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NMDA/glutamate mechanism of magnesium-induced anxiolytic-like behavior in mice

Ewa Poleszak[1], Piotr Wlaź[2], Andrzej Wróbel[3], Sylwia Fidecka[1], Gabriel Nowak[4,5]

[1]Department of Pharmacology and Pharmacodynamics, Medical University of Lublin, Staszica 4, PL 20-081 Lublin, Poland

[2]Department of Animal Physiology, Insitute of Biology, Maria Curie-Skłodowska University, Akademicka 19, PL 20-033 Lublin, Poland

[3]Second Department of Gynecology, Medical University of Lublin, Jaczewskiego 8, PL 20-090 Lublin, Poland

[4]Department of Neurobiology, Institute of Pharmacology, Polish Academy of Sciences, Smętna 12, PL 31-343 Kraków, Poland

[5]Department of Cytobiology and Histochemistry, Collegium Medicum, Jagiellonian University, Medyczna 9, PL 30-688 Kraków, Poland

Correspondence: Ewa Poleszak, e-mail: ewa.poleszak@am.lublin.pl

Abstract:
The anxiolytic-like activity of magnesium in mice during the elevated plus maze (EPM) has been demonstrated previously. In the present study, we examined the involvement of NMDA/glutamate receptor ligands on the magnesium effect on the EPM.
We demonstrated that low, ineffective doses of NMDA antagonists (the competitive NMDA antagonist CGP 37849, 0.3 mg/kg; an antagonist of the glycine$_B$ sites, L-701,324, 1 mg/kg; a partial agonist of the glycine$_B$ sites, D-cycloserine, 2.5 mg/kg; and the non-competitive NMDA antagonist MK-801, 0.05 mg/kg) administered together with an ineffective dose of magnesium (10 mg/kg) evoked a significant increase in the percentage of time spent in the open arm of the maze (an index of anxiety). Moreover, magnesium-induced anxiolytic-like activity (20 mg/kg) was antagonized by D-serine (100 nmol/mouse), an agonist of glycine$_B$ site of the NMDA receptor complex.
The present study demonstrates the involvement of the NMDA/glutamate pathway in the magnesium anxiolytic-like activity in the EPM in mice, and that this activity particularly involves the glycine$_B$ sites.

Key words:
magnesium, NMDA receptor ligands, anxiety, elevated plus maze, mice

Introduction

Anxiety disorders are the most common affective diseases and are the most chronic of the mental illnesses [12]. Benzodiazepines (BDZ) are still widely used for the treatment of several anxiety disorders [28, 49]. Unfortunately, this class of drugs induces many adverse side effects including tolerance, sedation, myorelaxation and memory disturbances [50]. Moreover, long-term BDZ administration induces dependence, sometimes resulting in severe withdrawal syndrome [20]. BDZ are effective only in 70% of patients and many patients are treatment-refractory, requiring more

effective drugs. The anxiolytic effect of BDZ is known to be connected with enhancement of inhibitory GABAergic neurotransmission. GABA is the predominant inhibitory neurotransmitter in the central nervous system (CNS). In contrast, the glutamate system is the major excitatory neurotransmitter system in the brain. Thus, it can be supposed that inhibition of glutamatergic transmission may produce a similar effect as enhancement of GABAergic transmission.

Glutamate acts *via* stimulation of ionotropic [N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid (AMPA), and kainate] and metabotropic receptors [21]. The NMDA receptor complex consists of an ion channel with regulatory sites for glutamate, glycine, phencyclidine, polyamines, zinc and magnesium [40]. These glutamate receptors are found in high concentrations in cortical and limbic regions, which accounts for its effects on cognition, perception, mood and psychiatric disorders [17]. Thus, NMDA ligands acting with NMDA receptor complex at different sites have been widely investigated as potential agents for the treatment of a variety of neuropsychiatric illnesses.

