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Effects of site-selective NMDA receptor antagonists in an elevated plus-maze model of anxiety in mice

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Abstract

NMDA receptor antagonists have been shown to be anxiolytic in animal models of anxiety, although they have not been tested extensively. These compounds bind to several specific sites within the NMDA-receptor complex, including the NMDA site itself, the phencyclidine site, and the strychnine-insensitive glycine site. The purpose of the present study was to examine potential anxiolytic effects of site-selective NMDA receptor antagonists in the elevated plus-maze. Drug-naive albino mice were placed in the center of an elevated maze shaped like a plus sign. Two opposing arms were enclosed by high walls; the crossing arms were open. Following injection with drug or vehicle, the number of entries and time spent in each type of arm were measured during 5-min tests. Analysis of results showed that the benzodiazepine, diazepam, and the competitive NMDA receptor antagonist, NPC 17742 (2*R*,4*R*,5*S* 2-amino-4,5-(1,2-cyclohexyl)-7-phosphono-heptanoic acid), increased number of open arm entries and open arm time. *N*-Nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor which may interfere with the transduction of NMDA receptor activation, also increased open arm entries and time; however, the magnitude of these increases was small. The phencyclidine-site NMDA receptor antagonist, phencyclidine, increased open arm entries, but failed to significantly increase open arm time. ACEA 1021 (5-nitro-6,7-dichloro-1,4-dihydro-2,3-quinoxalinedione), a putative glycine-site antagonist, had significant effects only on open arm entries at the highest dose tested. These results suggest that NMDA receptor antagonists show promise as potential anxiolytic agents, but that differences among antagonists acting at different cellular sites may be expected.

Keywords: NMDA receptor antagonist; NPC 17742; ACEA 1021; *N*-Nitro-L-arginine methyl ester; Phencyclidine; Anxiolytic

1. Introduction

The NMDA receptor is presently considered to be part of a receptor complex in which agonist binding to the NMDA site opens a channel gating the influx of Ca^{2+} and Na^{+} resulting in cellular excitation (Lodge and Johnson, 1990; Watkins, 1989). There are at least four different sites at which antagonists might block activation of this complex: the NMDA site, strychnine-insensitive glycine site, phencyclidine site, and polyamine site. In addition, stimulation of the NMDA receptor is believed to have a facilitory effect on the synthesis of nitric oxide, which may serve as a second messenger (Montague et al., 1994); hence, it is possible that blockade of nitric oxide synthesis by drugs such as *N*-nitro-L-arginine methyl ester could produce effects similar to those of NMDA receptor antagonists.

NMDA receptor antagonists share a number of pharmacological properties with classical central nervous system depressant drugs such as the benzodiazepines and barbiturates, including disruption of motor activity, anticonvulsant effects, and muscle relaxation (Balster and Wessinger, 1983; Willetts et al., 1990). Preclinical evidence suggests that NMDA receptor antagonists also may have utility for treatment of anxiety disorders (Wiley and Balster, 1992,1993).

The purpose of the present study was to examine potential anxiolytic effects of site-selective NMDA receptor antagonists in an elevated plus-maze procedure. Validation of the elevated plus-maze procedure has shown that it is sensitive to drugs that produce anxiolytic or anxiogenic effects in humans (Pellow et al.,

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1985), including some proposed anxiolytic drugs that have non-benzodiazepine sites of action (Pellow and File, 1986). In the present study, drugs that act at different sites within the NMDA-receptor complex were chosen for testing: NPC 17742 [2*R*,4*R*,5*S* 2-amino-4,5-(1,2-cyclohexyl)-7-phosphonoheptanoate] (competitive NMDA receptor antagonist), phencyclidine (non-competitive channel blocker), ACEA 1021 [5-nitro-6,7-dichloro-1,4-dihydro-2,3-quinoxalinedione] (glycine-site antagonist), and *N*-nitro-L-arginine methyl ester (nitric oxide synthase inhibitor). Although NPC 17742 and phencyclidine had previously been tested in punishment procedures (Porter et al., 1989; Willetts et al., 1993), little information was available on the effects of these drugs or other NMDA receptor antagonists in non-punishment procedures such as the elevated plus-maze.

2. Materials and methods

2.1. Subjects

When not being tested, experimentally naive male ICR mice (15–20 g) (Harlan, Dublin, VA) were individually housed in clear plastic cages with wood-chip bedding in a temperature-controlled (22–24°C) animal colony room (12-h light-dark cycle). Water and food were available in the home cages. Each mouse received a single dose of drug or vehicle and was tested once in the elevated plus-maze.

