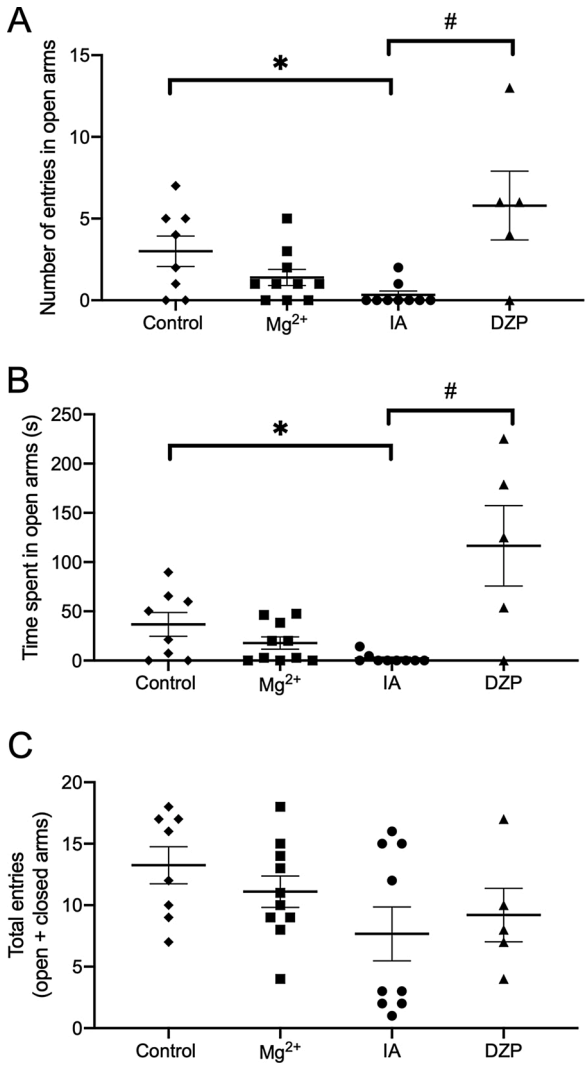

Fig. 1. Effect of Mg^{2+} supplementation, veratrine and diazepam on baseline anxiety. Rats were tested in the elevated plus maze immediately after s.c. injection of vehicle (Control and Mg^{2+} groups), veratrine (IA group) or diazepam (DZP group). **(A)** Number of entries to open arms *p = 0.049, #p = 0.0093. **(B)** Time spent in open arms. *p = 0.039, #p = 0.0047. **(C)** Total number of entries.

IA group compared to control group (p = 0.049). Rats from the control group spent 36.77 ± 12.06 s in open arms whereas rats from the IA group spent 2.1 ± 1.61 s (p = 0.039 vs control). These results confirm the anxiogenic effect of veratrine.

We did not find statistically significant differences in the total number of entries between control, Mg^{2+} and DZP groups, suggesting that baseline anxiety is not affected by Mg^{2+} supplementation or diazepam. However, we found that rats in the DZP group showed statistically significant more entries in open arms than rats in the IA group (p = 0.0093) and spent more time in open arms (116.6 ± 40.81 s, p = 0.0047 vs IA group, Fig. 1). This suggests that the anxiolytic effect of diazepam is unveil only when it is compared against the anxiogenic effect of veratrine.

3.2. Experiment 2. Mg^{2+} has an anxiolytic effect in the IA group

We tested the effect of the anxiolytic drug diazepam or 30-day magnesium supplementation to reduce the anxiety induced by veratrine. DZP + IA group showed a statistically significant increase in the number of entries in open arms (p = 0.0014 vs IA group). Rats that

