



# Anxiolytic Activity of Glycine-B Antagonists and Partial Agonists—No Relation to Intrinsic Activity in the Patch Clamp

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**Summary**—On the basis of animal models, anxiety was one of the first suggested clinical applications of partial agonists of the glycine<sub>B</sub> site coupled to the NMDA receptor. It is not certain, however, whether these findings can be extended to full glycine<sub>B</sub> antagonists and what is the relation between intrinsic activity (degree of NMDA receptor antagonism) and anxiolytic effect. In the present study several NMDA receptor antagonists, including several glycine<sub>B</sub> antagonists/partial agonists, were tested for anxiolytic activity in the Vogel conflict test and the elevated plus-maze. Additionally, the intrinsic activities of the glycine<sub>B</sub> partial agonists used [ACPC, (*R*,+)-HA-966 and D-cycloserine] were compared in patch-clamp experiments in cultured neurones. In the plus-maze the most striking increase in the time spent in open arms (index of anxiolytic effect) was seen after diazepam and D-cycloserine (at doses that did not change locomotion). Also reliable (dose-dependent), although weaker, anxiolytic activity was produced by the uncompetitive NMDA receptor antagonist (+)MK-801 and the competitive antagonist CGP 39551. Modest anxiolytic-like effect in the plus-maze was also observed after the glycine<sub>B</sub> antagonist L-701,324 and the partial agonist (+,*R*)-HA-966. Uncompetitive antagonists memantine and amantadine, the glycine<sub>B</sub> partial agonist ACPC (up to 600 mg/kg) or the full antagonists MRZ 2/570, MRZ 2/571 and MRZ 2/576 had no effect. In the Vogel conflict test neither memantine, nor any of the full glycine<sub>B</sub> antagonists tested (L-701,324 and MRZ 2/576), showed anxiolytic activity.

Patch-clamp studies revealed that the intrinsic activity of (+,*R*)-HA-966, D-cycloserine and ACPC was 13, 57 and 92%, respectively, as compared to that of glycine itself (100%).

In conclusion, for the agents tested there is no clear relation between the levels of intrinsic activity, i.e. degree of NMDA receptor inhibition, and anxiolytic activity. Moreover, L-701,324 and MRZ-type glycine<sub>B</sub> full antagonists do not exhibit anxiolytic activity in the elevated plus-maze and Vogel conflict test. © 1997 Published by Elsevier Science Ltd.

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At present it seems clear that a different profile can be expected from agents inhibiting NMDA receptors by channel blockade (uncompetitive antagonists), by competition with endogenous glutamate (competitive antagonists) or by blocking the glycine<sub>B</sub> site (Chiamulera *et al.*, 1990; Bubser *et al.*, 1992; Danysz *et al.*, 1994). On the basis of animal studies, the latter type of antagonist has been proposed as an attractive target for drug development due to lack of some effects that are often observed

after administration of antagonists that act at other sites. In short this concerns:

1. Lack of neurodegenerative changes in the cingulate/retrosplenial cortex even after high doses (Chen, 1993; Haggerty, 1993; Hargreaves, 1993; Berger, 1994);
2. Lack of psychotomimetic-like effects (Koek and Colpaert, 1990; Danysz *et al.*, 1994; Bristow *et al.*, 1996);
3. Lack of learning impairing effects at anticonvulsive doses (Chiamulera *et al.*, 1990; Murata and Kawasaki, 1993; Smith *et al.*, 1994; Faiman *et al.*, 1994);
4. Suggested favourable efficacy profile in stroke models

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(Globus *et al.*, 1991; Moroni *et al.*, 1992; E. Weber, presented at CoCensys meeting, 1994; Newell *et al.*, 1995).

Recent developments in the molecular biology of glutamate receptors have emphasized that the pharmacological properties of such receptors differ considerably, depending on the subunit composition of the ionic channel. Thus, although the glycine site is located on the NMDAR1 subunit, the sensitivity to agonists is low if the channel also contains NMDA2A or 2C subunits and high when NMDAR2B or 2D are present (Hollmann and Heinemann, 1994). This heterogeneity of NMDA receptors also finds expression *in vivo*, where the distribution of various receptor subtypes and, in turn, presumably their physiological role is different (Hollmann and Heinemann, 1994). It therefore seems likely that certain NMDA receptor antagonists might be favourable treatments for some diseases, but poor for others, depending on the brain region involved. Hence, at present the primary goal of the pharmaceutical industry is to select the most plausible therapeutic applications of glutamate antagonists on the basis of systemic administration in animal models to avoid both false positives and false negatives at the earliest possible stage of development.

The present paper focuses on verifying the anxiolytic activity of glycine<sub>B</sub> antagonists and partial agonists in animal models. Anxiolytic activity resulting from NMDA receptor antagonism was reported as early as 1986 when Stephens and colleagues (Stephens *et al.*, 1986) found that high doses of 2-amino-7-phosphonoheptanoic acid (APH, 160 mg/kg) increased locomotion in mice in the four plate test, and Bennett and Amrick (1986) showed anxiolytic effect in the conflict test in rats. Later, anxiolytic activity of uncompetitive and competitive NMDA receptor antagonists has been shown in the conflict test (Corbett and Dunn, 1993; Plaznik *et al.*, 1994), the social interaction test (Dunn *et al.*, 1989; Corbett and Dunn, 1991, 1993), the elevated plus-maze (Dunn *et al.*, 1989; Corbett and Dunn, 1993), separation-induced vocalization in rat pups (Kehne *et al.*, 1991) and by blockade of fear potentiated startle (Anthony and Nevins, 1993). In general, most data are in line with anxiolytic activity of NMDA receptor antagonists in rodents at rather low doses devoid of side effects (e.g. ataxia or myorelaxation, see Palfreyman and Baron, 1991). On the other hand, the status of our knowledge on the validity of glycine<sub>B</sub> antagonist as anxiolytic agents is far from certain, mainly because agents that penetrate to the brain following systemic administration have been introduced only recently (Rowley *et al.*, 1993; Pellegrini-Giampietro *et al.*, 1994; Woodward *et al.*, 1995; Obrenovitch and Zilkha, 1996). Most previous studies used high doses of agents where the blood-brain barrier penetration was questionable (Kehne *et al.*, 1991; Corbett and Dunn, 1993), central injections (Jessa *et al.*, 1996b) or, finally, utilized glycine<sub>B</sub> partial agonists (Trullas *et al.*, 1989; Corbett and Dunn, 1991; Przegalinski *et al.*,

1996). In all cases interpretation of the results, even if they are positive, is faced with serious pitfalls. Firstly, when very high doses of antagonists with no proven good blood-brain barrier penetration are used, interpretation of data is, at best, difficult. Secondly, even if central injections produce clear-cut anxiolytic activity, such effects are not therapeutically relevant since the same agents might have opposing behavioural effects depending on the site of injection. Thus, they cannot predict the net effect after systemic injection (see Turski *et al.*, 1991). Thirdly, the interpretation of the data obtained with partial agonists is confounded by their peculiar pharmacological profile. D-Cycloserine shows agonistic effects at lower doses, but antagonistic effects at higher doses (Lanthorn, 1994). Although aminocyclopropane carboxylic acid (ACPC) has a very high level of intrinsic activity *in vitro*, surprisingly it shows an antagonistic profile *in vivo* (Skolnick *et al.*, 1989). Thus, it is not clear whether anxiolytic effects of glycine<sub>B</sub> partial agonists relate directly to the degree of NMDA receptor inhibition (intrinsic activity) or are the consequence of other unknown features, e.g. selectivity for receptor subtypes. Due to these pitfalls, the validity of targeting the glycine<sub>B</sub> site with full antagonists for potential anxiolytic agents is uncertain. Recently, however, some novel glycine<sub>B</sub> antagonists with sufficient penetration to the central nervous system have been introduced and a first report indicates rather poor anxiolytic activity (Wiley *et al.*, 1995).

