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Involvement of NMDA receptor complex in the anxiolytic-like effects of chlordiazepoxide in mice

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Abstract In the present study, we demonstrated that low, ineffective doses of *N*-methyl-D-aspartic acid (NMDA) receptor antagonists [competitive NMDA antagonist, CGP 37849, at 0.312 mg/kg intraperitoneally (i.p.), antagonist of the glycine$_B$ sites, L-701,324, at 2 mg/kg i.p., partial agonist of glycine$_B$ sites, D-cycloserine, at 2.5 mg/kg i.p.] administered jointly with an ineffective dose of the benzodiazepine, chlordiazepoxide (CDP, 2.5 mg/kg i.p.), significantly increased the percentage of time spent in the open arms of the elevated plus-maze (index of anxiolytic effect). Furthermore, CDP-induced anxiolytic-like activity (5 mg/kg i.p.) was antagonized by NMDA (75 mg/kg i.p.) and by an agonist of glycine$_B$ sites of the NMDA receptor complex, D-serine [100 nmol/mouse intracerebroventricularly (i.c.v.)]. The present study showed a positive interaction between γ-aminobutyric acid (GABA) and glutamate neurotransmission in the anxiolytic-like activity in the elevated plus-maze test in mice and this activity seems to particularly involve the NMDA receptors.

Keywords Chlordiazepoxide · NMDA receptor ligands · Anxiety · Elevated plus-maze · Mice

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Introduction

γ-Aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the mammalian central nervous system (Sieghart 1995; Sieghart et al. 1999). Dysfunction of the central GABA system has long been associated with anxiety spectrum disorders (Nutt and Malizia 2001; Lydiard 2003; Nemeroff 2003). It is known that in both, humans and animals, positive modulators of GABA$_A$ ionotropic receptors produce anxiolytic-like activity, while the negative modulators evoke anxiety (Kalueff and Nutt 1996; Nutt and Malizia 2001). Thus, for many years, the leading treatment of anxiety disorders was benzodiazepines that enhance GABAergic inhibitory neurotransmission through allosteric modulation of GABA$_A$ receptors. They are still preferred due to their efficacy, rapid onset of action, and safety (Stahl 2002), but their adverse effects: sedation, cognitive impairments, undesirable interactions with other drugs, drug dependence and abuse mostly limited their use (Uhlenhuth et al. 1999; Stahl 2002). Moreover, these treatments are effective only in about 70% of patients and full remission is observed only in 40% of patients; thus, the novel therapeutic strategies are extensively sought.

Glutamate, which is the chief excitatory neurotransmitter in the mammalian central nervous system, is widely distributed throughout the brain and mediates its effects via stimulation of ionotropic and metabotropic receptors (Kew and Kemp 2005). The ionotropic glutamate receptor family is ligand-gated channels divided into three groups named after their selective agonists [*N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), and kainate] and their density is high in cortical and limbic regions being implicated in the mediation of fear and anxiety (Krystal et al. 1999). The preclinical data indicate that a number of different classes of NMDA receptor antagonists, acting at specific sites located on the NMDA receptor complex, produced anxiolytic-like activity in tests of anxiety in rodents (Dunn et al. 1989; Sharma and Kulkarni 1993; Płaźnik et al. 1994; Karcz-Kubicha et al. 1997; Kotlińska and Liljequist 1998; Przegaliński et al. 2000; Poleszak et al. 2004). In humans, memantine and D-cycloserine are effective in obsessive–compulsive disorder (Feusner et al. 2009; Abramowitz et al. 2009; Aboujaoude et al. 2009), and moreover, D-cycloserine was effective in post-traumatic stress disorder (Amiel and Mathew 2007). Despite these findings, the numerous adverse effects produced by competitive and non-competitive NMDA antagonist limited their potential clinical use (Tricklebank et al. 1989; Willetts et al. 1990). Profound side effects, typical for competitive and non-competitive NMDA receptor antagonist, do not occur after administration glycine_B site modulators (Parsons et al. 1998). This modulatory site of the NMDA receptor complex is a co-agonist site with affinity for glycine and D-serine (Wood et al. 1989, 1996). Antagonists and partial agonist of glycine_B sites inhibit the function of the NMDA receptor complex and produce effects which are similar to those produced by competitive and non-competitive NMDA receptor antagonist. It was shown that glycine potentiated the action of glutamate at NMDA receptors (Johnson and Ascher 1987), and antagonists and partial agonist of the glycine_B sites inhibited the function of the NMDA receptor complex and produced anxiolytic-like action in several experimental models of anxiety (Karcz-Kubicha et al. 1997; Przegaliński et al. 1998; Kotlińska and Liljequist 1998).

