

7-NI and ODQ Disturbs Memory in the Elevated Plus Maze, Morris Water Maze, and Radial Arm Maze Tests in Mice

Oguz Mutlu¹, Furuzan Akar¹, Ipek Komsuoglu Celikyurt¹, Pelin Tanyeri², Guner Ulak¹ and Faruk Erden¹

¹Department of Pharmacology, Kocaeli University Medical Faculty, Kocaeli, Turkey. ²Department of Pharmacology, Faculty of Medicine, Sakarya University, Sakarya, Turkey.

ABSTRACT: Nitric oxide (NO) is an atypical neurotransmitter that causes changes in cognition. Nitric oxide synthase (NOS) and guanylate cyclase (GC) inhibitors have been shown to exert some effects on cognition in previous studies; however, the findings have been controversial. This study was aimed at understanding the effects of an NOS inhibitor, 7-nitroindazole (7-NI), and a guanylate cyclase inhibitor, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), on spatial memory in modified elevated plus maze (mEPM), Morris water maze (MWM), and radial arm maze (RAM) tests. Male Balb-c mice were treated via intraperitoneal injections with 7-NI (15 mg/kg), ODQ (3, 10 mg/kg), L-arginine (100 mg/kg) + 7-NI (15 mg/kg), or physiological saline. ODQ (3 mg/kg) and 7-NI (15 mg/kg) significantly increased the second-day latency in the mEPM test. 7-NI (15 mg/kg) and ODQ (10 mg/kg) significantly increased the escape latency in second, third, and fourth sessions, decreased the time spent in the escape platform's quadrant, and increased the mean distance to the platform in the probe trial of the MWM test. ODQ (3, 10 mg/kg) and 7-NI (15 mg/kg) significantly increased the number of errors, whereas only 7-NI increased the latency in the RAM test. The administration of L-arginine (100 mg/kg) prior to 7-NI inverted the effects of 7-NI, which supports the role of NO on cognition. Our study shows that the NO/cGMP/GS pathway can regulate spatial memory in mice.

KEYWORDS: 7-NI, ODQ, memory, mice

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CORRESPONDENCE: oguzmutlu80@hotmail.com

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Introduction

Nitric oxide (NO) is synthesized from L-arginine by NO synthase (NOS) as a result of Ca²⁺ influx, which is activated by induction of *N*-methyl-D-aspartate (NMDA) receptors by excitatory amino acids. NO acts as an atypical neuromediator inside brain cells because it reacts with heme moieties of guanyl cyclase in the synaptic junction and induces cyclic guanosine monophosphate (cGMP)-mediated presynaptic glutamate release. NO is produced both presynaptically and postsynaptically in the brain as a result of the increase in cytosolic Ca²⁺ concentration; it subsequently diffuses outside and affects the neighboring neuronal structures and glial cells.¹ NO has several effects on behavior, cognition, and emotion, and has been shown to play role in depression, anxiety, locomotion, aggression, tolerance, addiction, and learning.^{2–4} NO has effects on the modulation of cognition;⁵ however, role of NO in learning is not completely understood.

Multiple mechanisms modulate synaptic efficacy and its actions, including the regulation of synaptic plasticity and the modulation of cGMP.⁶ There is evidence of soluble guanylyl cyclase (sGC) activation in memory formation.^{7–9} The activation

of sGC may represent a major pathway that regulates NO messenger function in the brain^{10,11} because it has been reported that the induction of long-term potentiation (LTP) in hippocampal slices can be blocked with sGC inhibitors.^{12,13}

In different rodent models, many studies have investigated drugs that affect NO levels to examine the role on cognition; however, the results have been controversial. In some studies, it was shown that compounds that block NOS inhibited learning,^{14,15} whereas some others did not support these findings.^{16,17} In the Morris water maze (MWM) test, systemic inhibition of NO had disturbing effects in some studies,^{18,19} whereas others showed different results.^{17,20} Besides, LTP playing a role in NO-mediated cognition was completely reversed after the administration of NOS inhibitors in some studies,^{21,22} whereas others studies have demonstrated a partial inhibition^{13,23} or no effect at all.^{24,25} Several studies regarding NOS inhibitors demonstrated that 7-nitroindazole (7-NI) exerted some impairing effects on cognition in rodents. Injections of 7-NI disturbed spatial memory and object recognition in rats and also had impairing effects in passive avoidance test in animal models.^{26–28}



