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Nitric Oxide Synthase Inhibition Impairs Delayed Recall in Mature Monkeys

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PRENDERGAST, M. A. , A. V. TERRY JR., W. J. JACKSON, AND J. J. BUCCAFUSCO. *Nitric oxide synthase inhibition impairs delayed recall in mature monkeys.* PHARMACOL BIOCHEM BEHAV **56**(1) 81–87, 1997.—The gaseous neuromodulator nitric oxide (NO) is formed in brain regions known to mediate learning and memory processes. In rodent models, pharmacologic inhibition of NO synthesis impairs such processes. In the present study, N^{ω}-nitro-L-arginine methyl ester (L-Name), an inhibitor of the constituitive form of the NO synthetic enzyme, was administered to seven non-aged, mature monkeys (*Macaca Fascicularis, Macaca Mulatta,* and *Macaca Nemestrima*) trained to perform a delayed matching-to-sample task (DMTS). L-Name (1,5, 25, and 50 mg/kg) produced marked decrements in task performance, as well as a reduction in the number of trials completed at the highest dose. This impairment of DMTS accuracy by the 50 mg/kg doses of L-Name appears to be associated with an aversive state marked by gastrointestinal disturbance and lethargy. The detrimental effects of the 25 mg/kg dose of L-Name on DMTS accuracy were completely blocked by concurrent administration of a mole-equivalent dose of the NO amino acid precursor L-arginine. As a whole, these data suggest that L-Name impairs processes involved in delayed recall in monkeys and that this impairment is associated with attenuated synthesis of NO. However, at higher doses ($\geq$ 25 mg/kg) this impairment is associated with aversive effects of L-Name, possibly at both central and peripheral sites.

Nitric oxide Learning and memory Non-human primates Delayed matching

NITRIC oxide (NO) is a free radical gas formed from the amino acid L-arginine by calmodulin-dependent activity of the enzyme NO synthase (NOS; 18). Immunohistochemical labeling of NO (6) and in situ hybridization detection of NOS mRNA (5) indicate a diffuse distribution of NO-generating neurons in the rat brain extending from the cerebral cortex to the cerebellum. Initially identified as an endothelial-derived relaxing factor in blood vessels and as a macrophage product associated with inflammatory processes, recent work indicates that one isoform of NO, produced in endothelial and neuronal cells, functions as a retrograde neuronal messenger which may be associated with learning and memory processes (4,11,20).

It has been suggested that the activity of NO during learning and memory tasks is induced by glutamatergic stimulation of *N*-methyl-D-aspartate (NMDA) receptors. Evidence that glutamate activation of NMDA receptors and subsequent cGMP activity is enhanced by L-arginine and attenuated by NOS inhibition (12) supports this conclusion and is particularly relevant to studies of long-term potentiation (LTP). A considerable body of data indicates that NMDA receptor activation is necessary for the development of LTP in hippocampal cells (10,26) and that NOS inhibition blocks the development of LTP in these cells (4,15,24). Further, NOS activity and NO have been shown to be essential to the production of LTP (24).

Behavioral data obtained from studies employing the NOS inhibitor N-nitro-L-arginine methyl ester (L-Name) also suggest a role for NO in mediating learning and memory processes. L-Name is an alkyl ester derivative of L-arginine which potently inhibits NOS activity in monkey and rat brain (IC_{50}s typically range from 0.5 to 5.0 μm; 22; 21) and has been widely used to study systemic vasoconstriction in both species (14, 19). With regard to learning and memory, L-Name has been shown to impair acquisition of a place-navigation task in rats (11); working memory in rats following hippocampal adminis-

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tration (20); and passive avoidance in the chick (16). Doses of L-Name eliciting such effects typically range from 1-20 mg/kg and correlate with ex vivo brain NOS inhibition to 50–80% of control levels between 30 min and 120 min after peripheral administration in rats (23). In monkeys, a dose of 60 mg/kg was reported to reduce ex vivo brain NOS activity by 90%, 20 min after IV administration (19).

