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Activation of hippocampal nitric oxide and calcium/calmodulin-dependent protein kinase II in response to Morris water maze learning in rats

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ABSTRACT

This study investigates the interactive roles of nitric oxide (NO) and CaM-kinase II (calcium/calmodulin-dependent protein kinase II) in Morris water maze learning. In Experiment I, experimental rats received 5 days of training on a Morris water maze, where the controls were trained in the water maze with no spatial cue condition or were trained via a visually guided landmark condition. The experimental rats showed improvement in their rate of spatial learning in the water maze. The escape latencies were significantly correlated with the Ca2+-independent activity of the hippocampal CaM-kinase II. Moreover, there was a significant increase in the endogenous phosphorylation of neuronal NOS and CaM-kinase II in the experimental group when compared to the controls. The intra-hippocampal infusion of 7-NI, KN-93, or AP5 did disrupt water maze learning. SDS–PAGE analysis showed that these drugs significantly depressed phosphorylation of hippocampal NOS. The Ca2+-independent activity of hippocampal CaM-kinase II was significantly lower in the KN-93 or the AP5 infused group when compared to the controls. Although these depressed activities were not reversed by the infusion of NO donor (sodium nitroprusside, SNP), the rats' water maze learning behavior were ameliorated significantly. These results, taken together, indicate that the NOS activation is essential for water maze learning, which may be triggered via the CaM-kinase II activation in hippocampus.

1. Introduction

Nitric oxide (NO) plays an important role in various biological systems (Garthwaite, 1991), including a critical role in learning and memory processes (Schuman and Madison, 1991). NO is synthesized from L-arginine via an NADPH-dependent reaction by nitric oxide synthase (NOS) (Bellamy et al., 2002; Dawson et al., 1998) and is present in several brain areas, including cerebellum, cortex, nucleus accumbens, and hippocampus (De Vente et al., 1998; Grohe et al., 2004; Prast, 1997). In the central nervous system, NO is postulated to act as an intercellular signaling molecule (Snyder, 1991) and has been shown to modulate various neurotransmitter systems (for a review see: Prast and Philippu, 2001).

The respective functional roles of NO in cerebellum, hippocampus, and amygdala have been reported in numerous studies (Baratti and Boccia, 1999; Boxall and Garthwaite, 1996; Teledgy and Kokavszky, 1997). Many lines of evidence have shown that NOS is a calcium/calmodulin-dependent enzyme, and its activity may be triggered through glutamate receptor activation (Bredt and Snyder, 1989; Fedele and Raiteri, 1996; Stanton et al., 2003). Evidence also suggests that NO is produced postsynaptically and retrogradedly diffuses to act on the presynaptic cells, which in turn modulate the neurotransmitters' release (Hawkins et al., 1998; O'Dell et al., 1991). This process plays an important role in activity-dependent potentiation (Arancio et al., 2000; Ko and Kelly, 1999; Son et al., 1998; Susswein et al., 2004).

NO may underlie the mechanism of synaptic plasticity, including long-term potentiation (Bon and Garthwaite, 2003; Ko and Kelly, 1999; Lu et al., 1999; Schuman and Madison, 1991), as well as long-term depression (Shibuki and Okada, 1991; Stanton et al., 2003). The interactions between nitrergic and glutamatergic systems may play significant roles in these neuronal plasticities (Son et al., 1998; Susswein et al., 2004; Yamada and Nabeshima, 1997). Evidence shows the crucial role of NO activation in various behavioral learning tasks including spatial learning, olfactory learning, inhibitory avoidance learning, conditioned eye-blinking response, etc. (Baratti and Boccia, 1999; Böhme et al., 1993; Chapman et al., 1992; Schafe et al., 2005; Yildiz Akar et al., 2007).

NO production and the levels of NOS in hippocampus can be induced by the activation of *N*-methyl-D-aspartate (NMDA) receptors by glutamate (Prast and Philippu, 2001; Yamada and Nabeshima, 1997). Many lines of evidence convergently suggest a strong link between glutamate receptors and specific protein kinase activations in LTP induction (Rodrigues et al., 2004; Yang et al., 2004), as well as behavioral learning (Lepicard et al., 2006; Silva, 2003; Tan and Liang, 1996; Tan, 2007). One of these protein kinases is Calcium/calmodulin-dependent protein kinase II (CaM-kinase II), which is abundantly present in the excitatory synapses (Kennedy et al., 1983). CaM-kinase II has been widely implicated in synaptic plasticity (for review, see Fukunaga and Miyamoto, 2000; Lisman et al., 2002). The mechanism

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underlying LTP induction in hippocampus, as well as in many brain areas, requires activation of *N*-methyl-D-aspartate (NMDA) receptors, which in turn trigger CaM-kinase II (Rodrigues et al., 2004; Yang et al., 2004). In the absence of bound calcium/calmodulin, CaM-kinase II is in inactive conformation. The influx of calcium ions results in the CaM-kinase II activation. CaM-kinase II phosphorylates on its inhibitory domain, in which it stays activated even after the calcium efflux.

