Nitric Oxide but Not Carbon Monoxide Is Involved in Spatial Learning of Mice

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ABSTRACT—The aim of the present study was to elucidate the role of carbon monoxide (CO) in learning and to compare it with that of nitric oxide (NO). Effects of an inhibitor of heme oxygenase which produces CO, Zn-protoporphyrin IX, on passive avoidance learning and spatial learning in mice were examined using step through, step down and water maze tests. Zn-protoporphyrin IX (10, 20 nmol, i.c.v.) affected neither type of learning. In contrast, *N*-ω-nitro-L-arginine (40 nmol, i.c.v.), an inhibitor of NO synthase, impaired spatial learning, but not passive avoidance learning. These results suggest that NO but not CO is involved in spatial learning.

Keywords: Carbon monoxide, Zn-protoporphyrin IX, Spatial learning, Passive avoidance learning, Nitric oxide

Long-term potentiation (LTP) in the hippocampus is a good model of synaptic plasticity and is widely believed to correlate with learning and memory (1). LTP is triggered by activation of postsynaptic *N*-methyl-D-aspartate (NMDA) receptors, and its maintenance requires both presynaptic and postsynaptic alternations (2), indicating that a retrograde messenger must be sent from the postsynaptic neuron to the presynaptic terminals. Nitric oxide (NO) is a good candidate for such a messenger (3), supported by many findings that inhibitors of NO synthase block the induction of hippocampal LTP (4–8) and impair some types of learning (9–11). NO, formed from L-arginine by NO synthase, is a short-lived free radical gas that can activate guanylyl cyclase. In 1993, Verma et al. (12) proposed that carbon monoxide (CO) is also a retrograde messenger that activates guanylyl cyclase. CO is produced by heme oxygenase through the metabolism of heme. Recent evidence suggests that CO is involved in the generation of hippocampal LTP (13–15). However, the association of CO and learning has not yet been fully elucidated. Therefore, we investigated the effects of Zn-protoporphyrin IX, an inhibitor of heme oxygenase, on passive avoidance learning (step through test, step down test) and spatial learning (water maze test) in mice. As a comparison, we also tested the effects of *N*-ω-nitro-L-arginine, an inhibitor of NO synthase.

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MATERIALS AND METHODS

Animals and surgery

Male ddY mice, 7- to 8-weeks-old, were purchased from Japan SLC (Hamamatsu). Each mouse was anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and fixed in a stereotaxic instrument. A stainless steel cylindrical cannula (0.6 mm o.d., 0.35 mm i.d., 5.0-mm-long) was implanted so that the tip of the cannula was placed in the left lateral ventricle (1.3 mm lateral to the midline, 0.3 mm posterior to the bregma, 2.0 mm ventral to the dura). The implanted cannula was fixed to the skull with a screw and dental acrylic cement and plugged with a stainless steel wire pin. The cannula served as a guide for i.c.v. injection of drug solutions. The operated mice were allowed 7–10 days to recover from the surgery. All mice were housed individually under conditions of controlled temperature and humidity (22℃, 55%) with ad libitum access to food and water. Body weights were monitored every day. The mice in the intact group were not subjected to the operation but were kept under the same conditions.

Drugs

The drugs used in this study were Zn-protoporphyrin IX (ZnPP; Aldrich Chemical Co., Milwaukee, WI, USA) and *N*-ω-nitro-L-arginine (L-NOArg; Sigma Chemical Co., St. Louis, MO, USA). ZnPP was diluted to the

desired concentrations with dimethyl sulfoxide (DMSO), and 0.5 μl of the drug solution was injected into the left lateral ventricle. L-NOArg was diluted with saline and a volume of 5.0 μl was injected. A stainless steel tube (0.35 mm o.d., 0.15 mm i.d.) was used to inject the drugs. The injection tube, the tip of which protruded 0.5 mm below the tip of the guide cannula, was connected to a Hamilton syringe via a polyethylene tube. The injection time was about 30 sec for 0.5 μl and 90 sec for 5.0 μl.