In this study, we investigated the interaction between glycine_B sites ligands and benzodiazepine/GABA_A receptor ligand, chlordiazepoxide (CDP) in the elevated plus-maze test in mice.

Materials and methods

Animals

The experiments were carried out on adult male Albino Swiss mice (25–30 g) purchased from the licensed breeder (Kołacz, Warsaw, Poland). The animals were kept in cages (up to 10 per cage) on a natural day–night cycle with free access to food and water and they were used after at least 7 days of acclimatization to laboratory conditions. Each experimental group consisted of 8–12 animals. The experimental protocol was approved by the Local Ethics Committee at the Medical University of Lublin (license no. 31/2007), and all the procedures were in strict compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Drug administration

Chlordiazepoxide (CDP, Polfa-Poznań, Poland) was administered intraperitoneally (i.p.) 60 min before the test. L-701,324 (7-chloro-4-hydroxy-3-(3- phenoxy)phenyl-quinolin-2[1*H*]-one, Sigma) was suspended in a 1% aqueous solution of Tween 80 (POCH, Gliwice, Poland) and administered intraperitoneally (i.p.) 60 min before the test. NMDA (Sigma), CGP 37849 (DL-(E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid, Tocris), D-cycloserine (D-4-amino-3-isoxazolidone, Sigma) were dissolved in 0.9% NaCl and administered i.p. and also administered 60 min before the test. D-Serine (Sigma) was also dissolved in 0.9% NaCl and administered intracerebroventricularly (i.c.v.) 15 min before the test. I.c.v. administration was performed according to a modified method described by Lipman and Spencer (1980). Control animals received an i.p. or i.c.v. injection of a respective vehicle. The volume of vehicles or drug solutions for i.p. and i.c.v. administrations was 10 ml/kg and 5 µl per mouse, respectively.

Elevated plus-maze test

The experiments were carried out on mice according to the method of Lister (1987). The plus-maze apparatus was made of black polyvinyl chloride 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 stable base raising it 38 cm above the floor and was illuminated by 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 maze. 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 entries into the open arms, 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 time spent in open arms or in a greater number of open arm entries. Total

number of entries into either type of an arm was used as a measure of the overall motor activity.

Statistical analysis

The obtained data were evaluated by two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. All results are presented as a mean ± standard error of the mean (SEM). A $p \leq 0.05$ was considered statistically significant.

Results

Anxiolytic-like effect of joint administration of CDP and CGP 37849 in the elevated plus-maze test

Chlordiazepoxide administered at a dose of 0.5 mg/kg or CGP 37849 administered at a dose of 0.312 mg/kg, both given alone, did not change the percentage of the time spent and also entries into the open arms with the exception of CDP, which significantly increased this latter measure (Fig. 1). The joint administration of CDP and CGP 37849 significantly increased the percentage of the time spent in the open arms (Fig. 1a) and enhanced the number of entries into open arms (Fig. 1b). A two-way ANOVA demonstrated lack of effect of CDP [$F(1, 28) = 1.91$, $p = 0.1777$], significant effect of CGP 37849 [$F(1, 28) = 26.38$, $p < 0.0001$] and significant interaction [$F(1, 28) = 8.02$, $p = 0.0085$] in the time spent in open arms, while a significant effect of CDP [$F(1, 28) = 15.301$, $p = 0.0005$], no effect of CGP 37849 [$F(1, 28) = 2.93$, $p = 0.09882$] and no interaction [$F(1, 28) = 0.0$, $p = 0.9492$] in the open arm entries.