The present paper compares: several glycine<sub>B</sub> full antagonists belonging to a new class tricyclic-pyridophthalazine-diones (Parsons *et al.*, in press) and 7-chloro-4-hydroxy-3-(3-phenoxy)-phenyl-2(H)quinolone (L-701,324); glycine<sub>B</sub> partial agonists ACPC, (+,*R*)-3-amino-1-hydroxy-2-pyrrolidone ((+,*R*)-HA-966) and D-cycloserine; uncompetitive antagonists (+)-5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine maleate ((+)MK-801), 1-amino-3,5-dimethyladamantane (memantine), amantadine and a competitive antagonist DL-(*E*)-2-amino-4-methyl-5-phosphono-3-pentanoic acid methyl ester (CGP-39551) in the elevated plus-maze and/or Vogel conflict test. Additionally, the intrinsic activities of the partial agonists used were compared in patch-clamp experiments in cultured cortical neurones.

## MATERIALS AND METHODS

### *Animals*

Naive male Sprague-Dawley rats (220–250 g) used for the elevated plus-maze test study were housed five per cage, with water and food *ad libitum*, in a 12 hr light-dark cycle (light on at 6 a.m.) and controlled temperature (23°C). The Vogel test experiment was performed on Wistar rats (200–220 g) that were deprived of water for 23 hr each day (see procedure description). All experiments were performed between 11.00 a.m. and 6.00 p.m.

### Plus-maze—procedure

The wooden plus-maze apparatus consisted of two open arms (50 × 10 cm) and two enclosed arms (50 × 10 cm, and 40-cm high walls) mounted 50 cm above the floor. The plus-maze was placed in a darkened room with a localized light (25 W) facing the apparatus and mounted 1 m above its surface (the light level was equal in all parts of the maze). The plus-maze was surrounded by black material to minimize distortion by external stimuli, including the experimenter. A camera connected to a monitor was placed 2 m above the maze to allow observation of the animal's behaviour without interference. Animals were brought to the experimental room 1 hr before the start of the experiment. Each rat was then placed in the centre of the plus-maze facing an open arm and the test continued for 5 min. During this period the number of entries into closed or open arms, as well as time spent in each type of arm, was monitored. The results were then expressed as mean ± SEM per cent entries or time spent in each arm type. Moreover, each arm was divided into three sectors, and crossing of dividing lines was used as a indicator of locomotor activity within the maze. Additionally, after the maze test, ataxia was monitored by visual inspection (present/absent) and myorelaxant activity was tested by lifting the animal by the tail and allowing it to grasp a horizontal string, placed 50 cm above the floor, with its forepaws and released. If the rat failed to hold for minimum of 4 sec in any of three consecutive trials, it was scored as showing myorelaxation.

### Vogel test—procedure

The experiment was performed according to the method described in detail by Plaznik *et al.* (1994). In short, thirsty rats were used, i.e. rats deprived of water for 23-hr daily, having free access to water only for 45 min immediately after daily 15-min training sessions in the test box. During the 15 min of training in the test boxes mild electric shocks (0.4 mA, 4-sec trains of impulses separated by 5-sec intervals) were applied while rats were drinking water. This experimental procedure takes 5 days of pre-training followed by a test session. Two control groups were used: control—not shocked and control—shocked rats, both injected with vehicle.

### Statistical analysis

The plus-maze and Vogel test data were calculated with the help of one-way ANOVA followed by Dunnett or Newman-Keuls test, respectively, for independent measures.

### Patch-clamp technique

Patch-clamp recordings were made from cultured rat superior collicular neurones (11–14 days *in vitro*; see Parsons *et al.*, 1993a for details) in the whole-cell mode with the aid of an EPC-7 amplifier (List) at a membrane potential of -70 mV. Patch-clamp electrodes were pulled

with a horizontal puller (DMZ) and had an internal tip diameter between 1.0 and 1.4 μM and a tip resistance of 4–6 MΩ. Cells were superfused continuously via one of eight channels of a custom-made fast superfusion system with a common outflow. Test substances then were applied by rapidly switching channels—complete exchange of the superfused solution was achieved within 10 msec. The application of solutions and the synchronized on-line electronic acquisition of data were controlled by the IBM program PCLAMP.

The contents of the intracellular (electrode) solution were as follows (mM): CsCl (120), TEACl (20), EGTA (10), MgCl<sub>2</sub> (1), CaCl<sub>2</sub> (0.2), glucose (10), ATP (2), cAMP (0.25). The extracellular solutions had the following basic composition (mM): NaCl (140), KCl (3), glucose (10), HEPES (10), CaCl<sub>2</sub> (0.2), sucrose (4.5). In addition, neurones were pharmacologically isolated from one another by the inclusion of 0.3 μM tetrodotoxin to block voltage-activated sodium currents. NMDA (200 μM), glycine (0, 1 or 10 μM) and glycine<sub>B</sub> partial agonists [(+,R)-HA-966 (1–3000 μM), D-cycloserine (0.3–3000) and ACPC (0.3–100 μM)] were added to this basic solution.

### Drugs

The following drugs were used: amantadine (Aldrich, Steinheim, Germany), ACPC (Tocris Cookson, Bristol, U.K.), memantine (Merz & Co., Frankfurt, Germany), (+,R)-HA-966 (Tocris Cookson, Bristol, U.K.), CGP 39551 (Ciba Geigy, Basel, Switzerland), D-cycloserine (Sigma, St. Louis, U.S.A.), L-701,324 (Merck Sharp & Dohme, Terlings Park, U.K.), diazepam (an ampoule of 5 mg/ml for intravenous injections, Ratiopharm, Ulm, Germany), (+)MK-801 (RBI, Natick, U.S.A.), and MRZ 2/570, MRZ 2/571, MRZ 2/576 (Merz & Co., Frankfurt, Germany).

(+)MK-801, memantine, amantadine, D-cycloserine, ACPC and (+,R)-HA-966 were dissolved in 0.9% saline; diazepam, CGP 39551, MRZ 2/570, MRZ 2/571 and MRZ 2/576 were dissolved in distilled water; L-701,324 was suspended in 0.5 % methyl cellulose. All solutions were prepared immediately before the injections and administered intraperitoneally at a volume of 2 ml/kg of body weight. Control animals were injected with the corresponding vehicle.

The drugs were administered *i.p.* as follows: CGP 39551—120 min; (+)MK-801, memantine, L-701,324, amantadine, D-cycloserine, (+,R)-HA-966—30 min; diazepam—20 min; all MRZ compounds and ACPC—15 min before the test.