Evidence that NOS inhibition does not affect visual discrimination learning implies no gross sensory or perceptual deficits, and suggests a selective role for NO in mediating acquisition and retention processes (11). Consistent with this notion is evidence that significant interactions exist between brain NO and acetylcholine (ACh), a neurotransmitter associated with the neuropathology of Alzheimer's disease (for review, see 2). For example, enhanced depolarization-induced release of ACh corresponds with the appearance of NOS catalytic activity in PC12 cells incubated with nerve growth factor (Hirsch et al. unpublished data). Moreover, release of ACh from these cells is suppressed by NOS inhibitors, an effect which is reversed by the introduction of L-arginine into the medium. Other researchers have provided corroborating evidence demonstrating neuronal colocalization of NO and ACh (3), as well as NO mediation of ACh-dependent cGMP formation (9).

Several recent reports, however, have provided evidence that NOS inhibition does not impair performance on spatial learning tasks in rats (1,27). These data have been interpreted as suggesting that NOS inhibition may induce transient, nonspecific physiologic effects which differentially alter performance on some tasks, but not others (27). This suggestion may indeed clarify discrepancies concerning the effects of NOS inhibition on learning and memory, but has not been specifically examined (eg. in a trial of conditioned taste aversion or in animals such as non-human primates which may provide more information regarding the untoward effects of NOS inhibition).

Detailed elucidation of the function of NO and NOS inhibition in learning and memory processes is necessary, then, to assess the role which it may have in pathological cognitive function. Little is known, however, about the effects of NO alterations on learning and memory in animal models other than the rat (e.g., 11,20). The present study examined the ability of the NOS inhibitor L-Name to alter performance of mature monkeys on a delayed matching-to-sample task (DMTS). Doses employed were within dose ranges previously shown to induce learning deficits in rats (11) and to significantly reduce NOS activity in rat ex vivo brain tissue (21,22). This study also assessed the ability of L-arginine to reverse monkey DMTS performance decrements induced by L-Name administration.

METHOD

Subjects

Seven mature monkeys (8–14 years), including 4 male pigtailed macaques (*macaca nemestrina*), 1 male and 1 female rhesus macaque (*macaca mulatta*), and 1 female cynomolgus macaque (*macaca fascicularis*), served as subjects. All monkeys were housed at the Animal Behavior Center of the Medical College of Georgia. The facilities of the Animal Behavior Center meet or exceed current Federal standards for nonhuman primate housing. Monkeys were housed within individual stainless steel cages composed of $50 \times 28 \times 26$ in units. Toys were provided routinely and monkeys were allowed to observe television programs each afternoon as a means of promoting psychological well-being. During periods when animals were not tested routinely, they were allowed access to an enclosed outdoor exercise facility on an individual or selected-group basis.

During the week, monkeys were allowed ad libitum access to water and maintained on a feeding schedule that allows approximately 15% of their normal daily food intake to be derived from 300 mg food pellets which served as rewards during experimental sessions (standard monkey chow and banana flakes, P.J. Noyes, Inc., Lancaster, NH). The remainder of their daily diet was obtained from standard laboratory monkey chow following completion of a test session. On days that the animals were not performing the DMTS task (e.g., weekends, holidays), the daily allotment of solid food was obtained from standard laboratory monkey chow supplemented with fruits and vegetables.

At the beginning of DMTS sessions, test panels were attached to the front of home cages. DMTS stimuli were 25 cm diameter colored disks (red, green, and yellow) presented via light-emitting diodes located behind clear push-keys. For six of the monkeys, sessions consisted of 96 trials each day. For one monkey, sessions consisted of 48 trials each day because of the considerable length of this monkey's individualized delay intervals (60-120-240 s).