Anxiolytic-like effect of joint administration of CDP and L-701,324 in the elevated plus-maze test

Chlordiazepoxide administered at a dose of 0.5 mg/kg did not alter the percentage of time spent and increased entries into the open arms (Fig. 2). L-701,324 given alone at a

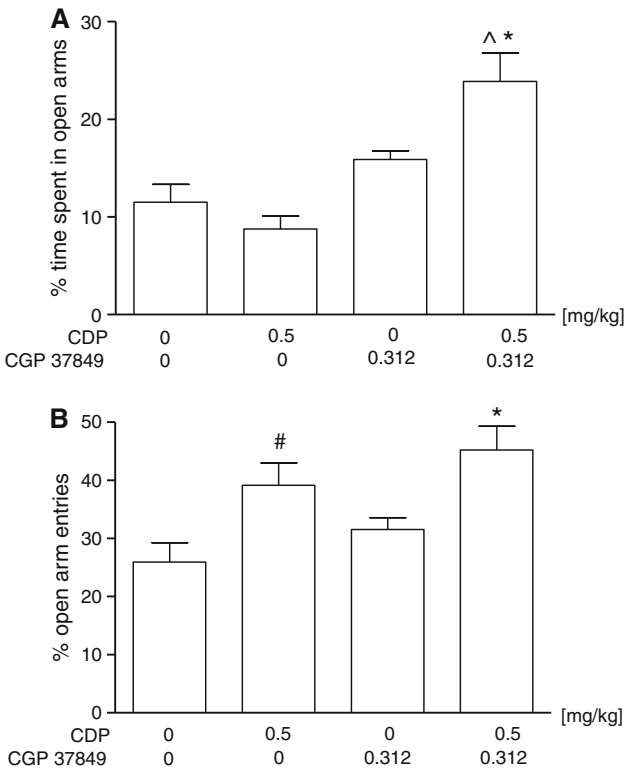

Fig. 1 Effect of joint administration of CGP 37849 and chlordiazepoxide (CDP) in the elevated plus-maze procedure in mice [the percentage of the time spent in the open arms (**a**), and the percentage of the open arms entries (**b**)]. CGP 37849 and CDP was administered i.p. 60 min before the test. The *values* represent the mean ± SEM ($n = 8$ mice per group) $^{*}p < 0.05$ versus CGP 37849, $^{\wedge}p < 0.001$ versus CDP, $^{\#}p < 0.05$ versus control (vehicle-treated group) (Bonferroni's test)

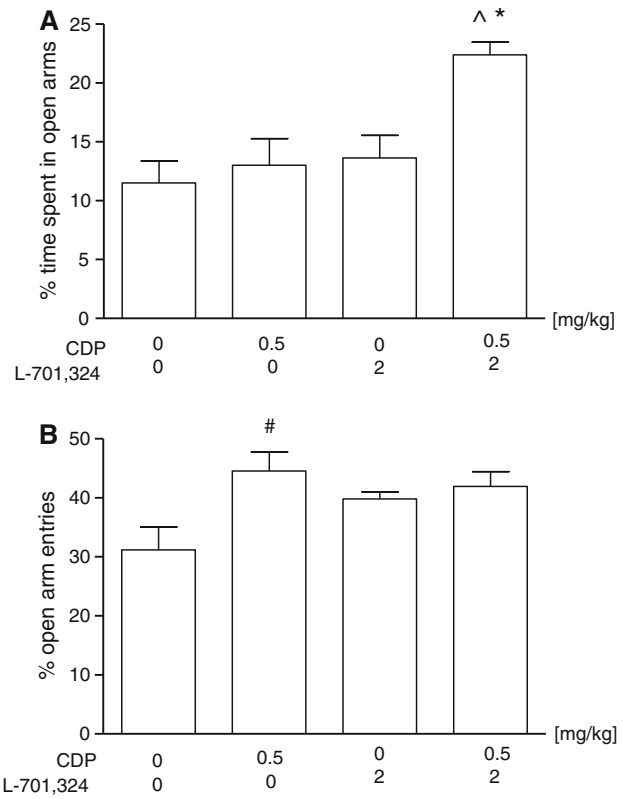

Fig. 2 Effect of joint administration of L-701,324 and chlordiazepoxide (CDP) in the elevated plus-maze procedure in mice [the percentage of the time spent in the open arms (**a**), and the percentage of the open arms entries (**b**)]. L-701,324 and CDP was administered i.p. 60 min before the test. The *values* represent the mean ± SEM ($n = 8$ mice per group) $^{*}p < 0.01$ versus L-701,324, $^{\wedge}p < 0.01$ versus CDP, $^{\#}p < 0.01$ versus control (vehicle-treated group) (Bonferroni's test)

