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Impaired learning in rats in a 14-unit T-maze by 7-nitroindazole, a neuronal nitric oxide synthase inhibitor, is attenuated by the nitric oxide donor, molsidomine

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Abstract

In previous experiments, it was demonstrated that systemic or central administration of the nitric oxide synthase (NO synthase) inhibitor, N^G-nitro-L-arginine (*N*-Arg), produced dose-dependent learning impairments in rats in a 14-unit T-maze; and that sodium nitroprusside, a NO donor, could attenuate the impairment. Since *N*-Arg is not specific for neuronal NO synthase and produces hypertension, it is possible that effects on the cardiovasculature may have contributed to the impaired maze performance. In the present experiment, we have investigated the maze performance of 3–4 months old male Fischer-344 rats following treatment with 7-nitroindazole, a NO synthase inhibitor that is selective for neuronal NO synthase and does not produce hypertension. In addition, we examined the effects of the NO donor, molsidomine, which is much longer acting than sodium nitroprusside. Rats were pretrained to avoid footshock in a straight runway and received training in a 14-unit T-maze 24 h later. In an initial dose–response study, rats received intraperitoneal (i.p.) injections of either 7-nitroindazole (25, 50, or 65 mg/kg) or peanut oil 30 min prior to maze training. 7-nitroindazole produced significant, dose-dependent maze acquisition deficits, with 65 mg/kg producing the greatest learning impairment. This dose of 7-nitroindazole had no significant effect on systolic blood pressure. Following the dose–response study, rats were given i.p. injections of either 7-nitroindazole (70 mg/kg) plus saline, 7-nitroindazole (70 mg/kg) plus the NO donor, molsidomine (2 or 4 mg/kg), or peanut oil plus saline as controls. Both doses of molsidomine significantly attenuated the learning deficit induced by 7-nitroindazole relative to controls. These findings represent the first evidence that impaired learning produced by inhibition of neuronal NO synthase can be overcome by systemic administration of a NO donor. © 1998 Elsevier Science B.V.

Keywords: Glutamate; NMDA (*N*-methyl-D-aspartate); Blood pressure; Neurotransmitter; Memory; Ca^{2+}

1. Introduction

Abundant evidence has emerged to demonstrate that glutamatergic transmission via the NMDA receptor is important for the formation of new memories (Morris et al., 1989; Muller et al., 1994; Rison and Stanton, 1995; Bear, 1996; Dragunow, 1996). The soluble gas, nitric oxide (NO), has been implicated in this system as a retrograde messenger that enhances presynaptic release of glutamate (Schuman and Madison, 1994; Segovia et al., 1994). Various behavioral studies have been conducted to investigate the involvement of NO in learning, but a definitive role for NO in memory formation remains unclear.

Previous behavioral studies have focused on blocking the formation of NO by inhibiting its synthetic enzyme, nitric oxide synthase (NO synthase). Systemic administration of N^{ω}-nitro-L-arginine (*N*-Arg) or N^G-nitro-L-arginine methyl ester (NAME), both of which inhibit NO synthase, has been found to significantly impair learning in various tasks and in several species of laboratory animals (Chapman et al., 1992; Estall et al., 1993; Papa et al., 1994; Robertson et al., 1994; Yamada et al., 1996). In contrast,

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other reports indicate that NO synthase inhibition produces no learning impairment or only minimal impairment (Okere et al., 1995; Holscher et al., 1995; Tobin et al., 1995). The results from these studies must be interpreted with caution, however, since *N*-Arg and NAME cause marked hypertension due to their effects on endothelial NO synthase (Boger et al., 1994).

Recent experiments conducted in our laboratory have demonstrated that *N*-Arg, when administered to rats systemically or centrally, impaired learning in a 14-unit T-maze (Ingram et al., 1997a; Ingram et al., 1997b). This learning impairment was attenuated by systemic injection of sodium nitroprusside, an NO donor. However, the improvement in learning following sodium nitroprusside treatment may well have been due to sodium nitroprusside (a potent vasodilator) counteracting the hypertensive effects of *N*-Arg. Furthermore, because *N*-Arg inhibits NO synthase both centrally and peripherally, it remains difficult to ascribe any of these behavioral findings, including those of previous studies using either NAME or *N*-Arg, directly to the inhibition of neuronal NO synthase.

