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THE EFFECTS OF NITRIC OXIDE WITHIN THE DORSAL HIPPOCAMPUS ON SPATIAL LEARNING AND MEMORY IN MALE NMRI STRESSED MICE

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Nitric oxide is an essential neurotransmitter in the hippocampus involved in spatial learning and memory and response to stress. This study investigated the effects of nitric oxide within the dorsal hippocampus on spatial learning and memory in male NMRI stressed mice. Male NMRI (Naval Medical Research Institute) mice (n= 7/group) were divided into; Saline, L-NAME (L-Nitro-Arginine Methyl Ester) (1, 5, 10 µg/mouse), and L-Arginine (1, 5, 10 µg/mouse) groups. Seven days after bilateral intra dorsal hippocampus cannulation, half of the animals received electro foot shock stress once/day, for four consecutive days. Barnes maze method was applied for the evaluation of stress effects on spatial learning and memory. Elapsed time and distance traveled to reach the target hole, and the numbers of errors were calculated as spatial learning and memory performance indicators. Stress increases the time and distance for reaching the target hole. The number of errors also was increased in the stress group. Intra dorsal hippocampus administration of L-arginine reduced the stress-induced spatial learning and memory deficit. The drug reduced the time and distance for reaching the target hole. L-NAME also reduced the stress effect. The drug reduced the time and distance for reaching the target hole. Since both the inhibition of NO synthesis and increasing the NO synthesis in the dorsal hippocampus resulted in learning and memory improvements in mice exposed to electro foot shock stress, NO in the dorsal hippocampus might be involved in stress-related spatial learning and memory deficits.

Key words: *Dorsal Hippocampus; L-Arginine; L-NAME; Nitric oxide; Spatial Learning and Memory*

INTRODUCTION

It is well established that chronic stress can impair spatial learning and memory in rodents[1&2] and declarative and working memory in humans[3]. It is also known that the effects of stress on spatial memory in rodent's deficits and declarative memory impairment in humans are due to the activity of glucocorticoid hormones released during stressful events from the adrenal gland on the hippocampal neurons[4&5]. In addition, stress response causes the release of catecholamines such as epinephrine and noradrenaline, both from the adrenal medulla and the locus coeruleus in the brain. Catecholamines provide the body for 'fight-or-flight' responses and quickly affect neural function in several areas of the brain essential to learning and memory[6].

Experiments indicated that glucocorticoid hormones (corticosterone in rodents and cortisol in humans) which are released during stress from the adrenal glands, readily pass the blood-brain-barrier and can activate their receptors located in the cell cytoplasm and cell membrane of the hippocampal pyramidal

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neurons (for rev see:[7]). The activated glucocorticoid receptors induce their effects by increasing the expression of particular genes and glutamate system activity in the hippocampus pyramidal and non-pyramidal neurons[8&9].

Stress exposure induces the release of glucocorticoids by activation of the hypothalamic-pituitaryadrenocortical axis. Glucocorticoids released into the circulation pass through the blood-brain barrier and activate glucocorticoid receptors at the synapse of hippocampal neurons.

In presynaptic neurons, glucocorticoid receptors control release of glutamate by genomic mechanisms. In Postsynaptic neurons, glucocorticoid receptors stimulate rapid non-genomic increases in synaptic GluA1 (Glutamate A1) expression and phosphorylation of CamKII (calcium/calmodulin-dependent protein kinase II), CREB (cAMP-response element binding protein), and TrkB (Tropomyosin receptor kinase B), as well as genomic-dependent increases in Arc expression. TrkB-mediated signaling pathways activated by Brain-derived neurotrophic factor (BDNF) converge on CREB phosphorylation[10].