Activated CaM-kinase II may serve as a link between the induction and expression of LTP as well as behavioral learning (Hudmon and Schulman, 2002; Mullasseril et al., 2007; Silva, 2003; Tan and Liang, 1996; Tan, 2007). Alpha-CaM-kinase II knock-out mouse studies clearly demonstrate its prominent role in hippocampal LTP and hippocampus-dependent behavioral learning (Mayford et al., 1996). It is worth noting that neuronal NOS can be regulated via phosphorylation by CaM-kinase II (Agostino et al., 2004; Fleming et al., 2001; Komeima et al., 2000). Therefore, this study examines the role of NO in spatial memory by directly infusing a specific NOS inhibitor and CaM-kinase II inhibitor in the hippocampus by using the paradigm of the Morris water maze learning task. The purpose of the experiments is to determine the interacting effects of NO and CaM-kinase II in rats' spatial memory when they undergo Morris water maze learning.

2. Experimental procedure

2.1. Subjects

245 male Sprague–Dawley rats (200–250 g) were used. They were housed in the animal center and maintained on a 12:12 h light/dark cycle with light on at 7:00 h. The care of the animals and the entire experimental procedure were in accordance with the Codes for Experimental Use of Animals based on Animal Protection Law of the Council of Agriculture, Taiwan. 45 rats were assigned by random into three groups in Experiment I ($N=15$ for each group) to receive various degrees of training in the Morris water maze. Another 120 rats were divided into six groups in Experiment II ($N=20$ for each group), which received pre-training intra-hippocampal infusion of one of the following drugs: 7-nitro-indazole (a NOS inhibitor), 2-[*N*-(2-hidroxyethyl)-*N*-(4-methoxy-benzenesulfonyl)]-amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine (a specific CaM-kinase II inhibitor), 2-amino-5-phosphonopentanoic acid (*N*-methyl-D-aspartic acid receptor antagonist), or a corresponding specific vehicle. In Experiment III (four groups, $N=20$ for each group), the rats were intra-hippocampally infused with AP5 + sodium nitroprusside (SNP, NO generator), AP5 + saline, KN-93 + SNP, or KN-93 + saline before the water maze training.

2.2. Surgery

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Atropine sulfate (0.4 mg/kg) was given 30 min before anesthesia to prevent respiratory congestion. The anesthetized rat was mounted on a stereotaxic apparatus and two guide cannulae made of 23-gauge stainless steel tubing were implanted bilaterally into the dorsal hippocampi. The coordinates were AP −3.6 mm, ML ±2.5 mm, and DV −3.0 mm according to Paxinos and Watson (1998). Three jewelry screws were implanted over the skull, serving as anchors, and the whole assembly was affixed on the skull with dental cement. The rats were monitored and handled daily. A week of recuperation was allowed before the behavioral training.

2.3. Behavioral tasks

2.3.1. Morris water maze

The water maze training was conducted in a circular pool of 2 m diameter, with 30 cm deep of water. A circular platform (8 cm diameter) made of Plexiglas was placed 1 cm beneath the water level at a specific location for the entire session. Water was made cloudy by the addition of milk. Distinctive visual cues were set up on the wall surrounding the pool. The behavioral training procedure was modified from that of earlier studies (Morris et al., 1986). Briefly, the rat was allowed to get accustomed to the water by swimming freely in the pool for 1-min and then removed from the water and placed in a holding cage for 30-s before the learning trial began. In each training trial, the rat was placed into the water at random positions. The time for the rat to get onto the platform was recorded. The time limit for each trial was 90-s. After reaching the platform, the rat was allowed to stay on it for 30-s in each trial. Each daily session consisted of 5 trials. In Experiment I, the rats were trained on the maze for 5 days. For the non-spatial learning control, the rats were trained in the water maze with no visual cues on the wall surrounding the pool. The visual guided rats were trained in the water maze where the platform was marked and elevated 2 cm above the water level. In Experiments II and III, the rats received intra-hippocampal infusion of a specific drug or its corresponding vehicle before spatial training in the water maze.

2.3.2. Locomotor activity assessment

To assess the effect of intra-hippocampal infusion of the drug on motor activity, all the injected rats were subjected to an open-field test at 10 min after drug infusion. An open-field platform (76×76 cm) was divided by lines into equal squares (4×4 cm) and elevated 65 cm above ground. The rat was placed at one corner and the number of lines it crossed within a 5-min period was recorded. The rats were subjected to Morris water maze learning 20 min after drug infusion.