Passive avoidance learning

Step through test: The apparatus (Model PA-M1; O'hara Co., Ltd., Tokyo) consisted of a lit compartment and a dark compartment with a electrifiable grid floor. The two compartments were separated by a black partition with a rectangular doorway in the center. For the learning trial, a mouse was placed in the lit compartment 30–60 min after the i.c.v. injection of either drug or vehicle. The latency before entering the dark compartment was recorded. When the mouse entered into the dark compartment and crossed an infrared beam placed 5 cm from the border, it received a 36 V AC footshock until it returned to the lit compartment. The mouse that received the shock was removed immediately so that it did not reenter the dark compartment. The testing trial was performed 24 hr later. The mouse was put into the lit compartment again. If the mouse did not enter the dark compartment within 300 sec, the test was terminated and a latency of 300 sec was recorded.

Step down test: The apparatus was a rectangular box (10 × 15 × 40 cm high) with an electrifiable grid floor and a rubber columnar platform (diameter: 3.5 cm, height: 4.0 cm) in one corner. For the learning trial, a mouse was placed on the platform 30–60 min after the i.c.v. injection of either drug or vehicle. When the mouse stepped onto the floor and received a 60 V AC footshock, it was countered as an error. The mouse was exposed to this condition for 10 min. The number of errors in the latter half of the 10 min was counted. The mouse was again placed on the platform 24 hr later, and the number of errors was counted for 3 min as the testing trial. The step down test was performed soon after the step through test.

Motor activity

Just before the passive avoidance tests, a mouse was put into a round tilting-type apparatus (Model GT-8450; O'hara; diameter: 18 cm, height: 18 cm), and the amount of motor activity was measured over a 30-min period.

Spatial learning

Morris-type water maze test: A circular blue pool (diameter: 74 cm, depth: 32 cm) was filled with water so that water depth was 27 cm. The temperature of the water was maintained at 18±1℃. The pool was surrounded by various prominent cues that remained throughout the experiment. Regions (1–4) and start positions (I and II) of the pool were decided as described in Fig. 4c. The movement of each mouse in the pool was recorded with a video camera, and a computerized tracking and analyzing system was used. On the day before the start of experiment, a mouse was placed in the pool and allowed to swim freely for 90 sec without a platform. On day 0, a circular black platform (visible platform, diameter: 10 cm) was placed in region 1, and its top surface was 0.5 cm above the water level. The mouse was placed into the water facing the wall at start position I. The mouse was allowed for a maximum of 90 sec to find the platform to escape from the water. If the mouse could not escape within 90 sec, it was picked up and placed on the platform. In any case, it was allowed to stay there for exactly 30 sec. On day 1 to day 6, a circular transparent platform (invisible platform, diameter: 10 cm) was placed in region 1, and its top surface was 0.5 cm below the water level. Two trials were conducted in a day at an interval of 5 min. As the first trial of the day, the start position of I or II was randomly chosen; and for the second trial, the other start position was employed. Other procedures were the same as day 0. On the final day (day 7), each mouse was placed in the pool at start position I without the platform, and its swimming pattern was recorded for 90 sec. On each day (day 0–day 7), the mice other than the intact group received an i.c.v. injection of vehicle or drug solution 30–60 min before the trials.

Statistics

The latencies in the step through test were analyzed by Dunnett's test. For all the other data, ANOVA followed by Duncan's multiple range test was employed.

RESULTS

Body weight

The operation decreased the body weights of almost all the mice, but by 7 to 10 days after the surgery, their body weights had recovered. A single injection of any drug did not affect the body weight measured on the next day. The mice used for the water maze test received either vehicle or the drugs for eight days, and we weighed all the mice every day. However, there were no significant differences among the groups, suggesting that the daily i.c.v. injection of ZnPP or L-NOArg did not affect the increasing rate of body weight (data not shown).