dose of 2 mg/kg exhibited no effect in this test (Fig. 2a, b). The joint administration of CDP and L-701,324 (both in ineffective per se doses) significantly increased the percentage of time spent in the open arms (Fig. 2a), but not in the number of entries into open arms (Fig. 2b). A two-way ANOVA demonstrated a significant effect of CDP [$F(1, 28) = 7.66$, $p = 0.0099$], a significant effect of L-701,324 [$F(1, 28) = 9.65$, $p < 0.0043$] and not quite significant interaction [$F(1, 28) = 3.83$, $p = 0.0603$] in the time spent in open arms, while a significant effect of CDP [$F(1, 28) = 7.25$, $p = 0.0118$], no effect of L-701,324 [$F(1, 28) = 1.09$, $p = 0.3056$] and not quite significant interaction [$F(1, 28) = 3.77$, $p = 0.0622$] in the open arm entries.

Anxiolytic-like effect of joint administration of CDP and D-cycloserine in the elevated plus-maze test

Chlordiazepoxide administered at a dose of 0.5 mg/kg did not change either the percentage of the time spent (Fig. 3a) or the number of entries into open arms (Fig. 3b).

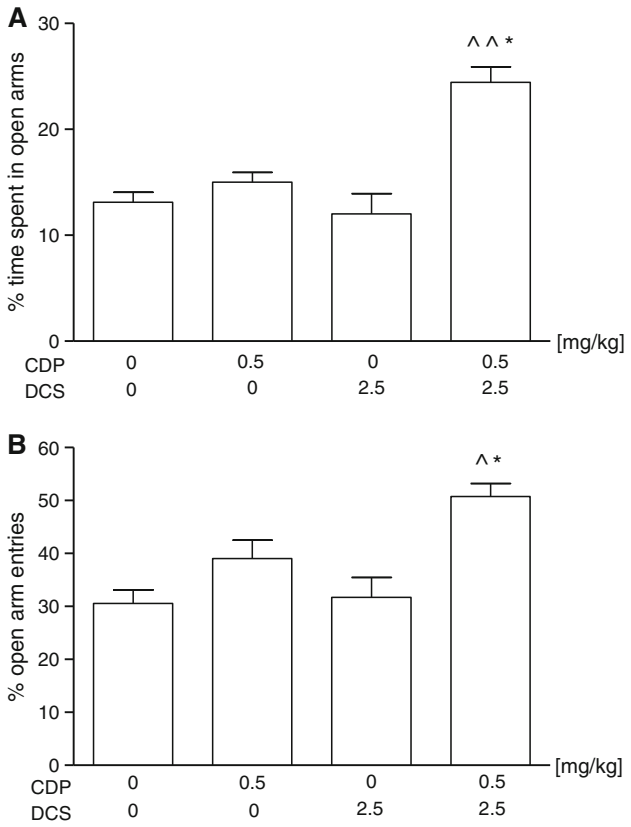

Fig. 3 Effect of joint administration of D-cycloserine (DCS) and chlordiazepoxide (CDP) in the elevated plus-maze procedure in mice [the percentage of the time spent in the open arms (**a**), and the percentage of the open arms entries (**b**)]. L-701,324 and CDP was administered i.p. 60 min before the test. The *values* represent the mean ± SEM ($n = 9$–10 mice per group) $^{*}p < 0.001$ versus DCS, $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.001$ versus CDP (Bonferroni's test)