2.4. Drug administration

A specific CaM-kinase II inhibitor: 2-[*N*-(2-hidroxyethyl)-*N*-(4-methoxy-benzenesulfonyl)]-amino-*N*-(4-chlorocinnamyl)-*N*-methyl-benzylamine (or KN-93), and negative control: 2-[*N*-(4-methoxy-benzenesulfonyl)]-amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine (or KN-92) were purchased from Calbiochem (U.S.A.); a specific brain NOS inhibitor: 7-nitro-indazole (or 7-NI) was purchased from Research Biochemicals (U.S.A.); *N*-methyl-D-aspartic acid (or NMDA)receptor antagonist: 2-amino-5-phosphonopentanoic acid (or AP5), and a NO generator: sodium nitroprusside (or SNP) were purchased from Sigma (U.S.A.). The rats received intra-hippocampal infusion of one of the following: KN-93 (5.0 μg/μl; 5.0 μg per site), KN-92 (5.0 μg/μl; 5.0 μg per site), 7-NI (25.0 μg/μl; 25.0 μg per site), AP5 (5.0 μg/μl; 5.0 μg per site), or specific vehicle controls 20 min prior to spatial training via a syringe pump at a rate of 0.5 μl per minute. The total volume of the infusion was 1.0 μl. The assigned drug dosages were tested earlier in our previous experiments and proven to be effective (Tan and Liang, 1996; Tan, 2007). The lower drug dosages produced inconsistent Morris water maze learning performance, while drug infusion at higher dosages caused confounding effects. Two and one rats died after four consecutive daily drug-infusions of KN-93 (10.0 μg/μl) and 7-NI (50.0 μg/μl), respectively. In addition, two rats showed locomotor deficit after infusion of AP5 at 10.0 μg/μl dosage. Saline was a common vehicle for all drugs, except 7-NI which was dissolved in 0.1%DMSO. The rats in Experiment III received two drug infusions (with 5 min of interval between the first and second drug) 20 min before training. The first drug infusion was KN-93 or AP5 followed by the second infusion of SNP (0.25 μg per site) or its vehicle. The rat was monitored for its locomotor activity at 10 min after drug infusion.

2.5. Biochemical assay procedures

The rats were sacrificed by decapitation immediately after the test trial. The hippocampi were quickly dissected out and frozen in liquid nitrogen. The samples were transferred to a −70 °C freezer for storage until the biochemical assays.

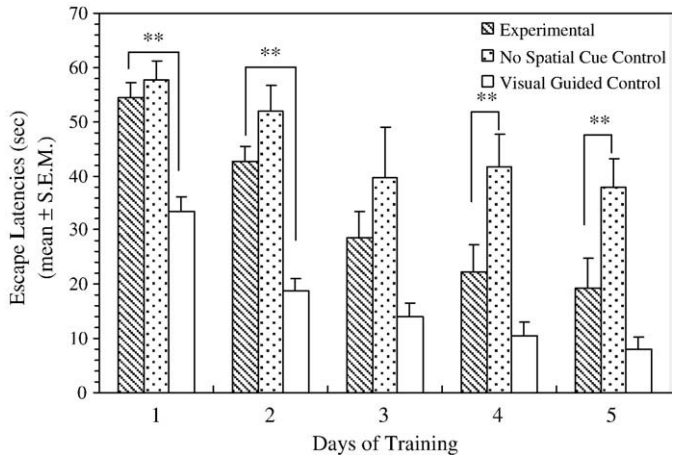

Fig. 1. Escape latencies of rats in the Morris water maze. There were statistically significant differences in the improvement rate of escape latency among the three groups ($F_{2,152}=5.361$, $P<0.01$). The escape latencies of the rats in the experimental group were significantly higher than those of the visual guided controls on Day 1 and Day 2. Conversely, the escape latencies of the rats in the experimental group were significantly lower than those of the no spatial cue controls on Day 4 and Day 5. $^{**}p<0.01$.

CaM-kinase II assay—Brain samples were homogenized in a 200 µl homogenization buffer: 50 mM HEPES (pH 7.5), 150 mM NaCl, 2 mM EGTA, 5 mM EDTA; 1 mM DTT; 0.1% Triton X-100; 25 mM NaF, 100 mM β-glycerophosphate, 15 mM NaPPi, 100 mM sodium vanadate, 0.4 mM microcystin; 5 µM leupeptin, 5 µM trypsin inhibitor, 5 µM aprotinin, 1 mM PMSF. Samples were centrifuged at 14,000 ×*g* for 5 min, and the supernatants were collected. The protein kinase assay was carried out at 30 °C for 2 min, and Syntide-2 was used as the CaM-kinase II substrate (Tan and Liang, 1996). The reaction mixture was: (final conc.) 50 mM HEPES (pH 7.5), 10 mM magnesium acetate, 1 mg/ml BSA, 5 µM PKA inhibitor, 2 µM PKC inhibitor, 40 µM [γ32P]-ATP (specific activity 2000 cpm/pmol) and 40 µM syntide-2; in a final volume of 25 µl. In the total activity condition 1 mM CaCl2 and 100 µg/ml calmodulin were added; for the calcium-independent activity condition 1 mM EGTA and 3 µM of mastoparon were added instead. Each sample was assayed in duplicates. The reaction was stopped by spotting onto a P81 filter paper. The filter papers were washed several times with 75 mM phosphoric acid, and were counted by liquid scintillation counter.