Motor activity

During the passive avoidance tests, motor acivity was measured with a tilting type ambulometor for 30 min just

before the learning and testing trials. Neither ZnPP nor L-NOArg influenced the motor activity (Fig. 1).

Passive avoidance learning (step through test and step down test)

To investigate the effects of drugs on the acquisition of passive avoidance learning, we administered drugs 30 to 60 min before the learning trial.

Step through test: Almost all the mice in any group learned the test very well and did not enter the dark compartment within 300 sec on the learning trial, although all the mice entered on the testing trial. Neither ZnPP nor L-NOArg affected the latencies to enter the dark compartment (Figs. 2a and 3a).

Step down test: The number of errors on the testing trial in the 10 nmol ZnPP-treated group (3.8 ± 0.7, $n=6$) was higher than that in the intact (1.1 ± 0.5, $n=10$) and vehicle-treated groups (1.7 ± 0.2, $n=7$), but 20 nmol ZnPP had no effect (1.3 ± 0.6, $n=6$) (Fig. 2b). We conducted an additional experiment in which the effects of 10 and 100 nmol ZnPP were tested. However, there was no significant difference among intact-, vehicle- and ZnPP-treated groups (data not shown). On the other hand, L-NOArg tended to increase the number of errors in the

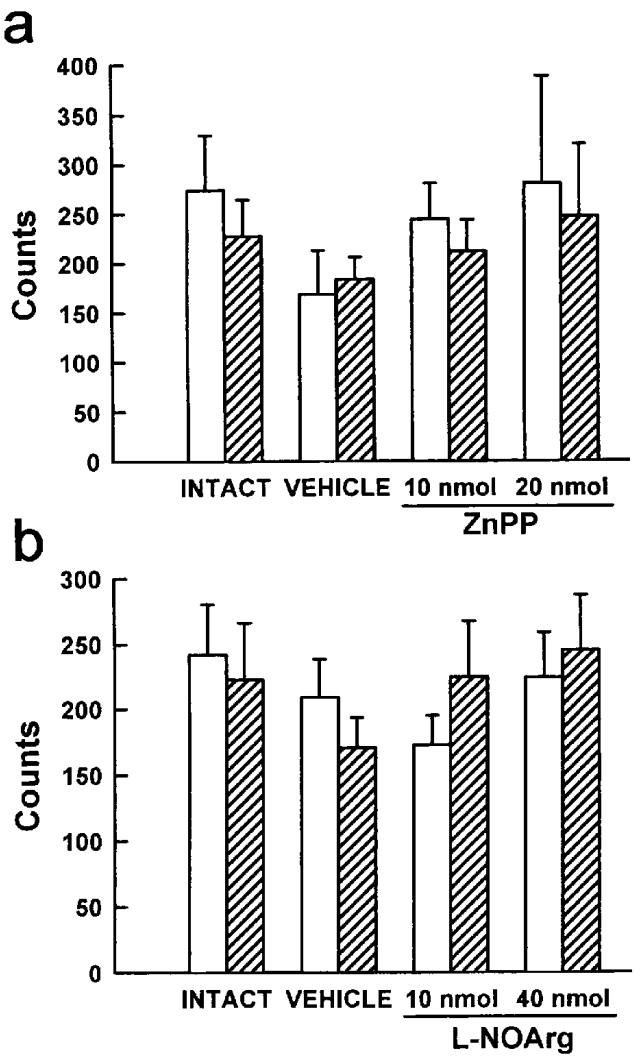

Fig. 1. Effects of ZnPP (a) and L-NOArg (b) on motor activity. Motor activities were measured for 30 min before the learning trial (immediately after the drug injection, □) and before the testing trial without drug injection (▨). Each value represents the mean±S.E.M. from 6 to 11 animals.

