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Discriminative Stimulus Properties of Ethanol in Rats: Studies on the Role of Nitric Oxide

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KOROS, E., W. KOSTOWSKI AND P. BIENKOWSKI. *Discriminative stimulus properties of ethanol in rats: Studies on the role of nitric oxide.* PHARMACOL BIOCHEM BEHAV **62**(4) 607–612, 1999.—The present study examined the role of the L-arginine–nitric oxide pathway in mediation of the ethanol interoceptive (discriminative) cue. Adult male Wistar rats (n = 16) were trained to discriminate ethanol (1 g/kg, 10% v/v) from saline under a fixed-ratio 10 (FR10) schedule of sweetened milk reinforcement. A nonselective nitric oxide synthase (NOS) inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; 10–540 mg/kg) did not substitute for ethanol. Similarly, a relatively selective neuronal NOS inhibitor, 7-nitroindazole (7-NI; 10–80 mg/kg), did not mimic the ethanol cue. However, both L-NAME and 7-NI produced significant reduction in the rate of operant responding. A nitric oxide precursor, L-arginine (100–500 mg/kg) neither substituted for nor antagonize the ethanol stimulus. Taken together, these results suggest that the L-arginine–nitric oxide pathway is not involved in mediation of the discriminative stimulus effects of ethanol in the rat. © 1999 Elsevier Science Inc.

Ethanol Nitric oxide Drug discrimination Rat

RESULTS of several in vitro experiments indicate that acute ethanol treatment attenuates the glutamate *N*-methyl-D-aspartate (NMDA) receptor-associated electrophysiological and biochemical responses [for review, see (17,28)]. For example, concentrations of ethanol as low as 10–25 mM produce pronounced inhibition of NMDA-evoked calcium influx (21). In agreement with the above, behavioral experiments show that NMDA receptors may contribute to ethanol intoxication, tolerance, and withdrawal syndrome in rodents (12,17).

A drug discrimination procedure is a particularly useful task for identifying potential receptor mechanisms that mediate drug effects in vivo. With this task animals learn a particular drug-induced interoceptive (discriminative) cue that can be tested for generalization to other drugs or antagonized by still other compounds (1,2,6,18). Notably, both competitive and uncompetitive NMDA receptor antagonists have been shown to substitute for ethanol in rats (2,4,18). These results indicate that also the discriminative stimulus effects of ethanol may be related to reduction in NMDA receptor conductance.

Nitric oxide (NO) is a simple free radical gas that is suggested to act as a transsynaptic messenger in the mammalian brain [for review, see (25,29)]. In neurons, NO is formed from L-arginine by neuronal nitric oxide synthase (nNOS) in a Ca^{2+}/calmodulin-dependent manner. NO can increase guanosine 3′,5′-monophosphate (cGMP) production by a direct stimulation of soluble guanylyl cyclase (29,34). It has been well documented that activation of NMDA receptors leads to increase in intracellular Ca^{2+}, which in turn, enhances nNOS activity and NO/cGMP formation (13,21,25,29). In contrast, NMDA receptor antagonists attenuate neuronal cGMP production (14,32).

There are several reports indicating that NO may mediate at least certain NMDA receptor-related biochemical and behavioral responses. Thus, different NOS inhibitors have been shown to prevent NMDA receptor-associated elevation of cGMP, release of neurotransmitters, neurotoxicity, and convulsions (9,13,29,34). In line with the above, NOS inhibitors have been reported to substitute for the discriminative stimulus effects of an uncompetitive NMDA receptor antagonist, phencyclidine (22).

Keeping in mind the role of NMDA receptors in ethanol intoxication and regulation of nNOS activity, one could spec-

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ulate that some central effects of ethanol are mediated by alteration in nNOS activity. Interestingly, it has been recently shown that acute ethanol exposure may decrease NMDA-stimulated NOS activity in cortical neurons (7). In contrast, chronic ethanol treatment increases NMDA-stimulated NO formation (8). At the behavioral level, NOS inhibitors have been shown to attenuate ethanol preference in both genetically selected ethanol-preferring (27) and nonselected outbred strain of rats (5). Recently, Green et al. (19) have tested the role of NO in the discriminative stimulus effects of ethanol. A NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) failed to substitute for ethanol in rats trained to discriminate ethanol from water (19). However, L-NAME did not alter the rate of operant responding in this latter study. Notably, rate-depressant effects are thought to reflect behavioral activity of drugs tested in drug discrimination experiments (3,18). Besides, L-NAME nonselectively inhibits different subtypes of NOS, and apart from central actions may produce significant cardiovascular effects associated with an inhibition of endothelial NOS (eNOS) (20,26).