A trial began with illumination of the sample key by one of the colored stimuli. The sample remained illuminated until the animal depressed the key, initiating a pre-programmed delay interval, during which no keys were illuminated. Following the delay interval, two choice lights located below the sample key were then illuminated. One of the choice stimuli always matched the hue of the previously presented sample light, while the non-matching choice was one of the other two colors. The choice stimuli remained illuminated until the animal depressed one of the choice keys. Responses to the choice key illuminated by the color matching the sample key were rewarded by a 300 mg banana-flavored pellet. Responses to the choice key illuminated by the non-matching color were not rewarded and another trial was initiated. Four possible delay intervals were employed between a monkey's response to the sample stimulus and the presentation of the two choice stimuli: zero delay and three longer delay intervals, referred to as short, medium, and long delays. Each stimulus color configuration occurred in conjunction with each delay interval an equal number of times. The location of correct response followed controlled sequences (13) to ensure chance reward ratios for possible strategies in choice responding. The monkeys were trained until performance for zero delay trials averaged 85–100% correct. Short, medium, and long delays were adjusted in duration to produce stable performance levels which approximated the following levels of accuracy: short delay, 75–85%; medium delay, 65–75%; and long delay, 55–65%. The length of delays for each animal was adjusted according to individual skill level and ranged from 0–15 s to a maximum of 0–240 s. The rationale for this procedure was to normalize DMTS performance for all monkeys given that monkeys exhibit considerable variability in baseline matching ability (17,25).

Drug Administration

L-Name, L-arginine (Sigma, St. Louis), or vehicle (sterile, normal saline) were administered IM (gastrocnemius muscle) in a volume of 0.035 ml/kg body weight. Baseline data were obtained following administration of vehicle and each monkey

served as its own control. Test sessions began 15 min after L-Name or vehicle administration. A minimum "drug washout" period of 2 days was allowed between sessions. During this period, a return to baseline DMTS performance was established in each monkey before L-Name was re-administered. Each monkey received the following ascending dose-series of L-Name: 1.0, 5.0, 10.0, and 25.0 mg/kg. The initial four monkeys used in the study received an additional dose of 50 mg/kg L-Name. The three remaining monkeys did not receive this dose because of its apparent toxicity.

Four weeks following administration of the last dose of L-Name, six of the seven monkeys were administered the lowest dose of L-Name which previously produced a DMTS performance decrement on both the short and medium delay trials (25 mg/kg) in conjunction with a mole-equivalent dose of L-arginine (19.8 mg/kg).

For statistical analysis, performance during vehicle-treated sessions was compared to drug-treated sessions and to sessions completed 24 h following treatment with L-Name using two-way analyses of variance and the Neuman-Keuls test of post-hoc multiple comparisons. In addition, latencies to respond to sample, and choice stimuli on correct and incorrect response trials were compared using one-way, repeated-measures analyses of variance. Drug effects were calculated as the % of correct DMTS trials compared to each individuals saline-derived baseline.

RESULTS

The average short, medium, and long delay intervals (in s) for all monkeys were, respectively, 9.5 ±; 2.39, 41.67 ± 10.46, and 83.33 ±; 20.92. Monkeys treated with saline displayed the following pattern of baseline DMTS accuracy (% correct) for each delay interval: zero delay = 97.8 ± 1.78; short delay = 84.1 ± 7.62; medium delay = 71.4 ± 2.40; and long delay = 60.4 ± 3.18. A one-way analysis of variance yielded a significant main effect for delay interval [$F(3, 24) = 41.40$, $p < 0.001$]. Multiple comparisons conducted using the Newman-Keuls method indicated significant differences between baseline performance on each delay interval ($p < 0.05$).

All groups represent data derived from seven monkeys, with the exception of those corresponding to 50 mg/kg L-Name, which were derived from four monkeys. Signs of systemic toxicity were observed following administration of 50 mg/kg L-Name in three of the four monkeys that received this dose. Approximately 20 min after the initiation of behavioral testing (35 min following drug administration), these monkeys displayed signs of lethargy (eg. inactivity, laying on floor of cage, and refusal to perform DMTS trials). Each of these monkeys failed to complete all of the pre-programmed DMTS trials (testing automatically ceased after a 700 s latency to respond to a sample key), completing 91.67, 81.25, and 60.40% of the total trials possible. One monkey vomited approximately 2 h following administration of this dose and another vomited at an unknown time between 1700 h of the testing day and 0800 h of the following day. Two of these three monkeys exhibited diarrhea approximately 3 h after administration. None of the three monkeys displaying initial toxicity to this dose completed all 96 trials 24 h after drug administration (76.00, 10.42, and 11.45% of possible trials were completed). Data derived from trials completed after administration of this dose were not included in statistical analyses. Given this apparent toxicity of L-Name at 50mg/kg, the remaining three monkeys were not administered this dose.