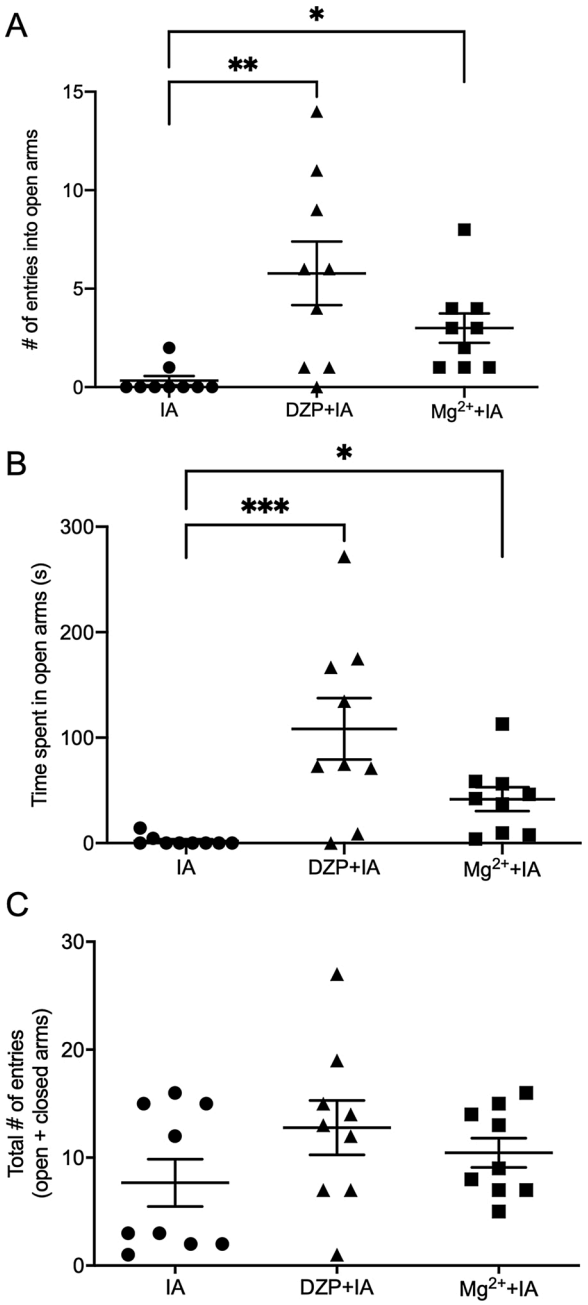

Fig. 2. Effect of 30 day-Mg^{2+} supplementation on veratrine-induced anxiety-like behavior. Rats were tested in the elevated plus maze immediately after s.c. injection of veratrine. Line and error bars represent mean ± SEM. n = 9 per group. Kruskal-Wallis test followed by a post-hoc Dunn's test. **(A)** Number of entries in open arms. *p = 0.0123, **p = 0.0014. **(B)** Time spent in open arms. *p = 0.02, ***p = 0.0003. **(C)** Total number of entries (open + closed arms). IA: induced anxiety. DZP + IA: diazepam + induced anxiety. Mg^{2+} + IA: magnesium supplementation + induced anxiety.

received chronic 30-day magnesium supplementation showed a statistically significant decrease in the number of entries (p = 0.012 vs IA group), suggesting an anxiolytic effect of the magnesium supplementation (Fig. 2A).

To further assess the anxiolytic effect of chronic magnesium supplementation and compare it to the well characterized anxiolytic drug diazepam, we quantified the time rats spent in the open arms. Rats in the IA group spent 2.1 ± 1.6 s, whereas rats in the DZP + IA group spent 108.4 ± 29 s (p = 0.0003 vs IA group) showing a clear anxiolytic effect of diazepam, which we used as positive control. Rats in the Mg^{2+} + IA