2.2. Apparatus

The elevated plus-maze was shaped like a plus sign with two unwalled (open) arms (35 cm × 5 cm) and two walled (closed) arms (35 cm × 5 cm × 15 cm). The floor of each arm was wooden and painted black; the walls of the closed arms were constructed of black Plexiglas. The entire maze was elevated to a height of 64 cm. During test sessions, two incandescent lights (40 W each) provided sufficient illumination of the maze area for video-taping of mouse movements.

2.3. Procedure

Upon arrival, mice were placed in individual cages and allowed to habituate to the vivarium environment for a minimum of 3 days. They were weighed and handled the day before testing. On the day of testing, each mouse was weighed and injected with a drug dose or vehicle and returned to its home cage until the pre-session injection interval had elapsed. Groups of four to eight mice were tested with each dose. Mice were placed in the center of the plus-maze facing the closed arms at the beginning of the 5-min test. An

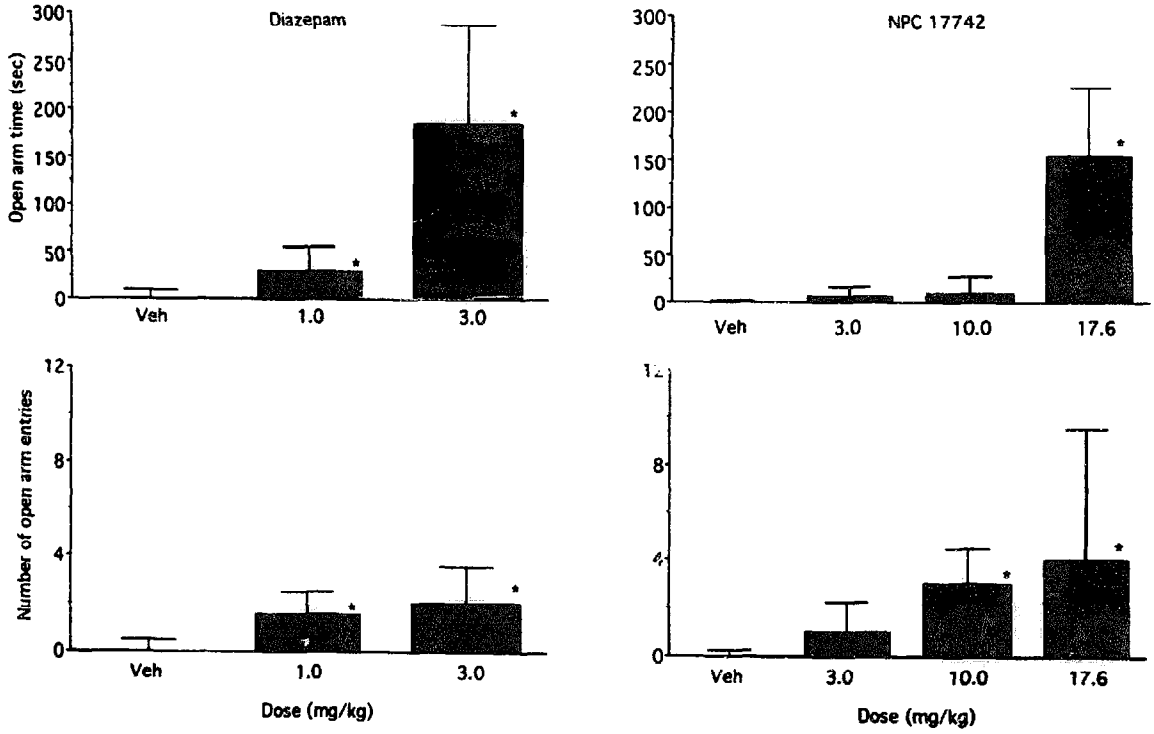

Fig. 1. Effects of diazepam (left panels) and NPC 17742 (right panels) on open arm time (s) [top panels], total number of arm entries [bottom panels], and number of open arm entries [bottom panels] in an elevated plus-maze procedure. Values at each dose represent medians (+semi-interquartile range). [For diazepam, $n = 10$ for all doses and for NPC 17742, $n = 8$ for vehicle and 3 mg/kg doses, $n = 7$ for 10 mg/kg dose, and $n = 6$ for 17.6 mg/kg dose].

observer recorded number of entries into each type of arm and, later, viewed a video-tape of the session and recorded time spent in each type of arm. An entry was defined as placing all four paws within the boundaries of the arm. The drug dose each mouse received was not identified during re-play of the video-tape. Anxiolytic activity was indicated by increases in open arm time or in number of open arm entries. Total number of entries into either type of arm was used as a measure of overall motor activity.