The effects of L-Name on DMTS performance were analyzed using a two-way analysis of variance treating drug (saline or dose of L-Name) and delay as separate factors. A significant interaction between treatment (saline or dose of L-Name) and delay was observed [$F(12, 120) = 1.90$, $p < 0.05$]. On the day of administration, L-Name had no effect on DMTS performance for trials with no delay (zero delay). On short-delay trials, multiple comparisons analysis indicated that administration of the 5 mg/kg and 25 mg/kg doses of L-Name produced a significant decrement in DMTS accuracy 15 min, but not 24 h, after drug injection ($p < .05$; Fig. 1). On medium delay trials, administration of the 1, 5, and 25 mg/kg doses produced significant decrements in DMTS performance ($p < .05$; Fig. 2). Accuracy on long delay trials was not significantly altered by L-Name administration, a finding possibly associated with the low level of baseline performance exhibited by all monkeys on long-delay trials (60.4 ± 3.18%).

Other Components of DMTS Performance

Two measures of response latencies were also recorded during DMTS testing: choice latency-the time interval between presentation of the two choice stimuli and depression of one of the choice keys, and sample latency- the time interval between initiation of a new trial (illumination of the stimulus light behind the sample key) and depression of the sample key by the animal. Examination of choice latency data revealed a significant main effect for the trial outcome, ie. correct or incorrect, [$F(1, 70) = 30.31$, $p < .001$]. This indicates that the latency to make a choice between the two possible matching stimuli was significantly greater on trials for which the choice was incorrect than on trials for which that choice was correct (3.22 ± 0.20 vs 1.96 ± 0.22 s, respectively). L-Name did not affect this pattern of responding. With regard to sample latency data, there were no significant differences between correct and incorrect trials and L-Name did not alter these latencies (1.84 ± 0.20 and 2.10 ± 0.21 s, respectively).

L-Name/L-arginine Co-administration

L-Name (25 mg/kg), administered concomitantly with L-arginine (19.8 mg/kg), did not alter DMTS performance, as it had when administered alone (Fig. 3). A significant effect for delay interval was observed [$F(3, 30) = 89.00$, $p < .001$]. Multiple comparisons analysis indicated that DMTS accuracy on zero delay trials was significantly higher than accuracy on trials of all other delays ($p < .05$). A similar, and significant, difference was observed between short and medium delay trials.

DISCUSSION

The results of previous studies in which the behavioral effects of NOS inhibition were examined have provided evidence that such inhibition impairs performance of tasks dependent on learning and memory processes (11,20). As noted, however, several reports have provided contradictory evidence and have suggested that task-specific impairment by NOS inhibitors may be associated with non-specific drug effects (27). The results of the present study suggest the possibility of both specific and non-specific effects of NOS inhibition on delayed recall. These data demonstrate that the decrements in DMTS performance of monkeys induced by L-Name administration are selective for those delays which are reliant on the extended retention and recall of sample stimuli. This is suggested by the observation that L-Name did not affect accuracy on zero-delay trials, but did reduce accuracy on short-