It is shown that stress-induced cell atrophy in the hippocampus is dependent on the glutamate system activity, especially its N-methyl-D-aspartate (NMDA) receptors activity in this brain region[10-12]. Other findings also revealed that NMDA receptors have a pivotal role in the synaptic plasticity and memory performance in the hippocampus (for rev see:[13]). On the other hand, it is well funded that glutamate induces its action on the NMDA receptors at least in part via activation of the enzyme nitric oxide synthase (NOS) and nitric oxide (NO) production[14]. Nitric oxide is one of the most important neurotransmitters in the nervous system that plays a vital role in the functions of the nervous system, such as transmitting pain signals, memory and learning, and drug abuse[15-17]. Regarding learning and memory, it is well established that nitric oxide plays an essential role in this phenomenon (for rev see:[18]). For example, it is shown that nitric oxide can affect spatial learning in rats[19]. In addition, it is shown that intra-cerebroventricular (icv) administration of an enzyme nitric oxide synthase inhibitor, NG-nitro-L-arginine methyl ester (L-NAME), can inhibit the promotional effects of four-week wheal running on spatial learning and memory in Sprague-Dawley rats. Those researchers also showed that the positive effects of wheal running on spatial memory are associated with an increase in the number of capillaries in the hippocampal CA1 and dentate gyrus areas, which were inhibited by i.c.v. L-NAME administration[20]. It is also revealed that nitric oxide can stimulate the release of noradrenaline and glutamate in the hippocampus *in-vivo*[21], the neurotransmitters in which are also involved in spatial learning and memory. Interestingly, it is claimed that nitric oxide may also be involved in the initiation and/or progression of Alzheimer's disease[22]. It must be noted that Alzheimer's disease is manifested by hippocampus degeneration and memory decline[23].

The possible role of nitric oxide in the dorsal hippocampus in spatial learning and memory during stress is poorly understood. Thus, the present study examined whether L-NAME or L-argenin administration in the dorsal hippocampus would affect spatial learning and memory in control and stressed mice.

MATERIALS AND METHODS

Animals

Male NMRI (Naval Medical Research Institute) mice (Pasture Institute, Tehran, Iran) (housed 3/cage, n= 7/group), were used in these experiments. The animals were kept in the sound-attenuated animal room (a soundproof window has been installed in the animal room to reduce noise) with a controlled light/dark cycle (lights on at 7:00) and free access to standard mouse chow (Pars dam Animal Food Co., Tehran, Iran) and tab water *ad lib*. All experiments were performed according to Baqiyatallah University of Medical Sciences for Animal Care (#431, Jan 12, 2015).

Animal groups

3-5 grouping of studied animals N7 male NMRI mice with a weight range of 20-25 were randomly divided into different groups: 1. Normal groups were tested without any interference. 2. The negative control group underwent surgery, and the cannulas were

placed inside the dorsal hippocampus and saline was injected into them and tested without further intervention. 3. Positive control group who underwent surgery and the cannulas were placed inside the dorsal hippocampus and saline was injected into them and after 5 min, they were subjected to electric shock of the sole of the foot (for four days) and from the day the fifth were tested. 4. The stress group that did not have surgery and saline was not injected, but they were subjected to the electric shock of the sole of the foot (for 4 days) and were tested from the fifth day. 5. The operated groups underwent surgery, and the cannulas were placed in the dorsal hippocampus and were injected with different doses of L-arginine (1, 5 and 10 µg/mouse)[24]. 6. The operated groups underwent surgery and the cannulas were placed in the dorsal hippocampus and were injected with different doses of L-arginine (1, 5 and 10 µg/mouse) and after 5 minutes they were subjected to the electric shock of the sole of the foot. 7. Experimental groups undergoing surgery and cannulas were placed inside the dorsal hippocampus and were injected with different doses (1, 5 and 10 µg/mouse) of L-NAME (L-Nitro-Arginine Methyl Ester)[24]. 8. Experimental groups undergoing surgery and cannulas were placed inside the dorsal hippocampus and were injected with different doses (1, 5 and 10 µg/mouse) of L-NAME, and after 5 min, they were subjected to the electric shock of the sole of the foot.

Stress procedure

Five minutes after bilateral intra-dorsal hippocampus infusion of L-arginine or L-NAME, the mice were transferred to a com-box (Borje Sanat, Iran). Electric foot shock stress was induced by placing the animals in a com-box. This device is divided into nine smaller compartments (16×16×50 cm), provided with a grid floor made of stainless-steel rods. In each session, mice were exposed to the electrical foot-shock (1mA, 0.2 Hz) for 2-s duration, the interval of 10 sec for 1 min through the stainless steel grids, controlled by a computer connected to the com-box[25]. Stress induction continued for four consecutive days.

