

Research report

Behavioral effects of D-cycloserine in rats: The role of anxiety level

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Abstract

It has been reported that the glutamatergic *N*-methyl-D-aspartate (NMDA) receptor is involved in stress responses and that anxiety is the primary response to stress. Although individual differences in anxiety levels of rats have been demonstrated by using the elevated plus-maze (PM) test, the role of NMDA receptor activity in such individuality of anxiety is not clear. Here, we examined whether low (LA) and high (HA) anxiety rats might respond differently to treatment with D-cycloserine (DCS), a partial agonist of the glycine binding site located on NMDA receptors. Male Wistar rats were screened by using the PM and divided into LA and HA subgroups. On the next day, these rats were again tested in the PM, 30 min after the treatment with DCS (5, 10, or 30 mg/kg ip). Five days later, the rats were subjected to a 2-day forced swim (FS) test, receiving the DCS treatment again 30 min before the second day session. The PM data showed that DCS had anxiogenic effects in LA but not HA rats. The immobility of LA or HA rats in the FS test was not affected by DCS. The results indicate that the behavioral effects of DCS depend on the anxiety level of rats and have task-dependent behavioral consequences, suggesting that glycine binding sites on NMDA receptors are involved in individual differences of anxiety level.

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1. Introduction

An increasing body of evidence has accumulated which shows that the glutamatergic system is involved in the modulation of stress responses [7,11,28,39]. Stress is correlated to the pathophysiology of both anxiety and

depression [9,18,31], and anxiety is the primary response to acute stress and precedes depression [18,35]. The *N*-methyl-D-aspartate (NMDA) receptor is a well-characterized glutamate receptor subtype [25], which has been reported to be involved in certain types of anxiety [21] and depression [26,29,30] in animal models. There are multiple regulatory sites on the NMDA receptor, for example a high affinity recognition site for glutamate and a glycine binding site [37]. The glycine binding site is activated by endogenous glycine and is required for receptor activation by glutamate [16,22]. D-Cycloserine (DCS) has the in vitro profile of a partial agonist acting at the glycine binding site

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[13,41]. The relationship between anxiety states and the glycine binding site on the NMDA receptor has been suggested by studies using animal models, where DCS has been shown to block ethanol-induced anxiolytic activity in the elevated plus-maze test using Wistar rats, with the lowest effective doses of 12 mg/kg [24].

Individual differences in serotonergic [38], cholinergic, and dopaminergic [40] as well as behavioral [12] activity have been reported. Although it is not yet clear whether glutamatergic activity may also differ individually, strain [33] and sex [5] differences in response to NMDA antagonist have been demonstrated. There are gender differences in the behavioral response to drugs that modulate the glycine binding site on NMDA receptors, since female rats are more sensitive than males to the anxiolytic effects of (+)-3-amino-1-hydroxy-2-pyrrolidone (HA-966), an NMDA receptor antagonist, in the elevated plus-maze [24]. Interestingly, co-administration of DCS blocked the effects of HA-966, also in a gender-dependent manner with a higher sensitivity of females than males to DCS [24].

In the elevated plus-maze test, the time spent in the open arm and risk assessment are used to evaluate unconditioned avoidance behavior as a measure of anxiety [4,34]; whereas in the forced swim test, immobility, or despair behavior, is used to investigate learned helplessness as an animal model of depression [36]. To our knowledge, work on the role of NMDA receptor function for individual differences in stress, anxiety, and depression has not yet been published. With the goal of understanding whether anxiety and depression responses to DCS treatment may differ between low (LA) and high (HA) anxiety rats, we screened a group of outbred male Wistar rats by using the elevated plus-maze and then measured their behavioral responses in the plus-maze as well as forced swim test after DCS treatment.

2. Materials and methods

2.1. Animals

All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of the Chung Shan Medical University. Ninety-six male Wistar rats (National Laboratory Animal Center, ROC; 245 ± 3 g) were used and housed in groups of five rats in acrylic cages ($35 \times 56 \times 19$ cm) in an animal room with a 12:12-h light–dark cycle (lights on at 07:00 h) with food and water provided ad libitum. Each animal was handled for 5 min on 3 consecutive days prior to the experiment.