D-Cycloserine administered singly at a dose of 2.5 mg/kg remained also without any effect on both of these measures (Fig. 3a, b). CDP administered at an ineffective per se dose of 0.5 mg/kg and D-cycloserine administered at such a dose of 2.5 mg/kg significantly increased the percentage of the time spent in the open arms (Fig. 3a) and enhanced the number of entries into open arms (Fig. 3b). A two-way ANOVA demonstrated a significant effect of CDP [$F(1, 34) = 24.83$, $p < 0.0001$], a significant effect of DCS [$F(1, 34) = 8.73$, $p = 0.0057$] and a significant interaction [$F(1, 34) = 13.35$, $p = 0.0009$] in the time spent in the open arms, while a significant effect of CDP [$F(1, 34) = 19.50$, $p < 0.0001$], a significant effect of DCS [$F(1, 34) = 4.31$, $p = 0.0451$] and no interaction [$F(1, 34) = 2.93$, $p = 0.0956$] in the open arm entries.

Effect of NMDA on the anxiolytic-like activity of CDP in the elevated plus-maze test

Chlordiazepoxide given at a dose of 2 mg/kg produced anxiolytic-like effect significantly increasing the percentage of the time spent in the open arms and increasing the percentage of the entries into the open arms (Fig. 4). The increase in percentage of the time spent in the open arms induced by CDP (2 mg/kg) was significantly reversed by NMDA (75 mg/kg) (Fig. 4a). The increase in the number of the open arm entries induced by CDP (2 mg/kg) was significantly decreased by NMDA (Fig. 4b). NMDA given alone had no effect on either the time spent or the entries into the open arms (Fig. 4). A two-way ANOVA demonstrated a significant effect of CDP [$F(1, 36) = 45.98$, $p < 0.0001$], a significant effect of NMDA [$F(1, 36) = 21.37$, $p < 0.0001$] and a significant interaction [$F(1, 36) = 21.19$, $p < 0.0001$] in the time spent in open arms, and a similarly significant effect of CDP [$F(1, 36 = 15.87$, $p = 0.0003$], a significant effect of NMDA [$F(1, 36) = 8.11$, $p = 0.0072$] and a significant interaction [$F(1, 36) = 11.91$, $p = 0.0014$] in the open arm entries.

Effect of D-serine on the anxiolytic-like activity of CDP in the elevated plus-maze test

Chlordiazepoxide given at a dose of 2 mg/kg produced an anxiolytic-like effect significantly increasing the percentage of the time spent in the open arms and increasing the percentage of the entries into the open arms (Fig. 5). The increase in the percentage of the time spent in the open arms induced by CDP (2 mg/kg) was significantly reversed by D-serine (100 nmol/mouse) (Fig. 5a). The increase in the number of the open arm entries induced by CDP (2 mg/kg) was significantly changed by D-serine (Fig. 5b). D-Serine given alone had no effect on either the time spent or the entries into the open arms (Fig. 5). A two-way ANOVA

