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Intracerebroventricular injection of N^{ω}-nitro-L-arginine in rats impairs learning in a 14-unit T-maze

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Abstract

We investigated whether intracerebroventricular (i.c.v.) infusion of the nitric oxide synthase inhibitor, N^{ω}-nitro-L-arginine (*N*-Arg), impairs learning in male Sprague–Dawley rats (2–3 months old) in a 14-unit T-maze. Rats were pretrained in one-way active avoidance to a criterion of 13/15 avoidances of foot shock in a straight runway. The next day, rats received i.c.v. injections of either artificial cerebrospinal fluid (aCSF) as controls or *N*-Arg (12 μg or 15 μg) 30 min before training in the 14-unit T-maze. The learning contingency was to negotiate each of 5 segments within 10 s to avoid footshock during 15 trials. Performance variables included errors (deviations from the correct pathway), runtime from start to goal, and shock frequency and duration. Compared to controls, the number of errors over the last 10 trials was higher in rats receiving 15 μg *N*-Arg and over the last 5 trials for those given 12 μg. Runtime, shock frequency and duration were increased in both *N*-Arg groups. The *N*-Arg-induced (15 μg i.c.v.) impairment could be attenuated when the nitric oxide donor, sodium nitroprusside (1 mg/kg), was administered intraperitoneally 1 min prior to maze learning. In a retention test, rats were treated with either aCSF or 15 μg *N*-Arg i.c.v. 30 min before being retested in the maze 7–10 d following acquisition training. Under these conditions, maze performance was not significantly affected. These results confirmed previous findings that inhibition of nitric oxide synthase impairs acquisition but not retention. Moreover, the *N*-Arg-induced learning impairment does not appear to be related to noncognitive aspects of performance. © 1998 Elsevier Science B.V.

Keywords: Nitric oxide (NO) synthase; Sodium nitroprusside; Blood pressure; Memory; Glutamate

1. Introduction

Several previous studies in rodents have demonstrated that systemic injections of nitric oxide (NO) synthase inhibitors, e.g. N^{ω}-nitro-L-arginine methyl ester (NAME) or N^{ω}-nitro-L-arginine (*N*-Arg), can impair performance in various learning tasks (Chapman et al., 1992; Holscher and Rose, 1992; Bohme et al., 1993; Estall et al., 1993; Mogensen et al., 1995a,b). This impairment is hypothesized to be due to neuronal mechanisms. Specifically, the inhibitors of NO synthase reduce calcium-mediated generation of nitric oxide that serves as a retrograde messesenger affecting presynaptic glutamate release (Garthwaite, 1991; Moncada et al., 1991). Results of several investigations, however, have called into question whether the observed impairments were due to noncognitive influences on performance (Bannerman et al., 1994b; Sandi et al., 1995). The effects of *N*-Arg or NAME on performance in learning tasks may not be specific to the inhibition of neuronal NO synthase. Systemic injections of these compounds also inhibit endothelial NO synthase to produce hypertension by vasoconstriction (Moncada et al., 1991; Sandi et al., 1995).

In a previous study (Ingram et al., 1997) conducted in this laboratory using a 14-unit T-maze, a learning impairment was observed in Fischer-344 (F344) rats following intraperitoneal (i.p.) injections of *N*-Arg (4–8 mg/kg) 30 min prior to training. Moreover, the *N*-Arg-induced im-

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pairment could be attenuated by treatment with the NO donor, sodium nitroprusside. The learning impairment was not likely due to noncognitive factors for several reasons. First, during acquisition training no significant effects of *N*-Arg on performance were observed during the first block of training trials. Second, when a high dose of *N*-Arg (6 mg/kg) was delivered prior to a retention test, no significant effects on performance were observed. In addition, this dose of *N*-Arg had no significant effect on the ability of rats to perform a rotarod task.

The present study was designed to explore further the effects of NO synthase inhibition on performance in this maze. Attempting to avoid peripheral drug effects and to target NO synthase in brain more efficiently, we examined the effects of central injections of *N*-Arg on performance of rats in the 14-unit T-maze, and tested whether *N*-Arg-induced effects could be attenuated by sodium nitroprusside. Results of previous studies using central injections of NO synthase inhibitors had demonstrated that behavioral effects, including decreased spontaneous motor activity and startle response (Sandi et al., 1995) and also reduced food intake (De Luca et al., 1995), could be produced through this route of administration. Few studies have been conducted using central injections of NO synthase inhibitors to examine effects on learning performance. Ohno et al. (1993) noted that hippocampal injections of NAME in rats impaired learning in a working memory component of a 3-panel runway task but not in a reference memory component. Fin et al. (1995) observed impaired retention in a passive avoidance task following hippocampal injections of *N*-Arg in rats before or contiguous with the training session. Holscher and Rose (1993) observed impaired passive avoidance learning in chicks following central NAME injections. Despite the evidence of learning impairments induced by central inhibition of NO synthase, Bannerman et al. (1994a) reported that i.c.v. infusion of NAME did not block long-term potentiation in the hippocampal gyrus. Thus, the question remained whether or not the learning impairments produced by NO synthase inhibitors are specific to memory processes.