Numerous experimental studies have demonstrated the antidepressant- and anxiolytic-like activity of various NMDA receptor antagonists. It has been shown that competitive and noncompetitive antagonists, polyamine site antagonists and the inorganic inhibitors of NMDA receptor function zinc and magnesium [6, 25] produced antidepressant-like effects in preclinical antidepressant screening procedures [3, 15, 16, 24, 46–48, 54]. Similarly, several lines of evidence suggest an anxiolytic-like profile of NMDA receptor ligands. In animals, competitive and noncompetitive receptor antagonists and glycine$_B$ partial agonists and antagonists demonstrated anxiolytic-like activity [11, 29, 38]. Despite these findings, a serous drawback of competitive and noncompetitive NMDA receptor antagonists is that they produce strong adverse effects (e.g., psychotomimetic-like symptoms), thus limiting their potential clinical use in humans [51, 56]. Thus, an alternative strategy to the use of competitive and noncompetitive NMDA antagonists may be to use other NMDA ligands that modulate NMDA receptor function. There is evidence that glycine strongly potentiates the action of NMDA receptors [10] and is required for receptor activation by glutamate [22]. Further studies have shown that antagonists and partial agonists of the glycine$_B$ site inhibit the function of the NMDA receptor complex and exhibit anxiolytic-like activity in many experimental models of anxiety [11, 14, 38]. Similarly, magnesium, an inorganic inhibitor of NMDA receptor function [25, 55], also evokes anxiolytic-like activity in the EPM test [32] and potentiates the anxiolytic-like activity of classical benzodiazepines (diazepam and chlordiazepoxide) [30] . The anxiolytic-like activity of magnesium was shown to be antagonized by flumazenil (a specific antagonist of the benzodiazepine/GABA$_A$ receptors), thus proving the influence of the benzodiazepine/GABA$_A$ system in the anxiolytic activity of magnesium [30]. Moreover, magnesium depletion leads to an increase in depression- and anxiety-related behavior in various behavioral tests in animals [23, 45]. Previous reports have demonstrated that magnesium evokes antidepressant-like activity in rodents [3, 32, 35] and that magnesium administration at doses ineffective *per se* with antidepressants produced synergistic effects [31, 36]. Moreover, magnesium at an ineffective dose of 10 mg/kg normalized the increased immobility time in mice subjected to restraint stress [34]. Furthermore, the antidepressant activity of magnesium involves the NMDA/glutamate pathway (as with organic NMDA antagonists) [33, 37].

In clinical trials, a low level of magnesium has been demonstrated in patients with anxiety and depression [7, 13, 42, 43]. Furthermore, magnesium therapy used supplementary to lithium in mania significantly reduced the doses of benzodiazepines and neuroleptics [8].

On the basis of the above data, in the present study we examined whether the anxiolytic-like activity of magnesium is mediated by the NMDA receptor complex. We investigated the effect of NMDA receptor ligands on anxiolytic-like effects of magnesium in the EPM test in mice.

Materials and Methods

Animals

All procedures were approved by the Ethical Committee of the Medical University, Lublin. The experiments were carried out on male Swiss Albino mice (25–30 g). The animals were kept on a natural day-night cycle with free access to food and water. Each experimental group consisted of 8–12 animals.

Drug administration

Magnesium hydroaspartate (Farmapol, Poznań, Poland) was always administered intraperitoneally (*ip*) 30 min before the test. The doses of magnesium refer to pure magnesium ions. 7-Chloro-4-hydroxy-3-(3-phenoxy)phenylquinolin-2[1H]-one (L-701,324, Sigma) was suspended in a 1% aqueous solution of Tween 80 and administered *ip* 60 min before the test. N-methyl-D-aspartic acid (NMDA; Sigma), DL-/E/-amino-4-methyl-5-phosphono-3-pentenoic acid (CGP 37849, Tocris), (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801, Sigma), and (R)-4-amino-3-isoxazolidone (D-cycloserine, Sigma) were dissolved in 0.9% NaCl and administered *ip* 60 min before the test. D-serine (Sigma) was also dissolved in 0.9% NaCl and administered intracerebroventricularly (*icv*) 15 min before the test. *Icv* administration was performed according to a modified method described by Lipman and Spencer [18]. Control animals received an *ip* or *icv* injection of saline (vehicle). The volume of vehicles or drug solutions for *ip* and *icv* administrations was 10 ml/kg and 5 μl per mouse, respectively.