2.2. General procedure

Three tests were performed in the following order: an open field test on the first day, then the elevated plus-maze

test on the following 2 days, and a 2-day session of forced swim test started on the ninth day. All behavioral tests were begun 3 h after the start of the lights on. First, the animals were weighed in the animal room. Then, they were placed individually in a clean cage ($25 \times 41 \times 19$ cm) and transported to a dim observation room (28 lx) for behavioral testing. The pieces of test equipment were thoroughly cleaned by using 20% alcohol followed by thorough drying before each rat was tested. The behavioral parameters of the open field test were analyzed by an automated computer program; behavior in the plus-maze and the forced swim test was scored from videotapes. DCS (Sigma, USA) was dissolved in saline (0.9% NaCl) immediately before usage and administered by intraperitoneal injection (ip) in a volume of 1 ml/kg of body weight 30 min before the second-day session of the plus-maze and swim test, respectively.

2.3. Behavioral tests

2.3.1. Open field

The open field consists of an acrylic box ($40 \times 40 \times 40$ cm) which was monitored by an automated activity monitoring system (Digiscan-16 Animal Activity Monitor System; model RXYZCM, Omnitech Electronics Columbus, OH, USA) [11]. The following measures were obtained by the automated computer program: (1) locomotion: the distance traveled in cm; (2) movement time; (3) rearing time; and (4) center time: defined as the animal's center of body being within the center area of the open field (20×20 cm). Behavior in the open field was tested for 10 min.

2.3.2. Elevated plus-maze

The elevated plus-maze apparatus was made of plastic and consisted of two opposed open arms (50×10 cm), two opposed enclosed arms with no roof ($50 \times 10 \times 40$ cm), and an open square (10×10 cm) in the center; it was elevated 50 cm above the floor as described before [12]. The animals were placed into the center of the plus-maze, facing one of the open arms. The following measures were analyzed from videotapes: (1) the number of entries into and (2) the time spent on center, open, or enclosed arms; (3) risk assessment, that is, the rat showed head dipping but its body was still in the enclosed arm during this behavior; (4) open arm latency, that is, the time from placing the rat into the plus-maze until it entered one of the open arms; and (5) within-arm activity, that is, the number that an animal crossed a virtual line which divided an arm into a proximal and a distal half. An entry into any of the compartments was defined as all four paws being placed on the arm. Each rat was tested on two consecutive days (5 min each). The open arm time in the first elevated plus-maze test was used to screen individual anxiety levels and to arrange treatment groups which were matched for equivalent numbers of high and low open arm responders. On the following day, 30 min

prior to the plus-maze test, the rats received intraperitoneal injections of either saline or DCS (5, 10, or 30 mg/kg), $n = 12$ for each group.

2.3.3. Forced swim

This test was carried out in a clear glass tank ($25 \times 25 \times 60$ cm) containing 39 cm clean water (26°C). The apparatus was cleaned thoroughly, and water was changed from rat to rat. A 2-day swimming session was conducted and videotaped: 15 min on the first day and 5 min on the second day. Thirty minutes before the second day session, rats received the injection treatment which was identical to what they had received in the plus-maze test. After each test, the rats were dried and kept warm under a heating bulb for 30 min before being returned to their home cages. The following parameters were measured from videotapes: (1) struggling, that is, strong movements of the limbs occurring during swimming, breaking the surface of the water, or scratching the walls; (2) duration of diving; and (3) immobility, which occurred when the rats remained motionless, or floating (including small limb movements to keep their heads above the water) [2].

2.3.4. Data analysis

Identically to our previous experiments [38,40], the animals were ranked using the time spent in the open arms and were assigned to two subgroups with either high (the 48 animals with shorter open arm time; HA rats) or low anxiety levels (the other 48 animals with longer open arm time; LA rats). These group assignments were used to present all other behavioral data. Statistical testing was performed to compare within or between groups using t tests for paired or unpaired data. The comparison of DCS effects was carried out by one-way analysis of variance (ANOVA), followed by Scheffé's test. All results were expressed as the mean \pm SEM. The level of significance was defined as $P < 0.05$.