In the present study, we wanted to further assess the role of NO in mediation of the ethanol interoceptive cue. For this purpose we assessed the effects of both L-NAME and a relatively selective nNOS inhibitor, 7-nitroindazole (7-NI) (10,11, 26) in rats trained to discriminate ethanol from saline (substitution tests). In addition, the effects of the NO precursor, L-arginine were tested both in the substitution and antagonism tests.

METHOD

Subjects

Sixteen male Wistar rats (HZL, Warsaw, Poland) were used. The rats were experimentally naive and weighed 330–360 g at the beginning of the study. The subjects were housed alone in plastic cages (40 × 20 × 25 cm), and kept under an artificial 12 L:12 D cycle (lights on at 0700 h) in a colony maintained at a constant temperature (22 ± 1°C) and humidity (∼50%). The rats were maintained at ∼80% of their free-feeding body weight by restriction of their daily food intake to 15–18 g of standard rat chow (Bacutil, Poland). Tap water was available ad lib. The experimental procedure was in accordance with Polish and European regulations on animal care and use.

Drug Discrimination Procedure

The animals were trained to press either left or right levers for sweetened milk reinforcement in standard operant chambers (Coulbourn Instruments, Inc., Allentown, PA). The chambers consisted of modular test cages enclosed within sound-attenuating and ventilated cubicles. A white house light was centered near the top of the front panel of the cage. Each cage was equipped with two response levers, separated by a liquid dipper, all positioned 4 cm above the floor. The start of a 15-min training (or test) session was signaled by turning the house light on. The liquid dipper presented sweetened milk in a 0.01-ml portion for 5 s during each operation. A computer, equipped with a Coulbourn L2T2 software package, was used to program and record all training and test sessions [for details, see (3,4)].

Discrimination training commenced once pressing on either the right or left lever was established on a fixed-ratio 10 (FR10) schedule of reinforcement. Fifteen minutes before the daily (Monday–Saturday) training sessions, the rats were injected IP with ethanol (1 g/kg, 10% v/v), and were required to press one of the levers ("ethanol-appropriate lever") to receive the milk reinforcement. After IP injection of saline (0.9% NaCl) the rats were required to press the opposite lever ("saline-appropriate lever"). Ethanol or saline were administered according to two alternating sequences: saline, ethanol, ethanol, saline, saline or ethanol, saline, saline, ethanol, ethanol. Dose response and other test sessions (1–2/week) were carried out when the rats had achieved discrimination criteria: (a) ⩽2 incorrect responses before completing first FR10; (b) ⩾90% of the total responses during the session on the appropriate level; (c) the response rate ⩾0.25/s—all in 9 out of 10 consecutive sessions. The test sessions were identical to the training sessions except that 10 responses on either lever resulted in sweetened milk delivery. To be tested in each subsequent test session the rat must have reached the discrimination criteria for at least 3 consecutive days. In the dose–response sessions the rats received different doses of ethanol (0.25, 0.5, 0.75, and 1 g/kg; 10% v/v) 15 min before the start of the session. The other test sessions were designed as the antagonism or substitution tests. In the substitution tests L-NAME (10, 30, 90, 180, 270, or 540 mg/kg; IP), 7-NI (10, 20, 40, or 80 mg/kg; IP), l-arginine (100, 500, or 1000 mg/kg; IP), or respective vehicle was injected 30 min before the start of the session. In the antagonism tests L-arginine (100, 500, or 1000 mg/kg; IP) or its vehicle was administered 30 min before the ethanol injection (1 g/kg), i.e., 45 min. before the start of the session. The drugs were tested in a balanced order in randomly selected groups of rats.