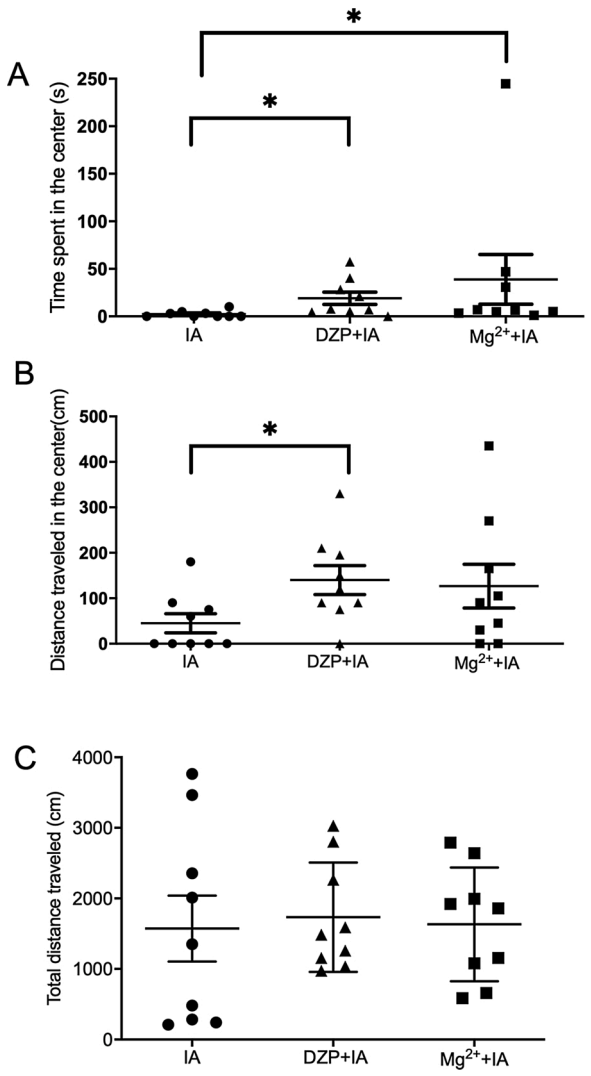

Fig. 3. Effect of 30 day-Mg^{2+} supplementation in open field test. Rats were tested in open field after inducing anxiety with veratrine. Line and error bars represent mean ± SEM. n = 9 per group. **(A)** Time rats spent in the center of the open field test. *p < 0.05. **(B)** Distance traveled in the center. *p < 0.05. **(C)** Total distance traveled. Groups as in Fig. 2.

Table 2
Concentration of magnesium in plasma (mg/dL).

Group	Pre- treatment	Post- treatment
IA	2.11 ± 0.09	2.11 ± 0.08
DZP + IA	2.38 ± 0.02	2.41 ± 0.2
Mg^{2+}+IA	2.01 ± 0.06	3.20 ± 0.03****

Data represent means ± SEM. n=9 per group. Paired t test ****p<0.0001 pre-treatment vs post- treatment. IA: induced anxiety; DZP+IA: diazepam + induced anxiety; Mg2++IA: magnesium + induced anxiety.

group spent 41.64 ± 11.3 s (p = 0.02 vs IA group). This shows that chronic Mg^{2+} supplementation has a statistically significant anxiolytic effect (Fig. 2B).

As an additional measure of anxiety, we quantified the time rats spent in the center of the open field test. Rats in the IA group spent 2.38 ± 1.16 s, whereas rats in DZP + IA spent 19.06 ± 6.51 s (p = 0.019 vs IA group) and rats in the Mg^{2+} + IA group spent 37.61 ± 26.44 s (p = 0.012 vs IA group) (Fig. 3A). These data confirm the anxiolytic effect of both diazepam and chronic Mg^{2+} supplementation. However, we did not find a statistically significant difference in the distance traveled in the center between the IA and the Mg^{2+} + IA groups, whereas rats in the DZP + IA group traveled a statistically significant larger distance than rats in the IA group (p = 0.042) (Fig. 3B).

We did not find a statistically significant change in plasmatic [Mg^{2+}] in rats that were not supplemented, whereas in rats that received supplementation we found a statistically significant increase from 2.01 ± 0.06 mg/dL to 3.2 ± 0.03 mg/dL (paired *t*-test, p < 0.0001, t = 13.31, dF = 8. Table 2). We quantified also the [Mg^{2+}] in CSF. Rats that were not supplemented (n = 5) had 1.95 ± 0.018 mg/dL of magnesium in CSF, whereas rats that were supplemented (n = 5) had 2.246 ± 0.012 mg/dL (unpaired *t*-test p < 0.0001; t = 13.13, df = 8).

3.3. Effect of treatments on locomotion

We were aware of the possibility that the injection of veratrine or diazepam, or the chronic magnesium supplementation had a sedative effect manifested as a reduction in locomotion. We tested this possibility by quantifying the number of total entries to open and closed arms in the EPM. We did not find statistically significant differences in the total number of entries among groups (Fig. 2C).