3. Results

3.1. First elevated plus-maze test

Based on the measure of time spent in the open arms of the plus-maze on the first day, animals were assigned to the LA and HA subgroups. These subgroups had the following profiles: The latencies until the first entry to an open arm differed between HA and LA rats, with shorter latencies in LA rats ($t = 7.4$, $df = 94$, $P < 0.001$). The behavior in the open arm, which was presented as the open arm time, open arm entry, and open arm activity, was significantly higher in LA rats than in HA rats (all P values < 0.001). The LA rats showed less behaviors in enclosed arm, which was expressed as less enclosed arm time, enclosed arm entry, enclosed arm activity, and risk assessment, compared to HA rats (all P values < 0.05). In addition, center time, total arm entry, and total arm activity were higher in LA rats than in HA rats (all P values < 0.05) (Table 1).

Table 1
Baseline of plus-maze behavior

	LA rats ($n = 48$)	HA rats ($n = 48$)
Open arm latency (s)	13.0 ± 3.6	$152.6 \pm 18.5^{***}$
Open arm time (s)	97.7 ± 6.3	$12.4 \pm 2.0^{***}$
Open arm entry (no.)	6.6 ± 0.4	$1.1 \pm 0.2^{***}$
Open arm activity (no.)	15.8 ± 1.1	$1.7 \pm 0.3^{***}$
Enclosed arm time (s)	136.6 ± 6.3	$233.7 \pm 5.5^{***}$
Enclosed arm entry (no.)	10.4 ± 0.5	$12.3 \pm 0.5^*$
Enclosed arm activity (no.)	28.7 ± 1.4	$36.8 \pm 1.5^{***}$
Risk assessment (no.)	1.5 ± 0.2	$2.8 \pm 0.3^{***}$
Center time (s)	61.0 ± 3.9	$47.4 \pm 5.1^*$
Total arm entry (no.)	17.0 ± 0.7	$13.4 \pm 0.6^{***}$
Total arm activity (no.)	44.4 ± 2.0	$38.5 \pm 1.6^*$

Mean \pm SEM are shown.

* *** Difference between LA and HA rats according to two-tailed t test.

* $P < 0.05$.

*** $P < 0.001$.

3.2. Open field test

In contrast to the plus-maze test, none of the measures obtained in the open field on the day before yielded significant group differences between LA and HA rats (unpaired t tests; all P values > 0.05) (Table 2).

3.3. Behavior in the plus-maze after drug injection

Most behavioral differences between HA and LA rats in plus-maze test were still present after injection of vehicle on the second day, since open arm time, open arm entries, open arm activity, total arm entry, and total arm activity were still higher in LA rats (all P values < 0.01) (Table 3). DCS did not influence the behavior of HA rats in the plus-maze. In contrast, DCS significantly changed the behavior of LA rats: all the dosage of DCS used in this study increased the enclosed arm time but decreased the center time, compared to the vehicle-treated group (all P values < 0.05). In addition, increased risk assessment was found with 10 mg/kg of DCS ($P < 0.01$). Moreover, the highest dose (30 mg/kg) of DCS significantly decreased the behavior of LA rats in the open arm, expressed as the attenuations of open arm time, open arm entries, and open arm activity (all P values < 0.05) (Table 3).

3.4. Forced swim test

No differences between LA and HA rats in the immobility time during the first 5-min period of forced swim were found in the day 1 session, and also after injection of vehicle in the day 2 session (Fig. 1). In addition, no between-group differences were noted in the time spent struggling and diving (data not shown). The immobility time of vehicle-treated rats increased from day 1 (LA rats: 140.0 ± 11.5 s; HA rats: 135.9 ± 38.6 s) to day 2 (LA rats: 201.7 ± 11.3 s; HA rats: 214.1 ± 9.6 s) (P values < 0.001 , paired t test). DCS did not affect the immobility in forced

Table 2
Open field behavior

	LA rats (<i>n</i> = 48)	HA rats (<i>n</i> = 48)
Distance (cm)	2799.0 ± 84.8	2763.0 ± 78.4
Movement time (s)	279.0 ± 29.2	250.0 ± 4.3
Rearing time (s)	147.0 ± 5.0	141.0 ± 5.0
Center time (s)	205.0 ± 7.0	202.0 ± 7.2

Mean ± SEM are shown.

swim test, compared to vehicle treatment (Fig. 1). In addition, the correlations of open arm time in the plus-maze to the immobility time in the forced swim test on day 1 (Pearson correlation = 0.144, *P* = 0.16) and that on day 2 (Pearson correlation = 0.028, *P* = 0.87) after saline injection were not significant.