The goal of this study was to further evaluate the effects of 7-NI (a nonselective inhibitor of NOS), L-arginine (an NO precursor combined with 7-NI), and 1*H*-[1,2,4]-oxadiazole[4,3*a*]-quinoxaline-1-one (ODQ, a highly selective, irreversible inhibitor of sGC) on spatial memory in the modified elevated plus maze (mEPM), MWM, and radial arm maze (RAM) tests. Furthermore, these studies were aimed at further understanding the effects of NO on cognition because of the controversy in the literature.

Methods

Animals. Ninety-six male inbred BALB/c ByJ mice (Uludağ University, Bursa, Turkey) aged 8 weeks were used in this study. The animals (4–5 per cage) were kept in the laboratory for 2 weeks prior to experimentation. The animal room had a temperature of $21 \pm 1.5^\circ\text{C}$ with 60% relative humidity, and a 12-hour light/dark cycle (light on at 8.00 p.m.). All procedures that involved animals were in compliance with the European Community Council Directive of November 24, 1986, and ethical approval was granted by the Kocaeli University Ethics Committee (Number: AEK 9/4-2010, Kocaeli, Turkey).

Modified elevated plus-maze test. Cognitive behavior was determined using the mEPM, which measures spatial long-term memory.²⁹ The maze was composed of wood; it comprised two open arms (29×5 cm) surrounded by a short (1 cm) Plexiglass edge to avoid falls and two enclosed arms ($29 \times 5 \times 15$ cm) arranged such that the two open arms were opposite to each other. The arms were connected by a central platform (5×5 cm). The maze was kept 40 cm above the floor. The principle of this experiment is based on aversion of rodents to open spaces and heights. The animals prefer the enclosed, protected areas of the maze.²⁹

The procedure was as previously described.^{29–32} In the acquisition session (day 1), each mouse was placed at the distal end of an open arm facing away from central platform. The time required for mice to move from the open arm to either of the enclosed arms (transfer latency) was recorded. Training (repeated exposure of the animals to the open arms) shortened this parameter, most likely as a result of learning acquisition and retention. After entering the enclosed arm, the mice were allowed to move freely in the maze regardless of open and enclosed arms for 10 seconds. The retention session followed 24 hours after the acquisition session. The mice were placed in the open arm, and the transfer latency was recorded again. The experiments were conducted between 10:00 and 14:00 hours in a dimly lit, semi-soundproof room under natural light.^{29–32}

Morris water maze test. The MWM comprised a circular pool (90 cm diameter and 30 cm height) filled with water (22°C) to a depth of 14 cm and rendered opaque by addition of small black balls. The pool was located in a dimly lit, soundproof test room with various visual cues, including a white/black colored poster on the wall, a halogen lamp, a camera, and the experimenter. The maze was divided into four quadrants,

and three equally spaced points served as starting positions around the edge of the pool. The order of the release positions was varied systematically throughout the experiment. A circular escape platform (6 cm diameter and 12 cm high) was located in one quadrant 1 cm above the water surface during the familiarization session and 1 cm below the water surface during the other sessions.^{17,20}

Video tracking was conducted with a video camera focused on the full diameter of the pool. Navigation parameters were analyzed using the Ethovision 3.1 video analysis system (Noldus). Mice were trained in MWM five times per day (familiarization session, S_1 , S_2 , S_3 , and S_4). One familiarization and four acquisition sessions were carried out using the MWM. During the familiarization session and acquisition phase of experiment, each mouse underwent three trials. The delay between trials was 60 seconds, and a 1-day interval was used between each session. For each trial, the mouse was removed from the home cage and placed in the water maze at one of three randomly determined locations with its head facing the center of the water maze. After the mouse had found and climbed onto the platform, the trial was terminated and the escape latency was recorded. If the mouse did not climb onto the platform in 60 seconds, the trial was terminated, and experimenter guided the mouse to the platform; an escape latency of 60 seconds was recorded.^{17,20}