Drugs

Ethanol solution was prepared from 95% stock ethanol and sterile physiological saline. L-NAME (RBI, Natick, MA) and L-arginine (Sigma, Poznan, Poland) were dissolved in saline and administered IP in a volume of 2 ml/kg. 7-NI (RBI) was suspended in 2% Tween, sonicated for 30 min, and injected IP in a 2-ml/kg volume. The doses of L-NAME, 7-NI and L-arginine were selected on the basis of published behav-

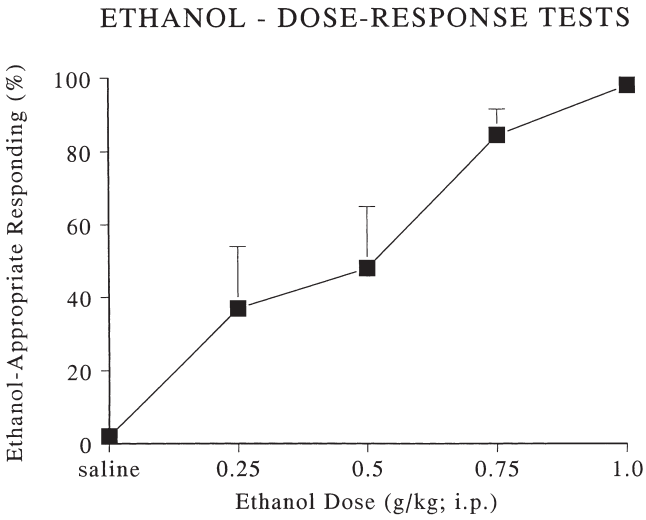

FIG. 1. Results are expressed as mean (±SEM) percentage of ethanol-appropriate responding following increasing doses of ethanol. n = 8–10 rats.

ioral studies (5,19,27,30,33). All solutions were prepared immediately prior to use.

Statistics

The percentage of ethanol-appropriate responding was calculated by dividing the responses made on ethanol-appropriate lever by the total number of responses made on both levers, and multiplying the result by 100. The operational definition of partial stimulus substitution was 40–79% responding on ethanol-appropriate lever after pretreatment with at least one dose of L-NAME, 7-NI, or L-arginine. The operational definition of complete stimulus substitution was ⩾80% of responding on ethanol-appropriate lever. Complete antagonism of the ethanol cue was defined as ⩽20% ethanol-appropriate responding after pretreatment with at least one dose of L-arginine. Values between 21–59% were defined as partial antagonism. Log-probit analysis (31) was used to determine the dose of ethanol predicted to elicit 50% ethanol-appropriate responding (ED_{50}). The response rate (1/s) was calculated by dividing the total number of responses by the total session time in seconds. A one-way analysis of variance (with Newman–Keuls test for individual post hoc comparison) was used for comparing the response rate data. The level selection data (but not the response rate data) were not included if the rat failed to complete at least one FR10 on either lever in 15 min.

RESULTS

Acquisition and Dose–Response Sessions

One out of 16 rats rat did not acquire the ethanol-saline discrimination even after an extended period of training (120 sessions) and was excluded from the study. The other subjects required an average (±SEM) of 49 ± 4.3 training sessions (range: 23–73) to reach the discrimination criteria. Once the discrimination was established, all rats responded with stable accuracy on both the ethanol- and saline-appropriate lever (a minimal mean accuracy for the training sessions performed between the test sessions was 91%).

The dose of ethanol predicted to elicit 50% ethanol-appropriate responding (ED_{50}) was 0.37 g/kg (Fig. 1). In agreement with our previous reports (2–4), neither the training dose nor