In-vitro protein phosphorylation and immunoblotting—in-vitro phosphorylations of the sample proteins were carried out in a water bath at 30 °C for 2 min. The reaction mixture (final conc.) was: 50 mM HEPES (pH 7.5), 75 mM NaCl, 1 mM EGTA, 2.5 mM EDTA; 0.5 mM DTT; 0.05% Triton X-100; 12.5 mM NaF, 50 mM β-glycero-phosphate, 7.5 mM NaPPi, 50 mM sodium vanadate, 0.2 mM microcystin; 2.5 µM leupeptin, 2.5 µM trypsin inhibitor, 2.5 µM aprotinin, 0.5 mM PMSF; 10 mM magnesium acetate, 2 mM CaCl2, 0.4 mM[γ32P]-ATP (specific activity 3000 cpm/pmol), in a final volume of 50 µl. Each sample was assayed in duplicate. The reaction was terminated by adding a Laemmli buffer and the sample was subjected to SDS–polyacrylamide gel electrophoresis. The 32P-incorporated phosphoproteins were visualized by autoradiography, and the phosphorylation activities were quantified by densitometric scanning (Bio-Rad). The nNos antibody and α-CaM-kinase II antibody were purchased from Calbiochem (U.S.A.) and were used in the immunoblotting experiments.

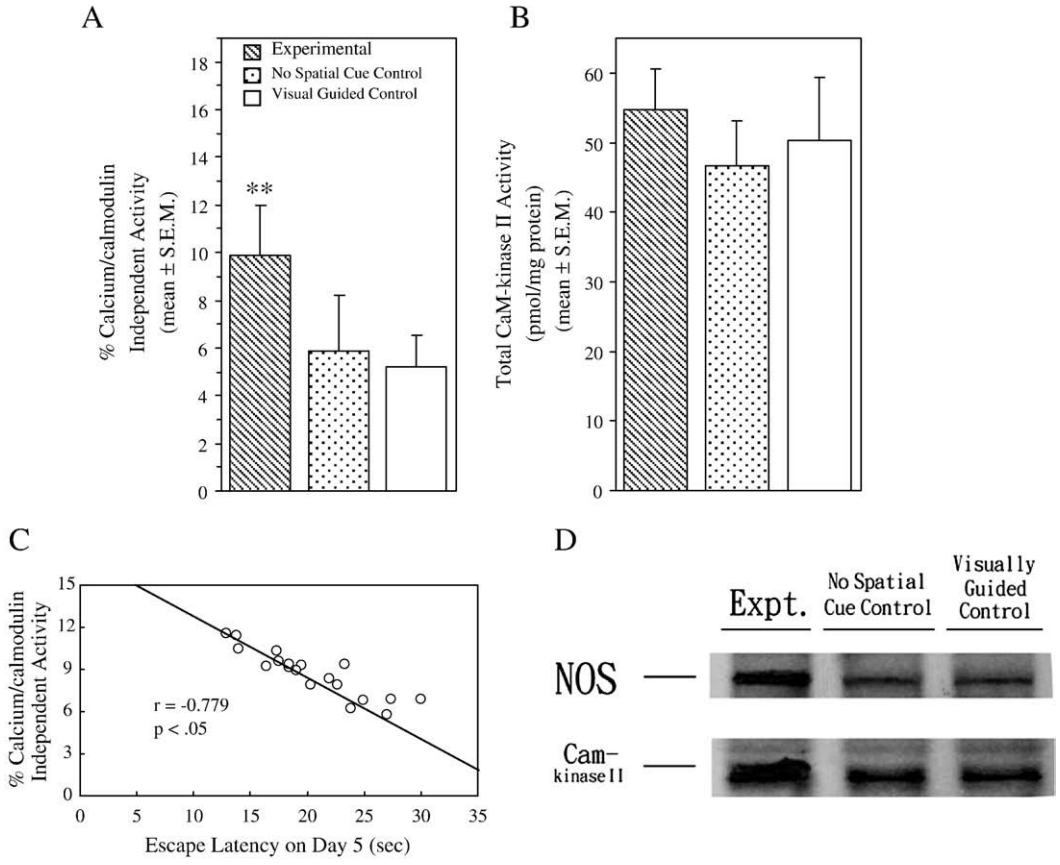

Fig. 2. The CaM-kinase II activity in the rat hippocampus after 5-day training in the water maze. A: There was a statistically significant difference in the percentage of Ca2+-independent activity of hippocampal CaM-kinase II among the groups ($F_{2,57}=5.952$, $P<0.01$). B: Total CaM-kinase II activity of the hippocampus; there were no differences in total CaM-kinase II activity after 5 days of training in the water maze. C: The correlation between the escape latencies and the hippocampal CaM-kinase II activities of the experimental rats after 5 days of training in the water maze. D. The SDS–PAGE analysis of endogenous phosphorylation of neuronal NOS and CaM-kinase II in the rat hippocampi. There was an increase in the phosphorylation of neuronal NOS and CaM-kinase II in the hippocampi of the experimental rats compared to those in the control groups.

2.6. Histological verification

Animals were sacrificed with an excess dose of sodium pentobarbital (i.p.). The brain was perfused through the heart with saline followed by 10% formalin. Brain sections (40 μm) were collected and stained with cresyl violet. Cannulae placements were identified as tracks on the stained brain sections.

2.7. Data analysis

To analyze the intensity of the protein phosphorylation in NOS, the autoradiograms were analyzed, where the amount of NOS 32P incorporation in the experimental groups was expressed as the percentage of those in the respective controls. The escape latency in the Morris water maze, the number of lines crossing in open-field activity, the counts of syntide-2 phosphorylation, and the percentage of 32P incorporation in the NOS band were analyzed by ANOVAs and Student's *t*-tests. The Pearson product-moment correlation coefficient between behavioral performance on Day 5 and the level of activated CaM-kinase II activity of the rats was calculated and statistically tested for significance.