2. Materials and methods

2.1. Subjects

Young (2–3 months old), virgin male Sprague–Dawley rats were obtained from Zivic Miller Laboratories (Pittsburgh, PA). Five days prior to their shipment to the Gerontology Research Center (GRC), these rats had been surgically implanted by the vendor with a cannula to the left or right lateral ventricle. We used only rats in which the presence of extruded cerebrospinal fluid through the implanted cannula was confirmed by visual inspection prior to shipment. Upon arrival at the GRC, the rats were housed individually in conventional plastic cages with wood shavings and were allowed to acclimate for at least 2 weeks before behavioral testing. A conventional diet (NIH-07) was provided ad libitum as was access to water through an automated and filtered system. In the vivarium constant temperature (22°C), humidity (70%), and a 12 h light; 12 h dark photocycle (lights on at 06.00) were maintained. All testing occurred during the light portion of the photocycle.

2.2. Apparatus

2.2.1. Straight runway

This runway was used during pretraining for shock avoidance and has been described previously (Ingram et al., 1997).

2.2.2. Maze

The construction and configuration of the 14-unit T-maze was identical to that described previously (Ingram et al., 1997).

2.3. Procedure

2.3.1. Maze pretraining

The procedure for pretraining was identical to that described previously (Ingram et al., 1997).

2.3.2. Maze acquisition

Maze training began 24 h following pretraining in the straight runway. The procedure was identical to that described previously (Ingram et al., 1997).

2.3.3. Drug treatment

Rats were assigned randomly to a control group or to experimental groups receiving intracerebroventricular (i.c.v.) injections of *N*-Arg (Sigma). *N*-Arg was mixed in freshly made, artificial cerebrospinal fluid (aCSF: 4.8 mM NaCl, 3 mM KCl, 1.2 mM $CaCl_2$, 0.85 mM $MgCl_2$) in two concentrations, 3.0 or 3.75 μg/μl. Treatments were delivered through the implanted cannulae via silastic tubing connected to a Hamilton microsyringe driven by a microinfusion pump (CMA/100, Acton, MA) over an 8 min period. In an initial experiment, rats received i.c.v. injections in a volume of 4 μl containing either aCSF as control ($n = 6$) or 12 μg *N*-Arg ($n = 5$) or 15 μg *N*-Arg ($n = 8$) 30 min prior to maze training. A related experiment determined whether a *N*-Arg-induced learning impairment could be attenuated with the NO donor, sodium nitroprusside. Control rats ($n = 13$) received i.c.v. delivery of aCSF 30 min prior to training followed by an intraperitoneal (i.p.) injection of saline (0.9% NaCl) 1 min before training. Two experimental groups received i.c.v. delivery of *N*-Arg (15 μg in a volume of 4 μl over 8 min) 30 min prior to training followed by i.p. injections of either saline ($n = 12$) or sodium nitroprusside (1 mg/kg; $n = 10$) 1 min prior to training. For all experiments, the location of the cannulae (right side versus left side) was balanced across conditions.

2.3.4. Blood pressure measurement

To assure that the dose of sodium nitroprusside used was biologically active, a pilot study was conducted to examine its effects on blood pressure. The procedure for measuring systolic blood pressure was identical to that described previously (Ingram et al., 1997). As controls, two rats were injected i.p. with saline and four with 1 mg/kg sodium nitroprusside, and blood pressure was monitored for 15 min at intervals of 5 min.

2.3.5. Maze retention

A group of 10 rats that received acquisition training as described above were retested in the 14-unit T-maze 7–10 d later. Thirty min prior to the retention test, half the group received i.c.v. infusion of *N*-Arg (15 μg in a volume of 4 μl over 8 min) and half received aCSF as controls. According to the procedures used during acquisition training, these rats received an additional 10 trials in the maze.