## RESULTS

### Plus-maze

Diazepam produced a dose-dependent increase in the time spent in the open arms (statistically significant at 2 mg/kg), but failed to affect the number of entries into the open arms (Table 1). Also it did not change locomotor

Table 1. Effect of diazepam and NMDA receptor antagonists on the anxious behaviour in the elevated plus-maze

Drug	Dose (mg/kg)	Per cent time in open arms (mean $\pm$ SE ( <i>n</i> ))	Per cent open-arm entries (mean $\pm$ SE)
Diazepam	0	14.83 $\pm$ 1.89 (8)	35.59 $\pm$ 4.27
	1	17.52 $\pm$ 0.87 (8)	35.12 $\pm$ 3.12
	2	47.71 $\pm$ 5.45 (8)*	51.47 $\pm$ 2.40
	3	41.13 $\pm$ 4.92 (8)*	45.80 $\pm$ 3.25
MK-801	0	13.97 $\pm$ 1.83 (10)	30.28 $\pm$ 2.98
	0.05	19.60 $\pm$ 2.57 (10)	35.93 $\pm$ 3.99
	0.1	25.10 $\pm$ 3.76 (10)*	34.41 $\pm$ 3.52
	0.15	27.96 $\pm$ 2.52 (8)*	37.84 $\pm$ 4.50
Memantine	0	22.11 $\pm$ 1.41 (10)	38.93 $\pm$ 1.83
	0.3	27.50 $\pm$ 2.09 (8)	41.10 $\pm$ 3.88
	1	31.54 $\pm$ 3.29 (8)	43.48 $\pm$ 2.16
	3	27.79 $\pm$ 2.19 (8)	37.86 $\pm$ 3.22
Amantadine	10	26.50 $\pm$ 2.71 (10)	39.57 $\pm$ 2.24
	0	15.77 $\pm$ 1.91 (10)	33.25 $\pm$ 1.89
	10	22.21 $\pm$ 3.77 (8)	36.46 $\pm$ 4.17
	30	29.75 $\pm$ 4.77 (8)	44.50 $\pm$ 3.41
CGP 39551	100	9.38 $\pm$ 4.12 (8)	41.82 $\pm$ 6.68
	0	13.06 $\pm$ 1.55 (10)	28.57 $\pm$ 2.28
	1	8.08 $\pm$ 1.54 (8)	23.75 $\pm$ 3.91
	3	20.75 $\pm$ 2.76 (8)*	34.24 $\pm$ 3.70
D-Cycloserine	10	30.13 $\pm$ 2.81 (8)*	43.11 $\pm$ 1.61*
	0	16.20 $\pm$ 1.95 (10)	36.26 $\pm$ 4.07
	10	28.90 $\pm$ 3.02 (7)*	41.42 $\pm$ 1.90
	30	35.71 $\pm$ 7.48 (7)*	45.94 $\pm$ 2.52
ACPC	100	38.76 $\pm$ 3.72 (7)*	51.23 $\pm$ 4.68
	300	44.52 $\pm$ 3.61 (7)*	47.05 $\pm$ 4.31
	0	14.38 $\pm$ 1.90 (8)	32.04 $\pm$ 4.91
	100	17.88 $\pm$ 2.92 (8)	31.68 $\pm$ 4.74
(+,R)-HA-966	300	22.04 $\pm$ 4.35 (8)	36.43 $\pm$ 4.28
	600	22.83 $\pm$ 2.91 (8)	40.41 $\pm$ 3.77
	0	14.72 $\pm$ 1.53 (10)	31.84 $\pm$ 2.71
	0.3	16.29 $\pm$ 2.86 (8)	32.28 $\pm$ 3.42
L-701,324	1	24.13 $\pm$ 0.16 (8)*	40.26 $\pm$ 3.71
	3	24.63 $\pm$ 0.82 (8)*	39.42 $\pm$ 4.77
	10	27.42 $\pm$ 1.89 (8)*	40.45 $\pm$ 3.44
	0	16.63 $\pm$ 1.59 (10)	29.53 $\pm$ 3.01
Mrz 2/570	1	16.46 $\pm$ 2.90 (8)	32.48 $\pm$ 3.10
	3	25.73 $\pm$ 1.27 (8)*	44.20 $\pm$ 2.62
	10	24.38 $\pm$ 1.96 (8)*	37.27 $\pm$ 3.47
	0	18.08 $\pm$ 2.57 (8)	40.47 $\pm$ 3.75
Mrz 2/571	1	15.29 $\pm$ 3.39 (8)	31.73 $\pm$ 4.88
	3	16.24 $\pm$ 2.64 (8)	32.72 $\pm$ 2.44
	10	14.83 $\pm$ 3.80 (8)	37.94 $\pm$ 5.70
	0	11.42 $\pm$ 2.28 (8)	23.88 $\pm$ 3.12
Mrz 2/576	1	11.90 $\pm$ 2.07 (8)	27.68 $\pm$ 3.89
	3	12.62 $\pm$ 2.69 (8)	26.12 $\pm$ 3.17
	10	11.46 $\pm$ 2.27 (8)	32.96 $\pm$ 2.68
	0	10.79 $\pm$ 0.84 (8)	26.63 $\pm$ 1.36
	1	15.24 $\pm$ 3.39 (8)	29.07 $\pm$ 3.87
	3	10.00 $\pm$ 1.54 (8)	30.40 $\pm$ 3.24
	10	7.92 $\pm$ 1.33 (8)	28.14 $\pm$ 3.54

\**p* < 0.05 vs vehicle treated group (Dunnett test).

activity as evidenced by the number of sectors crossed (data not shown).

Although (+)MK-801 also increased significantly the time spent in the open arms, the magnitude of this effect was modest (Table 1). At the doses used (0.025–0.15 mg/kg) no change in the number of arm entries or sectors crossed (data not shown) was observed. A similarly weak non-significant effect was seen after amantadine, and in this case it showed a bell-shaped dose–response relationship (Table 1). Amantadine at a dose of 100 mg/kg

significantly decreased locomotor activity, as evidenced by the number of sectors crossed. Memantine, on the other hand, had no effect at all (Table 1). A clear effect was observed with the competitive antagonist CGP 39551, which at a higher dose (10 mg/kg) increased the time spent in the open arms by over 130% and also increased the number of entries into the open arms (Table 1). Ataxic effects were observed after (+)MK-801 at the highest dose of 0.15 mg/kg in all animals tested.

The glycine<sub>B</sub> partial agonists D-cycloserine and (+,R)-HA-966 produced an increase in the time spent in the open arms (Table 1), which was much stronger in the case of the former agent. In contrast, ACPC was completely without effect (Table 1). The glycine<sub>B</sub> partial agonists tested produced neither muscle relaxation nor ataxia, nor a change in the number of sectors crossed (not shown).

Of the full glycine<sub>B</sub> antagonists tested, only L-701,324 increased the time spent in the open arms (Table 1), while other agents failed to change any of the parameters measured. The effect of L-701,324 was very modest and not clearly dose-dependent. No change in the number of sectors crossed was observed after any of the glycine<sub>B</sub> antagonists. Ataxia was observed at 10 mg/kg after MRZ 2/570, MRZ 2/571, MRZ 2/576 in seven, eight and three rats, respectively, out of the group of eight animals. Myorelaxation was observed at 10 mg/kg after MRZ 2/570, MRZ 2/571, MRZ 2/576 in two rats out of eight in each group.

#### Vogel test

Both memantine and L-701,324 failed to affect significantly the amount of water drunk during the Vogel test session under shock suppressed conditions (Fig. 1). Similarly, the MERZ glycine<sub>B</sub> antagonist Mrz 2/576 appeared to be ineffective in the Vogel test, neither attenuating nor potentiating the effect of shock across the dose-range tested (Fig. 1), although a non-significant tendency was seen toward attenuation of shock effects. Also, none of the agents tested significantly affected water intake under non-shock conditions.

#### Patch clamp

NMDA (200  $\mu$ M) evoked inward steady-state current responses in the continuous presence of glycine 1  $\mu$ M which were  $83.5 \pm 3.4\%$  of the maximal current achievable with glycine 10  $\mu$ M (data not shown). In the nominal absence of glycine, small steady-state current responses were still apparent ( $3.1 \pm 0.3\%$ ) due to the presence of residual contaminating glycine (probably around 20–40 nM, see Parsons *et al.*, 1993b). (+,R)-HA-966 antagonized NMDA responses in the presence of glycine 1  $\mu$ M ( $IC_{50}$   $28.6 \pm 1.2 \mu$ M) and, as expected, was 10 times weaker in the presence of glycine 10  $\mu$ M ( $IC_{50}$   $276 \pm 24 \mu$ M, Figs. 2 and 5). In the nominal absence of glycine, (+,R)-HA-966 potentiated NMDA responses with an  $EC_{50}$  of  $16.3 \pm 2.4 \mu$ M. The intrinsic activity was estimated to be 13.3%.