The objective of the present study was to further investigate NO in a learning paradigm without the confounding factor of hypertension. To this end, we used the NO synthase inhibitor, 7-nitroindazole, which is specific for neuronal NO synthase and thus does not produce hypertensive effects (Moore et al., 1993; Beierwaltes, 1995; Kelly et al., 1995; Vaupel et al., 1995). In addition, instead of using sodium nitroprusside as the NO donor, we used molsidomine (the active metabolite being 3-morpholinosydnonimine, or SIN-1). Molsidomine is easily absorbed and has a much longer duration of action than sodium nitroprusside (Boger et al., 1994). Using the 14-unit T-maze, the present study is relevant to current efforts being made to investigate age-associated memory impairment and to identify novel approaches to enhance cognition in patients with Alzheimer's disease (Ingram et al., 1996).

2. Materials and methods

2.1. Subjects

Subjects were 67 experimentally naive, 4–6 months old virgin male Fischer-344 rats. All rats were shipped from Harlan Sprague–Dawley (Indianapolis, IN) and housed in a vivarium at the Gerontology Research Center. Rats were housed in pairs in plastic cages with wood chip bedding, and were allowed to acclimate to the vivarium for 2–3 weeks prior to testing. Food (NIH-07) and water were available ad libitum, and the vivarium was maintained at 22°C with a 12:12 h light–dark cycle (lights on at 06:00 EST).

2.2. Apparatus and procedures

Apparatus and procedures are identical to those described in the previous papers (Ingram et al., 1997a,b).

2.2.1. Drug treatment

7-Nitroindazole (Research Biochemicals International, MA) was dissolved in peanut oil and stirred into solution under very low heat. Molsidomine (Alexis Biochemicals, CA) was dissolved in 0.9% saline. To determine the effective dose of 7-nitroindazole that would impair maze acquisition, a dose–response study was conducted. Approximately 30 min prior to training in the 14-unit T-maze, rats received intraperitoneal (i.p.) injections of either peanut oil (control, $n = 9$) or 7-nitroindazole 25 mg/kg ($n = 8$), 50 mg/kg ($n = 9$), or 65 mg/kg ($n = 9$).

Following the 7-nitroindazole dose–response study, other rats were assigned randomly to four different drug treatment groups. Approximately 30 min prior to maze training, rats received i.p. injections of either peanut oil plus saline (control, $n = 8$), 7-nitroindazole at 70 mg/kg plus saline ($n = 8$), or 7-nitroindazole at 70 mg/kg plus molsidomine at 2 or 4 mg/kg ($n = 8$).

2.2.2. Statistical analysis

Data on maze acquisition were analyzed in a manner similar to that used in the previous studies (Ingram et al., 1997a,b). All data from the four behavioral measures (errors, runtime, shock duration and shock frequency) were analyzed with a 4 (drug group) by 5 (blocks of 3 trials) factorial analysis of variance (ANOVA), with repeated measures on the second factor. Post hoc comparisons between vehicle controls and drug-treatment groups were assessed using Dunnett's procedure.

3. Results

3.1. 7-Nitroindazole treatment

Treatment with 7-nitroindazole produced dose-related performance deficits during acquisition training in the 14-unit T-maze (Fig. 1). A 4 (groups) × 5 (blocks)

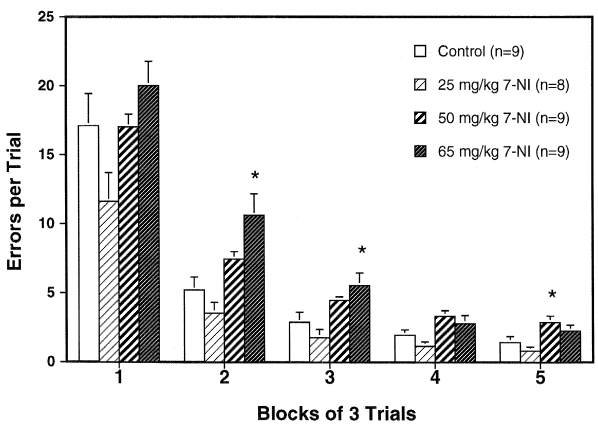

Fig. 1. Effects of 7-nitroindazole (7-NI) on mean (S.E.M.) number of errors (per block of 3 trials) during acquisition training in a 14-unit T-maze. * Significantly different from control group, $P < 0.05$.