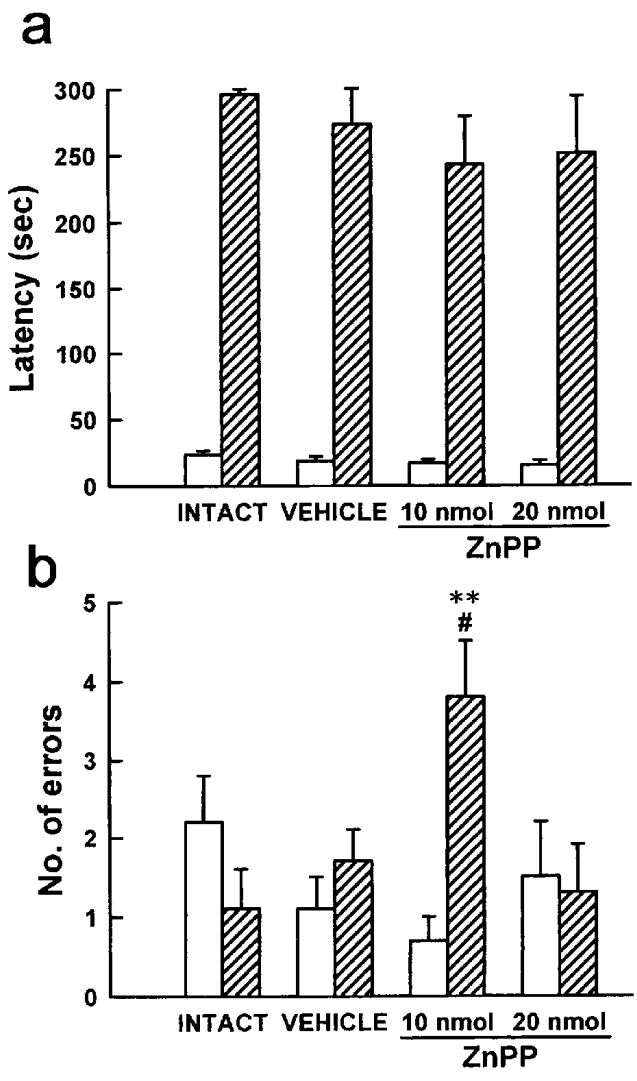

Fig. 2. Effects of ZnPP on passive avoidance learning. Open and hatched column represent the learning (□) and testing trial (▨), respectively. a: the latency for mice to enter the dark compartment in the step through test, b: number of stepping downs on the floor (errors) in the step down test. Except for the intact group ($n=10$), vehicle ($n=7$), 10 nmol ($n=6$) or 20 nmol ZnPP ($n=6$) was injected 30 min before the learning trial. Each value represents the mean±S.E.M. from 6 to 10 animals. $^{**}P<0.01$ vs intact group, $^{\#}P<0.05$ vs vehicle-treated group by Duncan's multiple range test.

testing trial dose-dependently (intact: 0.4±0.3, n=9; vehicle: 0.7±0.2, n=11; 10 nmol L-NOArg: 1.0±0.4, n=10; 40 nmol L-NOArg: 1.4±0.3, n=11), but this effect did not reach statistical significance (Fig. 3b).

Spatial learning (water maze test)

Free swimming for 90 sec on the day before the start of the experiment was conducted to allow the mice to become accustomed to water. All mice could swim well with the characteristic swimming posture.

In the visible platform task on day 0, there was no significant difference in escape latency among the groups (data not shown), suggesting that no treatment caused gross sensorimotor disturbance.