2.4. Drugs

Diazepam (Schein Pharmaceuticals, Port Washington, NY) was purchased commercially at a concentration of 5 mg/ml and diluted to desired concentrations with a vehicle of ethanol (10%), propyleneglycol (40%), and sterile water (50%). NPC 17742 (NOVA Pharmaceuticals, Baltimore, MD) was dissolved in sterile water with equimolar sodium hydroxide added for pH adjustment. ACEA 1021 (ACEA Pharmaceuticals, Irvine, CA) was mixed in a vehicle of Trizma pH 7.4 (10%), dextrose (5%), and sterile water (85%). Phencyclidine HCl (National Institute of Drug Abuse, Rockville, MD) and *N*-nitro-L-arginine methyl ester (Sigma Chemical Company, St. Louis, MO) were dissolved in saline. All drugs were administered intraperitoneally (i.p.) with an injection volume of 10 ml/kg. Doses of all drugs are expressed in mg/kg of the respective salts. Pre-session injection times were 15 min for diazepam, 30 min for NPC 17742 and *N*-nitro-L-arginine methyl ester, 10 min for phencyclidine, and 40 min for ACEA 1021.

2.5. Statistical analysis

Separate Kruskal-Wallis tests were performed on median open arm time, median number of open arm entries, and median number of total arm entries for each drug. When significant differences were indicated by results of the Kruskal-Wallis test ($\alpha < 0.05$), nonparametric post hoc analyses were conducted comparing median values for each drug dose to median values for the corresponding vehicle (Daniel, 1978; Siegel and Castellan, 1988).

3. Results

As illustrated in Fig. 1, diazepam and NPC 17742 produced dose-dependent increases in open arm time (top panels) and number of open arm entries (bottom panels). Total number of arm entries was not significantly affected by either drug, although 3 mg/kg diazepam produced a 57% decrease in the total number of arm entries (Table 1). The magnitude of the anxiolytic effects of diazepam and NPC 17742 were similar. Injection with 30 mg/kg NPC 17742 caused severe behavioral disruption in all mice (data not shown). At this dose, mice frequently fell off of the maze whenever entering an open arm; hence, open arm time and number of arm entries could not be reliably measured.

Fig. 2 shows the results of tests with phencyclidine (left panels), ACEA 1021 (center panels), and *N*-nitro-L-arginine methyl ester (right panels). Although phencyclidine increased open arm time at doses of 3.0 and