Barnes maze Test

Barnes maze apparatus is a plate made of milky Plexiglas with a diameter of 90 cm, which is located 2 cm from its edge with 20 holes with a diameter of 8 cm and a distance of 5 cm from each other. Below one of these holes (destination chamber) is a chamber made of black Plexiglas with dimensions of 10×10 cm in which the animal can be placed. In this room, there is 0.1 gram of animal food. At the beginning of the experiment, the animal is left in the center of the maze and moves freely in all directions until it finds the destination chamber. The time required finding the destination room, as well as the amount of movement and time spent in other holes, is recorded and used as a symbol of animal learning. The animal's food is reduced by 18% the day before the test begins to speed up the learning process. This allows the animal to remember the location of the destination room sooner on the day of the test. The test for each animal ends when they reach the destination room and receives food. Each animal is tested between 6 and 10 times a day. The 70% success rate is a sign of complete learning about each animal. This process continues for four days. The same thing is repeated on the fifth day with the animals, and the amount of memory is checked (Fig. 1).

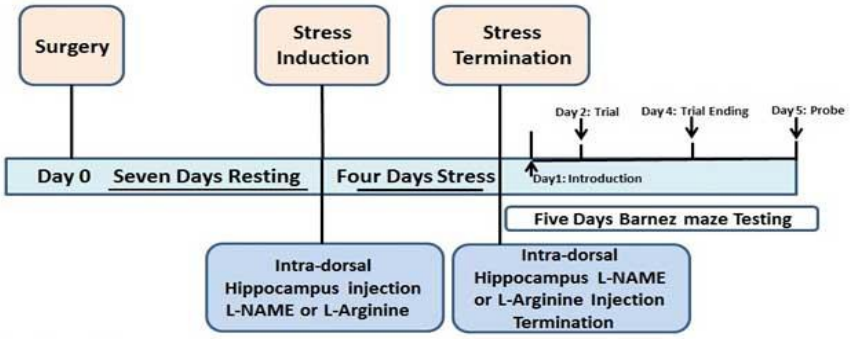

Fig. 1: Schematic timeline of the study for the intra-dorsal hippocampus L-NAME and L-Arginine injections.

Drug administration

The animals were first operated and two cannulas were placed symmetrically in their dorsal hippocampus using a stereotaxic device. Experiments were started one week after surgery and recovery. The drug was injected into the cannulated area through a Gage 30 injection cannula connected to a Hamilton syringe via a polyethylene connector. The injection cannula length µ500 longer than the guide cannula was chosen. First, the polyethylene tube and the injection cannula were filled with L-arginine / L-NAME. When injecting, the animals were gently restrained by hand and the steel wire was pulled out of the guide cannula. After the injection, the injection cannula remained in place for 60 seconds to release the drug, and then it was gently removed.

Statistical analysis

In this study, the extent of changes in the learning and memory of animals was studied. Data were expressed as mean ± standard error of variables. The data were analyzed using repeated measures variance analysis of variance with two factors of drug and stress and two-way analysis of variance with the same two factors and then Tukey test. Differences with $p< 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Results

Effect of stress on learning and spatial memory in stressed male mice

For this purpose, two groups of animals (group n= 7) were selected. The first group received saline as a control group and was placed in a silent stress device for four days without stress induction. After the fifth day, the Barnes maze test was performed on them. This test measured the three factors of elapsed time, distance traveled, and the number of errors. As a stress group, the second group first received saline and then received an electric shock from the sole of the foot as described in the Methods section for four days. After the fifth day, the Barnes maze test was performed on them. As shown in Fig. 2A, stressed mice spent more time finding the target chamber on training (learning) and test (memory) days (N= 7 group, $p< 0.05$) compared to the control group. Similarly, stress significantly changed the distance traveling on day 5 (N= 7) as compared to the control group ($p< 0.05$) (Fig. 2B). Although the error to find escape hole on days 1, 2, and 4 significantly increased in the stress group, no significant changes were observed in error on day five compared to the control group (Fig. 2C).