3. Results

3.1. Experiment I

The rats in general showed improvement in the escape latency over time in the Morris water maze learning (Fig. 1). The repeated measure ANOVA revealed a statistically significant difference in the improvement rate of escape latency among the three groups ($F_{2,152}=5.361$, $P<0.01$). Furthermore, the escape latencies of the rats in the experimental group were significantly higher than those of the visual guided controls on Day 1 (54.525±2.786 vs. 33.333±2.694; (Tukey's HSD, $P<0.01$)) and Day 2 (42.550±2.986 vs. 18.725±2.357; Tukey's HSD, $P<0.01$). This suggests that substantial learning had occurred in the first two days, if the escape response could rely on direct visualization of the marked platform. No significant differences were shown between the experimental and the no spatial cue control groups on Days 1 and 2. Conversely, the escape latency of the rats in the experimental group was significantly lower than those of the no spatial cue controls on Day 4 (22.435±4.935 vs. 41.550±6.074; Tukey's HSD, $P<0.01$) and Day 5 (19.385±5.341 vs. 37.825±5.246; Tukey's HSD, $P<0.01$). This indicates that with the no spatial cue control rats did not learn the location of the platform in the maze. Again, no significant differences were shown between the experimental and the visual guided control groups on Days 4 and 5. The overall results show that rats in the spatial learning experimental group, which had to rely on spatial cues to locate the submerged platform position, showed very little learning on the first two days of training, but accomplished much learning on Day 5.

The biochemical assays of the 5-day training rats from the three groups showed a statistically significant difference in the percentage of Ca2+-independent activity of hippocampal CaM-kinase II ($F_{2,57}=5.952$, $P<0.01$) (Fig. 2A). There were no differences in the total CaM-kinase II activity across the groups (Fig. 2B). Furthermore, the percentage of Ca2+-independent activity of hippocampal CaM-kinase II was inversely correlated with the escape latency of rats in the experimental group on Day 5 ($r=-0.779$, $P<0.05$) (Fig. 2C). The SDS–PAGE analysis shows a significant increase in the endogenous phosphorylations of two phosphoproteins (which had a molecular weight of around 50 kDa and 160 kDa) in the hippocampi of the experimental rats compared to the two control groups (Fig. 2D). These two protein bands are recognized by the antibodies of α-CaM-kinase II and neuronal NOS, respectively. The enhanced phosphorylation of hippocampal NOS and α-CaM-kinase II were 126±45% ($p<.001$) and 93±25% ($p<.001$) of the no spatial cue control's values, respectively, versus 133±85% ($p<.001$) and 88±15% ($p<.001$) of the visual guided control's values, respectively.

3.2. Experiment II

This experiment compares rats that received pre-training intra-hippocampal infusions of 7-NI, KN-93, and AP5 to those infused with the specific vehicle controls in the water maze learning. The differences in the escape latency of rats on Day 1 and Day 5 were calculated and then, used as a measurement of rats' improvement in spatial learning. There were statistically significant differences among the differences in the escape latency on Day 1 and Day 5 between the 7-NI and the vehicle infused groups ($t_{28}=3.815$, $p<.01$), KN-93 and KN-92 (control) infused groups ($t_{28}=3.562$, $p<.01$), and AP5 and saline infused groups ($t_{28}=4.432$, $p<.001$), respectively (Fig. 3A). In general, there were no differences for the rats' escape latencies on Day 1