We confirmed that locomotion was not affected by measuring the total distance traveled in the open field test. We did not find statistically significant differences among groups. Rats in the IA group traveled 1573 ± 467.3 cm. Rats in the DZP + IA group traveled 1733 ± 258.6 cm and rats in the Mg^{2+} + IA group traveled 1632 ± 268.7 cm (Fig. 3C). This suggests that administration of veratrine, diazepam or chronic magnesium supplementation had not sedative effect since rats in all groups were active. However, an important difference is that rats in the DZP + IA and Mg^{2+} + IA groups spent more of the traveled time moving in the center of the open field whereas rats in the IA group spent more time moving in the edges only. This result is consistent with a preference of anxious rodents for moving around the edges of the open field and avoid the center [34], and confirms that DZP and Mg^{2+} supplementation have an anxiolytic effect.

3.4. Experiment 3. Reaching a high plasmatic [Mg^{2+}] was not enough to reduce anxiety

We found that during Mg^{2+} supplementation, plasmatic [Mg^{2+}] increased in a statistically significant manner since day 6 of supplementation (P96, 2.95 ± 0.028 mg/dL, p < 0.05 vs baseline plasmatic [Mg^{2+}] in P90). In day 9 of supplementation (P99) plasmatic [Mg^{2+}] was 3.38 ± 0.02 mg/dL (p < 0.001 vs baseline), and in day 12 (P102) it reached values similar to those seen after 30 days of supplementation (3.52 ± 0.03 mg/dL, p < 0.0001 vs baseline) (Fig.4). In day 13, we

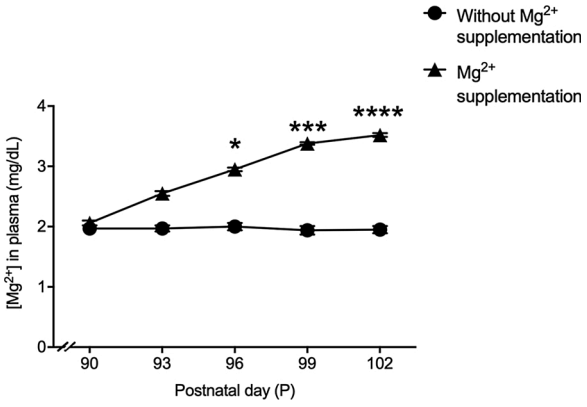

Fig. 4. Measurement of plasma Mg^{2+} concentration during 12 days of Mg^{2+} supplementation. Symbols and error bars represent mean ± SEM. Circles: group without Mg^{2+} supplementation (n = 5). Triangles: group with Mg^{2+}-supplementation (n = 9). Friedman test followed by a post-hoc Dunn's test: *p < 0.05, *** p < 0.001, **** p < 0.0001.