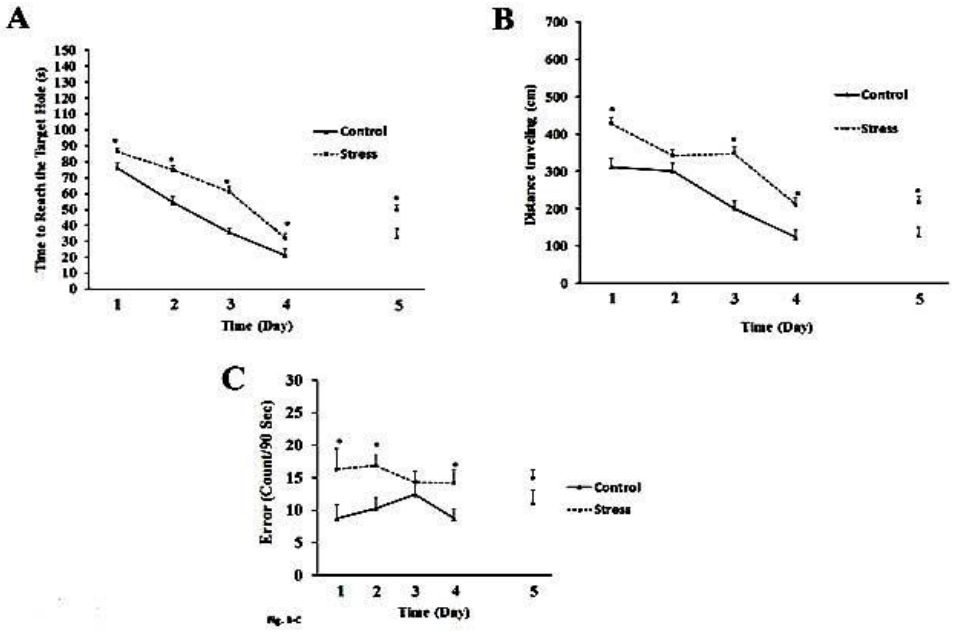

Fig. 2. Effects of stress on spatial memory (by Barnes test). **A)** The time elapsed for male mice to find the target chamber. **B)** Distance traveling. **C)** Error (N= 7). Values represent the mean ± SEM. * $p< 0.05$ as compared to the control group.

Effect of intra-dorsal hippocampus injection of L-arginine on spatial learning and memory in control and stressed mice

For this purpose, eight groups of animals were selected (n= 7 group). The first group received intra-dorsal hippocampus injection saline (10 µg/mouse) as a negative control or different doses of L-arginine (1, 5 and 10 µg/mouse intra-dorsal hippocampus injection, respectively), and 5 min later, they were placed in a silent stress induction device for 60 min. This was repeated for four days, and on the fifth day the Barnes maze test was performed on them.

The second group received different doses of L-arginine (1, 5, and 10 µg/mouse), in the dorsal hippocampus and 5 min later underwent foot-shock stress.

As shown in Fig. 3, intra-dorsal hippocampus injection L-arginine, significantly ($p< 0.05$) decreased the time elapsed for male mice to find the target chamber (Fig. 3A), distance traveling (Fig. 3B) and error (Fig. 3C) on day five as compared to the saline group. Also, intra-dorsal hippocampus injection L-arginine significantly ($p< 0.01$) decreased the time elapsed for male mice to find the target chamber (Fig. 4A), distance traveling (Fig. 4B), and error (Fig. 4C) on day five as compared to the stress group. However, dose 10 µg/mouse performed better than other doses.

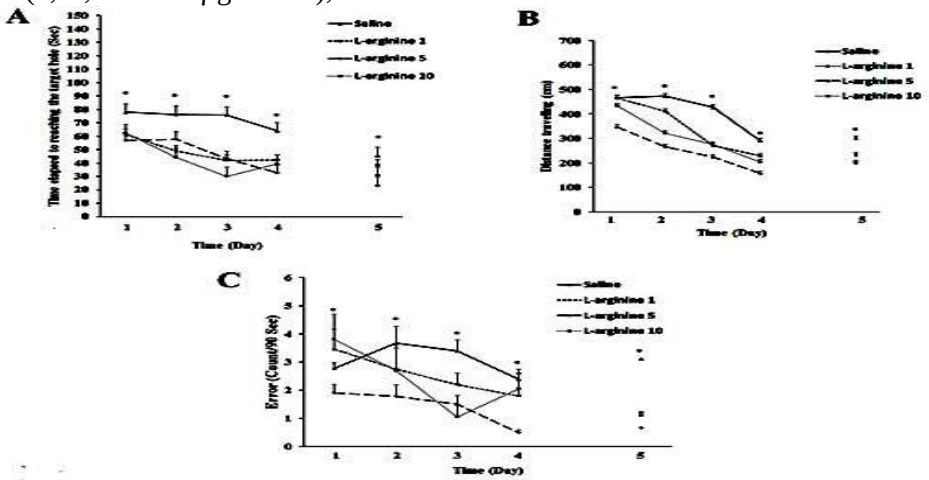

Fig. 3: Effect of intra-hippocampal administration of L-Arginine (1, 5 and 10 µg / mouse) on the **A)** time elapsed for male mice to find the target chamber, **B)** Distance traveling, and **C)** Error in the non-stress animal. N=7, Values represent the mean±SEM. * p <0.05 as compared to the saline group.