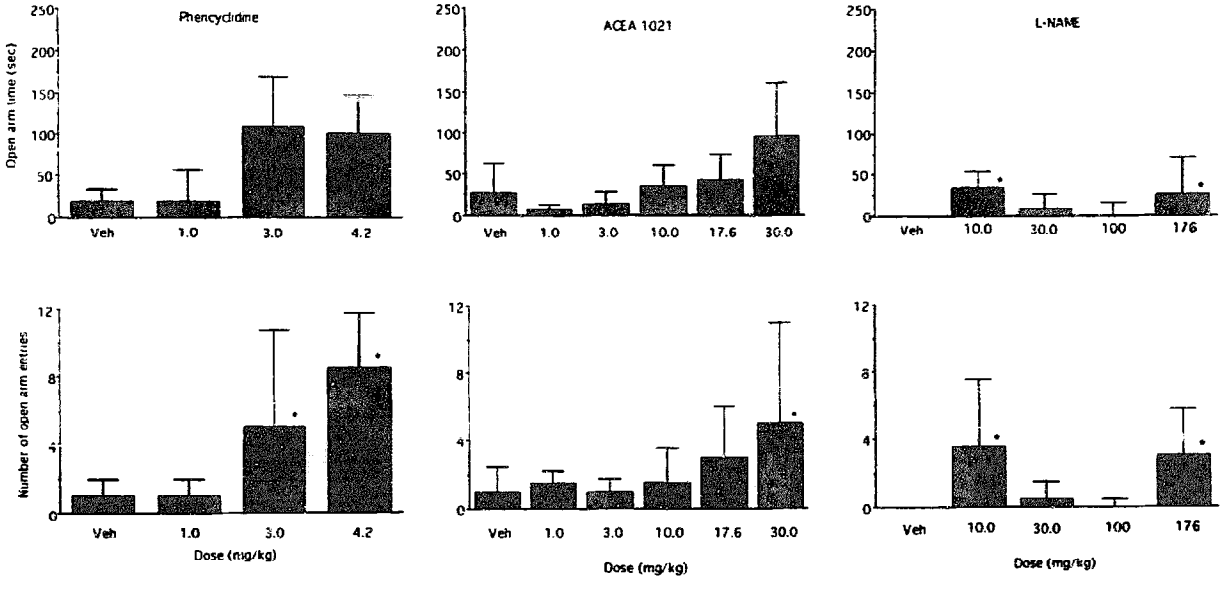

Fig. 2. Effects of PCP (left panels), ACEA 1021 (center panels), and *N*-nitro-L-arginine methyl ester (L-NAME) [right panels] on open arm time (s) [top panels], total number of arm entries [bottom panels], and number of open arm entries [bottom panels] in an elevated plus-maze procedure. Values at each dose represent medians (+semi-interquartile range). [For PCP, $n = 8$ per dose, except $n = 4$ for 4.2 mg/kg dose; for ACEA 1021, $n = 8$ per dose, except $n = 7$ for 17.6 mg/kg dose; and for *N*-nitro-L-arginine methyl ester, $n = 8$ per dose].

Table 1
Effects of site-selective NMDA antagonists on total number of arm entries in an elevated plus-maze procedure in mice

Compound	Dose (mg/kg)	Total number arm entries Medians (+SIR)	Ratio open/total entries	n
Diazepam	Veh	15.0 (3.5)	0	10
	1.0	17.5 (5.0)	0.16	10
	3.0	6.5 (7.0)	0.46	10
NPC 17742	Veh	12.5 (3.0)	0	8
	3.0	10.0 (2.8)	0.10	8
	10.0	13.0 (9.5)	0.31	7
	17.6	13.0 (8.0)	0.48	6
	30.0	mice fell off maze repeatedly		6
Phencyclidine	Veh	17.0 (6.8)	0.14	8
	1.0	19.5 (2.8)	0.08	8
	3.0	25.0 (5.0) *	0.36	8
	4.2	25.5 (10.5) *	0.45	4
	5.6	mice fell off maze repeatedly		4
ACEA 1021	Veh	13.0 (3.3)	0.13	8
	1.0	18.5 (1.3) *	0.08	8
	3.0	14.0 (2.0)	0.09	8
	10.0	13.5 (1.3)	0.11	8
	17.6	14.0 (3.0)	0.38	7
	30.0	14.5 (8.5)	0.44	8
L-NAME	Veh	13.5 (3.8)	0	8
	10	20.0 (7.0)	0.19	8
	30	14.5 (4.8)	0.03	8
	100	14.5 (4.3)	0	8
	176	10.5 (4.5)	0.20	8

* Based on Kruskal-Wallis test ($\alpha = 0.05$) and post hoc tests, median total arm entries significantly different from median at vehicle dose.

4.2 mg/kg, these effects were variable among animals and not statistically significant. Phencyclidine significantly increased number of open arm entries at the same doses (3.0 and 4.2 mg/kg) which also increased total number of arm entries (Table 1). When given a 5.6 mg/kg dose of phencyclidine, mice frequently fell off the maze and could not be tested (data not shown).

ACEA 1021 did not significantly increase open arm time at any dose and number of open arm entries was increased only by the 30 mg/kg dose. Although 1.0 mg/kg ACEA 1021 increased total number of arm entries, this effect did not occur at higher doses (Table 1). *N*-Nitro-L-arginine methyl ester produced small, but significant increases in open arm time and number of open arm entries; however, this effect was not dose-dependent and occurred only at the lowest (10 mg/kg) and highest (176 mg/kg) doses tested. Total number of arm entries was not affected at any dose (Table 1).