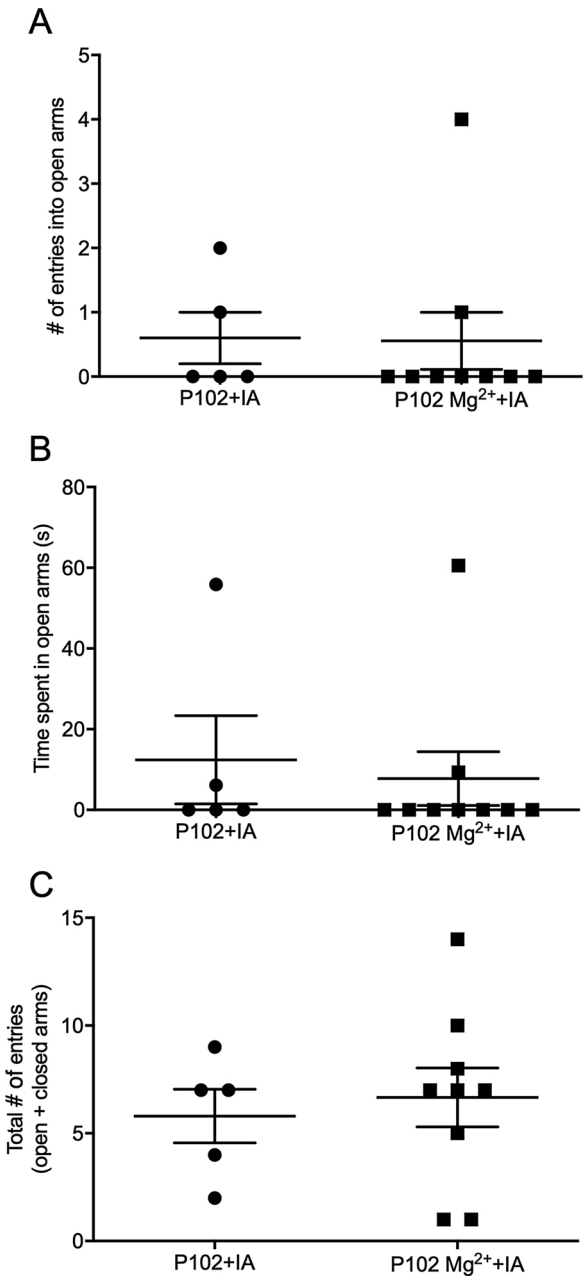

Fig. 5. Effect of 12 day-Mg^{2+} supplementation on veratrine-induced anxiety-like behavior (P102Mg^{2+} + IA). Rats were tested in elevated plus maze immediately after s.c. injection of veratrine. **(A)** Number of entries into open arms. **(B)** Time spent in open arms. **(C)** Total number of entries (open + closed arms). Line and error bars represent mean ± SEM. P102 + IA n = 5, P102Mg^{2+} + IA n = 9.

induced anxiety by injecting veratrine. We did not find statistically significant differences between P102 + IA and P102 Mg^{2+} + IA groups, showing that despite the fact that plasmatic [Mg^{2+}] was similar to that after 30-day supplementation, 12-day Mg^{2+} supplementation had not anxiolytic effect. (Fig. 5).

3.5. Experiment 4. Anxiolytic effect of chronic magnesium supplementation washed-out in 9 days

After 30-day supplementation, plasmatic [Mg^{2+}] was 2.17 ± 0.07 mg/dL in the group that did not received supplementation and 3.32 ± 0.07 mg/dL in the group that received magnesium supplementation (RM one-way ANOVA F [5,40] = 113, $p < 0.0001$. Dunnett's test q = 14.77, p

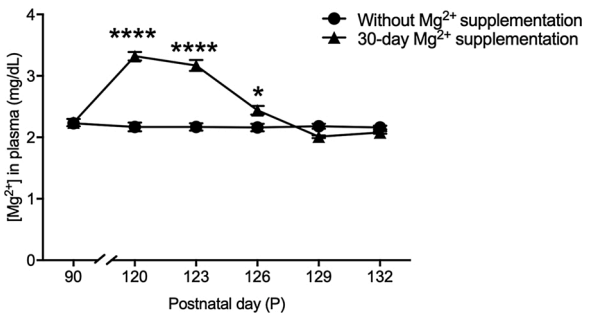

Fig. 6. Plasma Mg^{2+} concentration during 30 days of Mg^{2+} supplementation. Symbols and error bars represent mean ± SEM. Circle: group without Mg^{2+} supplementation (n = 5). Triangles: group with Mg^{2+} supplementation (n = 9). Repeated measures ANOVA F [5,40] = 113, $p < 0.0001$. Post-hoc Dunnett's test: q = 14.77 for P120, q = 12.72 for P123, q = 2.75 for P126. *$p < 0.05$ vs baseline, **** $p < 0.0001$ vs baseline.