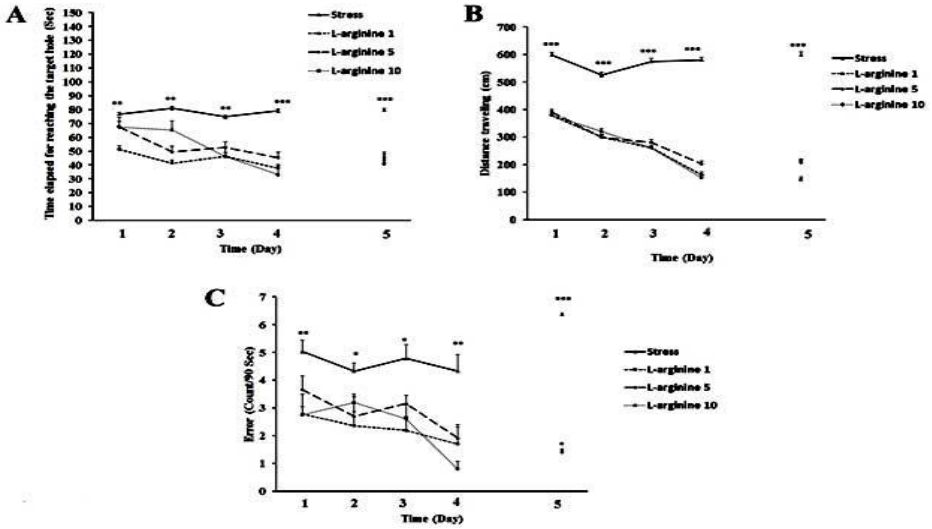

Fig. 4: Effect of intra-hippocampal administration of L-Arginine (1, 5 and 10 µg / mouse) on the **A)** time elapsed for male mice to find the target chamber, **B)** Distance traveling, and **C)** Error in the stressed animal. N=7, Values represent the mean±SEM. * p <0.05 as compared to the stress group. ** p <0.01 as compared to the stress group. *** p <0.001 as compared to the stress group.

Effect of intra-dorsal hippocampus injection of L-NAME on spatial learning and memory in control and stressed mice

The effect of intra-dorsal hippocampal administration of L-NAME on learning and spatial memory in control and stressed mice was investigated.

As depicted in Fig. 5, the time elapsed for male mice to find the target chamber significantly ($p< 0.05$) increased in the saline group (by two-way ANOVA followed by Tukey's test) as compared to different doses of L-NAME (1, 5, and 10 µg/mouse) (Fig. 5A). Furthermore, injection of L-NAME significantly ($p< 0.01$) reduced distance traveling compared with the saline group on probe day (Fig. 5B). However, intra dorsal hippocampus injection of different doses of L-NAME (1, 5 and 10 µg/mouse) could not significantly ($p< 0.01$) change the error to achieve the target hole on day 5 (N= 7, Fig. 5C) as compared to the saline group.

Foot shock stress significantly increased the time elapsed for male mice to find the target chamber ($p< 0.05$ by two-way ANOVA followed by Tukey's test) as compared with the control (saline) or different doses of L-NAME (1, 5 and 10 µg/mouse) (Fig. 6A) groups on day 5 (probe day) (i.e., foot shock stress damaged memory on the probe day). Also, intra-dorsal hippocampus injection of different doses of L-NAME (1, 5 and 10 µg/mouse) significantly ($p< 0.05$) decreased distance traveling (Fig. 6B) and error (Fig. 6C) to find the target hole compared with the stress group, respectively. On the other hand, intra-dorsal hippocampus injection of different doses of L-NAME improved foot shock stress-induced memory damages on the probe day.