Table 1
Effects of 7-nitroindazole (7-NI) on runtime (s) in the 14-unit T-maze

Group (n)	Runtime				
	block 1	block 2	block 3	block 4	block 5
Mean values ± S.E.M.					
Control (9)	153.47 ± 22.59	47.07 ± 6.78	26.91 ± 3.76	19.91 ± 3.67	16.25 ± 2.51
7-NI 25 mg/kg (8)	88.24 ± 13.6	40.18 ± 6.98	23.74 ± 3.54	16.02 ± 2.28	15.39 ± 1.95
7-NI 50 mg/kg (9)	135.78 ± 18.31	78.31 ± 11.62[a]	49.25 ± 6.81[a]	37.59 ± 3.39[a]	33.5 ± 3.99[a]
7-NI 65 mg/kg (9)	199.44 ± 23.46	100.72 ± 14.07[a]	59.49 ± 11.03[a]	38.34 ± 6.87[a]	25.85 ± 4.76

[a]Significantly different from control.

ANOVA on errors yielded significant main effects of both group, $F(3,31) = 11.25$, $P < 0.001$ and block, $F(4,124) = 153.78$, $P < 0.001$. One-way ANOVAs were conducted on each block, and comparisons between controls and drug-treated groups were assessed using Dunnett's procedure. During block 1, none of the groups given 7-nitroindazole differed significantly from controls, $P > 0.05$. At blocks 2 and 3, however, the group receiving 65 mg/kg of 7-nitroindazole was making significantly more errors than controls, $P < 0.05$. During blocks 4 and 5, rats given either 50 mg/kg or 65 mg/kg of 7-nitroindazole were still making nearly twice as many errors as controls, but these comparisons failed to reach statistical significance with the exception of those in block 5, in which the 7-nitroindazole 50 mg/kg group was significantly impaired relative to controls, $P < 0.05$. Although it appears that rats given 25 mg/kg of 7-nitroindazole made fewer errors than controls at each block, these comparisons did not reach statistical significance, $P > 0.05$. The enhanced performance displayed by this group is consistent with the link between NMDA receptor activation and NO production (Garthwaite et al., 1989), and with observations that very low doses of dizocilpine, a noncompetitive NMDA receptor antagonist, has facilitatory effects on learning whereas higher doses impair learning (Mondadori et al., 1989; Mondadori and Weiskrantz, 1993).

The mean (± S.E.M.) time (s) for each group of rats to navigate the maze is shown in Table 1. Each value reflects the mean (± S.E.M.) of 3 massed trials. A 4 (groups) × 5 (blocks) ANOVA of runtime revealed significant main effects of both group, $F(3,31) = 7.96$, $P < 0.001$, and block, $F(4,124) = 114.7$, $P < 0.001$. Consistent with the data on errors, no groups treated with 7-nitroindazole differed significantly from controls on block 1, $P > 0.05$. Groups given 50 and 65 mg/kg of 7-nitroindazole were significantly slower than controls in navigating the maze throughout the remaining blocks, with the exception of block 5, where only the 7-nitroindazole 50 mg/kg group was significantly slower than controls, $P < 0.05$.

The mean (± S.E.M.) shock duration and shock frequency are shown in Table 2. Data from these measures were averaged across the 5 blocks and analyzed by one-way ANOVA followed by Dunnett's tests between groups treated with 7-nitroindazole and controls. Although the mean for the 7-nitroindazole 65 mg/kg group was nearly twice that of controls, no significant differences in shock duration were found between any of the rats treated with

Table 2
Effects of 7-nitroindazole (7-NI) on shock duration and shock frequency in the 14-unit T-maze

Group (n)	Shock duration, blocks averaged	Shock frequency, blocks averaged
Mean values ± S.E.M.		
Control (9)	22.23 ± 5.1	1.52 ± 0.14
7-NI 25 mg/kg (8)	12.84 ± 4.39	1.43 ± 0.21
7-NI 50 mg/kg (9)	27.73 ± 6.48	2.54 ± 0.19[a]
7-NI 65 mg/kg (9)	41.71 ± 6.82	2.17 ± 0.12[a]

[a]Significantly different from control.

