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Nitric Oxide Synthase Inhibition Impairs Spatial Navigation Learning and Induces Conditioned Taste Aversion

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PRENDERGAST, M. A., J. J. BUCCAFUSCO AND A. V. TERRY, JR. *Nitric oxide synthase inhibition impairs spatial navigation learning and induces conditioned taste aversion.* PHARMACOL BIOCHEM BEHAV **57**(1/2) 347–352, 1997.—The free radical gas nitric oxide (NO) is formed from the amino acid precursor l-arginine in brain regions which are associated with learning and the formation of memory. We have previously reported that administration of the nitric oxide synthase (NOS) inhibitor Nω-nitro-l-arginine methyl ester (l-Name) impairs delayed recall in non-human primates but that, at higher doses, impairment is associated with aversive gastrointestinal side effects. The purpose of the present study was to examine the effects of l-Name on learning in a rat spatial navigation task and to assess the ability of l-Name to induce a conditioned taste aversion (CTA) to a novel sucrose solution in a two-bottle choice paradigm. In the Morris water maze, l-Name (5, 20, and 50 mg/kg) markedly impaired cued spatial learning required to locate a hidden platform on three consecutive days of testing, but did not affect general activity levels. These data also demonstrated the ability of l-Name to induce a potent CTA, though only with the 20 and 50 mg/kg doses. Both the impairment of learning and CTA were blocked by administration of a mole equivalent dose of l-arginine, indicating that attenuated NO activity was associated with both behavioral effects. These data demonstrate that inhibition of NO activity by l-Name induces significant and selective impairment of cognitive performance at low pharmacologic doses (< 20 mg/kg). However, with higher doses of NOS inhibitors, impairment may be a secondary effect of drug-induced malaise, possibly related to peristaltic dysregulation of gastrointestinal musculature. Therefore, conclusions as to the mediation of learning and memory processes by CNS NO may be difficult to interpret without the use of selective, centrally-acting compounds. © 1997 Elsevier Science Inc.

Nitric oxide Learning and memory Spatial navigation Conditioned taste aversion

THE free radical gas nitric oxide (NO) is formed from the amino acid precursor l-arginine by calmodulin-dependent activity of the enzyme NO synthase (NOS; 16). NOS and NO production are present throughout the mammalian nervous system and, in some regions, NO may function as a retrograde neuronal messenger associated with learning and the formation of memory (2,4,5,10,22). Systemic or intrahippocampal administration of the NOS inhibitors N-nitro-l-arginine methyl ester (l-Name), l-N-monomethylarginine, or l-nitro-arginine impairs learning in several different rodent paradigms (10,15,17,22), as well as, a passive avoidance task in chicks (14). Previous work conducted in this laboratory demonstrated the ability of l-Name to impair the performance of non-human primates on a delayed matching-to-sample task (24).

Though the specific mechanism involved in NO mediation of learning and memory is unclear, recent work suggests that this mediation may occur in response to glutamatergic stimulation of *N*-methyl-d-aspartate (NMDA) receptors. In a cellular model of learning, pharmacologic NOS inhibition blocks the formation of long-term potentiation in rat hippocampal cells induced by glutamate-stimulated activity of NMDA receptors (2,13,25). cGMP activity induced by glutamatergic stimulation of NMDA receptors is blocked by pharmacologic inhibition of NOS and enhanced by l-arginine (12). In addition, NOS activity and subsequent NO formation have been shown to be essential to the development of LTP (25). Mediation of learning and memory by NO may also be associated with potentiation of CNS acetylcholine (ACh) activity. Cholinergic

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neurotransmission has long been implicated in these processes (1) and NO has been demonstrated to potentiate ACh-dependent cGMP formation in rat cortical primary cultures (8). In addition, NO and ACh are significantly co-localized throughout the mammalian CNS (3). However, specific examinations of NO-ACh interactions in in vivo models of learning and memory have not been reported.