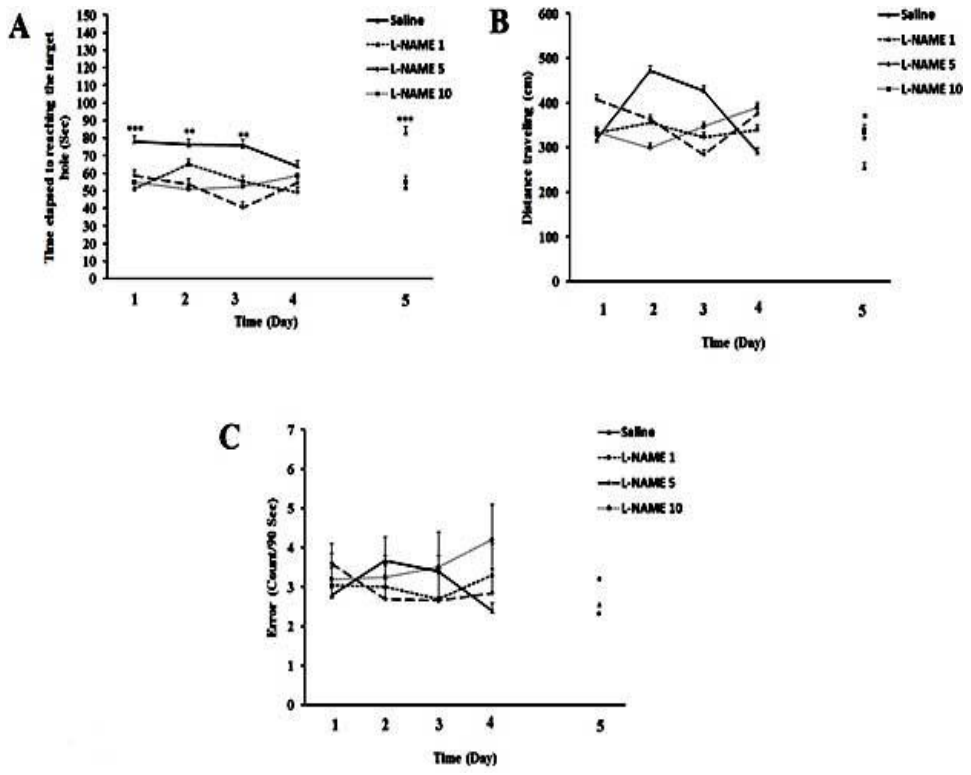

Fig. 5: Effect of intra-hippocampal administration of L-NAME (1, 5 and 10 µg / mouse) on the **A)** time elapsed for male mice to find the target chamber, **B)** Distance traveling, and **C)** Error in the non-stress animal. N=7, Values represent the mean±SEM. * p <0.05 as compared to the saline group. ** p <0.01 as compared to the stress group. *** p <0.001 as compared to the stress group.

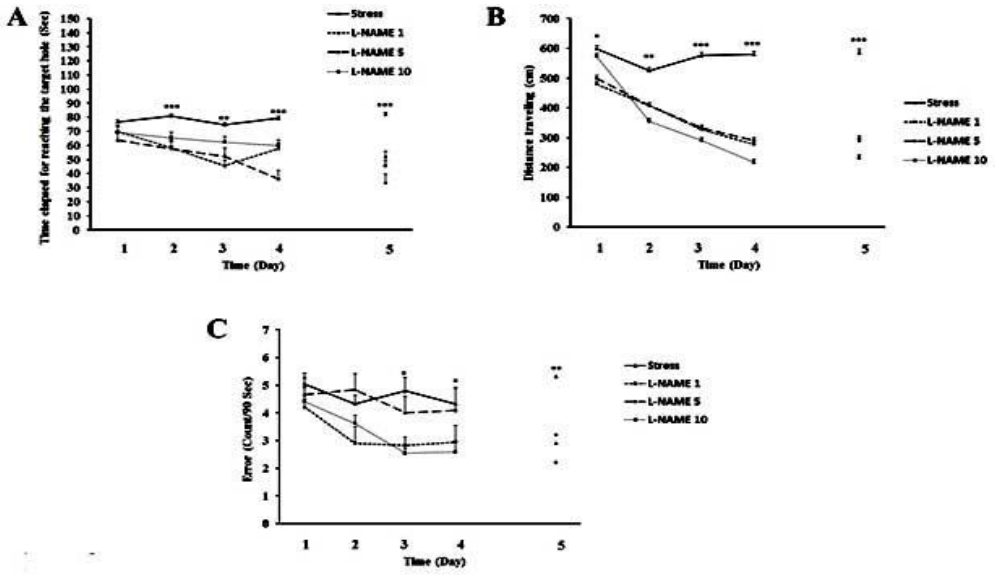

Fig. 6: Effect of intra-hippocampal administration of L-NAME (1, 5 and 10 µg / mouse) on the **A)** time elapsed for male mice to find the target chamber, **B)** Distance traveling, and **C)** Error in the stressed animal. N=7, Values represent the mean±SEM. * p <0.05 as compared to the stress group. ** p <0.01 as compared to the stress group. *** p <0.001 as compared to the stress group.