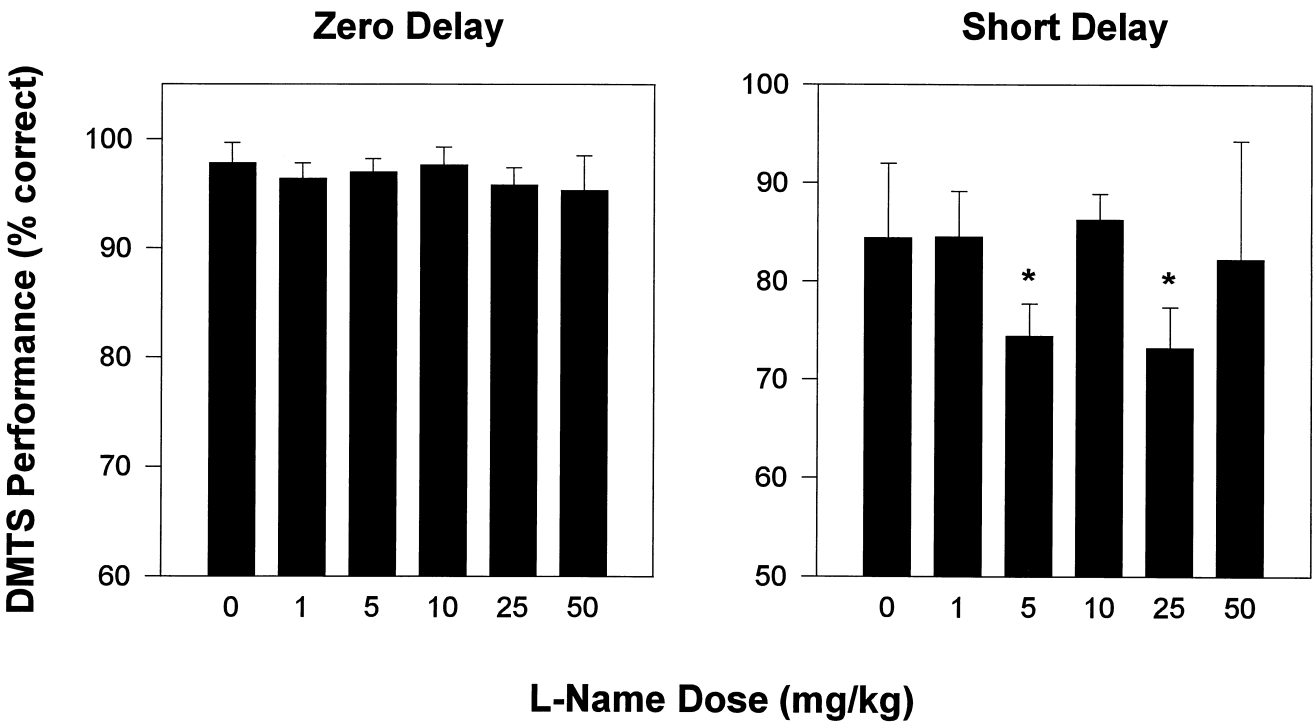

FIG. 1. Effects of L-Name on DMTS performance (mean + s.e.m.) for trials with zero and short delays initiated 15 min after drug administration. N = 7 monkeys for data derived from saline and 1,5,10, and 25 mg/kg L-Name. N = 4 monkeys for data derived from 50 mg/kg L-Name. * = $p < .05$ vs saline-derived baseline performance. Data from 50 mg/kg dose not included in analysis.