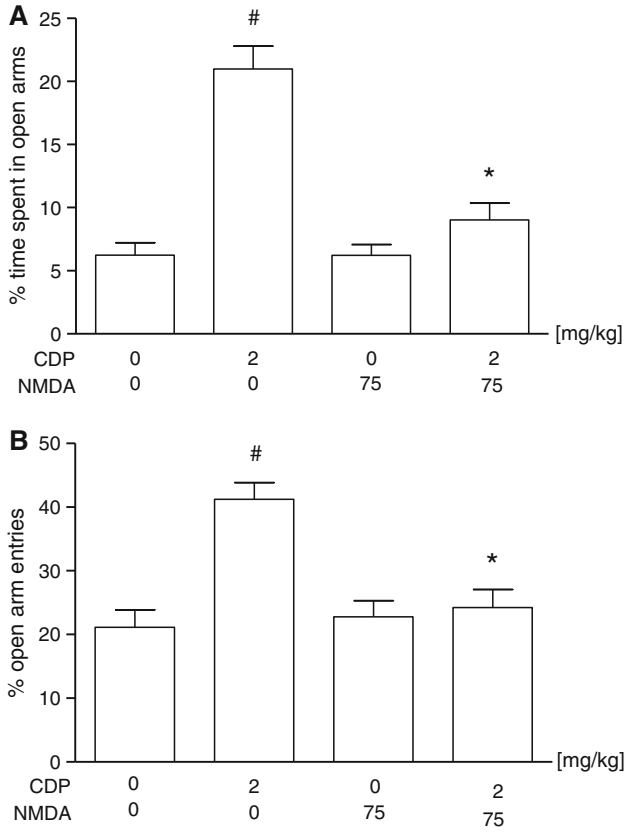

Fig. 4 Effect of *N*-methyl-D-aspartate (NMDA) on the action of chlordiazepoxide (CDP) in the elevated plus-maze procedure in mice [the percentage of the time spent in the open arms (**a**) and the percentage of the open arms entries (**b**)]. NMDA and CDP was administered i.p. 60 min before the test. The values represent the mean ± SEM ($n = 10$ mice per group) $^{*}p < 0.001$ versus CDP, $^{\#}p < 0.001$, versus control (vehicle-treated group) (Bonferroni's test)

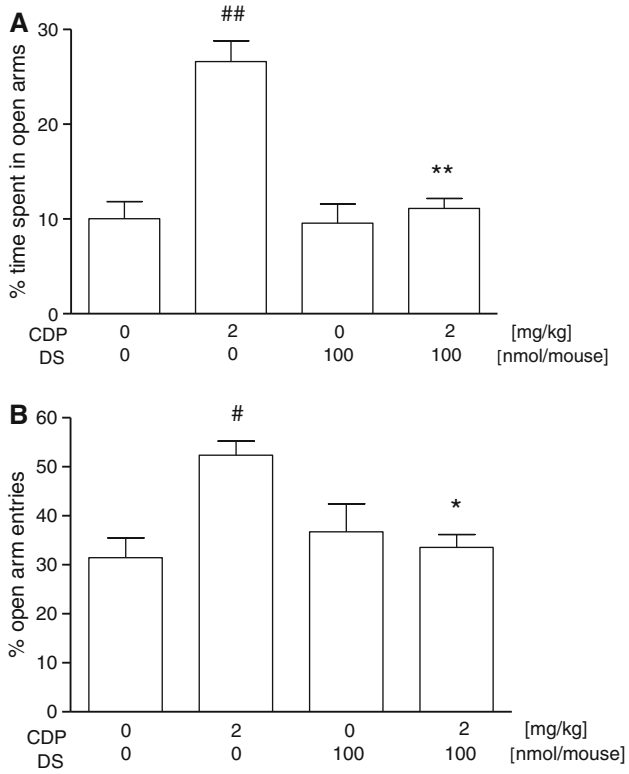

Fig. 5 Effect of D-serine (DS) on the action of chlordiazepoxide (CDP) in the elevated plus-maze procedure in mice [the percentage of the time spent in the open arms (**a**), and the percentage of the open arms entries (**b**)]. CDP was administered i.p. 60 min before the test, DS was administered i.c.v. 45 min after CDP injection. The *values* represent the mean ± SEM ($n = 9$–10 mice per group) $^{*}p < 0.01$, $^{**}p < 0.001$ versus CDP, $^{\#}p < 0.01$, $^{\#\#}p < 0.001$, versus control (vehicle-treated group) (Bonferroni's test)

demonstrated a significant effect of CDP [$F(1, 33) = 23.80$, $p < 0.0001$], a significant effect of D-serine [$F(1, 33) = 18.49$, $p = 0.0001$] and a significant interaction [$F(1, 33) = 16.33$, $p < 0.0003$] in the time spent in the open arms, while a significant effect of CDP [$F(1, 33) = 4.97$, $p = 0.0321$], no effect of D-serine [$F(1, 33) = 2.93$, $p = 0.0957$] and a significant interaction [$F(1, 33) = 9.11$, $p = 0.0046$] in the open arm entries.

Effect of CDP and NMDA ligands on the total arm entries

Chlordiazepoxide and all tested NMDA receptor ligands, administered singly or in combination, did not alter the number of the total arm entries (Table 1). A two-way ANOVA demonstrated the lack of the significant interaction between treatments in all analyzed experiments A: $F(1, 28) = 0.74$, $p = 0.3964$, B: $F(1, 28) = 0.53$, $p = 0.4723$], C: $F(1, 36) = 3.25$, $p = 0.0797$, D: $F(1, 36) = 2.59$, $p = 0.1164$, E: $F(1, 36) = 1.02$, $p = 0.3185$.

