Effects of *L*-arginine and N^{ω} -nitro-*L*-arginine methylester on learning and memory and $\alpha 7$ nAChR expression in the prefrontal cortex and hippocampus of rats

Xiao-Ming Wei^{1,*}, Wei Yang¹, Li-Xia Liu², Wen-Xiu Qi²

¹*Shanxi Medical University, Taiyuan 030001, China*

²*Department of Physiology, Fenyang College of Shanxi Medical University, Fenyang 032200, China*

*Present address: Department of Physiology, Nanyang Medical College of Henan, Nanyang 473000, China

Corresponding author: Wen-Xiu Qi. E-mail: fycqwx@163.com

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ABSTRACT

Nitric oxide (NO) is a novel type of neurotransmitter that is closely associated with synaptic plasticity, learning and memory. In the present study, we assessed the effects of *L*-arginine and N^{ω} -nitro-*L*-arginine methylester (*L*-NAME, a nitric oxide synthase inhibitor) on learning and memory. Rats were assigned to three groups receiving intracerebroventricular injections of *L*-Arg (the NO precursor), *L*-NAME, or 0.9% NaCl (control), once daily for seven consecutive days. Twelve hours after the last injection, they underwent an electric shock-paired Y maze test. Twenty-four hours later, the rats' memory of the safe illuminated arm was tested. After that, the levels of NO and $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) in the prefrontal cortex and hippocampus were assessed using an NO assay kit, and immunohistochemistry and Western blots, respectively. We found that, compared to controls, *L*-Arg-treated rats received fewer foot shocks and made fewer errors to reach the learning criterion, and made fewer errors during the memory-testing session. In contrast, *L*-NAME-treated rats received more foot shocks and made more errors than controls to reach the learning criterion, and made more errors during the memory-testing session. In parallel, NO content in the prefrontal cortex and hippocampus was higher in *L*-Arg-treated rats and lower in

L-NAME rats, compared to controls. Similarly, $\alpha 7$ nAChR immunoreactivity and protein expression in the prefrontal cortex and hippocampus were higher in *L*-Arg-treated rats and lower in *L*-NAME rats, compared to controls. These results suggest that the modulation of NO content in the brain correlates with $\alpha 7$ nAChR distribution and expression in the prefrontal cortex and hippocampus, as well as with learning and memory performance in the Y-maze.

Keywords: nitric oxide; *L*-arginine; N^{ω} -nitro-*L*-arginine methylester; learning and memory; $\alpha 7$ nicotinic acetylcholine receptor; cerebral cortex and hippocampus; Y-maze

INTRODUCTION

Nitric oxide (NO), a soluble, short-lived, and freely diffusible gas, is a novel type of neurotransmitter that is closely associated with synaptic plasticity, learning and memory in the central nervous system^[1,2]. Acetylcholine (ACh), another important chemical messenger in brain, also plays an essential role in mnemonic phenomena^[3,4]. Besides, the role of nicotinic ACh receptors (nAChRs) in cognitive function has been increasingly realized^[5,6]. So it is possible that there are links or interactions between the NOergic neuronal system and the cholinergic system in learning and memory.

It has been suggested that nAChRs play a role by affecting the NOergic neuronal system^[7,8] in learning and memory. For instance, the nitric oxide synthase (NOS) inhibitor N^ω-nitro-*L*-arginine methylester (*L*-NAME) markedly impairs spontaneous alternation behavior and decreases the NO_x level in the hippocampus; however, the cholinesterase inhibitor galantamine significantly attenuates this impairment and decreases NO_x levels, while the nAChR antagonist mecamylamine reduces the protective effects of galantamine^[9]. Hence it is proposed that the protection by galantamine against the *L*-NAME-induced impairment of spontaneous alternation behavior in the Y-maze task might be mediated mainly by NOergic activation via the related nAChR pathway. Under pathological conditions, increased α7 nAChRs also contribute to the enhancement of NO formation in astrocytes in the human hippocampus and entorhinal cortex^[10]. A recent study using conditioned place preference revealed interactions between the nicotinic and NO systems^[11]. For instance, inhibition of NOS by 7-nitro-indazole inhibits the development of place preference^[12] and behavioral sensitization induced by nicotine in the rat^[13]. Some morphological studies further indicated that NOS is often co-localized with ACh in the vertebrate brain^[14]. However, the effects of NO on the expression of nAChRs in the central nervous system and on learning and memory have seldom been reported.