3.5. Analysis of animals with low (LD) vs. high distance (HD) levels in the open field

In addition to the analysis based on plus-maze behavior, all animals were divided into LD and HD rats based on the movement distance in the open field test. When analyzing the behavior in plus-maze and forced swim test, there were no indications for substantial

differences between LD and HD rats; and also the DCS effects were not different between LD and HD groups (data not shown in detail).

4. Discussion

The present experiment shows that DCS, at doses of 10 and 30 mg/kg, has no effect in HA rats but exerts anxiogenic activity in LA rats by decreasing the open arm time, open arm entry, as well as open arm activity and increasing the enclosed arm time and risk assessment in the elevated plus-maze. These effects of DCS were not due to unspecific effects on activity, since the dosages used did not affect total arm entries nor total arm activity. Also, immobility in the forced swim test was not affected in LA or HA rats. The present study demonstrates for the first time that the individual differences of anxiety level play a critical role for the behavioral effects of DCS.

It has been demonstrated that the glutamatergic NMDA receptor is involved in stress responses [7,11,28,39]. Anxiety is the primary response to acute stress; chronic stress and anxiety precede depression [9,18,31]. Various stressors, like novel environments [11], tail pinch [3], or

Table 3
Plus-maze behavior 30 min after DCS treatment

	LA rats				HA rats			
	Vehicle (<i>n</i> = 12)	DCS, 5 mg/kg (<i>n</i> = 12)	DCS, 10 mg/kg (<i>n</i> = 12)	DCS, 30 mg/kg (<i>n</i> = 12)	Vehicle (<i>n</i> = 12)	DCS, 5 mg/kg (<i>n</i> = 12)	DCS, 10 mg/kg (<i>n</i> = 12)	DCS, 30 mg/kg (<i>n</i> = 12)
Open arm latency (s)	30.6 ± 24.6	12.7 ± 8.2	60.4 ± 28.7	52.1 ± 27.5	117 ± 39.3	96.5 ± 36.2	108.2 ± 41.1	156.7 ± 39.7
Open arm time (s)	89.0 ± 15.6	73.9 ± 14.1	69.7 ± 12.1	42.5 ± 8.8*	23.7 ± 7.1 ^a	19.1 ± 5.2	32.2 ± 9.6	19.4 ± 8.3
Open arm entry (no.)	7.9 ± 1.1	5.9 ± 1.0	5.4 ± 1.1	4.8 ± 0.9*	1.9 ± 0.5 ^a	1.8 ± 0.5	2.6 ± 0.7	1.8 ± 0.7
Open arm activity (no.)	19.5 ± 2.9	15.6 ± 2.4	13.7 ± 2.9	9.0 ± 2.4**	3.7 ± 1.3 ^a	2.8 ± 1.0	5.5 ± 1.6	3.4 ± 1.9
Enclosed arm time (s)	104.2 ± 14.7	148.1 ± 15.0*	169.5 ± 16.5***	173.1 ± 9.1***	189.2 ± 20.9 ^b	205.4 ± 20.2	215.1 ± 17.8	229.0 ± 12.9
Enclosed arm entry (no.)	9.4 ± 1.1	8.3 ± 0.8	9.6 ± 0.9	9.7 ± 0.7	9.1 ± 1.0	9.8 ± 0.8	10.9 ± 0.4	11.3 ± 1.0
Enclosed arm activity (no.)	24.5 ± 3.2	23.2 ± 2.4	26.8 ± 2.6	26.4 ± 2.1	24.9 ± 2.8	27.6 ± 2.1	30.6 ± 1.1	31.2 ± 2.9
Risk assessment (no.)	0.5 ± 0.2	1.3 ± 0.4	2.3 ± 0.5**	1.5 ± 0.4	1.8 ± 0.4 ^c	2.0 ± 0.4	1.8 ± 0.3	1.2 ± 0.3
Center time (s)	104.7 ± 9.7	76.2 ± 10.0*	56.2 ± 7.3***	76.5 ± 5.7*	81.9 ± 22.2	71.6 ± 17.1	48.9 ± 10.2	49.8 ± 9.7
Total arm entry (no.)	17.3 ± 1.4	14.2 ± 1.5	15.0 ± 1.7	14.4 ± 1.2	11.0 ± 1.4 ^b	11.6 ± 1.1	13.5 ± 0.7	13.1 ± 1.2
Total arm activity (no.)	44.0 ± 3.6	38.8 ± 3.3	40.5 ± 4.9	35.4 ± 3.3	28.6 ± 3.5 ^b	30.3 ± 2.6	36.1 ± 1.2	34.6 ± 3.4