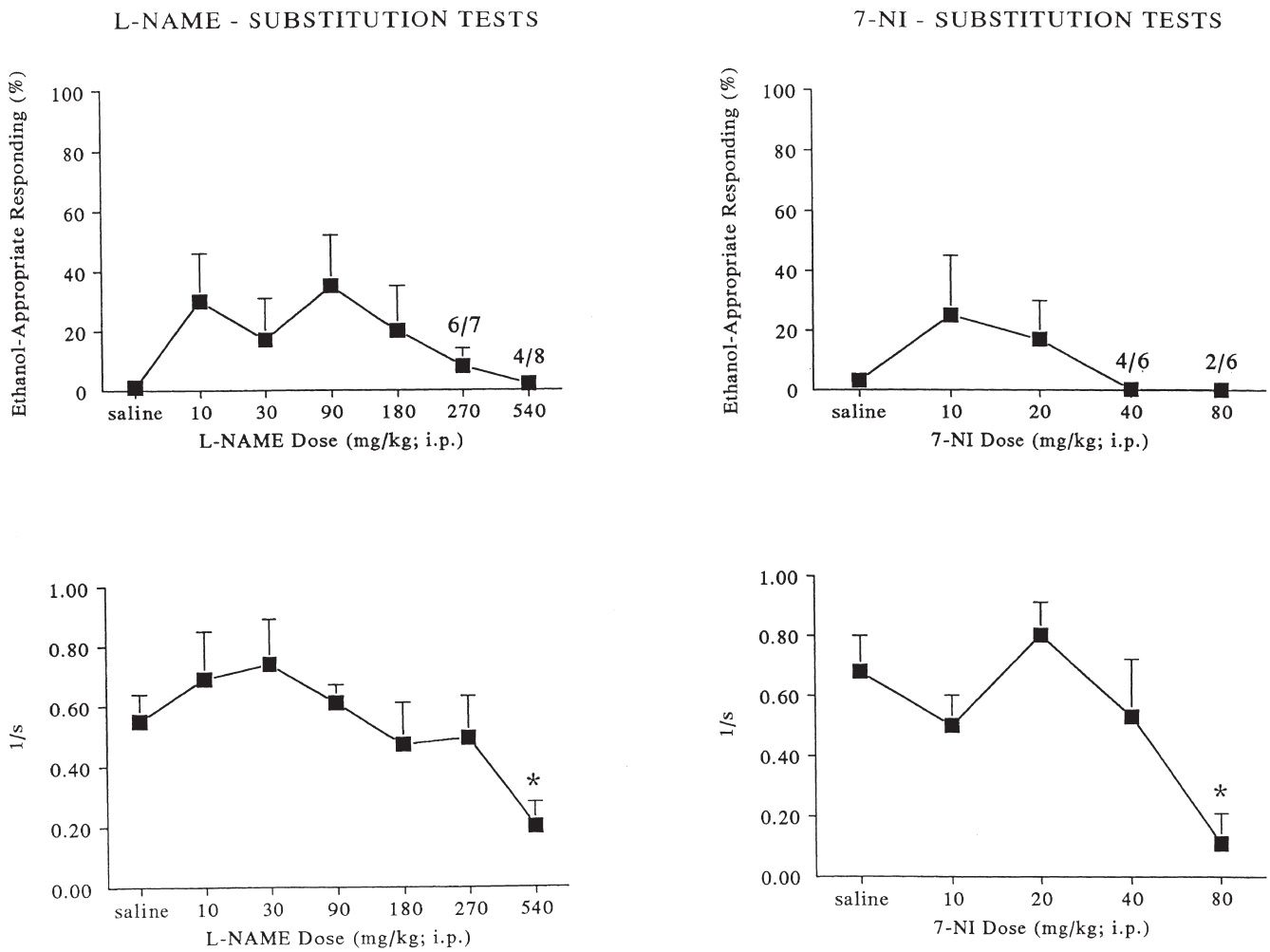

FIG. 2. Results are expressed as mean (±SEM) percentage of ethanol-appropriate responding (top) and mean (±SEM) response rate (bottom) following increasing doses of L-NAME or 7-NI. n = 6–8 rats; *n/n*—number of rats that obtained at least one reinforcement/number of rats tested; *$p < 0.05$ vs. the group treated with saline.

lower doses of ethanol influenced the rate of responding, $F(4, 24) = 0.48, p > 0.4$; data not shown).

Substitution Tests

Neither L-NAME nor 7-NI substituted for ethanol (Fig. 2). The ANOVA revealed that both L-NAME, $F(6, 39) = 2.43, p < 0.05$, and 7-NI, $F(4, 18) = 3.34, p < 0.05$, significantly affected the rate of responding. The highest doses of both compounds strongly reduced the ability of rats to respond even completely eliminating operant behavior in some animals (Fig. 2).