Twenty-four hours after the final acquisition session, a “probe trial” was used to assess the spatial memory retention of the location of the hidden platform. During this trial, the platform was removed from the maze and the mouse was allowed to search the pool for 60 seconds. The percent of time spent in each quadrant was recorded.^{17,20}

Radial arm maze test. The experimental device comprised an elevated maze with eight open arms (32 cm long and 5 cm wide) that led to an 8-cm square platform, which radiated from a central circular platform 44 cm in diameter with 1-cm high sides surrounding each arm.³³ A small cup, 1 cm in diameter, was embedded in each distal platform, and it contained a hidden 10-mg noodle used as reinforcement. The maze was oriented in a small room, which had four large black, white, or black and white striped patterns hung on walls; these patterns provided particularly salient visual extramaze cues. For further details on the apparatus, see Beuzen et al.³³ Twenty-four hours prior to training, the mice were deprived of food but not water; their weight loss reached 15%–20% of the initial body weight by the start of the testing.

The RAM procedure was applied according to Belzung et al.³⁴ The mice were first subjected to two pretraining sessions at 24-hour intervals. Groups of four mice were placed on the maze at the same time and for 20 minutes per session; the mice could freely explore the eight arms, which contained abundant food. Following pretraining, the mice were subjected to five training sessions at 90-minute intervals.

After baiting the eight arms with 10-mg noodles, a mouse was placed on the central platform. Sessions were terminated

when the animal had visited all eight arms and consumed the rewards, after 16 arms were visited (regardless of which arms), or after a maximum of 15 minutes. An error was recorded when the mouse entered an arm previously visited during the retention session. The total number of errors and the latency of the retention session (time taken to complete the task) were recorded.³⁴

Drug administration. 7-NI, ODQ, and L-arginine were procured from Sigma Chemical Company. ODQ and L-arginine were dissolved in saline, whereas 7-NI was dissolved in saline supplemented with 10% dimethylsulfoxide (DMSO). All drugs were freshly prepared and administered in a volume of 0.1 mL per 10 g body weight. The control groups received the same volume of vehicle. 7-NI (15 mg/kg), ODQ (3 and 10 mg/kg), L-arginine, or the vehicle was administered via an intraperitoneal (i.p.) injection 30, 30, and 60 minutes, respectively, prior to the first session (acquisition session, day 1) of mEPM test, prior to the retention trial of RAM test, and for 6 days prior to the acquisition trials and the probe trial of the MWM test. The number of animals per group ranged from 6 to 7. Effective dose of each drug was selected according to previous behavioral and neurochemical studies.^{3,35–37}

Statistics. A two-way analysis of variance (ANOVA) and post hoc Tukey test were used to analyze the mEPM, MWM, and RAM tests. The data are expressed as the mean \pm SEM. $P < 0.05$ was considered statistically significant.

Results

Effects of 7-NI, ODQ, and 7-NI \pm L-arginine on learning and memory in the mEPM test. When 7-NI (15 mg/kg), ODQ (3 and 10 mg/kg), or 7-NI (15 mg/kg) + L-Arg (100 mg/kg) was administered prior to the acquisition session (training; day 1), there was no significant effect of the drugs [$F(3,29) = 1.81$; Fig. 1A] or their combination [$F(1,29) = 1.71$; Fig. 1A] in the mEPM test. There was a significant effect of the drugs [$F(3,29) = 7.07$, $P = 0.001$; Fig. 1B] and their combination [$F(1,29) = 14.17$, $P < 0.001$; Fig. 1B] on the second-day latency in the mEPM test. ODQ (3 mg/kg) and 7-NI (15 mg/kg) significantly prolonged the latency (TL₂) on the second day compared with the control group when the drugs were administered prior to the acquisition session ($P < 0.05$ and $P < 0.01$, respectively; Fig. 1B). L-Arginine (100 mg/kg) combined with 7-NI (15 mg/kg) significantly shortened the TL₂ compared with 7-NI (15 mg/kg) alone ($P < 0.001$; Fig. 1B).