Discussion

The present study was performed to investigate the effect of nitric oxide in the dorsal hippocampus on behavioral changes including, spatial learning and memory in stressed mice. Findings in this study showed that foot-shock stress significantly reduced spatial learning and memory. The results also show an improvement in long-term memory by inhibiting nitric oxide production in the dorsal hippocampus. In contrast, inhibition of nitric oxide production did not significantly affect current memory and short-term memory. The production of NO under physiological conditions is one of the mediators required for normal learning. To date, many behavioral and neurochemical studies have been performed to elucidate the effect of NO on different learning devices, and there is a general convergence about the effective involvement of NO in learning and memory[26&27]. Holscher and Rose revealed that infusion of the N-nitro-L-arginine (NOS inhibitor) before training causes amnesia for the passive avoidance task. The amnesia may be conquered by infusing L-arginine together with the inhibitor[28]. Another study showed that intra-hippocampal infusion L-NAME impairs passive avoidance learning in rats[29]. It has also been observed that intracerebral injection of sodium nitroprusside (NO donor) after one week of training facilitates long-term memory formation in day-old chicks[30]. In the face of any stressors, the body activates compensatory mechanisms to control them. The two most important mechanisms of the body to cope with various stresses are the two main axes: sympathoadrenal (SA) and the hypothalamic-pituitary-adrenal (HPA) axis. When the brain interprets a phenomenon as stress, the activity of the central part of the amygdala sends messages to the paraventricular nucleus of the hypothalamus, which stimulates neurons in the nucleus[31-33]. There are two main groups of neurons in this nucleus: the macrocellular and the small cell (parvocellular). Large cells secrete a substance called vasopressin, or anti-urinary hormone, which induces binge drinking during stress. In contrast, small cells of a substance called CRF (corticotropin-releasing-factor) or corticotropin-releasing factor pass through the hypothalamus-pituitary gland to the anterior pituitary gland, increases the production and secretion of POMC protein, which is the precursor to ACTH. ACTH is secreted into the bloodstream and reaches the outside of the adrenal cortex (zona fasciculata). In this area, this hormone stimulates its receptors to increase the production of various glucocorticoids such as cortisol (in humans) and corticosterone (in rodents). This causes an increase in these hormones in the blood, followed by the effects of these hormones[34].

Ohno et al. showed that intra-hippocampal injection of L-NAME into the dorsal hippocampus after blood flow reperfusion increased the number of errors in current memory, while L-arginine minimized this number of errors, so they suggested processes mediated by NO synthesis in the dorsal hippocampus contributes to the postischemic damages of working memory [35]. Pourmotamed showed that inhibition of NOS enzyme in the CA1 region of the hippocampus suppresses learning and spatial memory in morphine-dependent male rats in the Morris blue model[36]. According to these studies, NO has a positive role in learning time, and inhibition of its synthesis leads to learning and memory deficits. While NO usually acts as a physiological neurotransmitter, excess NO production leads to brain damage. As a free radical, Nitric oxide is highly reactive and causes toxicity by damaging necessary metabolic enzymes and impairs nerve growth factor (NGF) function through the production of peroxynitrate. Any dysfunction of the NGF pathway is associated with cognitive disease and neuronal membrane damage[37]. On the other hand, it has been suggested that NO produced by nNOS has played a vital role in neuronal degeneration[38]. Stress alters nitric oxide production in disturbed areas of the brain. Chronic stress has been shown to increase the expression of nNOS and iNOS enzymes in the neocortex and hippocampus, leading to neurological disorders in animals [39]. The NO system mediates some of the effects of stress on behavior. In this regard, it has been shown that chronic mild stress (CMS) has led to changes in nitric oxide levels in BALB / c mice, followed by different behavioral responses in the animal[40]. Activation of glutamate receptors has also been reported to lead to activation of NOS and NO production and activation of target proteins that affect both LTP and learning and memory processes[41].