< 0.0001 vs baseline). We discontinued supplementation and measured plasmatic [Mg^{2+}] every three days. We found that plasmatic [Mg^{2+}] remained statistically significantly higher than control [Mg^{2+}] after 3 days (3.17 ± 0.09 mg/dL, Dunnett's test q = 12.72, $p < 0.0001$ vs baseline) and 6 days (2.44 ± 0.07 mg/dL, Dunnett's test q = 2.75, $p < 0.05$ vs baseline), and reached baseline values after 9 days (2.01 ± 0.02 mg/dL, n.s. vs baseline). After 12 days (P132, see Methods), plasmatic [Mg^{2+}] remained similar to that in baseline (2.08 ± 0.02 mg/dL, n.s. vs baseline) (Fig. 6). We induced anxiety at P132 by injecting veratrine to rats in both groups and we quantified the number of entries and time spent in open arms, and the total number of entries to open and close arms in the EPM test (Fig. 7). We did not find statistically significant differences between P132 + IA and P132 Mg^{2+} + IA groups. This shows that, when [Mg^{2+}] in plasma returns to baseline levels, the anxiolytic effect due to previous supplementation is not maintained, suggesting that a constant magnesium supplementation should be present to maintain a low anxiety level.

3.6. Effect of treatments on grooming

Novelty-induced grooming behavior has been used as a marker of anxiety. i.e., it increases in the presence of anxiogenic conditions and decreases in the presence on anxiolytics [35–37]. We tested the anxiolytic effect of diazepam or 30-day magnesium supplementation to reduce the anxiety induced by veratrine. Rats in the IA group groomed for 217.5 ± 29.1 s. Rats in the DZP + IA group groomed for 35.7 ± 13.4 s (Dunn's test $p < 0.001$ vs IA) and rats in the Mg^{2+} + IA group groomed for 36.2 ± 11.3 s (Dunn's test $p < 0.01$ vs IA; Fig. 8). This suggests that diazepam or chronic magnesium supplementation have an anxiolytic effect in rats injected with veratrine.

4. Discussion

Here, we studied whether magnesium supplementation can prevent or ameliorate acutely induced anxiety-like behavior in rat. We found that chronic Mg^{2+} supplementation via drinking water increased the concentration of magnesium in plasma and CSF and decreased anxiety-like behavior without affecting locomotion and exploration.

4.1. Magnesium and anxiety

Rodent studies link magnesium deficiency in diet and anxiety-like behavior. In mice, depletion of magnesium from diet during 3 weeks elicits anxiety-like behavior measured in behavioral tests such as open field, light/dark and EPM tests [38]. An anxiolytic effect of magnesium is seen in mice that received acutely intraperitoneally magnesium hydroaspartate (30 mg/ kg) 30 min before performing the EPM test.