In the invisible platform task on day 1 to day 6, ZnPP-treated groups learned to escape onto the platform as rapidly as the intact and vehicle-treated groups (Fig. 4a). In contrast, the 40 nmol L-NOArg-treated group learned a little more slowly; especially, on day 4, they took a significantly longer time to escape (54.3±15.0, $P<0.01$ vs intact group; $P<0.05$ vs vehicle-treated group, n=9) than the intact (15.7±3.2, n=10) and vehicle-treated groups (28.4±6.4, n=11) (Fig. 5a). A significant effect of 40 nmol L-NOArg was also observed when the total latency of day 1 to day 6 were compared ($P<0.01$ vs intact group) (Fig. 5b). However, the total latency was not modified by ZnPP (Fig. 4b).

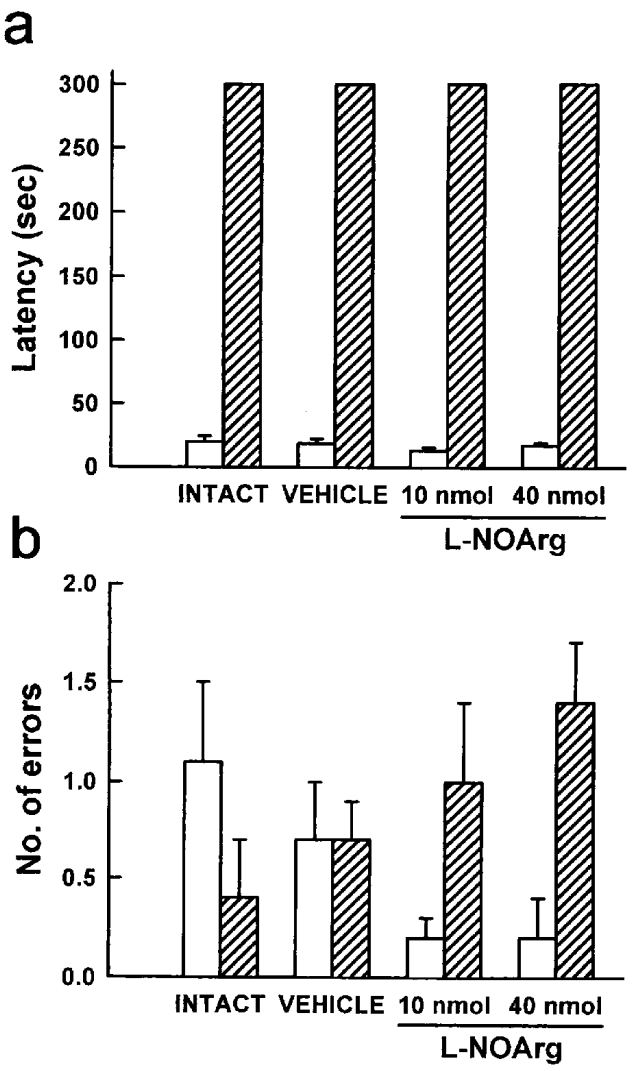

Fig. 3. Effects of L-NOArg on passive avoidance learning. Except for the intact group (n=9), vehicle (n=11), 10 nmol (n=10) or 40 nmol L-NOArg (n=11) was injected 30 min before the learning trial. For other explanations, see Fig. 2.

Memory retention of the platform location was assessed in the no platform task on day 7. Mice of all groups crossed region 1 of the pool more frequently where the platform had been located, suggesting that they remembered the former location of the platform, but the groups differed with respect to the extent of learning. The number of crossings of region 1 in the 10 nmol ZnPP-treated group (5.4±1.2, n=7) was slightly less than those of the intact (7.7±1.2, n=10) and vehicle-treated groups (7.0±1.0, n=7), but 20 nmol ZnPP had no significant effect (7.8±1.3, n=8) (Fig. 4c). In addition, no significant difference was observed in the invisible platform task. ZnPP, therefore, appeared to have no significant effect on the water maze test. On the contrary, i.c.v. injection of L-NOArg decreased the number of crossings of region 1 dose-dependently; and the 40 nmol L-NOArg-treated group crossed the four regions almost equally (region 1: 5.1±0.7, region 2: 5.0±0.8, region 3: 3.8±0.6, region 4: 4.7±0.7, n=9) (Fig. 5c), although they escaped to the platform as fast as the mice in the other groups on the last day of the invisible platform task, day 6. This indicates that the apparent learning level of the 40 nmol L-NOArg-treated group was lower than those of the intact and vehicle-treated groups.