On the other hand, L-NAME has been shown to reverse the beneficial effects of Pioglitazone on spatial memory acquisition in Alzheimer's mice[42]. It has also been reported that nitric oxide may be involved in normal aging and neurodegenerative processes. Other reports support this research, such as the fact that in 1996, high NO production in patients aged 47 to 87 years was involved in the pathogenesis of Alzheimer's disease[43]. Another study has shown that the simultaneous expression of NOS and p12ras in pyramidal neurons is responsible for the degeneration of nerve fibers in Alzheimer's disease[44]. Hyman *et al.*, also found that NO-producing neurons in Alzheimer's patients affect different areas of the brain, especially the hippocampal formation[45]. Miranda et al. (2000) suggested that NO acts directly as an antioxidant. Thus, in mammalian cells, it protects against cell damage caused by free radicals. Therefore, in the present study, stress probably increased NO in the dorsal hippocampus. In this regard, Olivenza et al. showed that the destructive effects of chronic limiting stress are mediated by increasing the amount of NO in the cerebral cortex. The findings also support the role of NOS enzyme inhibitors in neuroprotection in stressful situations[46]. Another report also states that dysregulation of the NO/NOS pathway in Huntington's disease is responsible for nerve damage[47]. Therefore, it can be assumed that nitric oxide plays an essential role in cognitive deficits related to stress. In this regard, studies showed that high nitric oxide production in the hippocampus of chronically exposed animal's leads to the cessation of neurogenesis, while in mice lacking the nNO gene and treated with nitric oxide inhibitor, reverse this effect[48]. In support of these findings, it has been suggested that repetitive limiting stress increases the expression of nNOS neurons in CA1, CA3, and EC regions. These results suggest that the dorsal hippocampus is involved in modulating the effects of stress[49]. Since nitric oxide production during learning is essential to memory recall, inhibition of its production can lead to defects in these processes. However, given that the inhibition of NO production in the pre-stress time and at a time interval relative to the learning phase was reminiscent of the effect, it probably did not interfere with learning and memory-related circuits. Effects on areas affected by stress mediated it. They have been able to moderate these effects and ultimately increase memory. Thus, it is clear that NO role in memory can vary according to stressful or non-stressful conditions.

In conclusion, it seems that nitric oxide in the mice dorsal hippocampus is involved in the learning and memory impairment induced by foot-shook stress. Therefore, it is suggested

that NO in the dorsal hippocampus promotes spatial memory under tonic and stress conditions.

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تأثيرات أكسيد النيتريك داخل الحُصَيْن الظهري على التعلم المكاني والذاكرة في ذكور فئران (NMRI) المعرضة للتوتر

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أكسيد النيتريك هو ناقل عصبي أساسي في الحُصين يشارك في التعلم المكاني والذاكرة والاستجابة للتوتر. بحثت هذه الدراسة في آثار أكسيد النيتريك داخل الحصين الظهري على التعلم المكاني والذاكرة لدى فئران (NMRI) المعرضة للتوتر. تم تقسيم فئران (NMRI) الذكور (عدد ٧/ مجموعة) إلى مجموعات كالتالي: محلول ملحي ، مجموعة إل–نيترو أرجينين ميثيل إستر (L-NAME) (١ ، ٥ ، ١٠ ميكروجرام / فأر) ، ومجموعة إل–أرجينين (١ ، ٥ ، ١٠ ميكروجرام / فأر). بعد سبعة أيام من إجراء إقناء ثنائي للحُصين الظهري ، تعرض نصف الحيوانات لصدمة كهربائية للقدم مرة واحدة في اليوم ، لمدة أربعة أيام متتالية ثم تم تطبيق طريقة متاهة بارنز لتقييم تأثيرات التوتر على التعلم المكاني والذاكرة. تم حساب الوقت المنقضي والمسافة المقطوعة للوصول إلى الفتحة المستهدفة وعدد الأخطاء كمؤشرات للتعلم المكاني وأداء الذاكرة. لوحظ أن الضغط يزيد من الوقت والمسافة المقطوعة للوصول إلى الفتحة المستهدفة. كما لوحظ أيضا زيادة في عدد الأخطاء في المجموعة التي تعرضت للصدمة الإجهاديه. خفض إعطاء إل–أرجينين داخل الحصين الظهري من النقص في التعلم المكاني وعجز الذاكرة الناتج من الصدمة الإجهاديه. كما قلل الدواء الوقت والمسافة اللازمة للوصول إلى الحفرة المستهدفة. يقلل إل–نيترو أرجينين ميثيل إسترأيضًا من تأثير التوتر حيث قلل الوقت والمسافة اللازمة للوصول إلى الحفرة المستهدفة. ونظرًا لأن كلا من تثبيط وزيادة تخليق أكسيد النيتريك في الحصين الظهري أدى إلى تحسين التعلم والذاكرة في الفئران المعرضة للتوتر بصدمة القدم الكهربائية ، فقد يكون أكسيد النيتريك في الحصين الظهري متداخلا في التعلم المكاني المرتبط بالتوتر وعجز الذاكرة.