L-Arginine did not substitute for ethanol, but significantly decreased the rate of responding, $F(3, 22) = 3.07, p < 0.05$. None of the rats tested with the highest dose of L-arginine (1000 mg/kg) was able to complete at least one FR10 (Fig. 3).

Antagonism Tests

L-Arginine given 30 min. before 1 g/kg ethanol did not change the percentage of ethanol-appropriate responding. However, the rate of responding was significantly altered, $F(3, 14) = 4.87, p < 0.05$. The highest dose of L-arginine significantly suppressed the operant behavior (Fig. 3).

Although L-NAME, 7-NI, and L-arginine strongly reduced the ability of the rats to respond, no signs of late toxicity were observed. As mentioned above, the discrimination accuracy for the training sessions performed between the test sessions remained high and stable. Similarly, the rate of responding for these training sessions remained unaffected.

DISCUSSION

The results of the present study clearly demonstrate that the NOS inhibitors, L-NAME and 7-NI, do not substitute for the ethanol cue even at the doses that significantly reduce the rate of operant responding. This finding contrasts with the ability of NMDA receptor antagonists to mimic the ethanol cueing effects (2,4,18). Thus, our results suggest that NO is not involved either in the formation of the ethanol cue or the ethanol-like cueing effects of NMDA receptor antagonists. This conclusion is further supported by the finding that the