D-Cycloserine also antagonized NMDA responses in

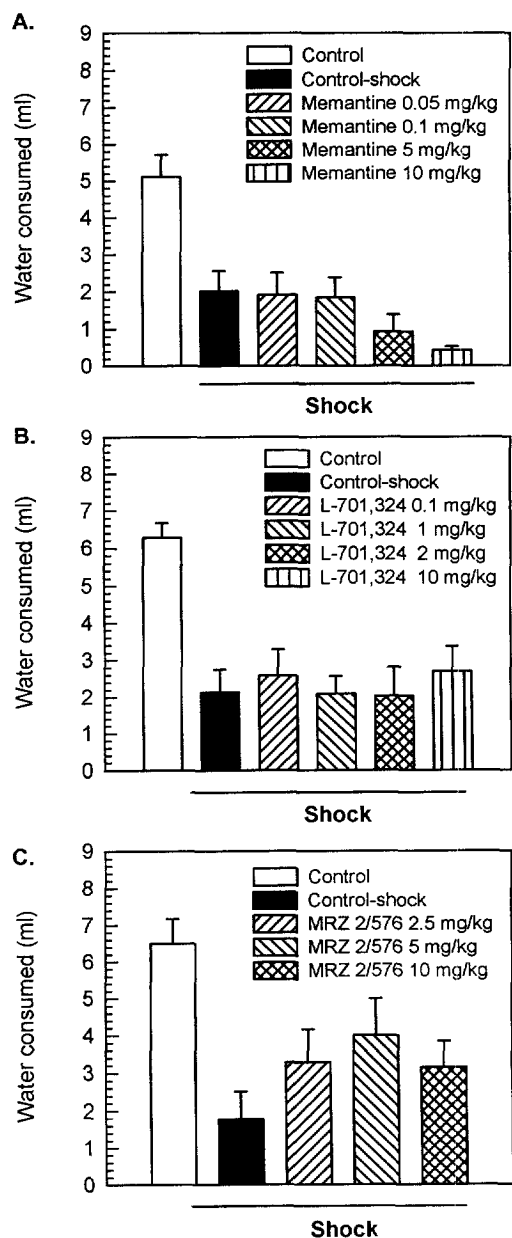


Fig. 1. Effects of the uncompetitive NMDA receptor antagonist, memantine (A) and the NMDA receptor-glycine<sub>B</sub> site antagonist, L-701,324 (B) and MRZ 2/576 (C) on rat behaviour in the Vogel test session. The results are means  $\pm$  SE for eight animals per group. All shocked groups drank significantly less water than non-shocked controls (\* $P$  < 0.05, Newman-Keuls test).

the presence of glycine 1  $\mu$ M ( $IC_{50}$  31.2  $\pm$  5.1  $\mu$ M) but was considerably more effective than (+,*R*)-HA-966 in potentiating NMDA responses in the nominal absence of glycine ( $EC_{50}$  of 9.8  $\pm$  1.2  $\mu$ M, Figs. 3 and 5). The intrinsic activity was estimated to be 57.4%. Similar results were obtained with D-cycloserine in cultured hippocampal neurons.

In the presence of various concentrations of both (+,*R*)-HA-966 and D-cycloserine, NMDA-induced currents lacked any obvious glycine-sensitive desensitiza-

tion in agreement with Kemp and Priestley (1991) (see Parsons *et al.*, 1993b).

The profile of activity of ACPC was more like a non-selective full agonist at the glycine<sub>B</sub> site. ACPC potentiated NMDA responses in the nominal absence of glycine ( $EC_{50}$  of 0.35  $\pm$  0.05  $\mu$ M) and some degree of glycine-sensitive desensitization was apparent at lower concentrations (Figs. 4 and 5).

The effects of ACPC in the presence of glycine 1  $\mu$ M were biphasic with maximal potentiation at 10  $\mu$ M followed by inhibition at higher concentrations. A true partial agonist should reach plateau potentiation at its level of intrinsic activity, and it therefore seems likely that the inhibition at higher concentrations of ACPC was due to antagonistic effects at a different recognition site. Nonetheless, the apparent intrinsic activity was estimated to be 92.2%.

## DISCUSSION

In the present study we used the elevated plus-maze and the Vogel conflict test to assess potential anxiolytic properties of compounds acting at different sites associated with the NMDA receptor complex, with particular attention to glycine site partial agonists and antagonists. Diazepam, used as a reference agent affecting benzodiazepine receptors, produced a consistent, dose-dependent increase in the percentage of time spent in the open arms in the plus-maze but had no significant effect on the percentage of entries into open arms. Our results are in agreement with some earlier investigations showing anxiolytic effects of diazepam and other benzodiazepines in different animal models of anxiety (Pellow and File, 1986; Dunn *et al.*, 1989; McAllister, 1990; Kehne *et al.*, 1991; Corbett and Dunn, 1991; Anthony and Nevins, 1993; Plaznik *et al.*, 1994; Wiley *et al.*, 1995; Jessa *et al.*, 1996a,b). Thus, the experimental procedure used for the plus-maze in the present study was able to detect the anxiolytic activity of an agent with established clinical efficacy.

Previously, both competitive and uncompetitive NMDA receptor complex antagonists have been shown to exhibit anxiolytic-like activities similar to benzodiazepines in preclinical screening tests in rodents, such as social interaction (Dunn *et al.*, 1989; Corbett and Dunn, 1993), elevated plus-maze (Stephens *et al.*, 1986; Dunn *et al.*, 1989; Corbett and Dunn, 1991; Wiley *et al.*, 1995), blockade of fear-potentiated startle response (Anthony and Nevins, 1993), conflict tests (Corbett and Dunn, 1993; Plaznik *et al.*, 1994; Jessa *et al.*, 1996a,b; Przegalinski *et al.*, 1996) and separation-induced ultrasonic vocalizations in rat pups (Winslow *et al.*, 1990; Kehne *et al.*, 1991). Moreover, the excitatory amino acid agonist NMDA produced anxiogenic effects in the social interaction and in the elevated plus-maze tests (Dunn *et al.*, 1989), supporting the involvement of the glutamatergic system in the modulation of anxiety.