Effects of 7-NI, ODQ, and 7-NI \pm L-arginine on learning and memory in the MWM test. There was a significant difference in the escape latency in the first, second, third, and fourth sessions during the evaluation of the drug groups [$F(3,35) = 6.58$, $P = 0.001$; $F(3,35) = 11.71$, $P < 0.001$; $F(3,35) = 7.41$, $P < 0.001$; and $F(3,35) = 10.24$, $P < 0.001$; respectively; Fig. 2A]. 7-NI (15 mg/kg) significantly increased the escape latency during the first, second, third, and fourth sessions ($P = 0.01$; $P < 0.01$; $P < 0.01$; and $P < 0.001$,

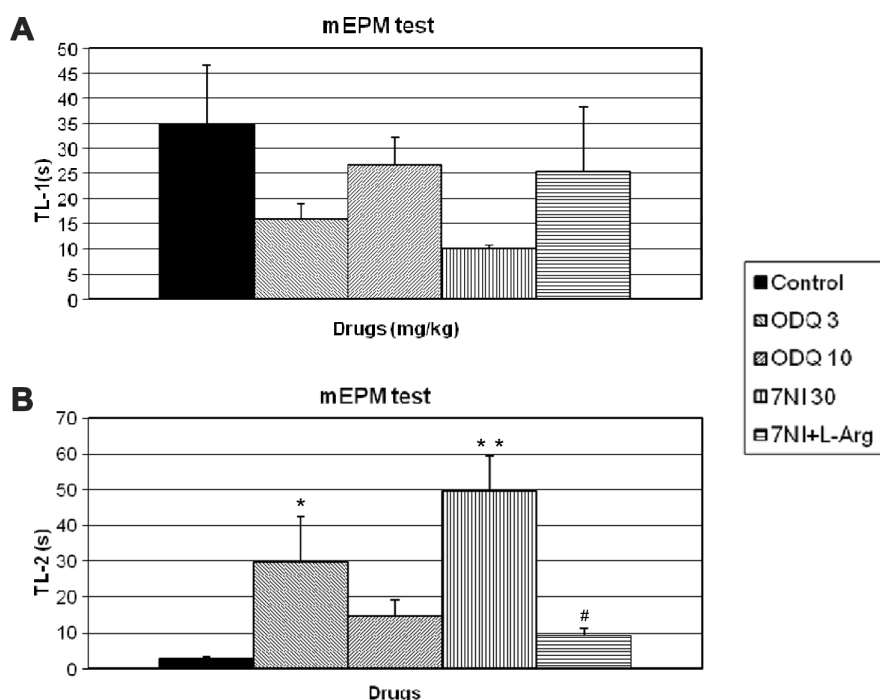


Figure 1. Drug effects on (A) transfer latency 1 (TL-1) and (B) transfer latency 2 (TL-2) ($n = 6$) in the mEPM test in mice. ODQ (3 and 10 mg/kg), 7-NI (15 mg/kg), or L-arginine (100 mg/kg) was administered 30, 30, and 60 minutes, respectively, prior to the acquisition trial of the mEPM test. The data are expressed as the mean \pm SEM values of the animals. * $P < 0.05$, ** $P < 0.001$ compared with the control group. # $P < 0.001$ compared with the 7-NI group.