Mean ± SEM are shown.

* ** *** Difference between a given dosage of DCS treatment and vehicle according to ANOVA followed by post hoc Scheffé's test.

^a, ^b, ^c, *** Difference between LA and HA rats according to two-tailed *t* test.

* *P* < 0.05.

** *P* < 0.01.

*** *P* < 0.001.

^a *P* < 0.001.

^b *P* < 0.01.

^c *P* < 0.05.

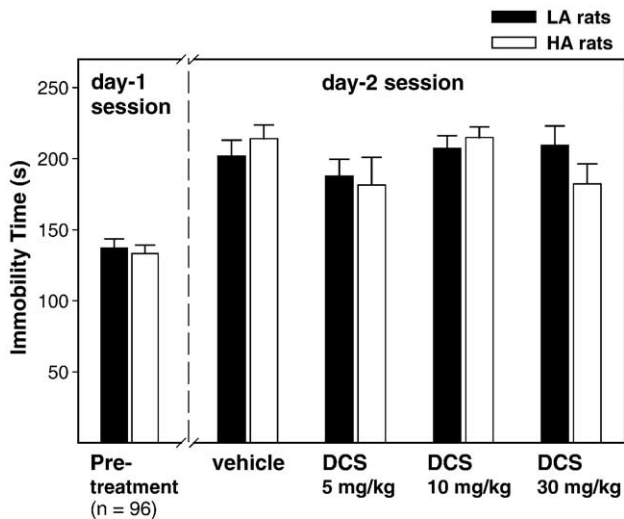


Fig. 1. Effects of DCS (0, 5, 10, and 30 mg/kg ip; $n = 12$ for each group) on the immobility time of rats in the forced swim test. Forty-eight LA and 48 HA rats were tested on the day 1 session. DCS was administered 30 min before the day 2 session. Immobility was not affected by DCS in both LA and HA rats. Data are expressed as mean \pm SEM.

restraint [20], are associated with increased extracellular glutamate concentrations in the brain. Nowak et al. demonstrated that the proportion of high affinity glycine binding site on the NMDA receptor is increased in the frontal cortex of suicide victims [27]. In addition, chronic administration of antidepressants is reported to induce adaptive changes in the glycine binding sites [32], suggesting a relationship between the function of NMDA receptors and the biological basis of psychological disorders.

DCS acts as a partial agonist, that is, a lower dose acts as an agonist but higher doses act as antagonists at glycine binding sites on the NMDA receptor [8,13,41]. In behavioral experiment, high dose of DCS (200–300 mg/kg) exhibited an anxiolytic-like activity in Sprague–Dawley rats, by decreasing the fear-potentiated startle response [1], and by increasing the number of shocks accepted in the conflict-drinking Vogel test [17]. The low dose (10–30 mg/kg) used in the current study showed an anxiogenic effect, which is in accordance with earlier reports showing that DCS at doses of 3–12 mg/kg significantly prevented ethanol-induced anxiolytic effects in the plus-maze test [24]. These results are consistent with the properties of partial agonists. Furthermore, because the anti-conflict effect of DCS is reduced by the administration of NMDA [17], the interactions between the glycine binding site and the glutamate recognition sites on the NMDA receptor may be important determinants of the behavioral effect of DCS. In agreement with previous results showing that DCS fails to affect spontaneous locomotor activity [10], all the dosages of DCS used in the current study did not affect motor activity since total arm entries and total arm activity in the plus-maze test were not influenced. Thus, the behavioral effects of DCS are unlikely to be accounted for by non-motivational factors.