DISCUSSION

In the present study, L-NOArg did not impair the acquisition of passive avoidance learning in both step through and step down tests. Although 10 nmol ZnPP appeared to impair acquisition of the learning in the step down test, 20 nmol ZnPP had no effect; and moreover, we found no significant difference in the additional experiment. Therefore, it is unlikely that this 10 nmol ZnPP effect was really due to the drug action of ZnPP itself, and we concluded that ZnPP does not affect passive avoidance learning.

The generation of hippocampal LTP in vivo was attenuated by ZnPP (15) or L-NOArg (8) administered 30 min before tetanic stimulation. If the mechanism of hippocampal LTP is necessary for the formation of passive

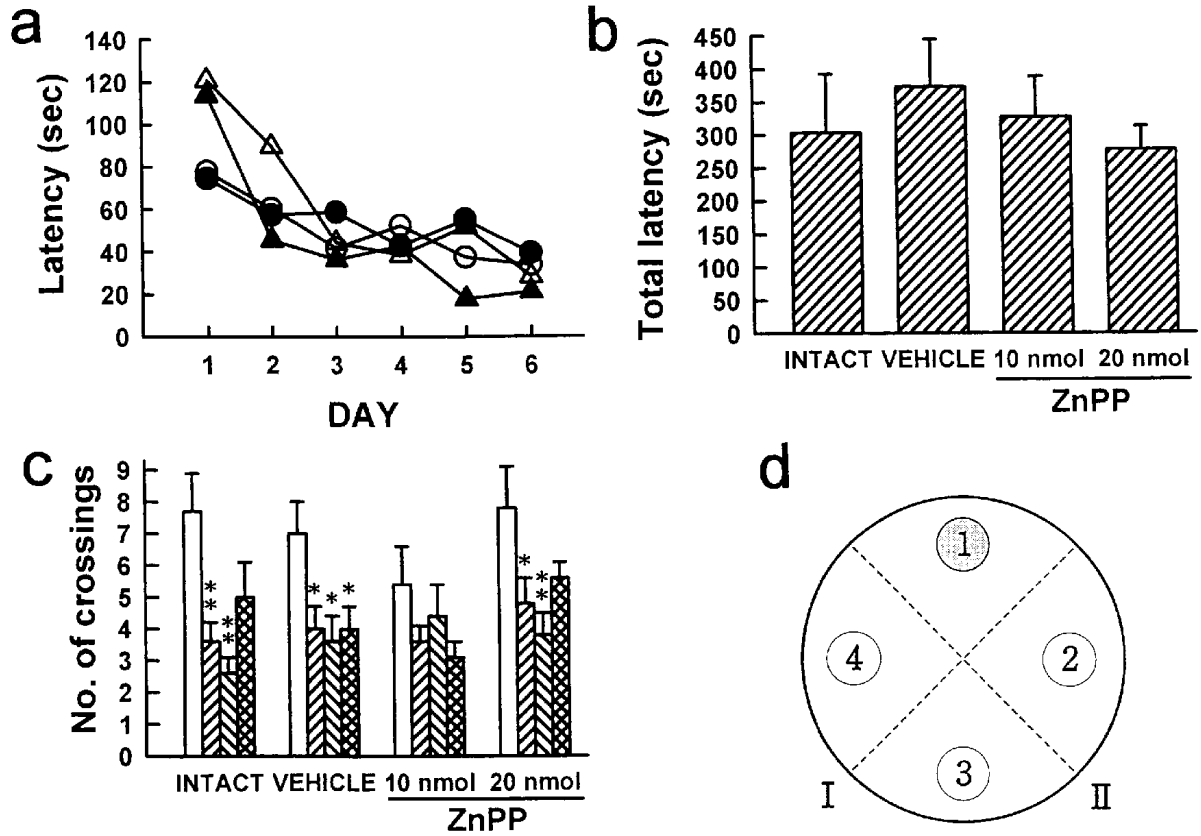

Fig. 4. Effects of ZnPP on spatial learning in the water maze test. a: sum of escape latency of two trials per day in locating the invisible platform on day 1 to day 6; (○) intact, n=10; (△) vehicle-treated, n=7; (●) 10 nmol ZnPP-treated, n=7; (▲) 20 nmol ZnPP-treated group, n=8. For clarity, only mean values are shown. b: the total escape latency for six days in the invisible platform task. Each value represents a mean±S.E.M. c: the number of crossings of each region, 1–4, on the last day, day 7, in the no platform task; (▯) region 1, (▨) region 2, (▧) region 3 and (▩) region 4. Each value represents a mean±S.E.M. *$P<0.05$, **$P<0.01$ vs region 1 of each group by Duncan's multiple range test. d: the pool for the water maze test. A platform was placed in region 1. Start positions I and II where a mouse was placed into the water facing the wall were as described above.

avoidance learning, ZnPP or L-NOArg administered 30 min before the learning trial should inhibit acquisition of the learning. However, the results were negative, suggesting that there is little relation between hippocampal LTP and passive avoidance learning.