In line with this evidence, the uncompetitive NMDA

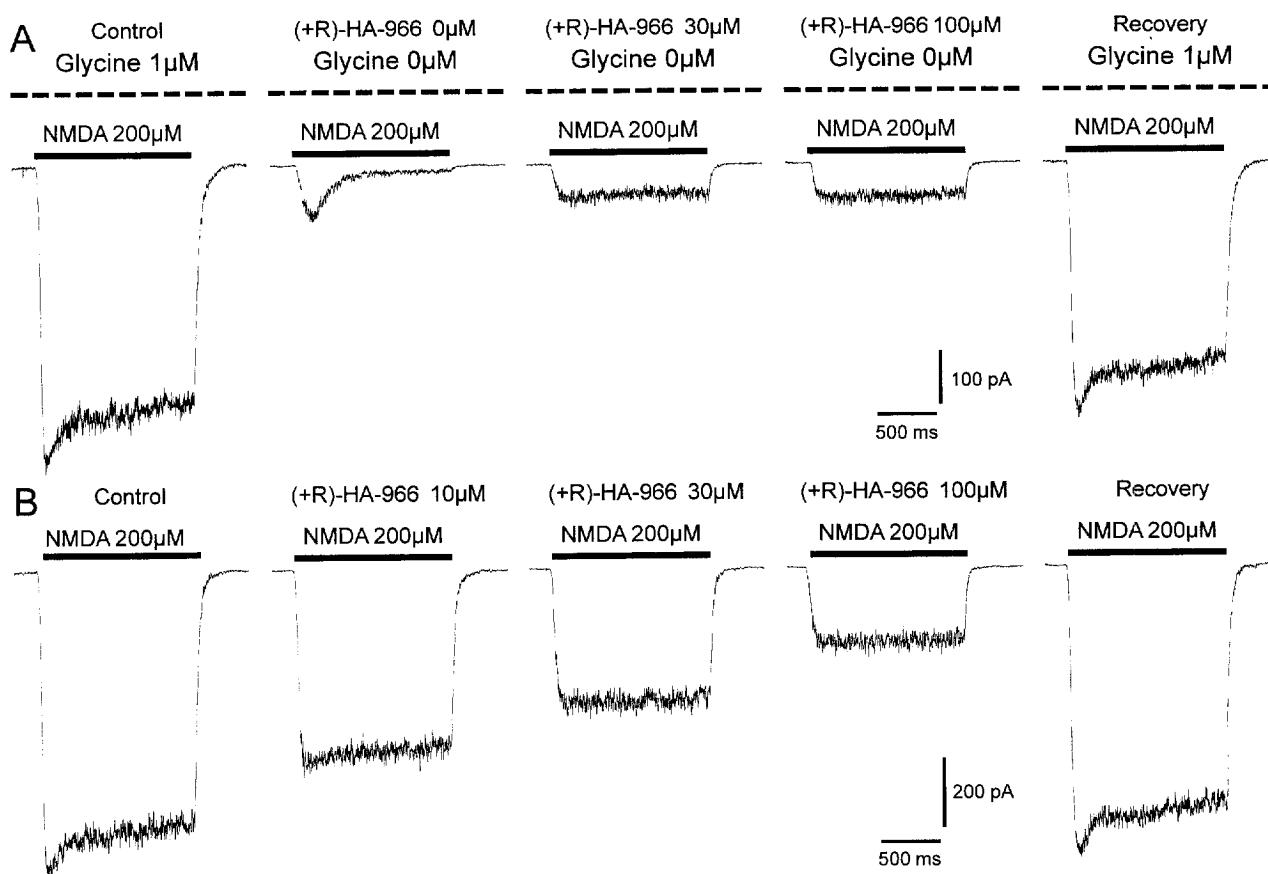


Fig. 2. Concentration-dependent effects of (+,*R*)-HA-966 on NMDA-induced inward current responses of cultured superior colliculus neurones. NMDA (200  $\mu$ M) was applied for 2.5 sec every 30 sec at a constant membrane potential of  $-70$  mV. The left and right panels show control and recovery responses to NMDA in the continuous presence of glycine 1  $\mu$ M. (A) The middle three panels show NMDA responses in the nominal absence of glycine and continuous presence of (+,*R*)-HA-966 (0, 30 and 100  $\mu$ M). (B) The middle three panels show NMDA responses in the continuous presence of glycine 1  $\mu$ M and (+,*R*)-HA-966 (10, 30 and 100  $\mu$ M).

receptor antagonist, (+)MK-801, in a rather narrow dose range of 0.1–0.15 mg/kg, increased the time spent in open arms; however, the observed effect was rather modest in comparison to that of diazepam. Anxiolytic activity of (+)MK-801 has also been reported previously by Dunn *et al.* (1989) and Corbett and Dunn (1991, 1993), where doses of 0.05 and 0.1 mg/kg significantly increased open-arm exploration time in the elevated plus-maze and social behaviour in the social interaction test in rats. Also, Plaznik *et al.* (1994) have shown that (+)MK-801 significantly increased the punished consumption of water in the Vogel conflict test. Surprisingly, in the present study no anxiolytic effects in the elevated plus-maze were observed with the other uncompetitive NMDA receptor antagonists memantine and amantadine. Memantine was also ineffective in the Vogel test. This apparent discrepancy in the effects of different uncompetitive NMDA receptor antagonists (e.g. (+)MK-801 vs memantine and amantadine) could relate to their different kinetics of channel blockade (Parsons *et al.*, 1993a) and/

or different selectivity for NMDA receptor subtypes (Bresink *et al.*, 1996). In contrast the competitive NMDA receptor antagonist, CGP 39551, reliably decreased anxiety in the plus-maze (present study) and in punished drinking in the Vogel test (Plaznik *et al.*, 1994).

Ataxia, sedation, amnesia and changes in locomotor activity are often associated with the application of NMDA receptor antagonists in animals (Koek and Colpaert, 1990; Willetts *et al.*, 1990; Carter, 1994; Danysz *et al.*, 1994, 1995). In line with such findings, at the highest doses used in the present study we observed ataxia after (+)MK-801 and sedative effects after amantadine. On the other hand, the competitive NMDA receptor antagonist CGP 39551 did not evoke obvious behavioural alterations and, in fact, agents belonging to this class have previously been proposed to be more promising for the treatment of anxiety (Plaznik *et al.*, 1994; see also Przegalinski *et al.*, 1996).

Similarly, glycine<sub>B</sub> antagonists and partial agonists have been suggested to show a favourable therapeutic