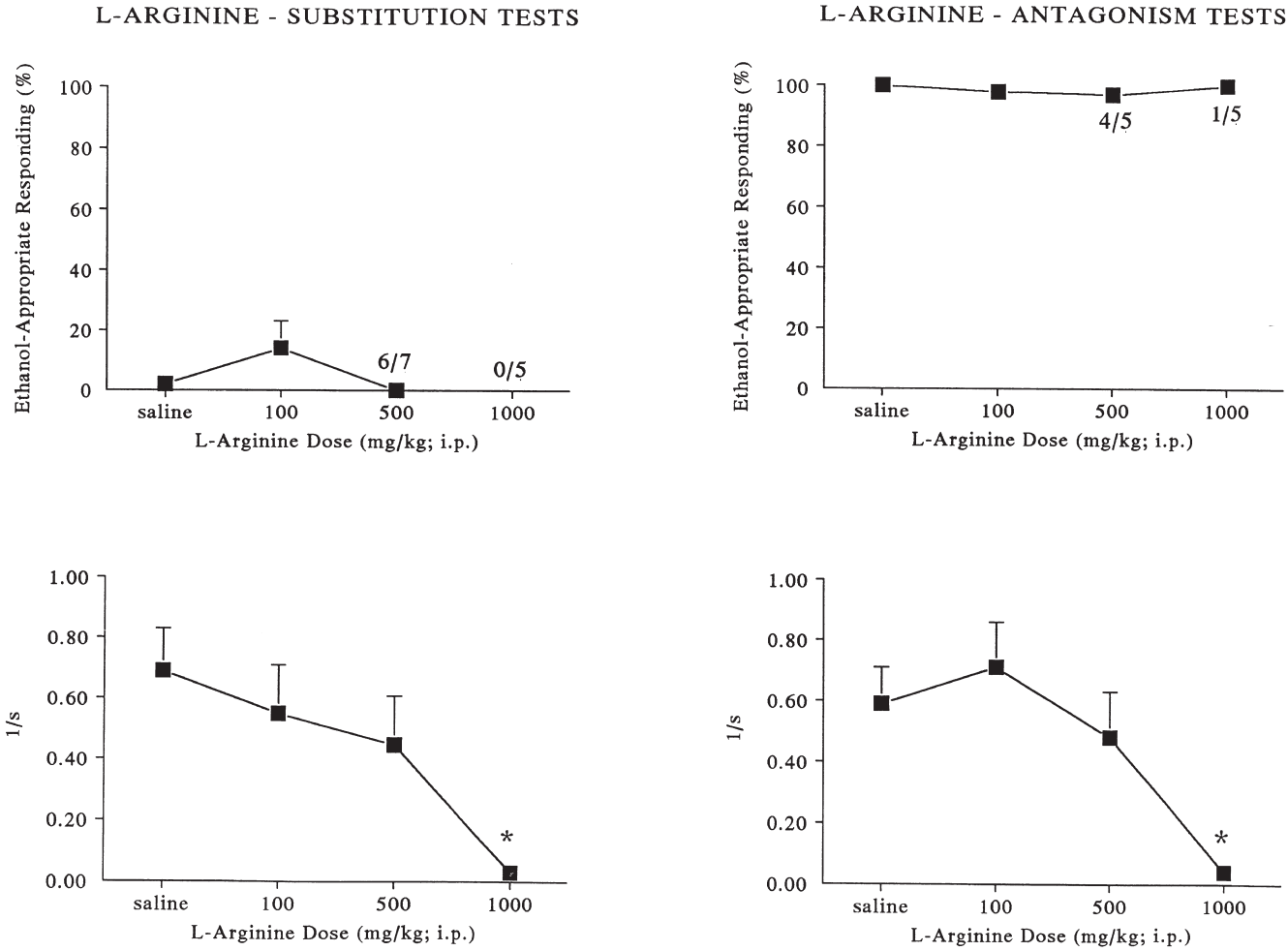

FIG. 3. Results are expressed as mean (±SEM) percentage of ethanol-appropriate responding (top) and mean (±SEM) response rate (bottom) following increasing doses of L-arginine injected either alone (substitution tests) or in combination with 1 g/kg ethanol (antagonism tests). n = 5–7 rats; *n/n*—number of rats that obtained at least one reinforcement/number of rats tested; $*p < 0.05$ vs. the group treated with saline.

NO precursor, L-arginine neither substituted for nor antagonized the cueing effects of ethanol. In line with the above, in our more recent experiment (Koros et al., unpublished) L-arginine did not affect the ethanol-like cueing properties of a NMDA receptor antagonist, D-(E)-2-amino-4-methyl-5-phosphono-3-pentanoate (CGP 40116).

The highest dose of L-arginine used in the present study almost completely eliminated the operant behavior both in the substitution and the antagonism tests. In the substitution tests none of the rats tested with 1000 mg/kg L-arginine completed at least one FR10. Thus, it should be stressed that the doses of L-arginine, which did not alter the operant responding (100–500 mg/kg), have been previously reported to reverse different pharmacological actions of NOS inhibitors (5,20,23,33).

Our results fully confirm recent finding of Green et al. (19), that L-NAME does not substitute for ethanol in male Long–Evans rats trained to discriminate either 1.5 or 2.0 g/kg ethanol (administered intragastrically) from water. Interestingly, in this latter study L-NAME up to the dose of 720 mg/kg did not consistently affect the rate of responding. In our hands, L-NAME (540 mg/kg) significantly decreased the rate of lever pressing. Different rat strain and training dose of ethanol are the factors that might explain the above difference. However, the rate-decreasing effects of L-NAME observed in the present study do not necessarily prove its central activity. L-NAME reduces activity of both nNOS and eNOS and at relatively low dose (20 mg/kg) increases mean arterial blood pressure (MAP) in the rat (20,26). On the other hand, similar doses of L-NAME induce some actions (e.g., learning impairment or anxiolytic-like effects), which are characteristic for centrally active drugs (35,36). In the case of 7-NI, the situation is more clear, as this compound does not change MAP up to the dose of 80 mg/kg (10,11,15,24,30), although a slight decrease in heart rate has been noted by some authors (24). Importantly, maximal inhibition of nNOS activity in the brain has been obtained after IP administration of 20–40 mg/kg of 7-NI (10,11).

More recently, Filip and Przegalinski (16) have studied the role of NO in the amphetamine and cocaine discrimination in rats. Interestingly, 7-NI enhanced and an NO donor, molsidomine, attenuated the cueing effects of both psychostimulants. Thus, NO may be involved in the discriminative stimulus effects of other abused substances, for example, psychostimulants (16) or phencyclidine (22).

Concluding, the results of the present study taken together with some previous findings (19) indicate that NO is not primarily involved in mediation of the discriminative stimulus effects of ethanol in the rat.

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