2.3.6. Statistical analysis

Maze performance variables analyzed included errors made (defined as deviations from the correct pathway of the maze), runtime from start to goal, and shock frequency and duration. Data on maze acquisition as well as on effects of sodium nitroprusside were submitted to a 3 (drug group) by 3 (blocks of 5 trials) repeated measures analysis of variance (ANOVA). Dose effects within blocks were assessed by one-way ANOVA followed by Dunnett's test comparing different treatment groups to controls. Data on maze retention were analyzed by two-tailed *t*-tests for data collapsed over 10 trials. For all comparisons, statistical significance was accepted as $P < 0.05$.

3. Results

3.1. Effects of N-Arg on maze acquisition

A single i.c.v. infusion of *N*-Arg impaired learning in the 14-unit T-maze. As presented in Fig. 1, no significant effects were evident during the first block of 5 trials, but over the last 2 blocks the number of errors increased with *N*-Arg dose. By the last block of training, the number of errors made by rats receiving 15 μg *N*-Arg was over 3 times as high as controls.

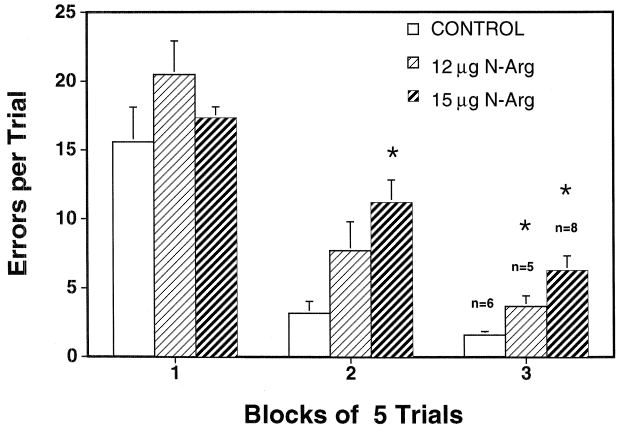

Fig. 1. Mean (S.E.M.) errors per trial in the 14-unit T-maze during acquisition training according to dose of N^{ω}-nitro-L-arginine (*N*-Arg) * significantly different from control group, $P < 0.05$.

Table 1
Effects of N^{w}-nitro-L-arginine (*N*-Arg) on noncognitive performance variables during acquisition training. Mean values (S.E.M.) per trial

Variable	Dose of *N*-Arg (μg)		
	control, $n = 6$	12, $n = 5$	15, $n = 8$
Runtime (s)	56.8 (6.8)	103.0[a] (11.6)	95.0[a] (6.3)
Shock frequency	2.0 (0.3)	3.3[a] (0.3)	4.2[a] (0.3)
Shock duration (s)	26.3 (7.9)	57.4[a] (10.5)	47.4[a] (5.1)

[a]Significantly different from control group, Dunnett's test, $P < 0.05$.

The ANOVA results provided statistical confirmation of these findings. Significant main effects were observed for group, $F(2,16) = 5.13$, $P < 0.02$, and blocks, $F(2,32) = 106.8$, $P < 0.001$. Group effects at each block were analyzed by one-way ANOVA followed by Dunnett's comparisons. Significant group effects were observed for block 2, $F(2,16) = 6.79$, $P = 0.007$ and block 3, $F(2,16) = 8.21$, $P < 0.004$. Dunnett's comparisons revealed a significant difference in errors between controls and the 15 μg dose over the last 2 blocks, and between controls and the 12 μg dose during the last block.

Analysis of the other performance variables indicated that i.c.v. infusion of *N*-Arg at both doses also increased runtime, shock duration and frequency. These data have been collapsed across trials and summarized in Table 1. Results of the 3 (drug group) by 3 (blocks of trials) ANOVAs revealed significant group effects for all variables, $F(2,16) > 4.1$, $P < 0.04$, as well as for blocks, $F(2,32) > 40.6$, $P < 0.001$.

3.2. Effects of N-Arg on maze retention

Infusion of 15 μg *N*-Arg i.c.v. had no significant effects on maze performance compared to controls in the retention test. These data are presented in Table 2 and were analyzed by *t*-tests. No significant differences were

Table 2
Effects of N^{w}-nitro-L-arginine (*N*-Arg) on maze performance variables during retention testing. Mean values (S.E.M.) per trial

Variable	Dose of *N*-Arg (μg)	
	control, $n = 5$	15, $n = 5$
Errors	3.1 (0.8)	1.8 (0.5)
Runtime (s)	31.2 (4.2)	34.4 (7.7)
Shock frequency	0.9 (0.33)	0.8 (0.31)
Shock duration (s)	4.8 (1.9)	5.8 (2.7)