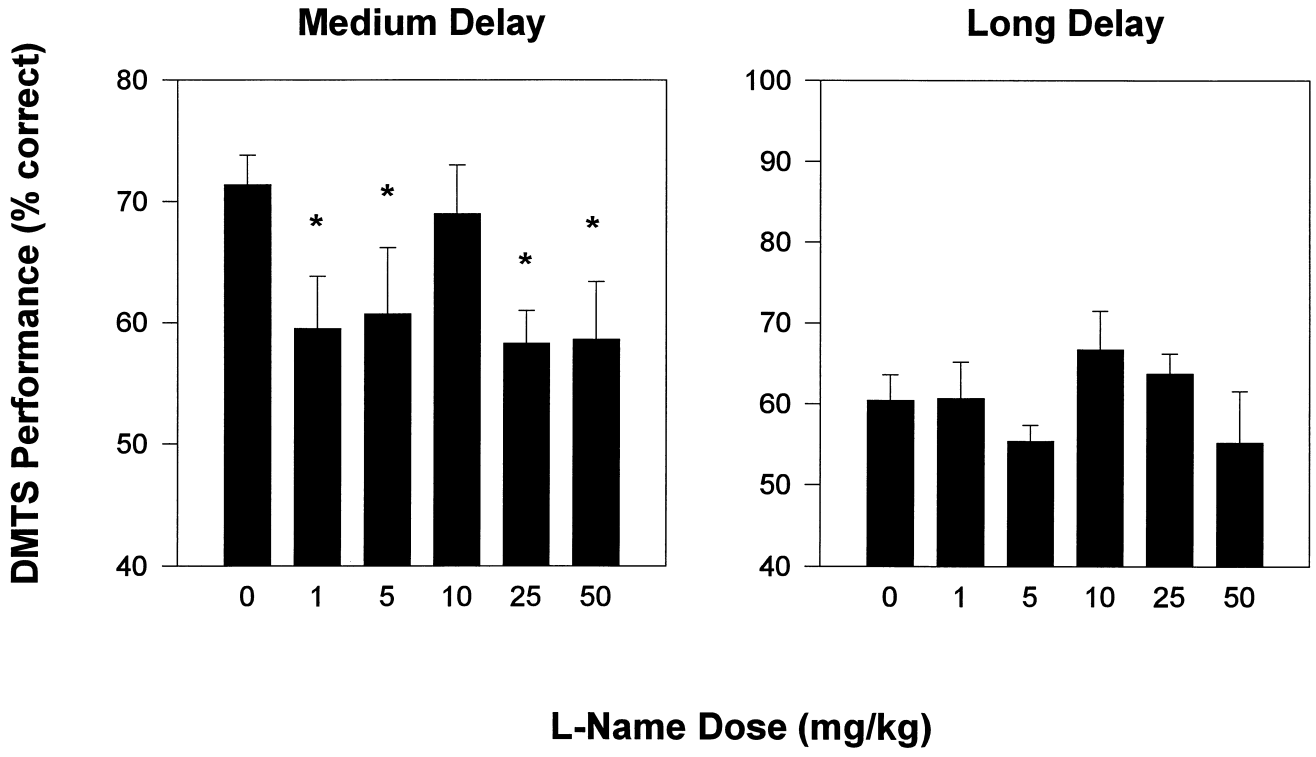

FIG. 2. Effects of L-Name on DMTS performance (mean + s.e.m.) for trials with medium and long delays initiated 15 min after drug administration. N = 7 monkeys for 1,5, 10, and 25 mg/kg doses of L-Name. N = 4 monkeys for 50 mg/kg L-Name. * = $p < .05$ vs saline-derived baseline performance. Data from 50 mg/kg dose not included in analysis.