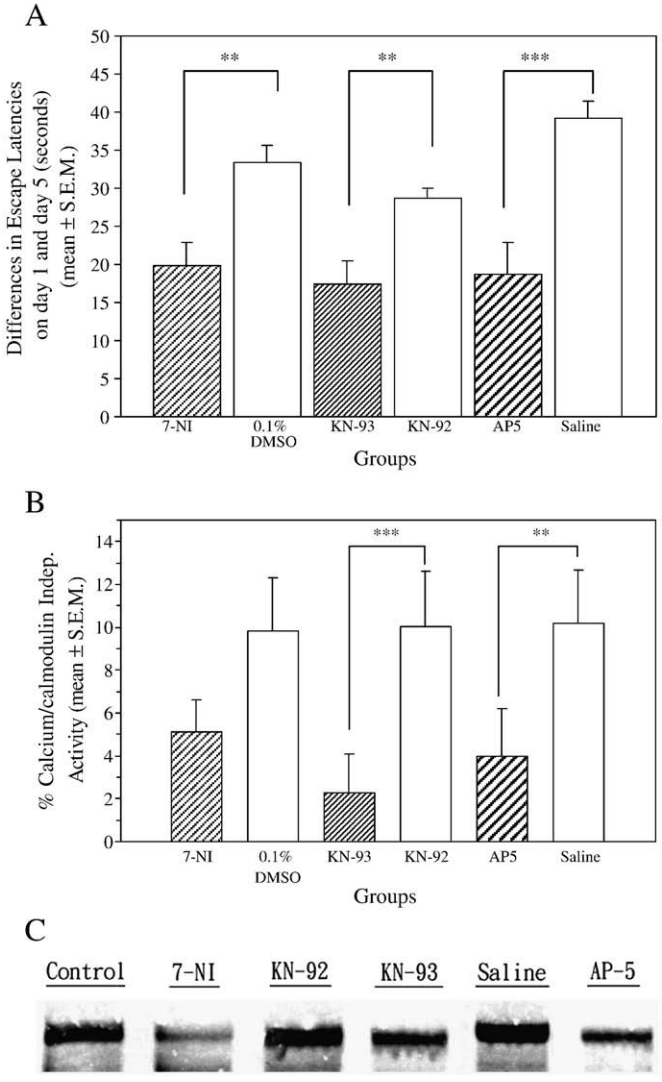

Fig. 3. A. The improvement in escape latencies of the intra-hippocampal infused rats in the Morris water maze. There were statistically significant differences in the improvement in escape latencies between the 7-NI and the vehicle infused groups, KN-93 and KN-92 (control) infused groups, and AP5 and saline infused groups, respectively. ***$p<0.001$; **$p<0.01$. B. The hippocampal CaM-kinase II activity of the intra-hippocampal infused rats. The percentage of Ca2+-independent activity of hippocampal CaM-kinase II was significantly lower in the KN-93 infused group and the AP5 infused group compared to the controls. ***$p<0.001$; **$p<0.01$. C. The SDS–PAGE analysis of endogenous phosphorylation of neuronal NOS in the rat hippocampi. The rats that received intra-hippocampal infusion of 7-NI, KN-93, and AP5 showed significantly depressed phosphorylation of NOS compared to the controls, respectively.

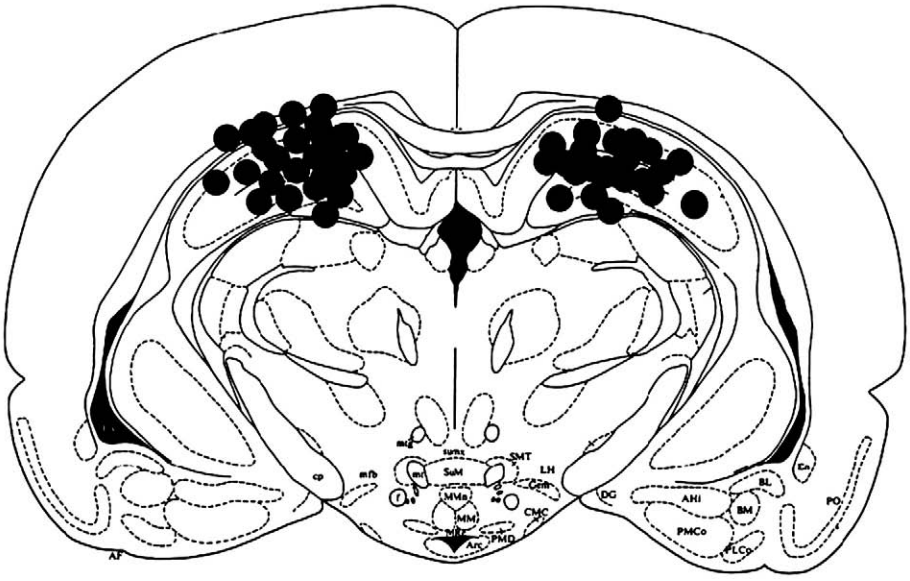

Fig. 4. Histological verification for cannulae placements in rats. The locations of cannulae tips in the stained slice were matched with the atlas of the rat brain.

among all groups, suggesting that the drug-infused conditions did not create any significant alteration in the rats' spontaneous reactivity compared to the controlled-infused conditions. Furthermore, the locomotor activity assessment after intra-hippocampal infusion did not show differences in the number of squares crossed in 5 min between the drug- and the vehicle-infused groups (the average number of squares crossed were 122.75, with a range of 107.45 to 127.52). Five rat brains from each group were selected by random for the histological verification of the cannula tips' positions, and indeed the locations of the cannula tips were targeted in the hippocampal formation (Fig. 4).

The hippocampal CaM-kinase II activity of the remaining intra-hippocampal infused rats (N=15 for each group) were assayed after Day 5. There were no differences in the total CaM-kinase II activity among the drug- and vehicle-infused groups. However, the percentage of Ca2+-independent activity of hippocampal CaM-kinase II was significantly lower in the KN-93 infused group compared to the KN-92 (control) infused group (2.289±1.804 vs. 10.037±2.590; t_{28}=4.867, p<.001). Similarly, the percentage of Ca2+-independent activity of hippocampal CaM-kinase II was significantly lower in the AP5 infused group compared to the saline infused group (4.007±2.189 vs. 10.208±2.478; t_{28}=3.367, p<.01). On the contrary, the percentage of Ca2+-independent activity of hippocampal CaM-kinase II was not significantly different in the 7-NI infused group compared to the vehicle infused group (5.121±1.497 vs. 9.843±2.448; t_{28}=1.933, NS) (Fig. 3B).