Elevated plus-maze test

The EPM studies were carried out on mice according to the method of Lister [19]. The EPM apparatus was made of Plexiglas and consisted of two open (30 × 5 cm) and two enclosed (30 × 5 × 15 cm) arms. The arms extended from a central platform of 5 × 5 cm. The apparatus was mounted on a Plexiglas base, raising it 38.5 cm above the floor, and illuminated by a red light. The test consisted of placing a mouse in the center of the apparatus (facing an enclosed arm) and allowing it to freely explore. The number of entries into the open arms and the time spent in these arms were scored for a 5 min test period. An entry was defined as placing all four paws within the boundaries of the arm. The following measures were obtained from the test: the total number of arm entries; the percentage of arm entries into the open arms; and the time spent in the open arms expressed as a percentage of the time spent in both the open and closed arms. Anxiolytic activity was indicated by increases in the time spent in open arms or in the number of open arm entries. The total number of entries into either type of arm was used as a measure of overall motor activity.

Statistics

The obtained data were evaluated by one way analysis of variance (ANOVA) followed by the Bonferroni's *post-hoc* test. All results are presented as the means ± SEM. $p < 0.05$ was considered as statistically significant.

Results

The anxiolytic-like effect of joint administration of magnesium and CGP 37849 in the EPM test

CGP 37849 given alone at a dose of 0.6 mg/kg significantly increased the percentage of time spent in the open arms and the percentage of the entries into the open arms (Fig. 1). Magnesium administered at a dose of 10 mg/kg or CGP 37849 administered at a dose of 0.3 mg/kg (given alone) did not change the percentage of time spent in or entries into the open arms (Fig. 1). The joint administration of magnesium and CGP 37849, however, significantly increased the percentage of time spent in the open arms [Fig. 1A; ANOVA: $F(4, 39) = 8.587$, $p < 0.0001$] and enhanced the number of entries into the open arms [Fig. 1B; ANOVA: $F(4, 39) = 3.736$, $p = 0.0115$].

The anxiolytic-like effect of joint administration of magnesium and MK-801 in the EPM test

Magnesium administered at a dose of 10 mg/kg did not change either the percentage of the time spent in or entries into the open arms (Fig. 2A and B). MK-801 given alone at a dose of 0.1 mg/kg significantly increased the percentage of time spent in the open arms (Fig. 2A), but, at a dose 0.05 mg/kg, it had no effect (Fig. 2A and B). The joint administration of magnesium and MK-801 (both at ineffective doses) significantly increased the percentage of time spent in the open arms [Fig. 2A; ANOVA: $F(4, 40) = 3.981$, $p = 0.0082$], but it did not significantly change the number of entries into the open arms [Fig. 2B; ANOVA: $F(4, 40) = 1.440$, $p = 0.2386$].

The anxiolytic-like effect of joint administration of magnesium and L-701,324 in the EPM test

Magnesium administered at a dose of 10 mg/kg did not change either the percentage of the time spent in

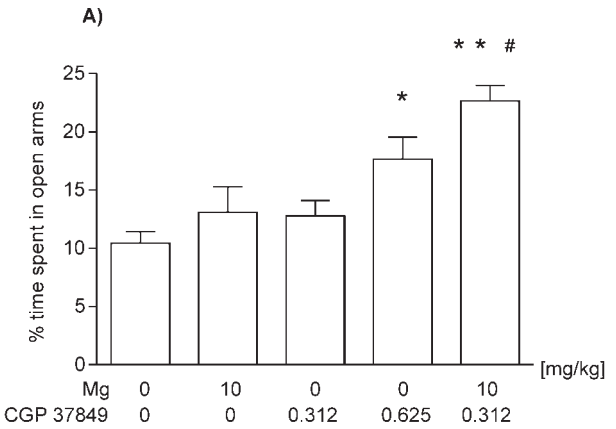

Fig. 1. Effect of joint administration of CGP 37849 and magnesium in the elevated plus-maze procedure in mice (percentage of time spent in open arms – **A**, and number of open arms entries – **B**). CGP 37849 was administered 60 min before the test, and magnesium was administered 30 min after the CGP 37849 injection. The values represent the means ± SEM (n = 8–9 mice per group). The absolute values in vehicle-treated mice were as follows: time, 30.00 ± 3.18, and number of open arm entries, 4.22 ± 0.70. * $p < 0.05$; and ** $p < 0.001$ *vs.* control (vehicle-treated group), # $p < 0.01$ *vs.* the magnesium- or CGP 37849 (0.3 mg/kg)-treated group (Bonferroni's test)