The α7 nAChR, one of the most widespread nAChR subtypes in the brain^[15], has been shown to be involved in improving cognitive function and spatial learning^[16,17]. It is prevalent in the hippocampus and cerebral cortex associated with learning and memory^[18]. Studies have also shown that the prefrontal cortex participates in identifying and temporarily maintaining spatial working memory^[19], and the hippocampus is well known to be involved in learning and memory. The Y-maze is an apparatus that combines electrical and light stimulation to assess spontaneous alternation behavior and the ability of learning and memory^[20,21].

In vivo, NO is synthesized from *L*-arginine (*L*-Arg) by NOS. Here, we investigated the effects of multiple intracerebroventricular (i.c.v.) administration of *L*-Arg or *L*-NAME on NO content and α7 nAChR expression in prefrontal cortex and hippocampus, as well as on learning and memory in rats.

MATERIALS AND METHODS

Animals and Treatments

Twenty-one 8-week-old male Wistar rats were used (200–230 g; Shanxi Medical University Experimental Center, Taiyuan, China). The animals were housed three per cage in Makrolon cages (47.5 cm long × 20.5 cm high × 27 cm wide) under a 12-h light/dark cycle (light on at 07:00) at 25 ± 1°C with 50–55% relative humidity, with free access to food and water. Animal handling and all related procedures were in accordance with international guidelines and were approved by the Shanxi Medical University Committee on Animal Research. Attempts were made to minimize the number of animals used and their suffering.

Surgery and Model Construction

One week before experiments, polyethylene cannulae (PE-10, OD 0.6 mm, ID 0.3 mm; Anlai Technology Co., Ltd., Ningbo, China) were implanted under chloral hydrate analgesia (300 mg/kg, i.p.), referring to the Paxinos rat brain stereotaxic atlas^[22]. Then the cranium was exposed and a cannula was implanted to the right lateral ventricle, 3.8 mm below the surface, 1.3 mm to the right of the sagittal suture, and 0.8 mm behind the coronal suture. The cannula was attached to the skull with dental cement. Then the rats were housed individually in standard plastic cages with sawdust bedding in a temperature-controlled room. Only animals exhibiting normal motor functions were used in further experiments. These animals were randomly divided into three groups (7/group). Two groups received i.c.v. injection of *L*-Arg (0.5 μmol/day; *L*-Arg group)^[23] or *L*-NAME (5 μmol/day; *L*-NAME group)^[24] in 5 μL both for seven days, followed by 5 μL saline to flush the catheter. The same amount of normal saline was given as control (NS group). Both drugs were diluted in physiological saline (0.9% w/v). *L*-Arg (A5006) and *L*-NAME (N5751) were from Sigma Chemical Co. (Santa Clara, CA).

Behavioral Tests

The behavioral response of learning in a Y-maze task was tested 12 h after the last injection. The Y-maze consisted of three arms (regions I–III) which converged to an equilateral triangular central area (region 0). Each arm had a lamp at the distal end. A safe region/arm was associated with illumination while other arms and region 0 became regions

with electrical foot stimulation. Each rat was first placed at the end of one arm (starting area chosen randomly) and allowed to move freely in the maze during a 4-min session for adaptation. In this period we did not change the orientation of the safe and stimulation regions. The rats preferred to stay in a dark arm, but they would finally escape to the illuminated arm when the dark arm had foot stimulation. Then the test was started, and the illuminated arm (safe region) became the new starting area. Further, we changed the orientation of the safe and stimulation regions using a randomization method. The time from foot stimulation delivery to escape into the safe region was measured, and was considered to be successful (learned) if the rat directly reached the safe region within 10 s. After each foot stimulation, we waited for the rat to reach the illuminated arm (the new starting area) before the next stimulation. If there were nine or more correct responses in 10 consecutive foot stimulations (9/10 standard)^[20,21], the rat was defined as having reached the learning criterion. The total number of stimulations to reach the criterion and the errors during training were recorded as the learning ability. Memory of the Y-maze task was tested 24 h after training. The procedure was identical with that on the previous day, except that the rat underwent a total of 30 foot stimulations, and the number of errors in the 30 stimulations was recorded as memory retention. After completion of the memory test, the rats were anesthetized with ether and the prefrontal cortex and hippocampus were located according to the rat brain atlas^[22], dissected and collected. The left

hemisphere was used for immunohistochemistry and the right hemisphere for NO content assessment and Western blot analysis.