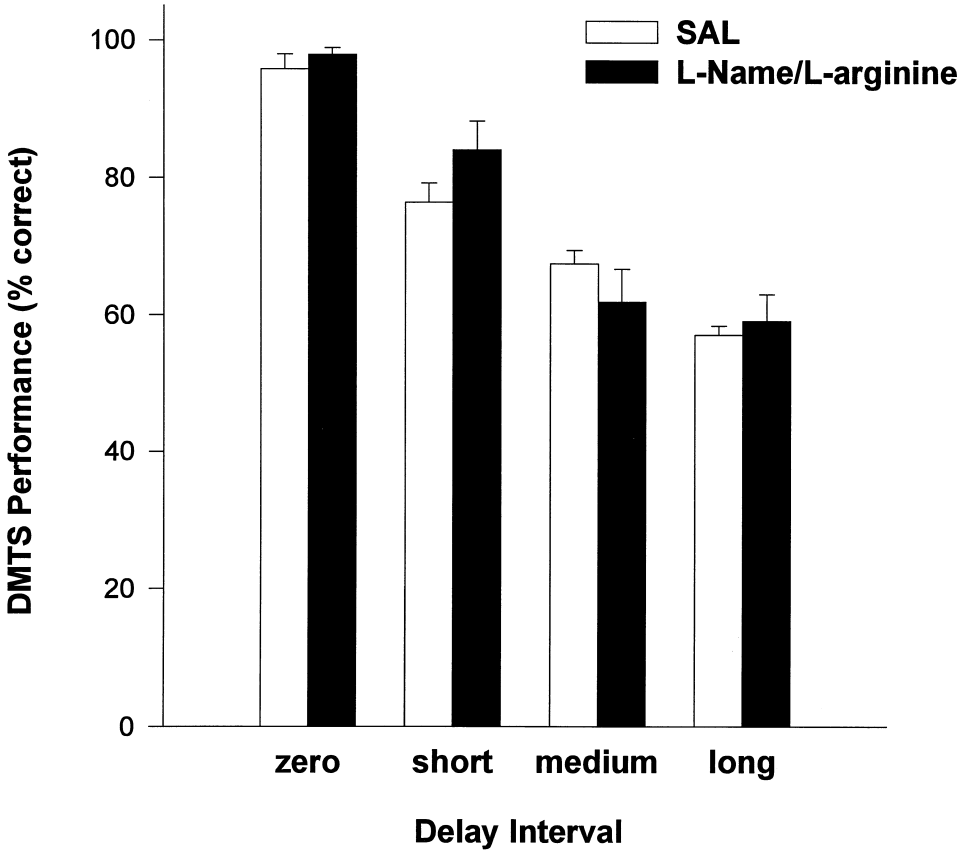

FIG. 3. Effects of saline and concurrent L-Name (25 mg/kg)/L-arginine (19.8 mg/kg) administration on DMTS accuracy (mean + s.e.m.) for all delay intervals. L-Name administration did not significantly alter DMTS performance in the presence of a mole-equivalent dose of L-arginine.

and medium-delay trials. It is reasonable to assume that performance on zero-delay trials is more dependent than short, medium, and long delay trials on immediate attentional processes than on processes of prolonged stimulus retention. With increasing delay intervals, however, prolonged retention and recall of sample stimulus characteristics is of increasing significance. It is likely that pharmacologic manipulations which selectively alter retention and recall processes would more dramatically affect performance on trials with longer delay intervals. Such a pattern was observed with regard to the effects of L-Name on DMTS accuracy. The 5 and 25 mg/kg doses of L-Name reduced accuracy on short-delay trials, while the lowest dose had no effect on performance of these trials. However, performance on medium-delay trials was markedly impaired (12-14% below saline levels) by the 1 mg/kg dose, as well as by the 5 and 25 mg/kg doses of L-Name.

The effect of L-Name on performance at both delay intervals appeared to be bi-phasic, given that the 10 mg/kg dose did not impair performance. This pattern of response to L-Name was observed on short, medium, and long delay trials. The ability of the lower dose of L-Name (1 mg/kg) to impair accuracy on medium-delay, as opposed to short-delay, trials appears to be associated with the drug's selective effects on prolonged retention and recall. It may be expected, then, that L-Name would induce the most significant impairment of DMTS performance on those trials with the longest delay intervals. However, performance on long-delay trials was not significantly altered by L-Name administration. Though accuracy on these trials is, theoretically, more dependent on retention and recall processes, baseline accuracy may have been too low (slightly greater than chance levels of accuracy) to reflect decrements induced by L-Name.

The selectivity of L-Name in disrupting retention and recall processes is further suggested by it's lack of influence on response latencies in monkeys. Altered latencies to respond to sample and/or choice stimuli may be an indicator of a drug-induced effect on psychomotor function, but was not observed with L-Name. Choice response latencies on DMTS trials which were completed incorrectly were significantly elevated, as compared to correct trials. This difference is likely due to an increased duration of attempted recall of the sample stimulus characteristics. L-Name had no affect on this pattern of responding. Sample response latencies were similar for both correct and incorrect trials and were also unaffected by drug administration.

