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Research report

Nitric oxide facilitates active avoidance learning via enhancement of glutamate levels in the hippocampal dentate gyrus

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HIGHLIGHTS

- Role of NO of DG in active avoidance learning is studied in freely moving rats.
- We examine the effects of L-NMMA or SNP on Glu and fEPSP in DG during avoidance learning.
- NO facilitates the learning via enhancements of glutamate level and synaptic efficiency in DG.

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ABSTRACT

The hippocampus is a key structure for learning and memory in mammals, and long-term potentiation (LTP) is an important cellular mechanism responsible for learning and memory. Despite a number of studies indicating that nitric oxide (NO) is involved in the formation and maintenance of LTP as a retrograde messenger, few studies have used neurotransmitter release as a visual indicator in awake animals to explore the role of NO in learning-dependent long-term enhancement of synaptic efficiency. Therefore, in the present study, the effects of L-NMMA (a NO synthase inhibitor) and SNP (a NO donor) on extracellular glutamate (Glu) concentrations and amplitudes of field excitatory postsynaptic potential (fEPSP) were measured in the hippocampal dentate gyrus (DG) region during the acquisition and extinction of active-avoidance behavior in freely-moving conscious rats. In the control group, the extracellular concentration of Glu in the DG was significantly increased during the acquisition of active-avoidance behavior and gradually returned to baseline levels following extinction training. In the experimental group, the change in Glu concentration was significantly reduced by local microinjection of L-NMMA, as was the acquisition of the active-avoidance behavior. In contrast, the change in Glu concentration was significantly enhanced by SNP, and the acquisition of the active-avoidance behavior was significantly accelerated. Furthermore, in all groups, the changes in extracellular Glu were accompanied by corresponding changes in fEPSP amplitude and active-avoidance behavior. Our results suggest that NO in the hippocampal DG facilitates active avoidance learning via enhancements of glutamate levels and synaptic efficiency in rats.

1. Introduction

The hippocampus is a critical site for several learning and memory processes including avoidance learning [1–3], and a number of studies have stressed the importance of long-term potentiation (LTP) in learning and memory formation [4–6]. Hippocampal LTP is a widely studied cellular model of synaptic plasticity and is believed to underlie declarative forms of learning [7]. In the hippocampus, the induction of LTP is typically dependent on the activation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, a consequent increase in Ca^{2+} influx into the postsynaptic cells [7,8], and a number of Ca^{2+}-activated biochemical processes in the postsynaptic neurons, including nitric oxide (NO) formation [9,10]. Furthermore, the induction and maintenance of LTP require that a retrograde messenger released from the postsynaptic cell acts on presynaptic terminals, where it enhances transmitter release [11].

Nitric oxide (NO), a gaseous free radical is an important neuronal messenger in the central nervous system (CNS) and is synthesized from L-arginine, molecular oxygen, and NADPH by NO synthases (NOS) [12,13]. Several lines of evidence indicate that NO is involved

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in various forms of synaptic plasticity, including hippocampal LTP, as an intercellular retrograde messenger [10,14–16]. However, because these experiments were conducted in in vitro hippocampal slices, in which neuronal connectivity is decreased and the extracellular milieu is significantly altered compared to in vivo preparations, the results may not faithfully reflect the natural functioning of the organism. Behavioral studies have revealed that NO has modulatory effects on learning and memory processes [17–19]. For instance, hippocampal NO is involved in spatial learning and inhibitory avoidance tasks in rats [3,20,21]. This raises the possibility that NO is involved in learning-dependent long-term enhancement of synaptic efficiency (LD-LTE) as a retrograde messenger. However, these studies did not consider the possibility of using both neurotransmitter release and synaptic efficiency as read-outs to elucidate the role of NO in conscious, freely moving animals. Although chronic *in vivo* recordings in freely moving animals are more difficult and time-consuming than in vitro recordings, the results obtained may provide the most accurate and holistic view of the functioning brain.

The dentate gyrus (DG), a hippocampal subregion, plays a critical role in learning and memory, and both computational modeling and physiological evidence indicate that the DG is important for encoding memory processes [22–25]. The perforant path (PP) is the main excitatory afferent pathway to the hippocampus. The response of DG granule cells to PP stimulation and the expression of LTP in this pathway vary with the behavioral state [26,27]. Although it has been demonstrated that nNOS is highly concentrated within the hippocampal DG region [12], the role of NO within the DG in the active avoidance learning is rarely studied. Therefore, in the present study, we microinjected N^G-methyl-L-arginine acetate salt (L-NMMA, an inhibitor of NO synthase) or sodium nitroprusside (SNP, a NO donor) directly into the DG region, then measured both glutamate (Glu) release – using an in vivo brain microdialysis technique – and synaptic efficiency in the DG, during the acquisition and extinction of active-avoidance behavior in conscious, freely-moving rats.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 180–220 g (Vital River Laboratories, Beijing, China) were used. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the animals' suffering, and the minimum number of animals was used.