NO Determination

NO is an active gas *in vivo*, and can be rapidly converted to NO_2^- and NO_3^- , then NO_2^- is further converted to NO_3^- . We converted NO_3^- to NO_2^- specifically with nitrate reductase, and then determined the NO content as optical density (OD) with a 752 - UV grating spectrophotometer (Third Analytical Instrument Factory, Shanghai, China) with 595 nm wavelength.

The prefrontal cortex and hippocampus were homogenized on ice in 10 volumes of 0.9% saline. The homogenate was then centrifuged at 2 000 rpm for 10 min at 4°C, and NO content in the supernatant was determined according to the instructions with the NO assay kit (A013-1; Nanjing Jiancheng Bioengineering Institute, China). The OD values of blank and standard tubes separately were determined with ddH₂O and a standard sample (20 $\mu\text{mol/L}$). The protein concentration of the sample was determined using Coomassie brilliant blue G-250, with bovine serum albumin as standard. The NO content in the homogenates of prefrontal cortex and hippocampus was calculated as follows: $\text{NO content } (\mu\text{mol/g}) = (\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}) / (\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}}) \times \text{standard concentration (20 } \mu\text{mol/L}) / \text{protein concentration of sample (g/L)}$.

Immunocytochemistry

The prefrontal cortex and hippocampus samples were

Fig. 1. A. Apparatus: one arm was the safe region (chosen randomly), for example, if it is arm I (illuminated), then arms II, III and region 0 became stimulation regions. The starting point was chosen randomly. Rats were allowed to move freely in the maze during a 4-min session to adapt. B. Training: rats preferred to stay in the dark arms (II and III), but they escaped to the illuminated arm (I) when the dark arm had electrical foot stimulation. Then arm I became the new starting point when the next stimulation was given (when we changed the orientation of the safe and the stimulation regions). C. Testing: arm II became the safe region (illuminated) which was chosen randomly. Then the rat would escape to avoid a shock. If the rat directly reached the safe region (arm II) in <10 s, the response was “correct”, if not, “incorrect”.

immersed in 4% paraformaldehyde overnight, followed by cryoprotection with 20% sucrose in 0.1 mol/L phosphate-buffered saline (PBS) (pH 7.4) for 24 h and with 30% sucrose in 0.1 mol/L PBS (pH 7.4) overnight at 4°C. Then samples were frozen separately to the optimal cutting temperature and cut into coronal sections 40 µm thick on a freezing microtome (Leica Biosystems Nussloch GmbH, Nussloch, Germany). Every fifth section was processed for immunohistochemistry. The sections were immersed in acetone for 20 min, followed by 3% hydrogen peroxide for 60 min. Then they were incubated with affinity-purified goat anti-α7 nAChR polyclonal antibody (1:200; Sc-1447, Santa Cruz Biotechnology, CA) for 48 h at 4°C, followed by incubation with rabbit anti-goat IgG (SA-1023, Boster-Bio) and avidin-biotin complex for 2 h each at room temperature. Finally, the sections were stained with 3, 3'-diaminobenzidine (ZLI-9032, ZSGB-Bio) for 5–7 min. After immunostaining, the sections were mounted on poly-L-lysine-coated slides, air-dried, dehydrated with ethanol, treated with xylene, and coverslipped. Staining controls were incubated with PBS instead of the primary antibody. The term "α7 nAChR-like immunoreactivity" (α7 nAChR-LI) is used here, since the cross-reactivity of antibody with proteins in the α7 nAChR family and other unknown structurally-related substances present in the tissue sections could not be excluded.

Sections were assessed using a light microscope (Olympus BH2, Tokyo, Japan), a Canon (BX41, Tokyo, Japan) camera and the Image-Pro Plus 6.0 image-analysis system. The α7 nAChR-LI neurons were identified by a brown cytoplasm or membrane. Five fields were randomly selected from each section for analysis of the distribution and number of labeled neurons. They were distinct from background at a magnification of 40×. Values were averaged separately for each animal and for each group.