While NO mediation of learning and memory processes appears to be associated, in part, with interactions at CNS NMDA and/or ACh sites, our previous report of l-Name-induced memory deficits in monkeys indicated that such deficits are associated, at higher doses, with aversive gastrointestinal (GI) side effects of l-Name (24). In this study, DMTS accuracy was impaired by 1, 5, 25, and 50 mg/kg doses of l-Name. However, impairment was accompanied by severe GI disturbance (e.g. vomiting, diarrhea) following administration of the 50 mg/kg dose. Therefore, impaired accuracy, at least with higher doses, may not be attributed to selective CNS effects of NOS inhibition on learning and memory formation. Further, it is unclear as to what degree impairment with lower doses of l-Name was associated with specific effects on cognition or drug-induced malaise. Evidence of aversive effects of l-Name may render conclusions as to the drug's effect on rat and chick behavior difficult without characterization of the aversive effects of l-Name. The purpose of the present study was to identify the dose-response characteristics of l-Name in a rat spatial navigation learning task and to compare these to the effects of l-Name in a trial of conditioned taste aversion using a two-bottle choice paradigm. The learning task employed, the Morris water maze (20), provides measures of both acquisition and recall processes, as well as, possible drug effects on locomotor behavior (swim speed) and visual acuity which may affect acquisition and recall.

METHOD

Morris Water Maze

Subjects. Fifty-four male, albino Wistar rats (Harlan Sprague–Dawley), approximately four months old (weighing 400–475 grams), were used in the water maze experiments. Each rat was housed individually in a stainless steel mesh cage in a temperature controlled room (25°C) with free access to food (NIH-07 formula) and water, and maintained on a 12 h light/dark cycle (lights on at 1800 h).

Testing Apparatus

Maze testing (20) was performed in a circular pool (diameter: 180 cm, height: 76 cm) made of plastic (Bonar Plastics, Noonan, GA) with the inner surface painted black. The pool was filled to a depth of 35 cm of water (maintained at 25 ± 1°C) which covered an invisible (black) 10 cm square platform. The platform was submerged approximately 1 cm below the surface of the water and placed in the center of the northeast quadrant. The pool was located in a large room with a number of extra-maze visual cues including brightly colored geometric images (squares, triangles, circles, etc.) hung on the wall, diffuse lighting and black curtains were used to hide the experimenter and the awaiting rats. Swimming activity of each rat was monitored via a ccTV camera mounted overhead, which relayed information including latency to find the platform, time and distance spent in each quadrant, and swim speed, to a video tracking system (Poly-Track, San Diego Instruments, San Diego, CA).

Procedure

Hidden platform test. Each rat was given four trials per day for five consecutive days. On days 1–4, a trial began by placing the rat in the water facing the pool wall in one of the four quadrants (designated NE, NW, SE, SW). The daily order of entry into individual quadrants was randomized such that all 4 quadrants were used once every day. For each trial, the rat was allowed to swim a maximum of 90 s in order to find the hidden platform. When successful, the rat was allowed a 30 s rest period on the platform. If unsuccessful within the allotted time period, the rat was given a score of 90 s and then physically placed on the platform and also allowed the 30 s rest period. In either case the rat was given the next trial (ITI = 30 s) after the rest period. On day 5, two trials were given (transfer test) in which the platform was removed from the pool to measure spatial bias (18). This was accomplished by measuring the time spent (dwell) in each of the 4 quadrants. Immediately following the transfer test, the platform was re-introduced into the pool in the quadrant opposite the original position (SW quadrant) with a highly visible, reflective cover attached to the platform which was raised above the surface of the water (approximately 1.5 cm). Lighting was changed such that extra-maze cues were no longer visible. Each rat was given one trial in order to acclimate to the new set of conditions and locate the platform visually. This was accomplished by lowering the rat into the water in the NE quadrant and allowing location of the platform. The rat was then immediately given a second trial in the same manner and the latency to find the platform measured as an assessment of visual acuity.

Drug Administration

The rats were placed in groups of 7–10 and administered one of the following drug regimens by ip injection 30 min before testing: saline (vehicle), or a dose of l-Name (5.0, 20.0, or 50.0 mg/kg). A separate group of rats received the combination of (l-Name) 20.0 mg/kg and a mole-equivalent dose (12.9 mg/kg) of l-arginine to assess the ability of l-arginine to block l-Name-induced learning impairment. All injections were administered in a volume of 1 ml/kg body weight.