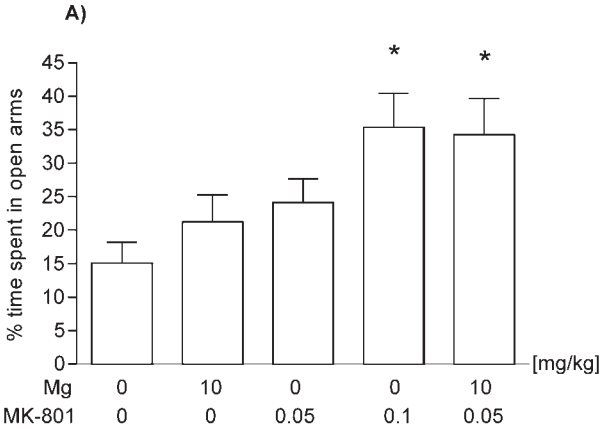

Fig. 2. Effect of joint administration of MK-801 and magnesium in the elevated plus-maze procedure in mice (percentage of time spent in open arms – **A**, and number of open arms entries – **B**). MK-801 was administered 60 min before the test, and magnesium was administered 30 min after the MK-801 injection. The values represent the means ± SEM (n = 8–10 mice per group). The absolute values in vehicle-treated mice were as follows: time, 25.22 ± 4.08; and number of open arm entries, 4.77 ± 0.81. * $p < 0.001$ *vs.* control (vehicle-treated group) (Bonferroni's test)

or entries into the open arms (Fig. 3A and B). L-701,324 given alone at a dose of 4 mg/kg significantly increased the percentage of time spent in the open arms (Fig. 3A), without a significant influence on the number of entries into the open arms (Fig. 3B). A L-701,324 dose of 2 mg/kg had no effect in this test (Fig. 3A and B). The joint administration of magnesium and L-701,324 (both at ineffective doses) significantly increased the percentage of time spent in the open arms [Fig. 3A; ANOVA: $F(4, 38) = 6.813$, $p = 0.0003$], with a marginal effect on the number of entries into the open arms [Fig. 3B; ANOVA: $F(4, 38) = 2.909$, $p = 0.0341$].

The anxiolytic-like effect of joint administration of magnesium and D-cycloserine in the EPM test

Magnesium administered at a dose of 10 mg/kg did not change either the percentage of time spent in or entries into the open arms (Fig. 4A and B). D-cycloserine, given alone at a dose of 5 mg/kg, significantly increased the percentage of time spent in the open arms, with no influence on the number of entries into the open arms. A dose of 2.5 mg/kg D-cycloserine was ineffective (Fig. 4A and B). Magnesium, administered at the ineffective dose of 10 mg/kg, and D-cycloserine, administered at the dose of 2.5 mg/kg,