Western Blot

The prefrontal cortex and hippocampus tissues were homogenized and protein extracted according to the instructions with the protein extraction kit (Applygen Technologies Inc., Beijing). Total protein in each sample was determined with Coomassie brilliant blue G-250 (0615; Amresco). Proteins (30 µg) were separated on a 10% SDS-PAGE gel, and transferred to nitrocellulose membrane by electro-blotting. Membranes were blocked

with 5% nonfat milk powder, 0.05% (v/v) Tween 20 in PBS. Primary antibody anti-α7 nAChR (Sc-1447; Santa Cruz Biotechnology) was applied in the same buffer at 1:200 at 4°C overnight. In order to confirm equal protein loading, the blots were also reacted with antibody anti-β-actin (1:600, TA-09, ZSGB-Bio). Immunoreactivity was detected using HRP-conjugated rabbit anti-goat (ZB-2306, ZSGB-Bio) or goat anti-mouse (ZB-2305, ZSGB-Bio) at 1:20 000 and enhanced chemiluminescence (P0018, Beyotime institute of Biotechnology). Quantification of immunoreactivity was carried out by densitometry using a computerized image-analysis system (Model JD801, Jieda, Jiangsu). We treated all gels the same way and all Western blot experiments were repeated at least three times. The integrated optical density of the bands was corrected by subtraction of the background values. The ratios of α7 nAChR to β-actin were expressed as a percentage of the average of the control in each blot.

Statistical Analyses

All data are expressed as mean ± SD, and were analyzed with one-way analysis of variance (ANOVA), followed by *post-hoc* LSD tests using SPSS 16.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

Behavioral Responses

The three groups differed in the number of stimulations [$F(2,18) = 16.367$, $P < 0.001$] and errors [$F(2,18) = 13.963$, $P < 0.001$] to reach criterion and the number of errors in memory retrieval [$F(2,18) = 17.676$, $P < 0.001$].

Compared with the control group, the numbers of stimulations and errors to reach the criterion as well as the number of errors in memory retrieval were significantly decreased in the *L*-Arg group, but increased in the *L*-NAME group (Table 1).

NO Content

The three groups differed in the NO content of prefrontal cortex and hippocampus [prefrontal cortex: $F(2,18) = 57.663$, $P < 0.001$; hippocampus: $F(2,18) = 215.397$, $P < 0.001$] (Table 2). Compared with the control group, the NO content was markedly increased in the *L*-Arg group in both areas ($P < 0.01$) while in the *L*-NAME group, the NO content

Table 1. Learning and memory performance of rats in Y-maze (mean $\pm$ SD)

Treatment	NS	L-Arg	L-NAME
Stimulations to criterion	41.6 $\pm$ 13.4	23.6 $\pm$ 10.5**	57.9 $\pm$ 9.4*
Errors to criterion	15.4 $\pm$ 6.2	8.6 $\pm$ 4.5*	23.7 $\pm$ 5.3**
Errors in memory retrieval	9.9 $\pm$ 4.0	5.7 $\pm$ 2.4*	16.6 $\pm$ 3.7**

* $P < 0.05$, ** $P < 0.01$ compared to the control (NS) group, $n = 7$ /group.

Fig. 2. Images showing the distribution of $\alpha 7$ nAChR-LI neurons (arrows) in the prefrontal cortex (A, a) and hippocampus (B, b) in the three groups. Scale bars: A and B, 250 μ m; a and b, 25 μ m.

Table 2. NO content in prefrontal cortex and hippocampus ($\mu\text{mol/g}$)

NO content	NS	<i>L</i> -Arg	<i>L</i> -NAME
Prefrontal cortex	3.03 ± 0.41	$5.41 \pm 0.28^{**}$	$1.60 \pm 0.33^{**}$
Hippocampus	3.10 ± 0.64	$4.64 \pm 0.56^{**}$	$1.11 \pm 0.64^{**}$

$^{**}P < 0.01$ compared to the control (NS) group, $n = 7/\text{group}$.

Table 3. Numbers of $\alpha 7$ nAChR-LI neurons in prefrontal cortex and hippocampal CA3 in the three groups

	NS	<i>L</i> -Arg	<i>L</i> -NAME
Prefrontal cortex	30.7 ± 5.35	$39.4 \pm 5.10^{**}$	$22.1 \pm 2.60^{**}$
Hippocampus	18.6 ± 0.83	$25.3 \pm 4.29^{**}$	$12.0 \pm 1.99^{**}$

$^{**}P < 0.01$ compared to control (NS) group, $n = 7/\text{group}$.