On the other hand, much evidence based on comparable effects of drugs on LTP and learning supports the relationship between hippocampal LTP and spatial learning. For example, the induction of LTP requires the activation of NMDA receptors (16), and the NMDA antagonist D-AP5 impairs spatial learning at a dose comparable to that inhibiting of hippocampal LTP in vivo (17). Moreover, transgenic alterations of the expression of key proteins in the LTP process such as *fyn* tyrosine kinase (18) also lead to concomitant impairment of LTP and deficit of spatial learning. Similarly, several studies have shown that inhibitors of NO synthase block the induction of hippocampal LTP both in vitro (4–7) and in vivo (8), and they impair certain forms of learning including spatial learning (9, 10). The inhibitors of heme oxygenase also prevent the induction of LTP in the CA1 region of hippocampal slices (13, 14) and in the dentate gyrus in vivo (15). Under the hypothesis that hippocampal LTP and spatial learning share a common underlying mechanism, therefore, ZnPP as well as L-NOArg may impair spatial learning. Heme oxygenase-2, the predominant form of heme oxygenase in the brain, is highly expressed in hippocampal CA1 pyramidal cells (12) and the dose of ZnPP used here is assumed to be sufficient to inhibit heme oxygenase fully in the brain (12, 13). Actually, we showed that i.c.v. injection of ZnPP attenuated the induction of LTP (15) under experimental conditions similar to those employed in the present study. In the present study, however, L-NOArg but not ZnPP impaired spatial learning.

It may be concluded from these results that NO but not CO is necessary for spatial learning. CO may be involved in spatial learning, but without CO, NO would be able to