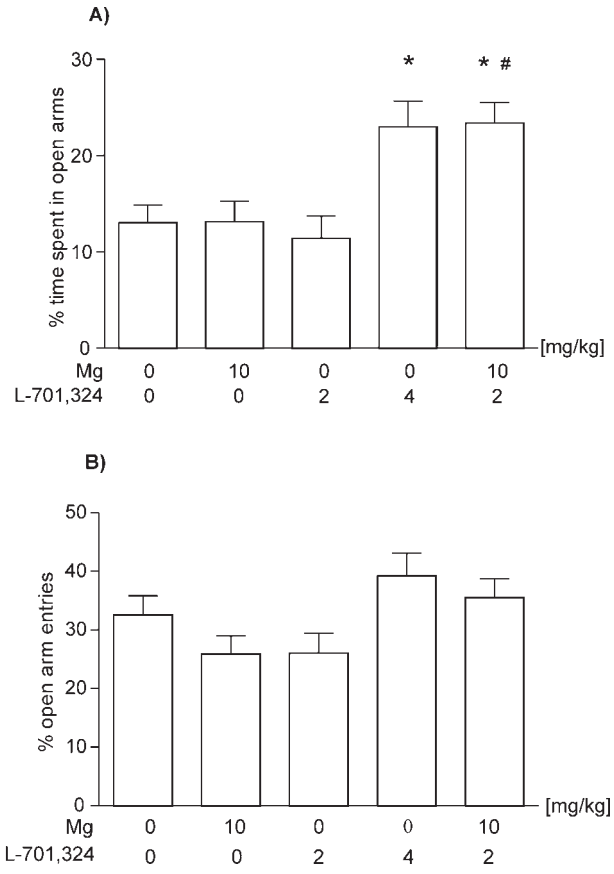

Fig. 3. Effect of joint administration of L-701,324 and magnesium in the elevated plus-maze procedure in mice (percentage of time spent in open arms – **A**, and number of open arms entries – **B**). L-701,324 was administered 60 min before the test, and magnesium was administered 30 min after the L-701,324 injection. The values represent the means ± SEM (n = 8–9 mice per group). The absolute values in vehicle-treated mice were as follows: time, 38.89 ± 5.50; and number of open arm entries, 5.77 ± 1.01. * $p < 0.05$ *vs.* control (vehicle-treated group), # $p < 0.05$ *vs.* magnesium- and $p < 0.01$ *vs.* L-701,324 (2 mg/kg)-treated group (Bonferroni's test)

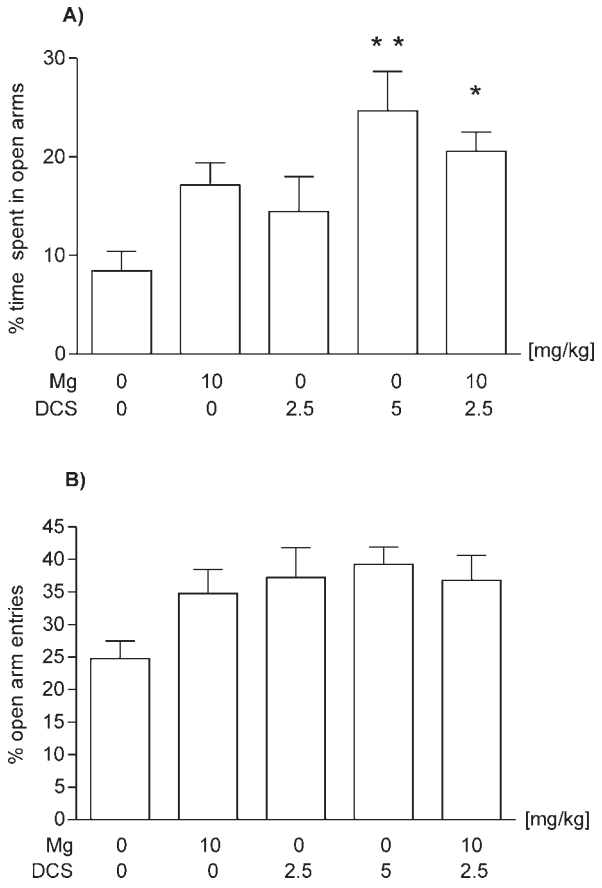

Fig. 4. Effect of joint administration of D-cycloserine and magnesium in the elevated plus-maze procedure in mice (percentage of time spent in open arms – **A**, and number of open arms entries – **B**). D-cycloserine was administered 60 min before the test, and magnesium was administered 30 min after the D-cycloserine injection. The values represent the means ± SEM (n = 8–9 mice per group). The absolute values in vehicle-treated mice were as follows: time, 25.44 ± 5.81; and number of open arm entries, 4.22 ± 0.72. * $p < 0.05$; ** $p < 0.01$ *vs.* control (vehicle-treated group) (Bonferroni's test)

significantly increased the percentage of time spent in the open arms [Fig. 4A; ANOVA: $F(4, 39) = 4.13$, $p = 0.0038$] with a marginal effect on the number of entries into the open arms [Fig. 4B; ANOVA: $F(4, 39) = 2.587$, $p = 0.0517$].