2.2. Drugs

L-NMMA and SNP (both from Sigma, USA) were dissolved in modified Ringer's solution (147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$; pH 6.5) at concentrations of 1 mM and 0.1 mM, respectively, and stored at 4 °C.

2.3. Surgical procedures

Rats were anesthetized with 10% chloral hydrate (300 mg/kg, i.p.) and placed on a stereotaxic frame (David Kopf, USA). A guide cannula (0.90 mm OD) affixed a 20-gauge stainless steel tubing (Terumo, Japan) was stereotaxically implanted 1.5 mm above the DG region according to the atlas of Paxinos and Watson [28], and sealed with a dummy cannula after implantation. The stereotaxic coordinates were 3.4 mm posterior to bregma, 2.2 mm lateral to the midline, and 2.5 mm ventral to the dural surface. A bipolar stimulating electrode (made of epoxylite-coated stainless steel; A-M Systems, Inc., USA) was lowered into the ipsilateral perforant path (PP; 6.8 mm posterior to bregma, 4.0 mm lateral to the midline, 5.0 mm ventral to the dural surface), and the guide cannula and stimulating electrode were fixed to the skull by dental cement. The animals were individually housed with access to food and water and allowed to recover from surgery for 3 days.

On the day before the experiment, the animal was anesthetized with isoflurane (2.5–3.0% in 100% oxygen) and the dummy cannula was replaced with a microdialysis probe bonded to microinjection tubing. To reach the DG region, the tip of the microdialysis probe, covered with a 1.5-mm long hollow fibers (200 μm OD, acetate cellulose membrane, cut-off 5.0×10^4 mol wt; Eicom, Japan), was set to extend 1.5 mm beyond the guide shaft. A monopolar recording electrode (stainless steel pin; A-M Systems, Inc, USA) inserted into the 20-gauge stainless steel tubing reached the DG region, where extracellular field potentials evoked by stimulation of the PP were recorded. Ground and reference electrodes consisting of uninsulated stainless steel machine screws were positioned contralaterally on the skull surface at locations corresponding to the parietal cortex. The recording electrode was lowered until the maximal evoked response was visually confirmed, and then fixed by dental cement.

2.4. Experimental procedure

The animals were divided into the following groups: (1) non-behavioral test groups: 1 μL of the drug solution (modified Ringer solution; L-NMMA; SNP) was microinjected into the DG region, and the extracellular concentrations of Glu and amplitudes of fEPSP were examined; (2) L-NMMA groups: on every session of behavioral test, 1 μL of the drug solution (modified Ringer solution; L-NMMA) was microinjected into the DG region 10 min before the test, and the Glu concentrations and fEPSP amplitudes were examined 10 min after behavioral test; (3) SNP groups: on every day of behavioral test, 1 μL of the drug solution (modified Ringer solution; SNP) was microinjected into the DG region 30 min before the test, and the Glu concentrations and fEPSP amplitudes were examined 10 min after behavioral test.

On the day of experiments, the collection of dialysates for Glu and measurement of fEPSP amplitudes were carried out under freely-moving conditions. The microdialysis probe was perfused with modified Ringer's solution at a constant rate of 1.5 μL/min, and the dialysate from the DG region was automatically collected by a fraction collector (EFC-82, Eicom, Japan) at 4 °C every 10 min. Three consecutive dialysate samples were collected to measure the Glu concentration. The measurement of fEPSP amplitudes was performed simultaneously: the PP was stimulated 10 times by single-phase square wave pulses (0.1 ms/phase, intensity was chosen to elicit 50% of the maximal fEPSP, interval was 30 s) generated with The Flexible Stimulus Isolator (ISO-Flex, A.M.P.I., Israel). Evoked responses were filtered (0.5–2.0 kHz) and amplified (1000×) by an AC amplifier (Neurolog, Digitimer, UK), digitized (Micro3, CED, UK), and analyzed on a computer with Spike2 software (CED, UK). 10 fEPSP traces were averaged to obtain the mean amplitude.

Glu levels were measured using high-performance liquid chromatography with electrochemical detection (HTEC-500, Eicom, Japan), as described previously [29]. Briefly, an *o*-phthalaldehyde (OPA) solution (40 mM) was made by adding 13.5 mg of OPA and 10 μL of 2-mercaptoethanol to 2.5 mL of 0.1 M K_2CO_3 buffer (pH = 9.5) with 10% ethanol. The solution was then stored at −4 °C and diluted in 0.1 M K_2CO_3 to yield a 4 mM OPA solution just before detection. The dialysate (12 μL) was mixed with 3 μL of the 4 mM OPA solution and allowed to react for 2.5 min at 25 °C incubation. After completing the reaction, 10 μL of the reaction mixture was