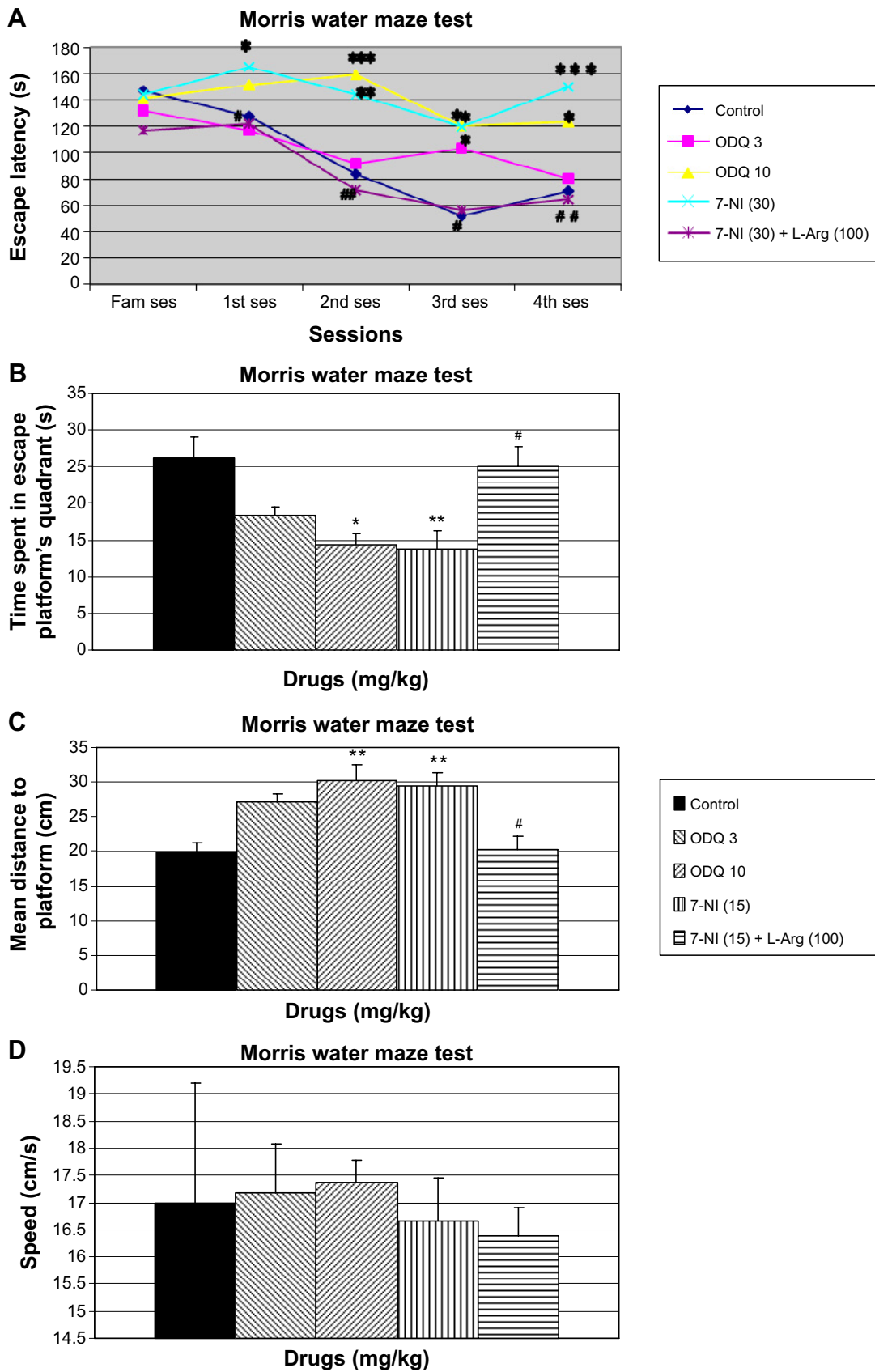


Figure 2. Drug effects on (A) the escape latency in five acquisition sessions of the MWM test, (B) the time spent in the escape platform's quadrant in the probe trial (60 seconds) in the MWM test, (C) the mean distance to the platform in the probe trial (60 seconds) of the MWM test, and (D) the swimming speed in the probe trial (60 seconds) of the MWM test. ODQ (3 and 10 mg/kg), 7NI (15 mg/kg), or L-Arg (100 mg/kg) was administered daily 30, 30, and 60 minutes, respectively, prior to the first trial of the day for 6 days. Results are expressed as the mean \pm SEM. $N = 7$ per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the control group; # $P < 0.001$ compared with the 7-NI group.

respectively), whereas ODQ 10 mg/kg significantly increased the escape latency during the second, third, and fourth sessions ($P < 0.01$; $P < 0.05$; and $P < 0.05$, respectively) compared with the control in naive mice. L-Arginine (100 mg/kg) significantly reversed the effects of 7-NI (15 mg/kg) on the escape latency in the first, second, third, and fourth sessions [$F(1,35) = 13.49$; $P < 0.001$; $F(1,35) = 23.29$; $P < 0.001$; $F(1,35) = 15.23$; $P < 0.001$; and $F(1,35) = 29.19$; $P < 0.001$, respectively; Fig. 2A].