Table 3
Effects of 7-nitroindazole (7-NI) on systolic blood pressure

Group (n)	Systolic blood pressure (mean percent of baseline)		
	time (min)		
	1	5	15
Mean values ± S.E.M.			
Control (3)	95.67 ± 1.45	98.33 ± 0.33	104.33 ± 3.18
7-NI 65 mg/kg (6)	101.33 ± 0.95	103.33 ± 1.2	101 ± 2.1

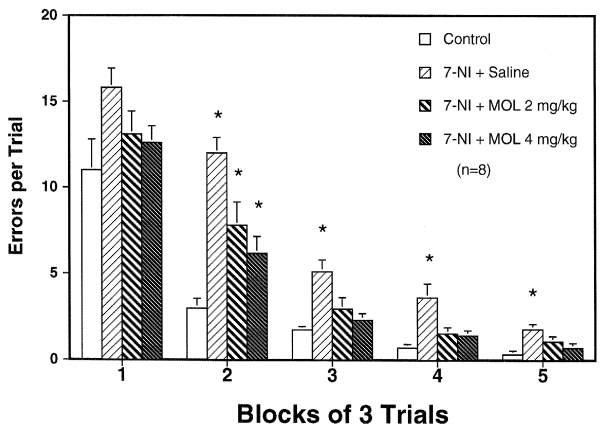

Fig. 2. Effects of 7-nitroindazole (7-NI, 70 mg/kg) and molsidomine (MOL) on mean (S.E.M.) number of errors (per block of 3 trials) during acquisition training in a 14-unit T-maze. * Significantly different from control group, $P < 0.05$.

Table 4
Effects of 7-nitroindazole (7-NI), in combination with molsidomine (MOL), on runtime (s) in the 14-unit T-maze

Group (*n*)	Runtime				
	block 1	block 2	block 3	block 4	block 5
Mean values ± S.E.M.					
Control (8)	70.52 ± 8.62	25.42 ± 4.01	17.6 ± 1.38	14.04 ± 2.06	9.66 ± 2.21
7-NI (8)	171.11 ± 30.29[a]	104.29 ± 17.19[a]	51.7 ± 9.14[a]	31.43 ± 4.82[a]	22.36 ± 3.38[a]
7-NI + MOL 2 mg/kg (8)	96.91 ± 14.18	57.55 ± 9.32[a]	29.6 ± 4.86	16.87 ± 3.06	15.62 ± 3.58
7-NI + MOL 4 mg/kg (8)	97.05 ± 10.53	57.74 ± 7.84[a]	28.44 ± 3.55	19.51 ± 2.93	12.31 ± 2.28

[a]Significantly different from control.

Table 5
Effects of 7-nitroindazole (7-NI), in combination with molsidomine (MOL), on shock frequency in the 14-unit T-maze

Group (*n*)	Shock duration					Shock frequency
	block 1	block 2	block 3	block 4	block 5	blocks averaged
Mean values ± S.E.M.						
Control (8)	26.4 ± 6.42	5.87 ± 1.37	2.42 ± 0.84	0.83 ± 0.39	0.25 ± 0.16	1.28 ± 0.15
7-NI (8)	120.81 ± 31.12[a]	68.71 ± 17.64[a]	20.83 ± 6.45[a]	8.79 ± 2.33[a]	4.54 ± 1.3[a]	2.69 ± 0.21[a]
7-NI + MOL 2 mg/kg (8)	53.08 ± 13.74	30.54 ± 5.39	13.21 ± 4.3	4.04 ± 1.53	1.92 ± 0.9	2.12 ± 0.21[a]
7-NI + MOL 4 mg/kg (8)	51.17 ± 10.6	28.89 ± 6.88	7.83 ± 2.09	3.42 ± 1.5	1.46 ± 0.76	1.94 ± 0.19[a]

[a]Significantly different from control.