The endogenous phosphorylation activities of hippocampal NOS were analyzed by the SDS–PAGE method (Fig. 3C). Three drugs—7-NI, KN-93, and AP5—depressed phosphorylation of hippocampal NOS to 21±55% (p<.001), 45±55% (p<.01), and 34±35% (p<.001) of the control values, respectively. However, there were no significant differences in the intensities of hippocampal NOS phosphorylation among the drug-infused groups, as well as those when compared among the three controlled-infused groups.

3.3. Experiment III

Those rats receiving an intra-hippocampal infusion of two drugs showed no significant difference in their locomotor activity compared to those of the rats in Experiment II (the average number of squares crossed were 122.75, with a range of 110.82 to 125.45). The rats showed significantly greater improvement in escape latency after a pre-training intra-hippocampal infusion of AP5+SNP and KN-93+SNP

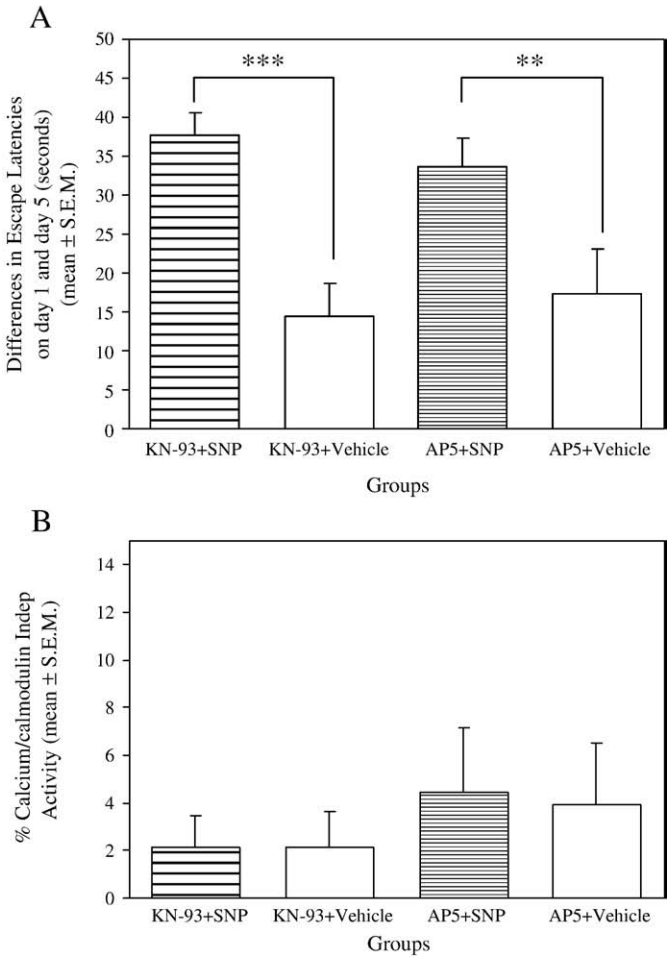

Fig. 5. A. The improvement in escape latencies of the intra-hippocampal infused rats in the Morris water maze. The rats showed significantly greater improvement in escape latency after a pre-training intra-hippocampal infusion of AP5+SNP and KN-93+SNP compared to those infused with AP5+saline and KN-93+saline, respectively. ***p<0.001; **p<0.01. B. The hippocampal CaM-kinase II activity of rats received an intra-hippocampal infusion of two drugs. There were no differences in the percentage of Ca2+-independent activity of hippocampal CaM-kinase II for the AP5+SNP infused group, as well as the KN-93+SNP infused group compared to the controls.

compared to those which were infused with AP5+saline and KN-93+saline, respectively (Fig. 5A). There were statistically significant differences in the improvement of escape latency between the AP5+SNP and AP5+saline infused groups (33.678±3.598 vs. 17.245±5.791; t_{28}=3.172, p<.01), and the KN-93+SNP and KN-93+saline infused groups (37.677±2.915 vs. 14.367±4.211, t_{28}=3.988, p<.001), respectively. The histological verification confirmed that the positions of the cannula tips were indeed in the hippocampal formation (Fig. 4).

The hippocampal CaM-kinase II activity was assayed after Day 5. There were no differences in the total CaM-kinase II activity among the drugs- and vehicles-infused groups (Fig. 5B). The percentage of Ca2+-independent activity of hippocampal CaM-kinase II was not significantly higher in the AP5+SNP infused group compared to the AP5-saline infused group (4.418±2.743 vs. 3.931±2.572; t_{28}=1.510, NS). Similarly, the percentage of Ca2+-independent activity of hippocampal CaM-kinase II did not increase significantly in the KN-93+SNP infused group compared to the KN-93+saline infused group (2.123±1.349 vs. 2.160±1.472; t_{28}=1.127, NS).

In the analysis of the endogenous phosphorylation activities of hippocampal NOS, the depression effects of AP5 and KN-93 on the NOS phosphorylations were not reversed by the infusion of NO donors SNP, where NOS phosphorylations did not show significant differences among the these groups.