A significant difference was noted between all drug groups in the time spent in the target quadrant [$F(3,35) = 6.04$; $P = 0.002$; Fig. 2B]. 7-NI (15 mg/kg) and ODQ (10 mg/kg) significantly decreased the time spent in the escape platform's quadrant ($P < 0.01$ and $P < 0.05$, respectively). L-Arg (100 mg/kg) combined with 7-NI significantly increased the decrease in the time spent in the escape platform's quadrant in the 7-NI only group [$F(1,35) = 12.06$; $P = 0.002$].

The mean distance traveled by the mice to the platform in the probe trial of the MWM test was significantly different between the drug groups [$F(3,35) = 6.94$; $P = 0.001$; Fig. 2C]. 7-NI 15 mg/kg and ODQ (10 mg/kg) significantly increased the mean distance traveled to the platform ($P < 0.01$). L-Arg combined with 7-NI significantly reversed the effects of 7-NI 15 mg/kg [$F(1,35) = 12.06$; $P = 0.002$; Fig. 2C].

The treatment groups were not significantly different in swimming speed [$F(3,35) = 0.06$; $P = 0.97$; Fig. 2D], and the combination had no effect on the speed of the animals in the probe trial of the MWM test [$F(1,35) = 0.02$; $P = 0.86$; Fig. 2D].

Effects of 7-NI, ODQ, and 7-NI ± L-arginine on learning and memory in the RAM test. In the evaluation of the effects of acute treatment with 7-NI (15 mg/kg) and ODQ (3 and 10 mg/kg) administered 30 minutes prior to the retention trial on the number of errors in the RAM test, a significant difference between the groups was identified [$F(3,29) = 19.08$; $P < 0.001$]. 7-NI (15 mg/kg) and ODQ (3 and 10 mg/kg) significantly enhanced the number of working memory errors in the RAM test in mice ($P < 0.001$). L-Arg (100 mg/kg) significantly decreased the number of working memory errors in the 7-NI-treated mice [$F(1,29) = 33.86$; $P < 0.001$] in the RAM test (Fig. 3A).

When the effects of 7-NI (15 mg/kg) and ODQ (3 and 10 mg/kg) on the latency (time taken to complete the task) of the animals in the RAM test were evaluated, there was a significant difference between the groups [$F(3,29) = 15.11$; $P < 0.001$]. 7-NI (15 mg/kg) significantly enhanced the latency of the animals ($P < 0.001$), whereas ODQ (3 and 10 mg/kg) had no significant effect ($P > 0.05$). L-Arg (100 mg/kg) combined with 7-NI (15 mg/kg) significantly decreased the latency