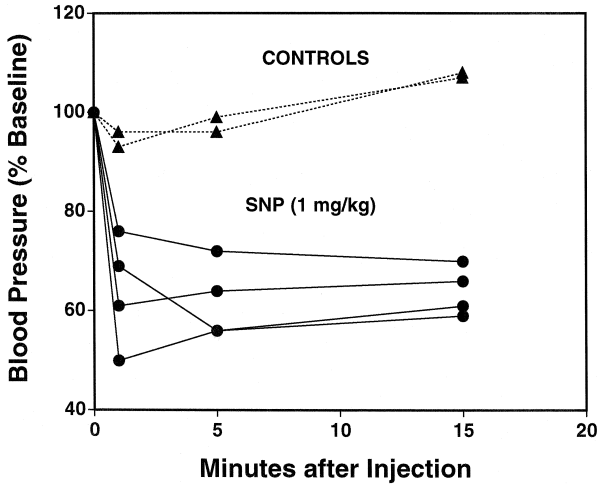

Fig. 2. Systolic blood pressure following injection of sodium nitroprusside (SNP).

found between *N*-Arg and control groups on any parameter ($P > 0.05$).

3.3. Effects of sodium nitroprusside

Replicating earlier observations in F-344 rats (Ingram et al., 1997), we observed that administration of 1 mg/kg sodium nitroprusside i.p. produced rapid and marked reduction of systolic blood pressure in Sprague–Dawley rats (Fig. 2). Reductions in blood pressure occurred as early as 1 min, were at levels 30–40% below baseline by 5 min, and were maintained over 15 min.

Injection of 1.0 mg/kg sodium nitroprusside 1 min prior to maze training significantly attenuated the learning impairment observed following 15 μg i.c.v. infusion of *N*-Arg. Error performance is shown in Fig. 3. Results of the ANOVA yielded a significant group effect, $F(2,32) = 7.43$, $P = 0.002$, and a significant block effect, $F(2,64) = 75.35$, $P < 0.001$. Group differences at each block were analyzed by one-way ANOVA, which yielded significant group effects only for block 2, $F(2,32) = 5.26$, $P < 0.02$, and block 3, $F(2,32) = 6.93$, $P < 0.004$. Dunnett's comparisons indicated that only the *N*-Arg-treated group made significantly more errors compared to controls at blocks 2 and 3.

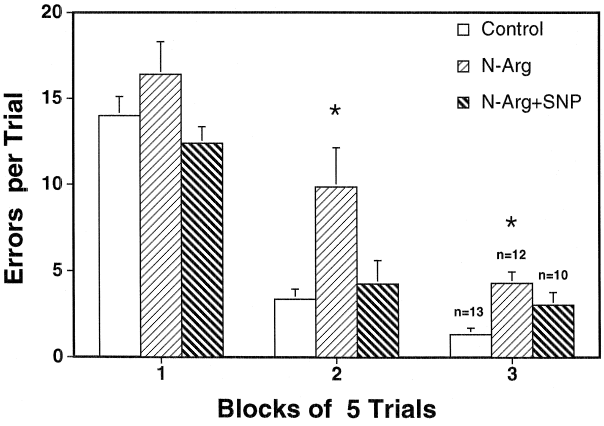

Fig. 3. Mean (S.E.M.) errors per trial in a 14-unit T-maze during acquisition training in control groups or groups receiving 15 μg N^{ω}-nitro-L-arginine (*N*-Arg) or *N*-Arg plus sodium nitroprusside (SNP) * significantly different from control group, $P < 0.05$.

Table 3
Effects of N^{w}-nitro-L-arginine (*N*-Arg 15 μg i.c.v.) alone or combined with sodium nitroprusside (SNP 1 mg/kg i.p.) on noncognitive performance variables during acquisition training. Mean values (S.E.M.) per trial

	n	Runtime (s)	Shock duration (s)	Shock frequency
Saline + saline	13	53.4 (7.9)	23.9 (7.2)	1.7 (0.2)
N-Arg + saline	12	77.9 (6.8)	35.0 (6.8)	2.9 (0.2)[a]
N-Arg + SNP	10	61.2 (8.9)	24.3 (7.6)	2.2 (0.3)

[a] Significantly different from saline + saline group, Dunnett's test, $P < 0.05$.

Collapsed across trials, data on other performance variables are presented in Table 3. Although the data would appear to parallel the findings with error performance, results of one-way ANOVAs for each variable yielded a significant main effect only for shock frequency, $F(2,32) = 6.51$, $P < 0.01$. Dunnett's comparisons indicated that only the *N*-Arg-treated group had received significantly more shocks compared to controls. Although runtime and shock duration were clearly elevated in the *N*-Arg-treated rats, results of the ANOVAs were not significant, $F(2,32) = 2.65$, $P > 0.05$, and $F(2,32) = 0.82$, $P > 0.05$, respectively.