During maze acquisition the data were collapsed across trials for each day and averaged to obtain a mean performance for each animal. A two-way analysis of variance with the post hoc Dunnett's test was used to compare daily group performance during days 1,2, and 3 of testing, as well as data from the transfer test, visual acuity test and swim speeds.

Conditioned Taste Aversion

Subjects. Thirty albino Wistar rats (Harlan Sprague–Dawley, Inc.) approximately five months old (weighing 400–475 grams) were used as subjects. Each rat was housed individually in a stainless steel mesh cage in a temperature controlled room (25°C) with free access to food (NIH-07 formula) and water except as described below, and maintained on a 12 h light/dark cycle (off at 1800 h).

Procedure

At 0930 h on the day of conditioning (Day 1), water bottles were removed from the home cages of all animals. Following 6 h of water deprivation (at 1530 h), rats were given access for 30 min to two bottles attached to the front of their home cages. One bottle contained 100 ml of a novel 10% wt/v sucrose

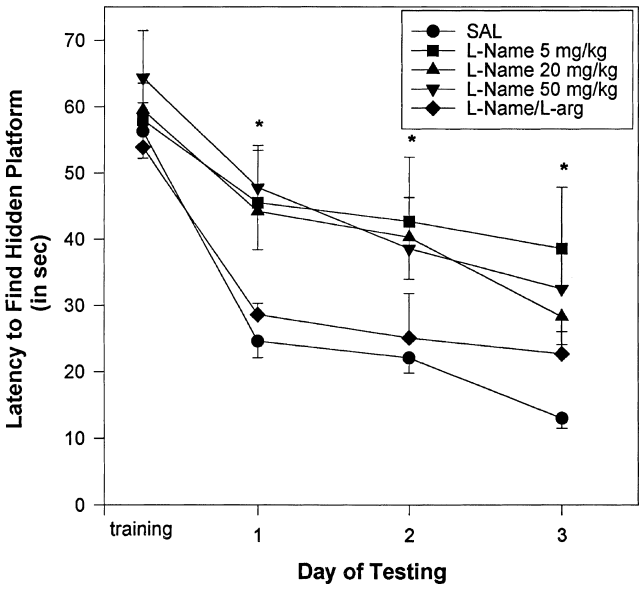

FIG. 1. Effects of l-Name and a combination of l-Name and a mole equivalent dose of l-arginine on latency (mean ± SEM) to find a hidden platform on a training day and three consecutive days of testing. Latencies were significantly higher for l-Name-treated rats on each day of testing for each dose of drug (* = $p < 0.05$). Impairment of learning was blocked by l-arginine treatment.

solution and the other contained 100 ml of tap water. The position of sucrose and water bottles on the front of cages was counterbalanced on each day of presentation. Immediately following the end of the 30 min, water bottles were removed and the amount of the different solutions consumed was measured (in ml). Each animal then received an injection (IP; $n = 6$ for all groups) of saline, l-Name (5, 20, or 50 mg/kg) dissolved in saline, or a combination of l-Name (20 mg/kg) and a mole-equivalent dose of l-arginine (12.9 mg/kg). Following injection, water bottles filled with tap water were placed on the front of home cages.

At 0930 h of the following day (Day 2), water bottles were removed from the home cages of all animals. Six hours later, rats were again given access to the two solutions for 30 min. Following this test of CTA, rats received an injection of saline or drug identical to that received the previous day. Water bottles filled with tap water were then returned to all home cages. Water deprivation and testing were completed in an identical manner on the following day (Day 3). No injections were given following consumption on Day 3. Two-way repeated measures analyses of variance were employed to compare sucrose and water consumption of saline- and drug-treated rats on the conditioning day (Day 1) to consumption on subsequent test days (Days 2 and 3).