4. Discussion

As expected, diazepam produced significant dose-dependent increases in open arm time and in number of entries into the open arms. These increases were not accompanied by statistically significant changes in motor activity, as indicated by the number of total arm entries; however, a non-significant decrease in motor activity was observed at the 3 mg/kg dose of diazepam. These data are consistent with the results of numerous previous studies which have shown that diazepam and other benzodiazepines produce robust effects at non-sedating doses in a variety of anxiolytic screening procedures, including conflict models (Cook and Davidson, 1973; Vogel et al., 1971), elevated plus-maze procedures (Pellow and File, 1986), other non-punishment procedures (File, 1980; Winslow and Insel, 1991), and drug discrimination models (Andrews and Stephens, 1990).

Similar to diazepam, the competitive NMDA receptor antagonist, NPC 17742, significantly increased open arm time and number of open arm entries without affecting motor activity. Previous research with rodents and pigeons has shown that competitive NMDA receptor antagonists, including (+)-CPP [(+)-2-carboxypiperazine-4-yl-propyl-l-phosphonic acid] (Corbett and Dunn, 1991), NPC 12626 [2-amino-4,5-(1,2-cyclohexyl)-7-phosphonoheptanoic acid] (Wiley et al., 1992), CGS 19755 [*cis*-4-phosphonomethyl-2-piperidinecarboxylic acid] (Bennett et al., 1989), and 2-amino-5-phosphonopentanoate (Dunn et al., 1989), produce anxiolytic effects in punishment and non-punishment procedures. In addition, Willetts et al. (1993) have reported dose-dependent NPC 17742-induced increases in punished responding. In the present study, the magnitude of NPC 17742's anxiolytic effect in the elevated plus-maze was similar to that produced by diazepam; similarly, the magnitude of increases in punished responding produced by NPC 17742 was at least as large as that produced by chlordiazepoxide (Willetts et al., 1993).

Although the magnitude of NPC 17742's effect in the present study was similar to that of diazepam, the dose range over which NPC 17742 produced its effect was narrower. Whereas both the 1.0 and 3.0 mg/kg doses of diazepam produced increase in open arm time and entries, only the 17.6 mg/kg dose of NPC 17742 produced increases in these measures. Raising the dose of NPC 17742 by one-quarter log unit to 30 mg/kg severely disrupted behavior in all mice. These results suggest that the 'therapeutic index' for NPC 17742 may be low; however, Willetts et al. (1993) have reported a larger separation between the lowest dose of NPC 17742 that increased punished responding and that which decreased unpunished responding, indicating that the separation between specific and non-specific effects of this drug may not be constant across procedures. Thus, NPC 17742 and other competitive NMDA receptor antagonists consistently exhibit anxiolytic effects in a variety of experimental models. The magnitude of these effects is similar to that of benzodiazepines in some studies (present study; Bennett et al.,

1989; Dunn et al., 1989), but not in others (Koek and Colpaert, 1991; Wiley et al., 1992).

Increases in open arm time produced by phencyclidine, a non-competitive NMDA receptor antagonist which acts by blocking the cation channel associated with the NMDA-receptor complex, were not statistically significant. Although the number of open arm entries was significantly increased at the two highest doses, these increases were accompanied by increases in number of total arm entries, possibly indicating non-specific locomotor stimulation. Phencyclidine and other phencyclidine-like NMDA receptor antagonists have been shown to produce anxiolytic effects in a punishment procedure (Porter et al., 1989), although another study failed to find this effect (Sanger and Jackson, 1989). Dizocilpine (MK-801), the only non-competitive phencyclidine-like NMDA receptor antagonist previously tested in a non-punishment procedure, exhibited anxiolytic effects in some studies (Corbett and Dunn, 1991; Dunn et al., 1989), but not in others (Criswell et al., 1994). Dizocilpine-induced anxiolytic effects, when they occurred, sometimes were accompanied by increases in locomotor activity (Criswell et al., 1994; Sharma and Kulkarni, 1993); however, in other studies, these changes in motor activity were not observed (Corbett and Dunn, 1991; Dunn et al., 1989). Overall, the effects of phencyclidine-like NMDA receptor antagonists do not appear to be as specific nor as consistent as those of competitive NMDA receptor antagonists. Drugs that act at the phencyclidine site produce also phencyclidine-like discriminative stimulus effects (Balster and Willetts, 1988; Willetts and Balster, 1988). Since substitution for phencyclidine in drug discrimination tasks is predictive of phencyclidine-like subjective effects of drugs in humans (Balster, 1990), it is unlikely that phencyclidine or dizocilpine would ever be used clinically as antianxiety agents. On the other hand, competitive NMDA receptor antagonists such as NPC 12626 (Ferkany et al., 1989) and NPC 17742 generally do not fully substitute for phencyclidine (R.L. Balster, unpublished observations) nor do phencyclidine or phencyclidine-like drugs substitute for competitive NMDA receptor antagonists (Bobelis and Balster, 1993; Willetts et al., 1989).