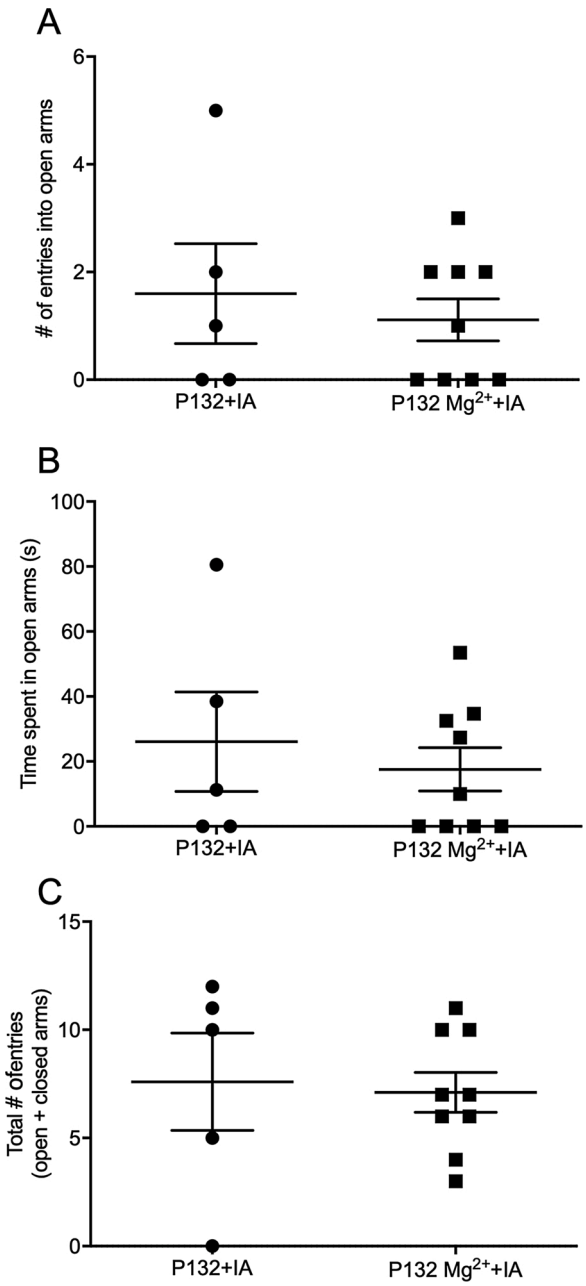

Fig. 7. Effect of 12-day washout period on veratrine-induced anxiety-like behavior (P132 Mg^{2+} + IA). Rats were tested in elevated plus maze. **(A)** Number of entries into open arms. **(B)** Time spent in open arms. **(C)** Total number of entries (open + closed arms). Lines and error bars represent mean ± SEM. P132 + IA group n = 5. P132 Mg^{2+} + IA group n = 9.

Such anxiolytic effect is associated with a 74 % increase in serum [Mg^{2+}] with respect to baseline concentration [23]. However, chronic intraperitoneal administration of magnesium hydroaspartate (30 mg/kg for 14 days) does not reduce the levels of anxiety despite the fact that the [Mg^{2+}] in plasma increases 39 % with respect to baseline. In our hands, chronic 30-day intake (50 mg/kg/day of elemental magnesium, from magnesium chloride) increased 59 % the [Mg^{2+}] in plasma and had an anxiolytic effect as shown in the EPM test, open field and grooming behavior. However, we found that reaching a high plasma [Mg^{2+}] is not enough to produce an anxiolytic effect, since 12-day supplementation did not reduce the induced anxiety. Further studies are needed to explain whether this discrepancy is due to dose, magnesium salt, method of administration, or duration of treatment.

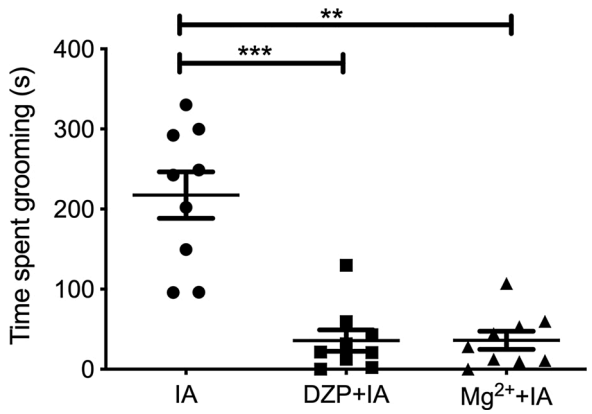

Fig. 8. Effect of diazepam and 30 day-Mg^{2+} supplementation on time spent grooming in rats with veratrine-induced anxiety-like behavior. Time rats spent grooming was quantify as an anxiety marker. Symbols and error bars represent mean ± SEM. IA: induced anxiety. DZP + IA: diazepam + induced anxiety. Mg^{2+} + IA: magnesium supplementation + induced anxiety. *** $p < 0.001$; **$p < 0.01$.

4.2. Magnesium supplementation: oral vs injected; acute vs chronic

In rodent models, therapeutic magnesium can be administered orally via gastric probe or drinking water, or it can be injected intraperitoneal, intramuscular, intravenous or subcutaneously. We decided to perform oral administration via the drinking water to avoid introducing the stress of rat manipulation as a variable. However, several studies using i.p. injections of magnesium show a reduction of anxiety- and depression-like behaviors [23,39]. In a model of traumatic brain injury, rats receiving magnesium intravenously 30 min after injury and evaluated for post-traumatic depression/anxiety behavior in open field test show only a 30 % incidence of depression/anxiety compared to 61 % in non-treated rats [21]. This shows the benefital effect of accute magnesium supplementation. Both, acute single dose and chronic administration of magnesium enhances long-term memory in rats [40].