RESULTS

Morris Water Maze

The latencies to find the hidden platform on the training trial and three test trials are illustrated in Fig. 1. Control animals (vehicle-treated) demonstrated a large decrease in latency to reach the hidden platform on Day 1 of testing, as compared to the training day. Latencies decreased further on Days 2 and 3, though these decreases were less pronounced. A significant portion of the learning which occurs in control animals appears, therefore, to occur following only a single day of exposure to the water maze. Animals treated with any dose of l-Name also exhibited decreased latencies to find the platform with each consecutive day of testing, but their latencies were significantly greater than were those of controls on each day of testing in that a significant main effect for drug was also observed [$F(3, 62) = 4.03, p < 0.05$]. Post hoc analysis indicated that administration of each dose of l-Name (5, 20, and 50 mg/kg) produced a significant increase in the latency to find the hidden platform, as compared to saline-treated controls ($p < 0.05$). There were no significant differences among doses of l-Name. Impaired performance induced by l-Name (20 mg/kg) administration was completely reversed by co-administration of a mole equivalent dose of the NO amino acid precursor l-arginine (12.9 mg/kg; Fig. 1).

A significant effect for day of testing was observed [$F(2, 62) = 7.27$, $p < 0.01$]. Post hoc comparisons using Dunnett's test indicated that a significant decrease in latency was observed on each day of testing, as compared to each previous day of testing, regardless of drug treatment ($p < 0.05$). Each group of animals, including those treated with even the highest doses of l-Name demonstrated an ability to gradually learn the task over the four days of training and testing.

Other Components of Water Maze Performance

Swim speed, calculated by dividing the total distance traveled by the latency to find the platform on the training day, was recorded during the first day of training and may provide information as to the effects of l-Name on general motor activity. The swim speed of rats treated with any dose of l-Name did not differ significantly from those of rats treated with saline (Table 1). In the test of spatial bias (dwell time in the quadrant which previously contained the platform), there were no significant differences among l-Name and saline-treated rats in the amount of time spent in the target quadrant (Table 1). Similarly, when the platform was again placed in the maze and made highly visible (a test of visual acuity), l-Name treatment did not significantly alter latency to reach the platform (Table 1). However, animals receiving the highest dose of l-Name (50 mg/kg) had a mean latency of 61.6 ± 10.96 s (compared to 31.9 ± 6.58 for controls) Though this difference is not significant due to considerable variability, a trend towards significance was observed ($p < 0.09$) and may indicate that some impairment in the ability to locate the visible platform was induced by administration of this dose (Table 1).

Conditioned Taste Aversion

The effects of l-Name (5, 20, and 50 mg/kg) on the development of conditioned taste aversion to the novel sucrose solution are illustrated in Fig. 2. A significant main effect for drug was observed [$F(3, 30) = 3.17\ p < 0.05$]. Multiple comparisons analysis indicated that rats treated with either 20 mg/kg or 50 mg/kg of l-Name following the first exposure to the novel sucrose solution consumed significantly less of the solution on Days 2 and 3 than did saline treated controls ($p < 0.05$), indicating an avoidance of the solution on these days. Sucrose consumption on Days 2 and 3 was unaffected by administration of the 5 mg/kg dose of l-Name. Sucrose solution consumption in saline-treated rats increased with each repeated exposure (8.30 and 5.50 ml above baseline levels, respectively), though this increase did not prove to be statistically significant.

TABLE 1

EFFECTS OF l-NAME ON SWIM SPEED, SPATIAL BIAS, AND VISUAL ACUITY IN THE WATER MAZE TEST

Treatment	Swim Speed (cm/s)	Spatial Bias (dwell time in s)	Visual Acuity (latency in s)
Saline	24.5 ± 1.12	39.8 ± 1.87	31.9 ± 6.58
l-Name 5mg/kg	24.3 ± 1.21	40.4 ± 5.11	44.8 ± 11.68
l-Name 20mg/kg	20.9 ± 1.23	34.6 ± 1.87	35.6 ± 6.25
l-Name 50mg/kg	25.0 ± 1.69	41.5 ± 2.13	61.6 ± 10.96*

* $p < 0.09$ vs. saline-treated controls.