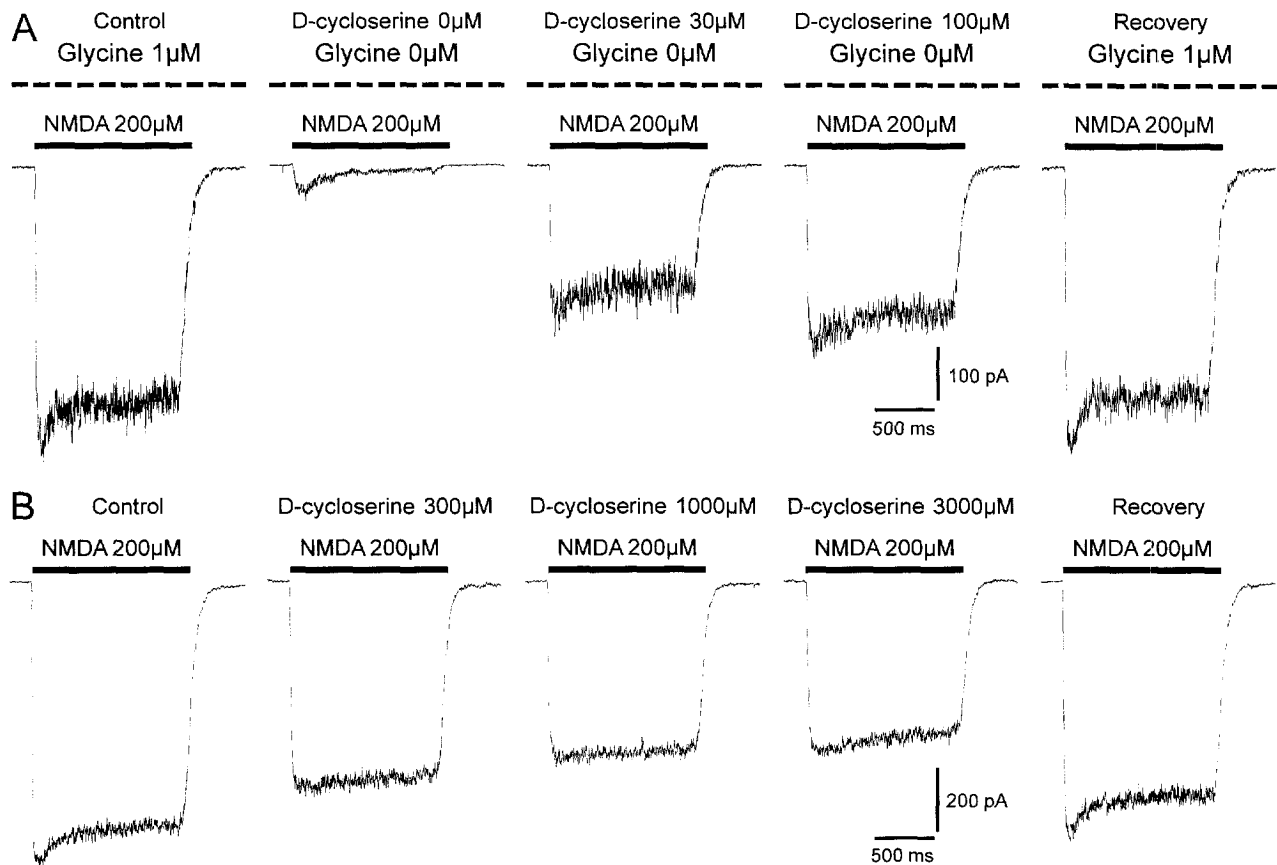


Fig. 3. Concentration-dependent effects of D-cycloserine on NMDA-induced inward current responses of cultured superior colliculus neurones. NMDA (200  $\mu$ M) was applied for 2.5 sec every 30 sec at a constant membrane potential of  $-70$  mV. The left and right panels show control and recovery responses to NMDA in the continuous presence of glycine 1  $\mu$ M. (A) The middle three panels show NMDA responses in the nominal *absence* of glycine and continuous presence D-cycloserine (0, 30 and 100  $\mu$ M). (B) The middle three panels show NMDA responses in the continuous presence of glycine 1  $\mu$ M and D-cycloserine (300, 1000 and 3000  $\mu$ M).

anxiolytic profile (Trullas *et al.*, 1989; Corbett and Dunn, 1991, 1993; Kehne *et al.*, 1991; Anthony and Nevins, 1993; Plaznik *et al.*, 1994; Kehne *et al.*, 1995). Furthermore, glycine<sub>B</sub> site full agonists, D-serine and glycine itself, injected into the dorsal peri-aqueductal grey matter (DPQG), have been reported to cause anxiogenic-like effects (Schmitt *et al.*, 1995). This indicates that the glycine<sub>B</sub> site of NMDA receptor is not fully saturated *in vivo* (see also Danysz *et al.*, 1989b) and that NMDA receptor-mediated neurotransmission in the DPQG may be related to the regulation of anxiety states. Comparing glycine<sub>B</sub> partial agonists in the plus-maze, we observed a clear, strong anxiolytic effect after D-cycloserine, but only a modest effect after (+,R)-HA-966 and no effect following ACPC administration.

The clear-cut anxiolytic effect of D-cycloserine was somewhat surprising. It has been shown that, while at the lower doses of D-cycloserine (below 20 mg/kg) agonistic effects are seen *in vivo*, at higher doses an antagonistic action predominates (Lanthorn, 1994; Peterson and Schwade, 1993; Emmett *et al.*, 1991). Although anxiolytic

activity of D-cycloserine has previously been reported by Anthony and Nevins (1993) in the fear-potentiated startle response test starting at a dose of 30 mg/kg, the present study represents the first demonstration of robust anxiolytic effects of D-cycloserine (of similar magnitude to diazepam) in the elevated plus-maze.

On the other hand, ACPC—a partial glycine<sub>B</sub> agonist with high intrinsic activity (92%, present paper; see also Marvizon *et al.*, 1989; Watson *et al.*, 1989)—failed to show anxiolytic activity in the elevated plus-maze in our experiments (doses up to 600 mg/kg). Thus, this contrasts to findings of Trullas *et al.* (1989, 1991), where ACPC at doses of 300–400 mg/kg (but not at 500 mg/kg) increased both the percentage of time spent in open arms and the percentage of entries into open arms in mice. However, even in these previous studies ACPC was significantly less efficacious than chlordiazepoxide (Trullas *et al.*, 1989). Anxiolytic properties of ACPC have been also shown in the fear-potentiated startle test starting at 200 mg/kg with complete blockade at 500 mg/kg (An-

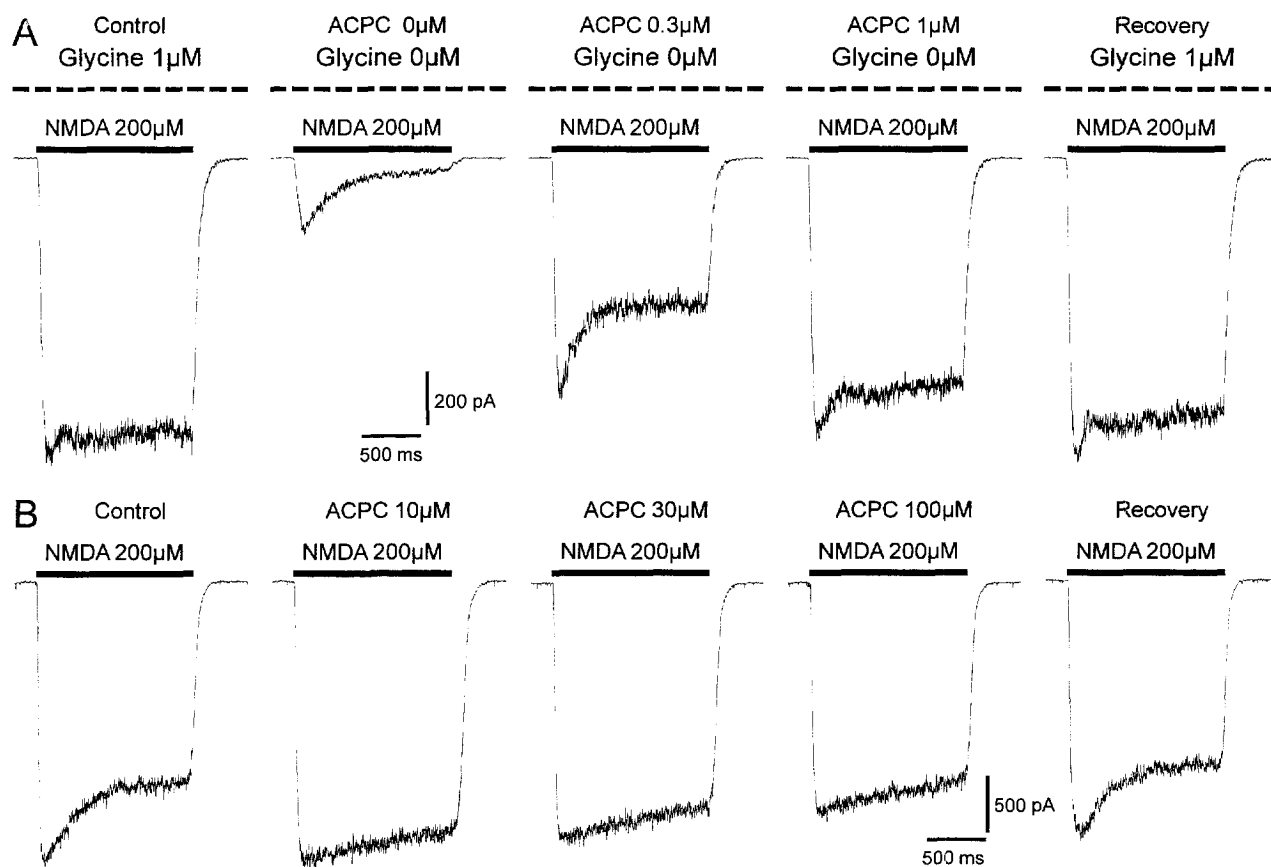


Fig. 4. Concentration-dependent effects of ACPC on NMDA-induced inward current responses of cultured superior colliculus neurones. NMDA ( $200 \mu\text{M}$ ) was applied for 2.5 sec every 30 sec at a constant membrane potential of  $-70 \text{ mV}$ . The left and right panels show control and recovery responses to NMDA in the continuous presence of glycine  $1 \mu\text{M}$ . (A) The middle three panels show NMDA responses in the nominal absence of glycine and continuous presence ACPC (0, 0.3 and  $1 \mu\text{M}$ ). (B) The middle three panels show NMDA responses in the continuous presence of glycine  $1 \mu\text{M}$  and ACPC (10, 30 and  $100 \mu\text{M}$ ).