Discussion

A number of behavioral data have suggested the involvement of the glutamate-mediated neurotransmission in an anxiolytic-like behavior. The anxiolytic-like activity was demonstrated for different modulatory sites of NMDA receptor complex: for a non-competitive NMDA antagonist, dizocilpine (MK-801) (Dunn et al. 1989; Sharma and Kulkarni 1993; Karcz-Kubicha et al. 1997), a competitive NMDA antagonist, 2-amino-7-phosphoheptanoic acid (AP7) (Płaźnik et al. 1994), CGP 37849 (Przegaliński et al. 2000), and partial agonists of glycine$_B$ sites, D-cycloserine (Karcz-Kubicha et al. 1997) and 1-aminocyclo-propane-carboxylic acid (ACPC) (Trullas et al. 1989; Trullas et al. 1991), and antagonist of glycine$_B$ sites: L-701,324 (Karcz-Kubicha et al. 1997; Przegaliński et al. 1998; Kotlińska and Liljequist 1998). The anxiolytic-like profile of different NMDA antagonists was similar to that of benzodiazepines (Płaźnik et al. 1994) and the excitatory amino acid agonist, NMDA, produced anxiogenic-like effects in the elevated plus-maze test (Dunn et al. 1989). Moreover, genetic

Table 1 The number of the total arm entries for all experimental groups

Treatment and dose	Number of total entries
A	
Vehicle	12.63 ± 0.46
CDP 0.5 mg/kg	12.00 ± 1.00
CGP 37849 0.312 mg/kg	13.25 ± 1.21
CDP 0.5 mg/kg and CGP 37849 0.312 mg/kg	14.75 ± 1.85
B	
Vehicle	14.38 ± 1.75
CDP 0.5 mg/kg	15.75 ± 1.46
L-701,324 2 mg/kg	13.50 ± 1.04
CDP 0.5 mg/kg and L-701,324 mg/kg	16.75 ± 0.59
C	
Vehicle	14.50 ± 0.74
CDP 0.5 mg/kg	11.44 ± 0.67
D-cycloserine 2.5 mg/kg	12.80 ± 0.70
CDP 0.5 mg/kg and D-cycloserine 2.5 mg/kg	12.00 ± 0.92
D	
Vehicle	11.80 ± 1.07
CDP 2 mg/kg	15.30 ± 1.90
NMDA 75 mg/kg	13.20 ± 0.53
CDP 2 mg/kg and NMDA 75 mg/kg	12.80 ± 0.92
E	
Vehicle	15.10 ± 1.65
CDP 2 mg/kg	19.30 ± 0.71
D-serine 100 nmol/mouse	12.40 ± 1.33
CDP 2 mg/kg and D-serine 100 nmol/mouse	17.40 ± 1.32

Data represent the mean ± SEM; $n = 9$–12. Chlordiazepoxide (CDP), CGP 37849, L-701,324, D-cycloserine and *N*-methyl-D-aspartate (NMDA) were administered i.p. 60 min before the test. D-serine was administered i.c.v. 15 min before the test

studies indicated an anxiolytic-like activity of NR2A subunit of NMDA receptor knockout mice (Boyce-Rustay and Holmes 2006). Validation of the elevated plus-maze procedure has shown that it is sensitive to drugs that produce anxiolytic or anxiogenic effects in human (Pellow et al. 1985), including drugs that have non-benzodiazepine sites of action (Pellow 1986). Moreover, in the clinical study showed useful of memantine (non-competitive NMDA receptor antagonist) augmentation in treatment-resistant obsessive–compulsive disorder (Feusner et al. 2009; Aboujaoude et al. 2009), D-cycloserine (partial agonists of glycine$_B$ sites) (Hood et al. 1989; Emmett et al. 1991) as a potential therapeutic agent for post-traumatic stress disorder and specific phobia (Heresco-Levy et al. 2002; Ressler et al. 2004), and riluzole in the treatment of symptoms obsessive–compulsive disorder (Coric et al. 2005) and general anxiety disorder (Mathew et al. 2005).