4. Discussion

As an extension of previous results reported for this paradigm using i.p. injections of *N*-Arg (Ingram et al., 1997), impaired learning in the 14-unit T-maze can be induced in rats when NO synthase is inhibited by either systemic or central *N*-Arg treatments. Importantly, we replicated previous observations (Ingram et al., 1997) that *N*-Arg increased the number of errors made only during the last 2 blocks of training with no significant effects observed during the first block. Moreover, this finding was extended to a different rat strain, Sprague–Dawley compared to the F-344 rats that were studied previously (Ingram et al., 1997). We again observed that the *N*-Arg dose which impaired learning had no significant effects on retention performance. Thus, the ability of rats to perform in the maze as measured by runtime and shock avoidance was not impaired by *N*-Arg treatment per se. As a further replication of Ingram et al. (1997), we demonstrated that the *N*-Arg-induced impairment could be attenuated by systemic treatment with the NO donor, sodium nitroprusside.

The current results also agree with the findings of at least two other studies using central injections of NO synthase inhibitors to impair learning. Fin et al. (1995) reported that bilateral hippocampal injections of *N*-Arg (2 μg) impaired acquisition of a shock-motivated passive avoidance task when delivered prior to training or immediately after but not 30 min afterward. Ohno et al. (1993) found that bilateral hippocampal injections of NAME (10–32 μg) impaired performance of rats in a working memory component of a 3-panel runway task. A major difference between the latter study and the current one was that their task was food-motivated while ours was aversively motivated and thus similar to that used by Fin et al. (1995). Since De Luca et al. (1995) have reported that systemic as well as central inhibition of NO synthase reduces food intake in rats, a precaution would have to be applied to the results of any food-motivated task. However, it should be noted that Sandi et al. (1995) also reported that central injections of *N*-Arg would diminish an acoustic startle response in rats. When these findings were combined with observations of diminished behavioral response in rats receiving systemic *N*-Arg treatment, they argued that NO synthase inhibition impairs reactions to novel situations, an observation which might account for impaired performance in learning tasks. This contention was similar to the argument by Bannerman et al. (1994b) that learning impairments produced by NO synthase inhibitors were nonspecific in their effects. However, it should be noted that in studies using systemic (Estall et al., 1993) or central injections (Ohno et al., 1993) of NO synthase inhibitors, learning is not impaired in tasks in which performance has been shown to be independent of hippocampally associated spatial memory processing. Septo-hippocampal systems are involved in learning the 14-unit T-maze (Ingram et al., 1996); thus, *N*-Arg-induced impairment would be expected.

Two other caveats regarding the current results should also be addressed. Because shock was used as the aversive stimulus in the current study, one concern is possible *N*-Arg-induced changes in nociception. Sandi et al. (1995) reported reduced startle response to an electrical stimulus following systemic NAME treatment. Shibuta et al. (1995) used i.c.v. infusion of NAME and reported reduced responses to a thermal stress. The only data that we can provide to counter this argument is that i.c.v. infusion of *N*-Arg did not significantly affect avoidance of shock during retention trials. In addition, Fin et al. (1995) did not observe significant impairment in passive avoidance learning when *N*-Arg was injected into hippocampus immediately after the training trial, an event which occurred in the absence of the shock stimulus. Moreover, they also observed that performance was enhanced in rats receiving hippocampal injections of the NO donor, *S*-nitroso-*N*-acetylpenicillamine, from 30–150 min after training. This facilitative effect on memory processing would be independent of any interaction with shock.

A second caveat is whether we actually avoided a centrally mediated vasoconstriction following i.c.v. infusion of *N*-Arg. Injection of NAME i.c.v. in doses of 1.0 μM or less has been shown to increase arterial blood pressure in rats (Cabrera and Bohr, 1995). Because blood pressure was not measured after central *N*-Arg treatment in the current study, we cannot be certain that the issue of hypertension has been avoided. Thus, further studies using inhibitors specific for neuronal NO synthase would assist in addressing whether a learning impairment can be produced in the absence of a hypertensive state. In addition, because knockout mice without neuronal NO synthase have been reported to be unimpaired in long-term potentiation (O'Dell et al., 1994), it is important to examine various conditions under which learning impairments are observed following treatment with neuronally specific NO synthase inhibitors. The effect of the neuronal specific NO synthase inhibitor, 7-nitroindazole, on performance in the 14-unit T-maze has been investigated by Meyer et al. (1997).

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