7-nitroindazole and controls, $P > 0.05$. However, rats given either the 50 mg/kg or 65 mg/kg dose of 7-nitroindazole received a significantly higher frequency of shocks relative to controls, $P < 0.05$.

3.2. Blood pressure measurement

The mean (±S.E.M.) systolic blood pressure readings (converted to percent-of-baseline) obtained from rats given either saline or 65 mg/kg of 7-nitroindazole are shown in Table 3. ANOVA of these readings yielded no significant main effects of either group, $F(1,7) = 5.3$, $P > 0.05$, nor time, $F(2,14) = 2.13$, $P > 0.05$.

3.3. 7-Nitroindazole plus molsidomine

As shown in Fig. 2, treatment with 70 mg/kg of 7-nitroindazole increased errors in the maze, and molsidomine markedly attenuated this learning deficit. A 4 (groups) × 5 (blocks) ANOVA revealed significant main effects of both group, $F(3,28) = 14.24$, $P < 0.001$, and block, $F(4,112) = 178.04$, $P < 0.001$. No significant differences were observed between any groups on the first block of trials. At blocks 2 through 5, the 7-nitroindazole group made significantly more errors than controls, $P < 0.05$. In contrast, groups receiving 7-nitroindazole plus molsidomine (2 mg/kg or 4 mg/kg) made significantly more errors than controls only on block 2, $P < 0.05$. During the last 3 blocks of trials, the 7-nitroindazole plus molsidomine groups did not differ significantly from controls, $P > 0.05$.

The mean (±S.E.M.) time (s) for each group of animals to navigate the maze is shown in Table 4. Each value reflects the mean (±S.E.M.) of 3 consecutive trials. A 4 (groups) × 5 (blocks) ANOVA on runtime yielded significant main effects of group, $F(3,28) = 10.4$, $P < 0.001$, and block, $F(4,112) = 82.97$, $P < 0.001$. The 7-nitroindazole group was significantly impaired in maze runtime relative to controls on all 5 blocks of trials, $P < 0.05$. Groups receiving 7-nitroindazole plus molsidomine (2 or 4 mg/kg) had significantly longer runtimes than controls on block 2, $P < 0.05$, but were not significantly different from controls on blocks 3–5, $P > 0.05$.

The mean (±S.E.M.) shock duration and shock frequency are shown in Table 5, and these data are similar to the pattern of results observed for errors and runtime. ANOVA of shock duration yielded significant main effects of both group, $F(3,28) = 6.65$, $P < 0.01$, and block, $F(4,112) = 40.98$, $P < 0.001$. The 7-nitroindazole group received significantly more shocks than controls on all 5 blocks of trials, $P < 0.05$, whereas rats receiving 7-nitroindazole plus molsidomine (2 or 4 mg/kg) were not significantly different from controls at any block of trials, $P > 0.05$. The number of shocks received by each group during training in the 14-unit T-maze were averaged across the 5 blocks of trials (Table 5). Groups receiving either 7-nitroindazole, 7-nitroindazole plus molsidomine (2 mg/kg), or 7-nitroindazole plus molsidomine (4 mg/kg) received significantly more shocks than controls, $P < 0.05$.

4. Discussion

7-Nitroindazole impaired maze performance in a dose-related manner on all four behavioral measures without producing hypertensive effects. Thus, the deficit in maze acquisition likely reflects an impairment in learning pro-

duced by inhibiting neuronal NO synthase. Furthermore, systemic administration of the NO donor, molsidomine, significantly attenuated the learning deficit at very low doses (2 or 4 mg/kg). To our knowledge, this is the first behavioral study to demonstrate that a learning deficit produced by selective inhibition of neuronal NO synthase can be overcome by a NO donor, thus providing evidence for specificity of the effect.