Consumption in rats treated with a combination of the 20 mg/kg dose of l-Name and a mole equivalent dose of l-arginine (12.90 mg/kg) was lower on Day 2 than on the conditioning day, but this decrease did not prove significant. By Day 3, consumption in these animals was similar to that observed on the conditioning day, indicating a significant attenuation of CTA (7.60 ± 2.20 vs 2.15 ± 0.65 ml for l-Name-treated animals).

Consumption of the alternate choice water solution following saline, l-Name, or l-Name/l-arginine administration is illustrated in Fig. 3. A significant main effect for day of testing was observed [$F(2,31) = 8.34, p < 0.01$] was observed. Multiple comparisons analyses indicated that on Days 2 and 3, rats consumed significantly more water than they did on the conditioning day, prior to drug treatment ($p < 0.05$). These effects appear to result largely from the marked increase in water consumption by animals treated with the 20 mg/kg and 50 mg/kg doses of l-Name. Water consumption in saline treated animals did not differ markedly on any of the test days. The increase in water consumption observed in animals treated with 20 mg/kg of l-Name was attenuated by co-administration of the mole equivalent dose of l-arginine.

A significant effect of drug treatment on water consumption was also observed [$F(3, 31) = 6.20, p < 0.01$]. Post hoc analysis indicated that animals treated with any dose of l-Name (5, 20, or 50 mg/kg) consumed more water than did saline treated controls ($p < 0.05$). This is due, in part, to the considerable variability in baseline water consumption observed in different groups of animals. However, this effect appears also to be associated with a marked increase in water intake on Days 2 and 3, which corresponds to the development of CTA in animals treated with the two highest doses of l-Name (Fig. 3).

DISCUSSION

In the water maze task, administration of each dose of l-Name impaired spatial navigation learning on each day of testing. There were no apparent differences among doses of the drug, precluding the identification of a dose-response relationship. These data are consistent with several previous reports demonstrating that l-Name impairs acquisition and retention processes in several species of experimental animals (10,22,24). Estall and colleagues (10) demonstrated an impairment of water maze acquisition following administration of 10 and 20 mg/kg doses of l-Name but not with a 5 mg/kg doses, as was observed in the present study. The discrepancy between the present findings and those of Estall (10) appears to be related to differing methodologies. For example, in the

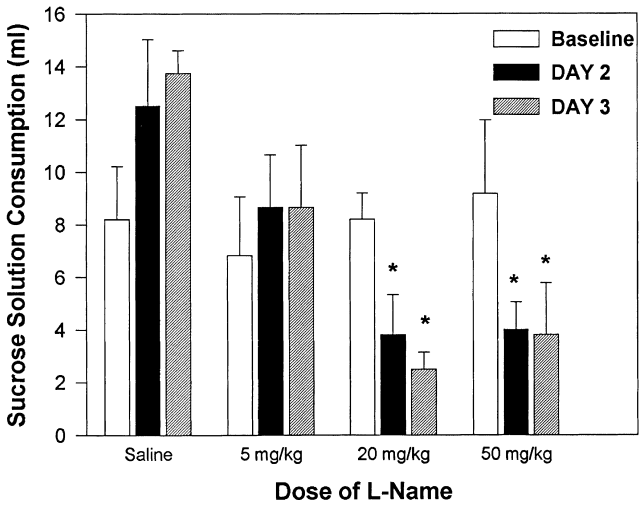

FIG. 2. Effects of l-Name and a combination of l-Name and a mole equivalent dose of l-arginine on the development of conditioned taste aversion to a novel 10% sucrose solution. Data represented as ml consumed (mean ± SEM). Treatments were administered following baseline consumption on Day 1. * $p < 0.05$ vs. saline-treated controls.