4. Discussion

The results of Experiment I suggest that activities of CaM-kinase II and NOS in the rat hippocampi were significantly increased after 5-day spatial learning in the Morris water maze. The rats trained in either the visually guided condition or no spatial cue condition shows a lower hippocampal activities of CaM-kinase II and NOS. Furthermore, the percentage of Ca2+-independent activity of hippocampal CaM-kinase II is inversely correlated with the escape latency of rats in the experimental group on Day 5. Taken together, the establishment of spatial learning in the water maze is associated with the activation of hippocampal CaM-kinase II and NOS.

The rats receiving intra-hippocampal implants and infusions showed no effect on their swimming activities, as their escape latencies from all drug- and vehicle-infused groups on Day 1 are 50.135±1.274 s compared to the non-implanted experimental group (54.525±2.786 s) from Experiment I. In addition, the spatial learning rate of the vehicle-infused controls shows no difference when compared with the non-implanted from the experimental group in Experiment I (20.192±3.016 vs. 19.385±5.341 s on Day 5). The results from Experiment II establish a possible causal relationship between hippocampal NOS activation and spatial learning, which was acted via an excitatory glutamatergic system. The rats show significantly slower in the improvement of escape latencies after a pre-training intra-hippocampal infusion of 7-NI, KN-93, and AP5. These results are consistent with and extend previous studies on the intra-hippocampal infusion of 7-NI, CaM-kinase II inhibitor, or AP5 that impair the rats' behavioral learning (Baratti and Boccia, 1999; Liu et al., 2005; Tan and Liang, 1996; Wass et al., 2006). There are no differences in the escape latencies for all the intra-hippocampal infused rats on Day 1. Furthermore, the rat locomotor activity assessment shows that pre-training drug-infusion did not create any significant alteration behavioral reactivity, suggesting that the drug-infused condition did not jeopardize spatial learning. However, one study has reported that 7-NI could affect blood pressure through intra-peritoneal injection (Prickaerts et al., 1997). Even though they concluded that NO synthase and cGMP are involved in recognition memory processes independently of their cardiovascular effects, 7-NI is shown to be not a selective neuronal NO synthase inhibitor. The present study administered the drug via intra-hippocampal infusions, which presumably affects a discrete site where the drug may diffuse into the neurons directly without affecting the blood flow. Moreover, the drug infusion did not alter the behavioral activity as well as the response latency during the training trial. Nevertheless, the blood flow alteration in the brain by intra-hippocampal 7-NI infusions should be considered regardless of its slight possibility.

The kinase assays reveal that the endogenous hippocampal CaM-kinase II activities of the KN-93- and AP5-infused groups are significantly depressed, except for the 7-NI-infused group. This suggests that NOS inhibition impairs spatial learning without suppressing the endogenous CaM-kinase II activity. Interestingly, the endogenous phosphorylation activities of hippocampal NOS were suppressed for all three drug-infused groups compared to their respective controls. It is tempting to postulate that hippocampal NOS is phosphorylated by CaM-kinase II (Agostino et al., 2004; Komeima et al., 2000; Yamada and Nabeshima, 1997) via NMDA receptor activation.

The results from Experiment III reveal the specificity of NOS activation during spatial learning. The rats show impairments in spatial learning after a pre-training intra-hippocampal infusion of AP5+saline and KN-93+saline compared to those infused with AP5+SNP and KN-93+SNP, respectively. Interestingly, the intra-hippocampal infusion of the NO donor did not reverse the depression effect of AP5 or KN-93 on CaM-kinase II activation and NOS phosphorylation, but did ameliorate the effect on spatial learning. This suggests that hippocampal NO has an effect on spatial learning which might not act through CaM-kinase II activity.

It is tempting to speculate how the mechanism of hippocampal enzymatic processes is involved in spatial learning. Many lines of evidence suggest the critical role of NMDA receptor activation and postsynaptic CaM-kinase II actions in LTP (Hardingham et al., 2003; Ko and Kelly, 1999), as well as in behavioral learning (Tan and Liang, 1996; Yang et al., 2004). The present study shows that endogenous phosphorylation of hippocampal NOS is enhanced after behavioral training. Hippocampal NOS phosphorylation decreases with an NMDA receptor antagonist or with a CaM-kinase II inhibitor. This suggests that hippocampal NOS may be regulated by excitatory synaptic processes, presumably via the route of NMDA receptor/CaM-kinase II transductional activity (Agostino et al., 2004; Ko and Kelly, 1999; Ledo et al., 2005; Schuman and Madison, 1991). This also confirms the reports on the studies of NOS phosphorylation (Fleming et al., 2001; Komeima et al., 2000; Stanton et al., 2003).

The impairment of spatial learning by intra-hippocampal infusion of either the NMDA receptor antagonist or CaM-kinase II inhibitor can be reversed by the NO donor. The fact that hippocampal NOS inhibition impairs spatial learning without suppressing endogenous CaM-kinase II activity suggests the possibility of upstream action by CaM-kinase II in the memory processing of affective memory. Although the present study reveals enhanced phosphorylation activity of nNOS via NMDA receptor and CaM-Kinase II activations, the precise cellular enzymatic pathway in hippocampus during spatial learning is not known. The significance of this enzymatic interaction for the delineation of the acquisition of spatial learning should be explored in the future.

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