Nonspecific NO synthase inhibitors (e.g., NAME or *N*-Arg) produce marked hypertension following systemic administration (Kelly et al., 1995; Swislocki et al., 1995; Granger et al., 1996). Thus, it difficult to rule out the possibility of cardiovascular effects contributing to the performance deficits observed in laboratory animals treated with these compounds. Bannerman et al. (1994) reported an early acquisition deficit in water maze performance of rats treated with NAME; however, in follow-up experiments NAME did not disrupt learning in previously tested rats that were retrained in a water maze with entirely different cues. The authors concluded that the original acquisition deficit did not necessarily reflect a learning impairment, but rather was the result of noncognitive effects of NAME, such as cardiovascular compromise or perhaps even an aversive psychological state that distracted the animal from the motivational component of the task (also see Sandi et al., 1995). The latter findings are in contrast to a study by Estall et al. (1993), in which NAME-treated rats were severely impaired during the initial acquisition trials in a water maze, but did not exhibit any sensorimotor or motivational deficiencies.

In two experiments reported previously using the 14-unit T-maze (Ingram et al., 1997a,b), it is unlikely that the *N*-Arg induced learning deficits observed were caused by noncognitive factors, since performance in retention and rotarod tests was not disrupted by the same dose of *N*-Arg that impaired maze acquisition. Furthermore, in both of the previous experiments as well as in the present study, no significant differences were found during the first block of trials in the number of errors made between any of the groups treated with a NO synthase inhibitor. However, given that *N*-Arg produces marked effects on vascular function, and since our previous behavioral findings are still subject to this alternative interpretation, it was important to begin to investigate NO synthase inhibitors which are more specific for neuronal NO synthase. This was the rationale of the present study in which we used 7-nitroindazole. It would be premature to conclude that NO is specifically involved with memory formation based upon results using nonspecific NO synthase inhibitors (i.e. NAME and *N*-Arg) that are known to profoundly affect cardiovascular function.

7-Nitroindazole has consistently been reported to be devoid of hypertensive effects when blood pressure is measured peripherally (Moore et al., 1993; Beierwaltes, 1995; Kelly et al., 1995; Vaupel et al., 1995). Recent evidence suggests that central blood pressure is likewise unaffected by 7-nitroindazole (Pajewski et al., 1996; Montecot et al., 1997). Several newer compounds are more potent, more soluble, and even more specific for neuronal NO synthase than 7-nitroindazole (Handy et al., 1995). We have begun testing these compounds in our laboratory.

Despite the increased availability of neuronal NO synthase specific inhibitors for use in learning and memory experiments, it should be acknowledged that recent evidence has suggested that neuronal NO synthase may not be the only isoform of the enzyme that is involved in memory formation. In a study by Son et al. (1996) using mice with targeted deletions of endothelial NO synthase, neuronal NO synthase, or both endothelial and neuronal NO synthase, long-term potentiation in field CA1 of the hippocampus was reduced only when both forms of NO synthase were deleted. Thus, endothelial and neuronal NO synthase appear capable of substituting for each other so far as establishing long-term potentiation, but the precise mechanism and the functional degree to which endothelial NO synthase can compensate for neuronal NO synthase are unclear.

Our findings in young rats treated with molsidomine suggest that this drug may serve as a useful therapeutic agent for elevating neuronal levels of NO in brains of aged animals. Evidence already exists that molsidomine increases brain glutamate levels in vivo. Specifically, Segovia et al. (1994), infused molsidomine into the striatum and hippocampus (area CA1) of conscious rats and observed a significant increase in glutamate levels. Molsidomine also appears to be quite potent, as effective doses were in the range of 2–4 mg/kg in the present study. In addition, molsidomine is easily absorbed and can be administered orally. Given these qualities, and lacking any deleterious side-effects, molsidomine appears to be a suitable candidate for administration to aged animals, and could prove to be a valuable tool to investigate further the role of NO in age-related cognitive decline. Currently, our laboratory is conducting behavioral studies involving both acute and chronic administration of molsidomine to aged animals.

In conclusion, our results lend support to previous studies that documented learning deficits induced by either NAME (Chapman et al., 1992; Estall et al., 1993) or *N*-Arg (Papa et al., 1994; Robertson et al., 1994). Moreover, the present study provides more direct evidence that neuronally generated NO is involved in the formation of new memories. Future experiments in our laboratory will be aimed at investigating alterations in NO production, that may be correlated with age-related learning deficits in the 14-unit T-maze (Ingram et al., 1996).

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