The effect of D-serine on the anxiolytic-like activity of magnesium in the EPM test

Magnesium given at a dose of 20 mg/kg produced an anxiolytic-like effect, significantly increasing the percentage of the time spent in the open arms and increasing the number of entries into the open arms (Fig. 5A and B). The increase in the percentage of time spent in the open arms induced by magnesium (20 mg/kg) was significantly reversed by D-serine (100 nmol/mouse) [Fig. 5A; ANOVA: $F(3, 38) = 9.578$, $p < 0.0001$]. The increase in the number of open arm entries induced by magnesium (20 mg/kg) was not significantly changed by D-serine [Fig. 2B: ANOVA: $F(3, 38) = 4.251$ $p = 0.0110$]. D-serine given alone had no effect on either the time spent in or the entries into the open arms of the EPM test (Fig. 5).

The effect of magnesium and NMDA ligands on the total arm entries

Magnesium and all tested NMDA receptor ligands, administered alone or in combination, did not alter the number of total arm entries (Tab. 1).

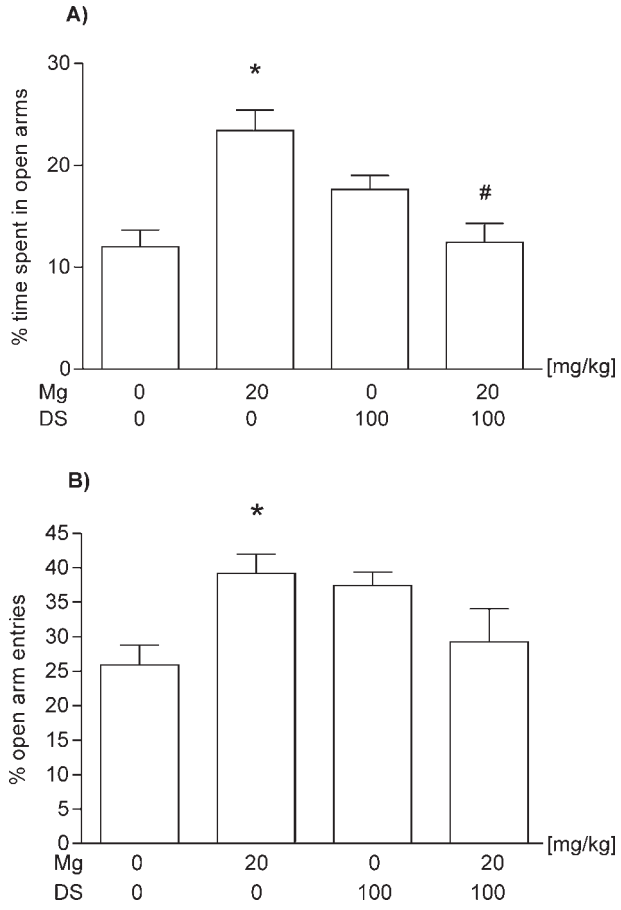

Fig. 5. Effect of D-serine on the action of magnesium in the elevated plus-maze procedure in mice (percentage of time spent in open arms – **A**, and number of open arms entries – **B**). Magnesium was administered 30 min before the test, and D-serine was administered 15 min after magnesium injection. The values represent the means ± SEM (n = 9–12 mice per group). The absolute values in vehicle-treated mice were as follows: time, 36.36 ± 4.95; and number of open arm entries, 4.54 ± 0.66. * $p < 0.001$ *vs.* control (vehicle-treated group), # $p < 0.001$ *vs.* the magnesium-treated group (Bonferroni's test)