thony and Nevins, 1993) and in the conflict test at 100 and 200 mg/kg (Przegalinski *et al.*, 1996). Similarly, Winslow *et al.* (1990) reported positive effects of this agent (12.5–200 mg/kg) in separation-induced ultrasonic vocalization model in rat pups. In contrast, in pigeons ACPC did not produce statistically significant anxiolytic effects in a conflict test (Koek and Colpaert, 1991). Thus, the lack of anxiolytic activity of ACPC in the plus-maze (present study) was rather unexpected and needs further elucidation, but may relate to its peculiar pharmacokinetic profile, i.e. a half-life time of less than 5 min (Rao *et al.*, 1990).

The low efficacy (13%) glycine site partial agonist, (+,*R*)-HA-966 (present study; see also Danysz *et al.*, 1989a; Kemp and Priestley, 1991; Priestley and Kemp, 1994), produced an anxiolytic effect in the elevated plus-maze starting at a dose of 1 mg/kg, but this effect was rather modest in comparison to that of diazepam and D-cycloserine. Previously Corbett and Dunn (1991) demonstrated anxiolytic-like activity of racemic HA-966 in the

conflict model, social interaction and plus-maze tests. However, only the (+,*R*)-HA-966 enantiomer shows affinity for the glycine<sub>B</sub> site whereas the (–,*S*)-enantiomer is inactive at the glycine<sub>B</sub> site but is responsible for sedative-like effects *in vivo* (Singh *et al.*, 1990). (+,*R*)-HA-966 has been also shown to decrease fear-potentiated startle responses at doses of 10 and 30 mg/kg (Anthony and Nevins, 1993). In line with this result, Matheus *et al.* (1994) reported that (+,*R*)-HA-966 injected into DPQG increased both the percentage of time spent in open arms and the percentage entries into the open arms in the elevated plus-maze test.

In view of the very different anxiolytic effects of these three partial agonists in the elevated plus-maze, we decided to test their partial agonistic profile at the glycine<sub>B</sub> site using path clamp recordings from cultured neurones. In agreement with previous reports (+,*R*)-HA-966 and D-cycloserine were moderately potent true partial agonists at the glycine<sub>B</sub> site. The intrinsic activity of (+,*R*)-HA-966 was quite low (13.3%) whilst that of D-