In the present study, we have demonstrated the influence of NMDA receptor ligands on anxiolytic-like activity of CDP in the elevated plus-maze in mice. We have shown that a competitive NMDA antagonist (CGP 37849), partial agonist of the glycine$_B$ site (D-cycloserine) and glycine$_B$ antagonist (L-701,324) enhanced the anxiolytic-like activity of CDP in the elevated plus-maze. The previous study has shown that CGP 37849 evoked potentially anxiolytic-like effects in the Vogel conflict drinking test, an open field test (Płaźnik et al. 1994) and in the elevated plus-maze test in rats and its anxiolytic-like activity was abolished by flumazenil (Przegaliński et al. 2000). In our study, 0.312 mg/kg CGP 37849 produced synergistic effects with CDP (0.5 mg/kg) in the elevated plus-maze test in mice. Unfortunately, the side effects produced by competitive and uncompetitive NMDA antagonist (motor impairment, hyperactivity, stereotypy and psychotomimetic actions (Willetts et al. 1990) limited their potential use for the treatment in humans. After the discovery that the NMDA receptor activity is regulated by co-agonist (glycine or D-serine), numerous studies concentrated on this pathway. It is known that a co-agonist site exerts major regulatory roles in the activity of NMDA receptor complex. The binding of the co-agonist (glycine or D-serine) is an obligatory requirement for NMDA receptor/channel activity (Johnson and Ascher 1987; Kleckner and Dingledine 1988). More recently, studies have shown that selective blockers of the co-agonist site abolish NMDA receptor activity (Kessler et al. 1989; Kleckner and Dingledine 1989) and produced pharmacological effects similar to those exerted after the administration of a competitive and non-competitive NMDA antagonist. D-Cycloserine has a profile of a partial agonist acting at the glycine binding site (Hood et al. 1989; Watson et al. 1990). In the preclinical data D-cycloserine exhibits anxiolytic-like effects in the Vogel conflict test (Kłodzińska and Chojnacka-Wójcik 2000), in the elevated plus-maze (time in open arms) (Karcz-Kubicha et al. 1997; Ho et al. 2005; Poleszak et al. 2008), and in a fear-potentiated startle response test (Anthony and Nevins 1993) (Campeau et al. 1992; Fendt et al. 1996). The glycine$_B$ receptor antagonist (L-701,324) has produced anxiolytic-like effects in the elevated plus-maze test and the four-plate test (Karcz-Kubicha et al. 1997; Przegaliński et al. 1998; Kotlińska and Liljequist 1998).

In our study, the combined treatment with CDP and L-701,324 (a glycine$_B$ site antagonist) or D-cycloserine (a glycine site partial agonist), all at low, ineffective doses, increased the time spent in the open arms of the elevated plus-maze, with no effects on the number of total entries (Table 1). Because the obtained data indicate a lack of effect on the locomotor activity, we can assume that this combination produced an anxiolytic-like activity. The

anxiolytic-like activity of CDP was reduced by NMDA or by D-serine, what suggests the involvement of NMDA receptor complex in the anxiolytic activity of CDP in the elevated plus-maze in mice. The interaction between NMDA and GABAergic neurotransmission in anxiety assessments in animals was shown in other experiments. The anxiolytic-like activity of MK-801 was enhanced by diazepam, antagonized by the benzodiazepine receptor antagonist (Ro-15-1788) and reversed by the anxiogenic agent, β-carboline FG-7142 (Sharma and Kulkarni 1993).

To summarize, the present study indicates the involvement of the NMDA receptor complex in the anxiolytic-like activity of CDP in the EMP in mice. Our results also suggest that full anxiolysis might be possible with a reduced dose of benzodiazepines when co-administered with NMDA receptor antagonists and this might substantially reduce side effects and the risk of the benzodiazepine dependence. Clinical trials are necessary to determine if such a combination is of a potentially therapeutic value in humans with anxiety disorders.

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Conflict of interest The authors declare they have no conflict of interest.

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