Tab. 1. The number of total arm entries for all experimental groups

Treatment and dose	Number of total arm entries
Vehicle	16.89 ± 0.58
Magnesium 10 mg/kg	14.44 ± 1.01
CGP 37849 0.3 mg/kg	18.00 ± 1.57
CGP 37849 0.6 mg/kg	19.56 ± 3.67
Magnesium 10 mg/kg and CGP 37849 0.3 mg/kg	15.38 ± 1.32
	F(4, 39) = 3.736, p = 0.0115
Vehicle	20.00 ± 2.31
Magnesium 10 mg/kg	19.30 ± 1.52
MK-801 0.05 mg/kg	23.38 ± 2.79
MK-801 0.1 mg/kg	27.67 ± 2.08
Magnesium 10 mg/kg and MK-801 0.05 mg/kg	23.89 ± 3.62
	F(4, 40) = 1.829, p = 0.1422
Vehicle	16.78 ± 1.52
Magnesium 10 mg/kg	17.75 ± 1.42
L-701,324 2 mg/kg	14.89 ± 0.71
L-701,324 4 mg/kg	18.69 ± 2.27
Magnesium 10 mg/kg and L-701,324 2 mg/kg	17.13 ± 1.39
	F(4, 38) = 0.8323, p = 0.05131
Vehicle	16.78 ± 2.01
Magnesium 10 mg/kg	16.00 ± 1.40
D-cycloserine (DCS) 2.5 mg/kg	14.67 ± 1.88
D-cycloserine (DCS) 5 mg/kg	18.33 ± 1.19
Magnesium 10 mg/kg + DCS 2.5 mg/kg	16.22 ± 1.42
	F(4, 39) = 0.6843, p = 0.6071
Vehicle	16.0 ± 2.11
Magnesium 20 mg/kg	20.75 ± 0.98
D-serine 100 nmol/mouse	19.90 ± 1.56
Magnesium and D-serine	14.67 ± 1.17
	F(3, 37) = 3.653, p = 0.0208

Data represent the means ± SEM; n = 9–12. CGP 37849, MK-801, L-701,324 and D-cycloserine were administered 60 min before the test, and magnesium was administered 30 min before the test. D-serine was administered *icv* 15 min before the test. The obtained data were evaluated by one way analysis of variance (ANOVA) followed by the Bonferroni test *post-hoc*

Discussion

Several lines of evidence suggest an involvement of glutamate in anxiety. It has been shown that NMDA receptor antagonists exhibit anxiolytic-like activity in animals. The preclinical animal studies demonstrated that an uncompetitive NMDA antagonist, dizocilpine (MK-801) [4, 11, 44]; a competitive NMDA antagonist, CGP 37849 [39]; partial agonists of the glycine$_B$ sites, D-cycloserine [11] and ACPC (1-aminocyclopropanecarboxylic acid) [52, 53]; and an antagonist of glycine$_B$ sites of the NMDA receptor, L-701,324 [11, 14, 38], demonstrated anxiolytic-like activity. Thus, different NMDA antagonists showed an anxiolytic-like profile, similar to that of the benzodiazepine di-

azepam [29]. Also, recent genetic studies indicate anxiolytic-like behavior in mice harboring a genetic deletion in the NR2A subunit of NMDA receptor [2]. Additionally, the excitatory amino acid agonist NMDA produced anxiogenic effects in the EPM test [4].

Validation of the EPM procedure has shown that it is sensitive to drugs that produce anxiolytic or anxiogenic effects in humans [26], including drugs that have non-benzodiazepine sites of action [27]. Clinical studies have shown that memantine (a non-competitive NMDA receptor antagonist) and D-cycloserine (partial agonists of glycine$_B$ sites) are potential therapeutic agents for anxiety disorders (phase III and II clinical trials, respectively) [28].

In the present study, we demonstrate the influence of NMDA antagonists on the anxiolytic-like activity of magnesium in the EPM test in mice. We have shown that competitive (CGP 37849) and uncompetitive (MK-801) antagonists, a partial agonist of the glycine$_B$ site (D-cycloserine), and a glycine$_B$ antagonist (L-701,324) produced anxiolytic-like activity in the EPM test, the results that support previously published data [4, 11, 14, 39, 44]. The uncompetitive NMDA receptor antagonist (MK-801), at a dose 0.1 mg/kg, increased the open-arm exploration time, but a dose 0.05 mg/kg was ineffective. Our observations are in line with those obtained by Karcz-Kubicha et al. [11] in rats. The anxiolytic-like activity of MK-801 was also observed over a wide dose range (0.025–0.15 mg/kg) [4, 11, 44]. It should be noted, however, that the anxiolytic effect of MK-801 was less than or equal to the benzodiazepines [4, 11]. The observed variation between the doses used between our and other studies may be the result of the use of different species of animals (e.g., mice and rats). In the present study, in order to evaluate the effect of MK-801 on the anxiolytic-like activity of magnesium, we chose a dose of 0.05 mg/kg, which by itself was ineffective in the EPM test. The joint administration of this low dose of MK-801 with an ineffective dose of magnesium increased the time spent in the open arms of the EPM.