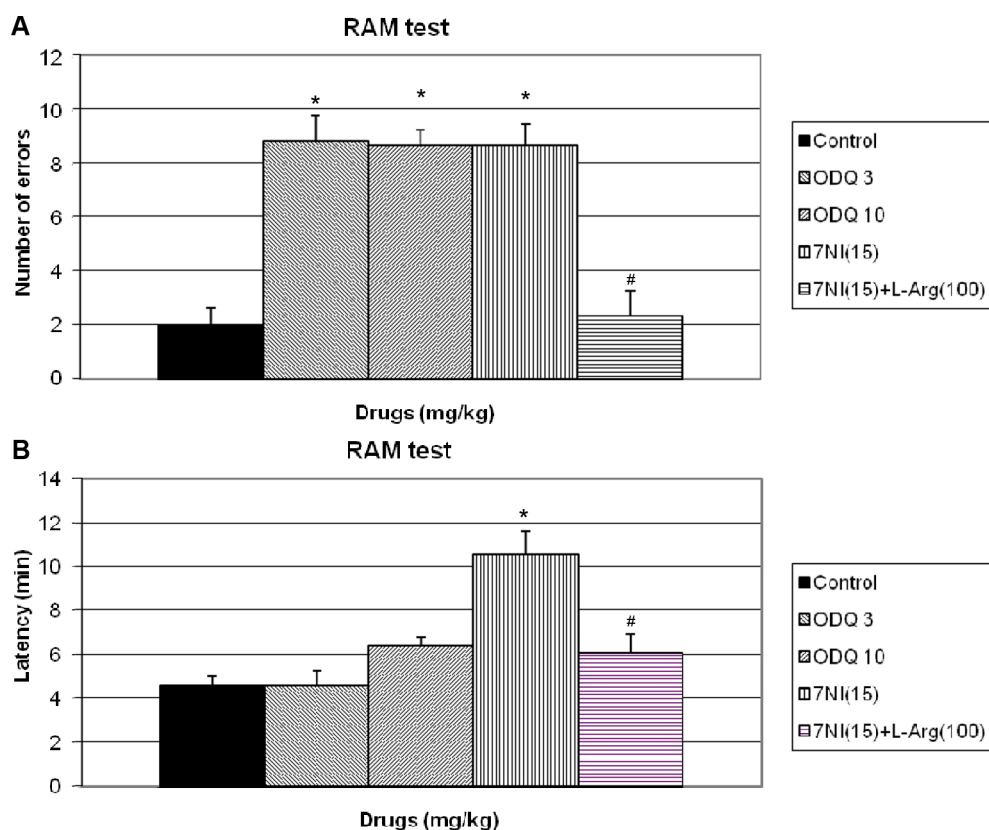


Figure 3. (A) Drug effects on working memory errors in the RAM test. (B) Drug effects on the latency in the RAM test. ODQ (3 and 10 mg/kg), 7-NI (15 mg/kg), or L-arginine (100 mg/kg) was administered 30, 30, and 60 minutes, respectively, prior to the retention trial of the RAM test. Results are expressed as the mean \pm SEM. $n = 6$ per group. * $P < 0.001$ compared with the control group; # $P < 0.001$ compared with the 7-NI group.



of the RAM test compared with 7-NI alone [$F(1,29) = 19,01$; $P < 0.001$; Fig. 3B].

Discussion

In our study, both the GC inhibitor ODQ (3 mg/kg) and the NOS inhibitor 7-NI (15 mg/kg) increased the retention latency in the mEPM test. 7-NI (15 mg/kg) and ODQ (10 mg/kg) significantly increased the escape latency in the second, third, and fourth sessions, diminished the time spent in the escape platform's quadrant, and enhanced the mean distance to the platform in the probe trial of the MWM test. ODQ (3, 10 mg/kg) and 7-NI (15 mg/kg) significantly increased the number of errors, whereas only 7-NI increased the latency in the RAM test. The 7-NI (15 mg/kg)-induced effects in the mEPM, MWM, and RAM tests were reversed by the NO precursor, L-arginine (100 mg/kg).

MWM is a spatial, long-term memory evaluation test.³⁸ The daily decrease in escape latencies reflects learning, and it is related to long-term reference memory. This study revealed that ODQ (10 mg/kg) and 7-NI (15 mg/kg) increased the escape latency during the acquisition sessions, diminished the time spent in the escape platform quadrant, and enhanced the mean distance traveled to the platform during the probe test. Therefore, both ODQ and 7-NI disturbed the spatial memory. The drug treatment did not alter the swimming speed of the mice, which suggests ODQ and 7-NI did not alter their motor activity. Because the position of the platform did not change throughout the experiment, these results indicate that ODQ and 7-NI disturbed the reference spatial memory.

It has been shown that the ability to make a series of correct choices in the RAM test depends on the spatial information from extramaze cues.³⁹ In our protocol, the effects of the drugs on spatial memory were evaluated because performance in the maze requires sufficient memory of the spatial environment. Each arm was baited with food, and reentry to a previously visited arm was categorized as an error; thus, spatial working memory was thought to be examined.³⁹ Both ODQ (3 and 10 mg/kg) and 7-NI (15 mg/kg) disturbed the spatial working memory in the RAM test in our study.

The mEPM test is a simple method that evaluates spatial memory. A shortened transfer latency in the second trial is used as a parameter to measure the retention or consolidation of memory, and drug treatment prior to the first day may be utilized to determine the effects on memory acquisition.⁴⁰ The evaluation of drug effects in the first trial may be confounded by nonspecific effects, such as effects on anxiety, locomotion, and motility.⁴¹ Since there was no significant difference between groups for the first-day latency in the mEPM test, we can exclude these nonspecific effects. Both ODQ (3 mg/kg) and 7-NI (15 mg/kg) treatment led to memory deterioration in the EPM test in our study, which is in accordance with previous studies.^{28,37,42} The effects of ODQ did not follow a typical dose-response curve of a drug. The experimental condition can cause some differences between the results. We studied

with standard experimental conditions and standard equipment and obtained these results.