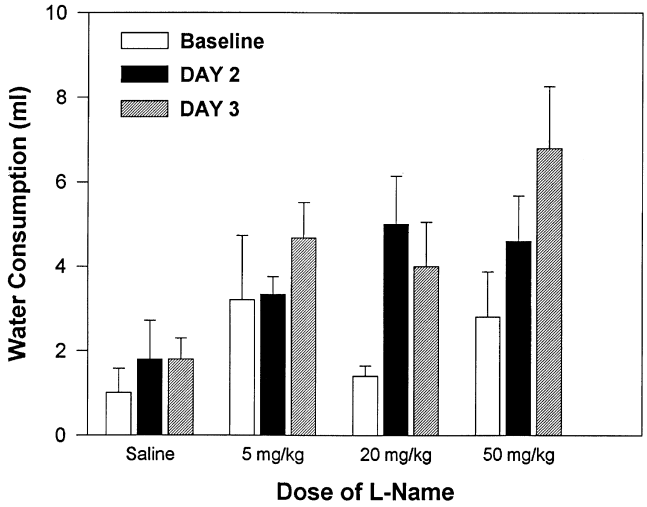

FIG. 3. Effects of l-Name and a combination of l-Name and a mole equivalent dose of l-arginine on water consumption (mean ml ± SEM) during three consecutive days of testing. Consumption was markedly elevated on Days 2 and 3 in rats which received the 20 and 50 mg/kg doses of l-Name.

previous report, animals had participated in 4 trials of a visible platform test two h prior to testing in the hidden platform test, on each day of testing. This provides animals with some exposure to navigational cues for subsequent testing. In the present study, no animals had a pre-exposure to the visible platform prior to hidden platform testing. In addition, different spatial cues and drug administration schedules were employed in the two studies. Therefore, direct comparison of the dose-response characteristics of l-Name in the two studies is difficult, though both readily demonstrate the ability of NOS inhibition to impair acquisition of this task.

While the specific mechanism associated with NOS inhibition-induced impairment is unclear, these data are similar to water maze impairments induced by administration of the NMDA receptor antagonists AP5 and MK-801 (18,21). It is possible, therefore, that l-Name-induced deficits are indeed associated with disruption of glutamate-NMDA receptor interactions, as has been postulated (2,13,25), possibly at hippocampal sites (17,22). Others have suggested that l-Name and other NOS inhibitors function in the CNS as ACh muscarinic receptor antagonists (7). Muscarinic antagonists have previously been shown to induce cognitive deficits (26), therefore, this possibility is consistent with the present data. However, Buxton and colleagues (7) reported that l-Name exhibited μM affinity for brain muscarinic receptors *in vitro*. Further, work in this laboratory indicated that l-Name displaced [^{3}H]methylscopolamine binding to spinal cord membranes only at concentrations above 1 mM, while atropine displaced [^{3}H]methylscopolamine with an IC_{50} of 1.5 nM (6). Finally, the NOS inhibitor NG-monomethyl-l-arginine acetate exhibited even less affinity for muscarinic receptors that did l-Name in this study. Therefore, it is not likely that l-Name produced significant blockade of brain muscarinic receptors under the conditions of our experiment. This contention is supported by our finding that the amnestic actions of l-Name were completely blocked by l-arginine.

Swim speed and visual acuity in the water maze task were not significantly altered by l-Name administration. With regard to swim speeds, this suggests that the drug induced no gross psychomotor deficits. Therefore, elevated latencies, relative to controls, in l-Name-treated animals are not attributable motor impairments (eg. sedation, muscular weakness). Though visual acuity was not significantly altered by drug administration, those animals which received the highest dose of l-Name displayed a mean 30 s elevation, relative to controls, in latency to find the visible platform. Though difficult to interpret given the considerable variability present, this may indicate the presence of visual impairment or malaise induced by the 50 mg/kg dose. This has not previously been identified as an effect of l-Name at high doses but may warrant further examination.