To test whether DCS might have different effects in HA versus LA rats on coping behavior in stressful environments, behavior in the forced swim test was tested. In this test, the stressor is inescapable and rats acquire helplessness during the training session, in contrast to the plus-maze, where the aversive open arm is avoidable. The present results are consistent with our previous observations [12], in showing that HA and LA rats display different coping strategies in escapable but not in inescapable stress environments, since open arm time in the plus-maze test was significantly different between LA and HA rats, whereas immobility in the forced swim test did not differ between them. In addition, the behavioral effects of DCS depended on the anxiety level and/or experimental situation because DCS increases anxiety levels in LA but not HA rats in plus-maze test, whereas DCS did not affect the despair behavior in either subgroups in the forced swim test. This view is also supported by the finding that when divided animals into HD and LD subgroups based on their movement distance in the open field, the DCS effects were not different between HD and LD rats. Such task-dependent function has also been reported in previous literature; the glycine_B antagonist, L-701,324, showed an anxiolytic-like effect in the plus-maze test but did not affect the behavior in Vogel conflict test [15].

Although the pathophysiology of both anxiety and depression is related to stress, the drugs used clinically for these two disorders are not the same, suggesting that their mechanisms are different. The present data are comparable to this view because the anxiety behavior, expressed as open arm time in the plus-maze test, is not correlated to the immobility in the depression model, forced swim test. Furthermore, DCS, at least at the current dosages, affects anxiety but not the despair response. Although one might argue that the repeated administration of DCS in the present study, 6 days apart, may produce residual effects or behavioral tolerance on the swim test, the fact that DCS in plasma and brain is rapidly eliminated, with plasma half-life about 70 min [19], indicates that it may be not the case. Moreover, it should be noted that some drugs are effective in clinical therapy but show no relevant response in animal screening tests. For example, the atypical anxiolytic buspirone does not have a reliable anxiolytic effect in the plus-maze test [35]. Thus, it is necessary to clarify the role of DCS in depression by using other models.

A possible explanation for the lack of effects of DCS on the anxiety level in HA rats is the weak efficacy of DCS as an agonist for the glycine binding site on NMDA receptor: DCS shows only 64% of the agonistic potency as compared to the endogenous agonist glycine [6,41]. Thus, the effect of DCS on the NMDA receptor depends on the occupancy of the glycine binding site by glycine: if these sites are not fully saturated, DCS at low dose could enhance the stimulating effects of glutamate on the NMDA receptor [13]. In fact, low dose of DCS (0.3–15 mg/kg) appeared to be able to stimulate the NMDA receptor, as measured in

several memory paradigms in humans and animals [14,23], indicating that endogenous glycine does not fully occupy the glycine binding site in vivo and that this receptor can therefore be stimulated by low dose of DCS. Thus, the DCS doses used in the current experiment might be too low for HA rats to bind to the glycine binding site to reveal the expected agonistic properties of DCS. Another explanation for the lack of behavioral effects of DCS in HA rats might be full occupancy of the glycine binding site on NMDA receptor by endogenous glycine. Therefore, the present result suggests that glutamatergic activity and/or the occupancy of glycine binding site may be higher in HA than in LA rats.

The result of the current study is consistent with our earlier observation [12] showing that male Wistar rats exhibit substantial variations in their anxiety response. The biological basis of anxiety and depression is not identical since DCS affected anxiety but not despair behavior. Furthermore, there was no correlation between these two behaviors. In addition, the behavioral effects of DCS depended on the anxiety level of rats and have task-dependent behavioral consequences, suggesting that the function of the glycine binding site on NMDA receptor is involved in individual differences of anxiety levels.

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