Fig 3. $\alpha 7$ nAChR expression in the prefrontal cortex and hippocampus of the three groups of rats. A: Western blots of $\alpha 7$ nAChR protein. Upper: prefrontal cortex (PFC); lower: hippocampus. β -actin served as the internal control. B: quantitative representation of the protein levels of $\alpha 7$ nAChR. $n = 7/\text{group}$, $^{}P < 0.01$ compared to the control (NS) group.**

was decreased in both ($P < 0.01$).

$\alpha 7$ nAChR-like Immunohistochemistry

$\alpha 7$ nAChR-LI neurons were present in every layer of the prefrontal cortex (Fig. 2A), CA1 and CA3 as well as the dentate gyrus of the hippocampus (Fig. 2B); they were more abundant in CA3. The number of $\alpha 7$ nAChR-LI neurons differed among the three groups in prefrontal cortex and CA3 [prefrontal cortex, $F(2,18) = 25.685$, $P < 0.001$; hippocampus, $F(2,18) = 40.233$, $P < 0.001$]. These numbers in the *L*-Arg group were greater than in the control group, while the numbers in the *L*-NAME group were less than in control (Fig. 2 and Table 3).

Western Blot

The expression of $\alpha 7$ nAChR in prefrontal cortex and hippocampus was higher in the *L*-Arg group than in the control group (prefrontal cortex, 0.44 ± 0.01 vs 0.35 ± 0.03 ; hippocampus, 0.76 ± 0.02 vs 0.69 ± 0.02 , both $P < 0.01$); while in the *L*-NAME group, $\alpha 7$ nAChR expression was lower than that in the control group (prefrontal cortex 0.25 ± 0.02 vs 0.35 ± 0.03 ; hippocampus 0.41 ± 0.01 vs $0.69 \pm$

0.02 , both $P < 0.01$) (Fig. 3).

DISCUSSION

Previous studies have shown that nAChRs play a role in learning and memory by affecting the NOergic neuronal system^[7-9]. In recent years, studies using conditioned place preference suggested interactions between the nicotinic and NO systems in mice^[11]. However, no studies have shown that the NOergic neuronal system affects the cholinergic system or nAChRs and its effects on learning and memory.

Our results indicate that 7-day application of the NO precursor *L*-Arg induced a long-term high level of NO. This also increased the number of $\alpha 7$ nAChR-LI neurons and strengthened the expression of $\alpha 7$ nAChR protein in the prefrontal cortex and hippocampus, as well as improving learning and memory; while 7-day application of the NOS inhibitor *L*-NAME decreased the NO content, the number of $\alpha 7$ nAChR-LI neurons and $\alpha 7$ nAChR protein level, and weakened learning and memory. NO is synthesized from

L-Arg by NOS in the nervous system. Thus, the present results indicated that multiple applications of the NO precursor *L*-Arg increased the $\alpha 7$ nAChR expression and the learning and memory through a high level of NO for a prolonged period. Therefore, we infer that the NOergic neuronal system affects nAChRs and further affects learning and memory performance.

This conclusion is supported by previous studies showing that NO facilitates the release^[14] and inhibits the reuptake of neurotransmitters^[25] such as ACh. Both actions increase the availability of ACh in the synaptic cleft, which in turn may affect postsynaptic AChR modulation^[26]. In addition, NO has neuroprotective effects by acting as a potent antioxidant or an inhibitor of apoptosis-related enzymes^[27,28], and then could modulate the function and expression of $\alpha 7$ nAChR directly or indirectly. As noted, $\alpha 7$ nAChRs are thought to improve spatial learning and memory and neuronal plasticity^[6,29,30]. Therefore, it is possible that some of the effects of NO on learning and memory are mediated through an increase of $\alpha 7$ nAChR expression.

In conclusion, our results suggest that multiple applications of the NO precursor *L*-Arg increased exogenous NO levels and promoted the expression of $\alpha 7$ nAChRs in the prefrontal cortex and hippocampus. Meanwhile, the learning and memory performance of rats was improved. However, multiple applications of the NOS inhibitor *L*-NAME reduced the production of endogenous NO, then inhibited the expression of $\alpha 7$ nAChRs, and weakened the performance in learning and memory. Whether NO enhances learning and memory through the $\alpha 7$ nAChR mechanism needs further investigation.

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