CGP 37849, the competitive NMDA antagonist, also shows possible anxiolytic-like effects. The results described by other authors indicate that a single injection of CGP 37849 evokes an anxiolytic-like activity in the Vogel conflict drinking test [29], open field test [29] and EPM test in rats [39]. In our study, 0.325 mg/kg CGP 37849 produced synergistic effects with magnesium in the EPM test in mice.

The fact that competitive and uncompetitive NMDA antagonists induce severe side effects, including motor impairment, hyperactivity, stereotypy and psychotomimetic actions [56], limits their potential therapeutic use in humans. After the discovery that *in vivo* inhibition of NMDA receptor activity *via* a blockade of glycine$_B$ sites causes pharmacological effects similar to those observed after administration of specific NMDA receptor antagonist, it has been suggested that glycine$_B$ receptor antagonists could act as functional NMDA receptor blockers [53]. This hypothesis has been confirmed in several studies. In one study, it was shown that antagonists and partial agonist of glycine$_B$ sites of the NMDA receptor produce behavioral effects similar to those obtained by competitive and noncompetitive NMDA receptor antagonists [1, 11, 29, 53]. It was also shown that glycine$_B$ antagonists such as 7-chlorokinurenic acid (7-CKA), 5,7-dichlorokinurenic acid (5,7-DCKA) and L-701,324, as well as with the partial agonists ACPC and D-cycloserine, increased the quantity of time spent in the open arms of the EPM [11, 52, 53]. Our study demonstrated the results similar to those described above with competitive and uncompetitive NMDA antagonists; i.e., combined treatment with magnesium and L-701,324 (a glycine$_B$ site antagonist) or D-cycloserine (a glycine$_B$ site partial agonist [5, 9]), all at low, ineffective doses, increased the time spent in the open arms of the EPM, with no effect on the number of total entries. Most of the NMDA receptor ligands administered together with magnesium did not significantly affect open arm entries, either. Since our previous [33] and present (Tab. 1) data indicate a lack of effect on the locomotor activity of these combined treatments, we can assume that this combination of treatment specifically influences only the time the mice spent in the open arms of the EPM.

This apparent synergism between all of the studied ligands acting at various modulatory sites on the NMDA receptor complex suggests a common site of action: the NMDA receptor complex. Moreover, D-serine reduces the anxiolytic-like action of magnesium. Thus, the present data concerning the effect of the NMDA ligands on magnesium activity indicate the involvement of the NMDA receptor complex (particularly the glycine$_B$ sites) in the anxiolytic activity of magnesium in the EPM test.

Several studies have indicated an interaction between NMDA and GABAergic neurotransmission in anxiety assessments in animals. The anxiolytic-like

activity of MK-801 was enhanced by diazepam, antagonized by a benzodiazepine receptor antagonist (Ro-15-1788) and reversed by the anxiogenic agent β-carboline FG-7142 [44]. Also, the anxiolytic-like effect of CGP 37849 was abolished by flumazenil [39]. It should be noted that the anxiolytic-like effects of magnesium seem to be mediated, at least in part, through an interaction with the benzodiazepine/$GABA_A$ system [30, 41]. Thus, the functional relationships between the NMDA and the benzodiazepine/$GABA_A$ systems may play a crucial role in magnesium anti-anxiety mechanism(s).

To summarize, the present study indicates the involvement of the NMDA/glutamate pathway in the anxiolytic-like activity of magnesium in the EPM in mice and further suggests the anxiolytic activity of this ion in human anxiety disorders.

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