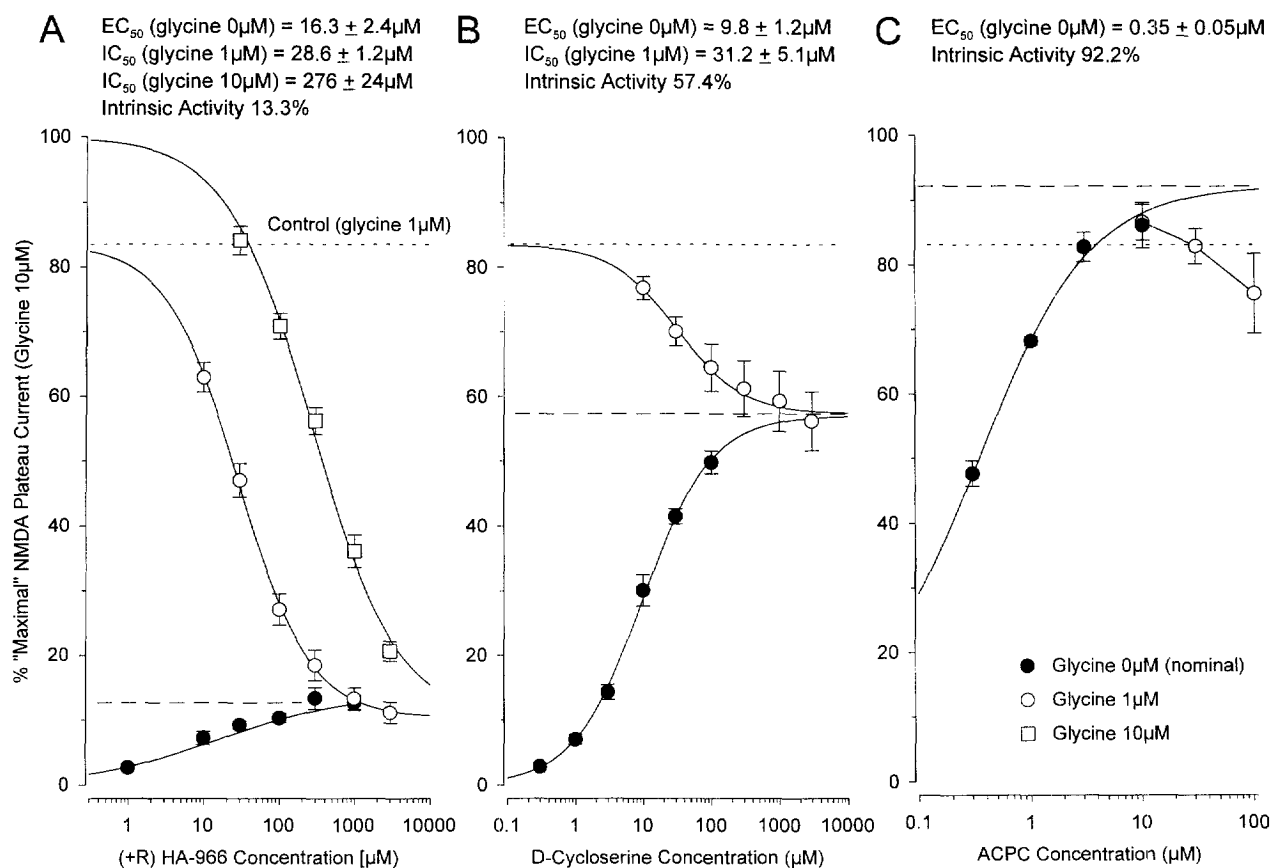


Fig. 5. Concentration-dependence of the modulation of NMDA receptors by glycine<sub>B</sub> partial agonists. Control plateau (steady state) NMDA-induced inward current responses recorded in the continuous presence of various concentrations of glycine (nominal  $0\mu\text{M}$ ,  $1\mu\text{M}$  and  $10\mu\text{M}$ ) were normalized with respect to "maximal" responses assessed with glycine  $10\mu\text{M}$  ( $3.1 \pm 0.3\%$ ,  $83.5 \pm 3.4\%$ ,  $100\%$ , respectively). The effects of glycine<sub>B</sub> partial agonists were then assessed relative to this normalized control level and plotted as means ( $\pm$  SEM) against partial agonist concentration. Estimation of  $IC_{50}$ s and curve fitting were made according to the four-parameter logistic equation (GrafFit, Erithacus Software). At least six cells were tested at each concentration. (A) (+,R)-HA-966 antagonized NMDA responses in the presence of glycine  $1\mu\text{M}$  ( $IC_{50}$   $28.6 \pm 1.2\mu\text{M}$ ) and, as expected, was 10 times weaker in the presence of glycine  $10\mu\text{M}$  ( $IC_{50}$   $276 \pm 24\mu\text{M}$ ). In the nominal absence of glycine, (+,R)-HA-966 potentiated NMDA responses with an  $EC_{50}$  of  $16.3 \pm 2.4\mu\text{M}$ . The intrinsic activity was estimated to be 13.3%. (B) D-Cycloserine antagonized NMDA responses in the presence of glycine  $1\mu\text{M}$  ( $IC_{50}$   $31.2 \pm 5.1\mu\text{M}$ ) and potentiated NMDA responses in the nominal absence of glycine ( $EC_{50}$  of  $9.8 \pm 1.2\mu\text{M}$ ). The intrinsic activity was estimated to be 57.4%. (C) ACPC potentiated NMDA responses in the nominal absence of glycine ( $EC_{50}$  of  $0.35 \pm 0.05\mu\text{M}$ ). Its effects in the presence of glycine  $1\mu\text{M}$  were biphasic with some potentiation at  $10\mu\text{M}$  but inhibition at higher concentrations. The intrinsic activity was estimated to be 92.2%.

cycloserine was somewhat higher (57.4%). This is in line with previous studies indicating that (+,R)-HA-966 does not produce a complete inhibition of NMDA currents or [<sup>3</sup>H]MK-801 binding (Danysz *et al.*, 1989a; Priestley and Kemp, 1994) and in certain conditions (added 7-chlorokynurenic acid) an enhancement of binding can be observed (Danysz *et al.*, 1989a). However, Priestley and Kemp (1994) reported higher intrinsic activity for D-cycloserine (86%) than in the present study (57%), and the report by Watson *et al.* (1990; 40–50%). Similarly, D-cycloserine has been shown to enhance or inhibit [<sup>3</sup>H]TCP binding in rat forebrain membranes and

NMDA-induced currents in *Xenopus* oocytes depending on glycine concentration (Hood *et al.*, 1989; Watson *et al.*, 1989). However, both could be expected to cause some degree of functional antagonism of NMDA receptors *in vivo* at receptors near saturating levels of glycine, i.e. above 300 nM at NMDAR1/2A or 2B (see Parsons *et al.*, 1993b). At other heteromeric NMDA receptor complexes, functional antagonism could also be expected at even lower glycine concentrations. In contrast, ACPC had much higher potency at the glycine<sub>B</sub> site and, in our hands, appeared to be a full glycine<sub>B</sub> agonist at lower concentrations (see also Priestley and

Kemp, 1994) with additional functional antagonism of NMDA receptors at higher concentrations, probably mediated at a different site. On the basis of our *in vitro* data, little functional antagonism at the glycine<sub>B</sub> site would be predicted with acute ACPC *in vivo* and the lack of acute anxiolytic effects are, therefore, not entirely surprising. However, it should be noted that in cerebellar granular cells ACPC seems to be less efficacious (60%) than glycine itself (cGMP stimulation, Fossum *et al.*, 1995b); moreover, prolonged exposure to ACPC has been shown to cause long-term changes in NMDA receptor expression (Fossum *et al.*, 1995a). Taken together with the NMDA receptor antagonistic effects observed with higher concentrations *in vitro* in the present study, such effects could theoretically account for the anxiolytic activity reported in previous studies, although such concentrations probably can never be achieved *in vivo* (see Rao *et al.*, 1990). Thus there is apparent lack of relationship between the degree of NMDA receptor antagonism (intrinsic activity) and anxiolytic activity in the plus-maze.

In contrast to glycine<sub>B</sub> partial agonists, less is known about anxiolytic activity of full antagonists, mainly due to their poor blood-brain barrier penetration (see Introduction). 7-Chlorokynurenic acid, given i.p. at a dose of 25 mg/kg or directly into DPQG, decreases anxiety in the elevated plus-maze (Trullas *et al.*, 1989; Matheus *et al.*, 1994). Another glycine site antagonist, 5,7-dichlorokynurenic acid injected i.p. significantly increased social interaction behaviour (doses 30 and 100 mg/kg), open-arm exploration time (100 mg/kg) and significantly increased conflict responding. It is worth noting that at the highest dose of 100 mg/kg, 5,7-dichlorokynurenic acid significantly decreased locomotor activity (Corbett and Dunn, 1993). This compound was also tested with positive effects for anxiety in separation-induced ultrasonic vocalization in rat pups (Kehne *et al.*, 1991), and in the Vogel conflict test (Plaznik *et al.*, 1994). In our hands, the specific and high affinity glycine site antagonist L-701,324 (Grimwood *et al.*, 1995) had very weak anxiolytic effects in the elevated plus-maze and was without effects in the Vogel test. Similarly, no effect of tricyclic-pyridao-phthalazinodiones belonging to structurally novel class of glycine site antagonists (Parsons *et al.*, in press) was seen in either model. We tested three of these compounds, i.e. MRZ 2/570, MRZ 2/571 and MRZ 2/576 in the elevated plus-maze, and MRZ 2/576 in the Vogel conflict test. These agents show ca. 0.1–1  $\mu$ M affinity for the glycine<sub>B</sub> site *in vitro* and selectively inhibit responses to microelectrophoretic NMDA *in vivo* with low mg/kg i.v. potency (Parsons *et al.*, in press). They also inhibit convulsions induced by NMDA, pentylenetetrazol and maximal electroshock with ED<sub>50</sub> ranging from 5 to 30 mg/kg and show other actions typical for inhibition of central NMDA receptors (Parsons *et al.*, in press). Finally, MRZ 2/570, MRZ 2/571 and MRZ 2/576 at the highest doses produced signs of ataxia and muscle relaxation which are also typical for

“classical” NMDA receptor antagonists. The present results correspond to a report by Wiley *et al.* (1995) showing that 5-nitro-6,7-dichloro-1,4-dihydro-2,3-quinolineolone (ACEA 1021), another novel glycine<sub>B</sub> site full antagonist, failed to exhibit consistent anxiolytic activity in the elevated plus-maze (significant but modest increase was seen only in open arm entries at the highest dose of 30 mg/kg).

Although it has been suggested that full antagonists at the strychnine-insensitive glycine site of the NMDA receptor complex might be useful in the therapy of anxiety, our results did not support these assumptions. However, the lack of clear effects of the compounds tested in the present study does not necessarily question the concept of glycine<sub>B</sub> antagonists as anxiolytic agents in general. It could well indicate that the antagonists studied have preference for NMDA receptor subtypes that are not involved in fear regulation. Previously, Kehne *et al.* (1995) compared two glycine<sub>B</sub> antagonists, i.e. MDL-100,458 (3-(benzoylmethylamino)-6-chloro-1H-indole-2-carboxylic acid) and MDL-102,288 (5,7-dichloro-1,4-dihydro-4-([4-[(methoxycarbonyl)-amino]-phenyl]sulfonyl]imino)-2-quinoline-carboxylic acid) and found that the former was 100 times more potent as an anticonvulsant in DBA/2 mice, whilst the latter was 13 times more potent as an anxiolytic in a separation-induced vocalization model.

In conclusion, of the NMDA receptor antagonists tested, the strongest anxiolytic activity was observed after D-cycloserine, while full glycine<sub>B</sub> antagonists failed to show consistent effects. In this context, the clinical usefulness of the types of glycine<sub>B</sub> antagonist tested may be questionable, although species differences should always be taken into account.

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