4.3. Baseline vs induced anxiety

Most studies test the effect of magnesium deficiency and magnesium supplementation on the baseline anxiety elicited by the open field test [19,38,39,41]. We studied the anxiolytic effect of chronic magnesium supplementation on anxiety-like behavior induced by s.c. injection of veratrine. Veratrine acts as anxiogenic presumably by enhancing NMDA receptor-mediated glutamatergic transmission [30,42]. Magnesium is an extensively characterized voltage-dependent blocker of NMDA receptor [43,44]. We hypothesize that the most likely mechanism of action of magnesium supplementation is via blockade of NMDAR during the acute actions of veratrine. Such blockade may be achieved only when [Mg^{2+}] in CSF reaches a threshold, e.g., the one achieved after 30-day supplementation. This could be the reason why we did not see an anxiolytic effect after 12 days of supplementation despite the fact that [Mg^{2+}] in plasma increased. However, the fact that magnesium crosses the brain blood barrier (BBB) in piglets in an age-dependent manner, i.e., the younger the subject, the more permeable the BBB is to magnesium [45], suggests that age of treatment may be an important variable to consider.

4.4. Magnesium bioavailability

In human adults, the ratio of [Mg^{2+}] in CSF with respect to serum is 1.3 [46]. In Wistar rats, the ratio in baseline conditions ranges from 1.05 and 1.12 [47] to 1.3 [48]. In our hands, the ratio was 0.92, i.e., [Mg^{2+}] in plasma was higher than in CSF. This discrepancy may be due to detection methods, i.e., atomic absorption spectrophometry vs

colorimetry or to the baseline [Mg^{2+}] in the potable water and the period it was administered.

A variety of commercially available magnesium supplements for human intake are recommended for having high bioavailability, e.g., magnesium chloride, magnesium oxide, magnesium citrate, magnesium L-threonate [31,49]. The effects of magnesium L-threonate on behavior and the mechanisms involved have been systematically characterized in rodent models [31,50,51]. In our study, we used the low-cost magnesium chloride for supplementation in the drinking water. We did not observe a decrease in the consumption of water or food or changes in body weight, and stools remained firm during the time of the study, suggesting that magnesium did not disturb gastric health of rats. We controlled the mineral concentrations of drinking water such that they resembled the average tap water in North American Cities [52].

In our hands, magnesium chloride supplementation decreased the anxiety-like behavior induced pharmacologically. In rats feed with a Mg-deficient diet, chronic 49-days magnesium chloride supplementation via orally inserted intragastric intubation restores to baseline some acute responses such as locomotor, anxiety-like and depression-like behaviors caused by agonists and blockers of dopamine and serotonin receptors [19]. We consider our work as an addition to the current knowledge in the magnesium supplementation field because we study the time course of magnesium supplementation via water intake to increase the [Mg^{2+}] in plasma and CSF of rats with normal [Mg^{2+}], as well as the time course for the increase to be washed out. We also contribute to the anxiety field because, instead of studying baseline anxiety, we studied an extreme form of pharmacologically-induced acute anxiety.

4.5. Caveats

Anxiety is a complex behavior influenced by genetic and environmental factors. A limitation of our study is the use of Wistar rats that do not display a high innate anxiety. The use of rat strains selectively bred or genetically modified mice to study the anxiolytic effect of Mg^{2+} would provide more conclusive data. Another limitation of our study is that the experimental groups magnesium supplementation alone, diazepam alone and veratrine alone were not carried out in parallel with the rest of the experimental groups. We recognize this as a limitation because we cannot perform statistical comparisons among all groups since we consider inappropriate to compare experimental groups that did not run in parallel despite the fact that they have equal n. Last, in our study we used only two behavioral tests, both using the natural aversion of rodents to open spaces as main trigger for anxiety. To evaluate the anxiolytic effect of Mg^{2+} supplementation on other forms of anxiety, more behavioral tests such as the black-white box and the elevated T maze are needed.

5. Conclusion

This study shows that oral magnesium supplementation via the drinking water for one month decreased the anxiety-like behavior induced by veratrine in rats.

We propose that oral magnesium supplementation via water intake is an effective anxiolytic with a non-invasive administration, an affordable cost and without causing side effects.

Statment

CMV and LBP contributed to the study conception, design and analysis. Material preparation, data collection and analysis were performed by MMC, SRN and MLLM. The first draft of the manuscript was written by MMC and all authors commented on following versions of the manuscript. All authors read and approved the final manuscript.

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