Recent studies suggest that both neuronal (nNOS) and endothelial (eNOS) nitric oxide isoforms have a role in memory formation. Additionally, in a passive avoidance test, nNOS cannot substitute for the role of eNOS on LTP.⁴³ In research using "knock-out" mice, it has been demonstrated that both neuronal and endothelial NOS isoforms were expressed in hippocampal CA1 pyramidal cells, and both endothelial and neuronal NOS deletion resulted in the loss of LTP.⁴⁴ In contrast, when only one isoform mutant was used, the mice had preserved their normal LTP capacity.⁴⁴ Taken together, these results support the claim that both isoforms have a role in LTP and can substitute for each other; however, the mechanisms that underlie their actions and the degree to which eNOS can compensate nNOS remain unknown. These findings can explain why the nonselective nNOS and eNOS inhibitor 7-NI impaired learning and memory in our study, as well as the discrepancies in the studies with NOS inhibitors on memory. The differences between the animal strains, types, and protocols of the test, drug type, pharmacokinetics, specificity, dose, and administration route of the drugs can explain, in part, the discrepancies between different studies.

NO inhibitors or donors are known to exert effects on blood pressure.⁴⁵ NO donors can cause hypotension, whereas NOS inhibitors can lead to hypertension. The effects of these drug groups on cognitive functions can be the result of their effects on cardiovascular functions. There is controversy regarding the effects of 7-NI on blood pressure, although Prickaerts et al¹⁴ reported that the disruption of 7-NI effects was not correlated with its effects on blood pressure. Hölscher et al²⁶ observed that 30 mg/kg 7-NI impaired spatial learning without changing blood pressure in both the water maze and eight-arm radial maze tests. In our study, we administered the drugs systematically; thus, we could not avoid nonspecific effects, such as hypertension. However, in recent studies, it was shown that 7-NI did not have a significant hypertensive effect at the doses used in our study.²⁶

NO affects the release and reuptake of various neurotransmitters,⁴⁶ which results in the LTP of synaptic transmission and the neuronal basis of memory formation. Some studies have reported that NOS inhibitors do not change the learning performance or memory,^{47,48} whereas other studies have reported that these drugs inhibited the retention trial of the passive avoidance test,^{49,50} spatial learning in the water maze test,^{51,52} or object recognition¹⁴ in the radial arm maze.⁵³ 7-NI has been shown to impair learning and memory in most tests,^{26,28,37} and these findings are similar to the results observed in our study. Our study is in accordance with the studies that demonstrated the disturbing effect of NOS inhibitors on learning and memory.

The reversal of the 7-NI effects by L-arginine supports the theory that these effects are specific to NOS. NO is known



to play a role in some forms of memory processing,^{14,52,53} however, the exact function is not known. The activation of the GC/cGMP/protein kinase G (PKG) pathway causes most NO-mediated physiological processes. Edwards et al⁵⁴ reported that the inhibition of GC and PKG impairs retention for the passive avoidance task. Kleppisch et al⁵⁵ reported that PKGs are not involved in LTP in mice, but NO induces LTP through an alternative cGMP-independent pathway, possibly adenosine diphosphate (ADP) ribosylation. Our study supports the role of cGMP on cognition and the disruptive effects of ODQ on learning and memory.

In conclusion, the present study demonstrates that both the GS inhibitor ODQ and the NOS inhibitor 7-NI disturbed spatial memory in the mEPM, MWM, and RAM tests. L-Arginine, the NO precursor, reversed 7-NI-induced changes, which confirms that the effects of 7-NI were NO dependent. Our findings suggest that the nNOS/sGC/cGMP pathway is involved in the pathophysiology of memory functions.

Author Contributions

Conceived and designed the experiments: OM, FA, IKC, PT. Analyzed the data: OM, FA, IKC. Wrote the first draft of the manuscript: OM, FA, GU. Contributed to the writing of the manuscript: OM, GU, FE. Agree with manuscript results and conclusions: OM, FA, IKC, PT, GU, FE. Jointly developed the structure and arguments for the paper: FE, GU, PT. Made critical revisions and approved final version: FA, IKC, PT. All authors reviewed and approved of the final manuscript.

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