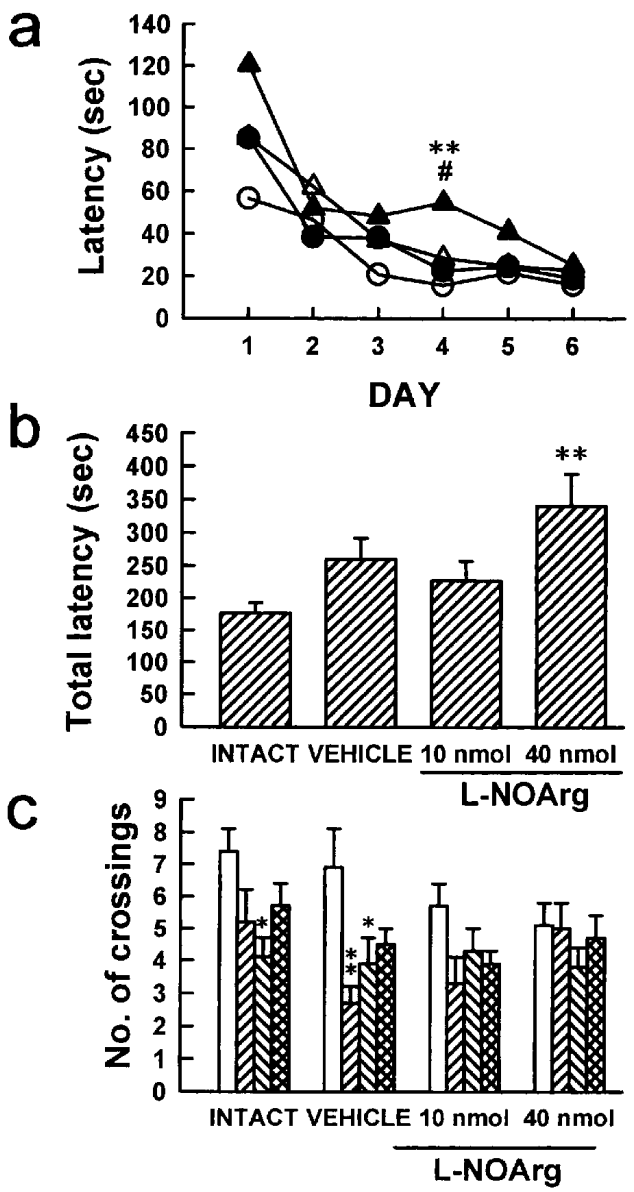

Fig. 5. Effects of L-NOArg on spatial learning in the water maze test. a: (○) intact, n=10; (△) vehicle-treated, n=11; (●) 10 nmol L-NOArg-treated, n=10; (▲) 40 nmol L-NOArg-treated group, n=9. **P<0.01 vs intact group, #P<0.05 vs vehicle-treated group on each day by Duncan's multiple range test. b: **P<0.01 vs intact group by Duncan's multiple range test. Other explanations are the same as in the legend of Fig. 4.

activate guanylyl cyclase in compensation for CO and support spatial learning. In spatial learning, therefore, the necessity of NO would be higher than that of CO.

The present results that L-NOArg impaired spatial learning but not acquisition of passive avoidance learning in mice confirmed the previous report in rats (9). This suggests that NO is involved in some but not all forms of memory formation and that there is distinction between processes of spatial learning and passive avoidance learning. It remained to be proved whether the impairment of spatial learning by L-NOArg is really due to the inhibition of NO production. To clarify this point, more direct evidence would be required; e.g., no impairment of spatial learning by D-NOArg, reversal of the L-NOArg-induced-impairment with coadministration of L-arginine, impairment by other NO synthase inhibitors. Recently, Salter et al. (19) have reported that hippocampal NO synthase activity in rats was significantly lowered 30 min after i.c.v. administration of L-NOArg. Comparing our conditions with those of this report, the dose used in present study seems to be sufficient to inhibit hippocampal NO synthase in mice. The impairment by L-NOArg is unlikely to result from a nonspecific performance deficit because L-NOArg did not influence body weights and motor activity of mice, and the 40 nmol L-NOArg-treated group finally reached the same latency level as the other groups on day 6.

We could not find how CO is related to learning in these experiments, in spite of the advocacy of CO in addition to NO as retrograde messengers in the brain (20, 21) and the recent report that bilateral intrahippocampal infusion of ZnPP (2 μg/side) caused amnesia for the passive avoidance task in rats (22, 23). Although we cannot completely contradict the involvement of CO in LTP and learning, it appears that CO is less important for the memory formation of learning than NO and that CO is a less likely candidate for a retrograde messenger than NO.

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