The lack of pervasive influence of l-Name on motor or visual functioning may appear to stand in contrast to its ability to induce CTA. CTA is a learned taste avoidance developed after associative pairing of a novel taste cue and nausea or a more general gastrointestinal malaise and is used frequently to assess the ability of pharmacologic agents to induce such states (11). The present data demonstrate that a single pairing of a novel sucrose taste and administration of 20 or 50 mg/kg of l-Name is sufficient to produce subsequent avoidance of the sucrose solution, relative to controls. CTA induced by the 20 mg/kg dose of l-Name was blocked by concurrent administration of a mole equivalent dose of l-arginine, indicating a selective role for NOS inhibition in the development of this CTA. On Day 1 of testing, sucrose solution consumption was markedly greater than was water consumption, evincing the palatability of this sucrose solution. On Days 2 and 3, however, animals treated with the two highest doses of l-Name consumed significantly less sucrose solution than did control animals. Control animals consumed greater quantities of the solution with each successive day of testing. This pattern of increasing consumption with repeated exposures to a novel taste (hyponeophagia) in control animals has previously been described and appears to be dependent upon developing familiarity with the taste (23,28). Though animals which received the 5 mg/kg dose did not demonstrate an apparent learned aversion to the solution, consumption on Days 2 and 3 was decreased, as compared to controls. This may suggest that the 5 mg/kg dose of l-Name induced a mild aversive state which attenuated the daily increase in consumption seen in controls, but is not severe enough to induce avoidance of the solution.

The marked reduction in sucrose solution consumption after pairing of the solution with administration of 20 or 50 mg/kg of l-Name suggests that IP administration of this drug induces a potent aversive state which is associated with nausea or a more general form of gastrointestinal malaise. Given that these animals were water deprived, the marked increase in water consumption that paralleled avoidance of the sucrose (on Days 2 and 3) appears to represent a compensation for this avoidance. CTA induced by l-Name, therefore, does not appear to be associated with drug-induced hypophagia or hypodypsia, but with an otherwise aversive state induced by IP administration of this drug. This suggestion is supported by our previous report of severe gastrointestinal distress following administration of higher doses of l-Name to non-human primates (24).

Evidence of malaise induced by l-Name administration is relevant to behavioral studies employing similar administration methods for l-Name given that this aversive state may influence cognitive performance, rendering interpretation difficult. It is quite possible, if not likely, that the presence of drug-induced malaise during cognitive testing may have impaired the animal's attention to spatial cues which aid in platform location or the consolidation of cue locations in the formation of a spatial navigation strategy. Further, overt symptoms of malaise such as lethargy, vomiting, and diarrhea in monkeys treated with l-Name was invariably associated with impairment of delayed recall in our previous study (24). It appears likely, therefore, that malaise does indeed interfere with delayed recall and, possibly, spatial navigation learning, though the nature of this impairment is unclear (i.e. impaired attention or impaired recall).

These data are also relevant to recent work which has demonstrated the ability of NOS inhibitors to suppress feeding in rats (19), in that hypophagia may be induced by malaise independently of alterations in activity at CNS sites. Our finding that CTA induced by l-Name was blocked by administration of l-arginine indicates that CTA was associated with a NOS-selective inhibition. While is unclear as to what involvement CNS NO systems may have in the observed CTA, it is likely that peripheral NOS inhibition following l-Name administration is closely related to the development of CTA. Recent work has indicated that NO release is associated with reflexive relaxation of gastrointestinal musculature (eg. fundus, lower esophageal sphincter) in response to intragastric pressure (9,27). Further, NOS inhibitors such as l-Name, *N*-monomethyl-l-arginine, and *N*-nitrol-arginine prevent relaxation of gastrointestinal smooth muscle induced by electrical stimulation (9,27). It is possible, if not likely, then that high doses of l-Name ($>$ 20 mg/kg) may induce GI constriction

and/or peristaltic dysregulation, either of which may serve as a salient aversive GI cue in a trial of CTA. In addition, either or both of these processes may explain the vomiting and diarrhea observed in non-human primates which received l-Name.

In sum, acute inhibition of NOS by l-Name produces marked decrements in spatial navigation learning which are blocked by concurrent administration of the NO amino acid precursor l-arginine and are, therefore, likely to be associated with attenuated synthesis of NO.

In addition, higher doses of l-Name (> 20 mg/kg) induce a potent, dose dependent CTA to a novel sucrose solution. Therefore, impairment of water maze performance with these doses appears to result, in part, from drug-induced malaise in addition to, or instead of, a CNS effect on learning and memory formation. Careful interpretation of the dose-response effects of NOS inhibitors on learning and memory, as well as on other behaviors, is warranted, then, as drug-induced aversive states may alter behavior independently of alterations in targeted CNS activity.

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