The present data support the suggestion that NOS inhibition may induce aversive physiological effects which are independent of learning and memory. At a high dose (50 mg/kg), L-Name administration was associated with significant side effects which were likely to have contributed to DMTS performance decrements displayed by the monkeys. Symptoms of gastrointestinal disturbance (eg. vomiting and diarrhea) and lethargy were observed in several monkeys following administration of the 50 mg/kg dose. Though McPherson and col-

leagues (19) reported the use of a 60 mg/kg dose of L-Name in monkeys, these animals were anesthetized, precluding the identification of many signs of toxicity. It is apparent, therefore, that DMTS performance deficits induced by these doses of L-Name were associated with this aversive state and resultant distractibility and lethargy, possibly in addition to disrupted retention and recall processes. Disruption of DMTS performance after administration of the 1, 5, and 25 mg/kg doses of L-Name was not associated with observable signs of side effects, though the absence of such effects can not be confirmed. Lower doses of L-Name may, therefore, alter cognitive performance in these animals independently of a drug-induced aversive state.

Though the extent of NO inhibition was not assessed in the present study, similar doses of L-Name (1-60 mg/kg) have previously been shown to induce ex vivo reductions in monkey and rat brain NO synthesis of 30-90%, depending on dose and route of administration (19,23). In vitro concentrations of L-Name eliciting similar reductions in NO synthesis range from 1.0-100 μm (22). Using conservative estimations of L-Name bioavailability between 10% and 80% following IM administration in monkeys, the doses employed in the present study likely resulted in in vivo concentrations within a range of 1.0-100 μm or possibly greater, similar to those observed in in vitro studies.

Our finding that DMTS performance deficits induced by L-Name administration were blocked by concurrent administration of L-arginine strongly suggests that these deficits were associated with the attenuated biosynthesis of NO from L-arginine. Similarly, Ohno et al. (20) employed hippocampal injections of L-Name and induced profound deficits in working memory in rats that were attenuated by concurrent intrahippocampal administration of L-arginine. d-Arginine, the inactive isoform, had no effect on working memory performance. These data suggest a possible localization of NO neurons which mediate learning and memory processes in the hippocampus. This is consistent with evidence that L-arginine and NOS inhibitors potentiate and inhibit, respectively, LTP in hippocampal cells that is induced by glutamatergic stimulation of NMDA receptor activity (10,12,26). This mediation may also be associated with NO mediation of ACh activity (9), a general effect which enhances cognitive performance on a variety of tasks (for review, see 2). Therefore, NOS inhibition may attenuate hippocampal neuronal activity during task acquisition and recall which is induced by glutamate and/or ACh activity.

Alternatively, other data suggest that learning and memory deficits induced by L-Name administration may be associated with alterations in muscarinic receptor activity (8). These authors demonstrated the ability of alkyl ester derivatives of L-arginine, such as L-Name, to bind brain muscarinic receptors at μm concentrations. Inhibition of muscarinic receptor activity produced by L-Name would attenuate the postsynaptic effects of ACh and, theoretically, induce learning and memory deficits similar to those observed following administration of the muscarinic antagonist scopolamine (25). However, work conducted in this laboratory demonstrated that L-Name displaced [3H]methylscopolamine binding to spinal cord membranes only at concentrations above 1 mM, whereas atropine displaced [3H]methylscopolamine with an IC_{50} of 1.5 nM (7). The NOS inhibitor NG-Mono-methyl-L-arginine acetate (L-NMMA) exhibited even less affinity than L-Name. In addition, it is unlikely that an effect of L-Name mediated by muscarinic receptors would be reversible by L-arginine. Therefore, conclusions as to the effects of NOS inhibition on CNS muscarinic activity appear to be contentious. Further, other definitive conclusions as to the specific mechanisms associated with learning and memory deficits induced by NOS inhibition remain difficult in light of the paucity of data concerning NO function in other CNS regions associated with learning and memory processes.

In sum, L-Name induced performance deficits in monkeys completing a DMTS tasks appear to be associated with the attenuated synthesis of NO from L-arginine. Administration of high doses of L-Name (>25 mg/kg) induces severe gastrointestinal disturbance and lethargy in monkeys. DMTS performance deficits observed with administration of these high doses, may be a secondary or partial result of inducing an aversive state. Examination of the role of NO in mediating learning and memory processes appears to be relevant to understanding mechanisms of cognitive pathology, such as Alzheimer's disease and related dementias. With the development of novel pharmacologic agents which affect NO activity, this system may represent one to be exploited in the treatment of such conditions.

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