ACEA 1021, a glycine-site antagonist (Cai et al., 1993; Woodward et al., 1993, 1995), did not significantly increase open arm time at any dose and increased number of open arm entries only at the highest (30 mg/kg) dose. Doses tested were similar to those which produced a neuroprotectant effect (Warner et al., 1993) and decreased operant rates of responding (Balster et al., 1995) in rodents. These results are consistent with those of previous studies which have shown that other glycine-site NMDA receptor antagonists, such as (+)-HA-966 [(+)-3-amino-1-hydroxy-2-pyrrolidinone], 5,7-dichlorokynurenic acid, and 7-chlorokynurenic acid, produce anxiolytic effects in both punishment (Dunn et al., 1992) and non-punishment (Kehne et al., 1991; Trullas et al., 1989) procedures. Since only one dose of ACEA 1021 produced effects in only one of the two indices of anxiolytic activity, further studies with these quinoxalinediones will be needed to determine the reliability of these effects.

Although it has not yet been demonstrated conclusively that ACEA 1021 serves as a glycine-site NMDA receptor antagonist in vivo, Woodward et al. (1993, 1995) have demonstrated that ACEA 1021 is a high potency, competitive glycine site antagonist within the NMDA receptor complex in binding and electrophysiological assays. Further, ACEA 1021 is not active at the competitive NMDA receptor site (Woodward et al., 1995) nor does it bind to other standard non-glutamate sites in the brain (E. Weber, unpublished data). ACEA 1021 also does not substitute for phencyclidine in a drug discrimination assay (Balster et al., 1995), suggesting that it does not act via the phencyclidine site. Since other quinoxalinediones bind to non-NMDA glutamate receptors and non-NMDA glutamate receptor antagonists have recently been reported to have anxiolytic effects in animal tests (Benvenga et al., 1993), actions at these sites cannot be ruled out as the basis for the results obtained here with ACEA 1021; however, electrophysiology experiments have indicated that ACEA 1021 is 200-fold less potent at non-NMDA glutamate receptors than at the glycine-site NMDA receptor (Woodward et al., 1995). Hence, ACEA 1021's most likely site of action is the glycine-site of NMDA receptors.

N-Nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor, produced significant increases in open arm time and in number of open arm entries without affecting total number of arm entries. Although this effect occurred at two doses, the doses were widely separated; hence, the effect was not dose-dependent. In addition, the magnitude of the observed effect, although significant, was small. Interestingly, a previous study reported that L-N^G-nitro arginine, another nitric oxide synthase inhibitor, antagonized the anxiolytic effects of chlordiazepoxide in an elevated plus-maze procedure (Quock and Nguyen, 1992). This antagonistic effect was reversed by i.c.v. administration of L-arginine, a drug which increases nitric oxide synthesis. The effect of L-N^G-nitro arginine alone was not examined. Combined with the results of the present study, these results suggest that the nitric oxide levels may play a role in modulation of anxiety, perhaps via a NMDA-or γ-amino-butyric acid-related second messenger process. A recent report that *N*-nitro-L-arginine methyl ester and 7-nitroindazole, another nitric oxide synthase inhibitor, partially substitute for phencyclidine in a drug discrimination procedure in pigeons (Jewett et al., 1994) offers further support for the idea

that the behavioral effects of nitric oxide synthesis inhibition may share some similarities with those of NMDA receptor antagonists. Given the preliminary nature of these findings, however, additional tests of drugs that affect nitric oxide synthesis in anxiolytic screening procedures is warranted.

In summary, diazepam, NPC 17742, ACEA 1021, and *N*-nitro-L-arginine methyl ester were effective in the elevated plus-maze. The anxiolytic effects of diazepam and NPC 17742 were selective and of similar magnitude. In contrast, phencyclidine, ACEA 1021, and *N*-nitro-L-arginine methyl ester were not as clearly effective or selective in this model. The results of the present study provide additional support for consideration of NMDA receptor antagonists as antianxiety agents, but, combined with the results of previous research, suggest that differences among antagonists acting at different cellular sites can be expected.

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