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The Role of Mitochondrial Biogenesis and A2-Adrenergic Receptors of the Hippocampal CA1 Region in Morphine-Induced Memory Impairment

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ABSTRACT

Background and Objective: Mitochondrial biogenesis and the adrenergic system play an important role in cognitive processes. The dorsal hippocampus (CA1) has a high distribution of α2-adrenergic receptors. Given that morphine can cause memory impairment, the aim of the present study was to investigate the role of mitochondrial biogenesis and α2-adrenergic receptors in the CA1 region in morphine-induced memory impairment.

Methods: This experimental study included four experimental groups: 1) morphine, 2) clonidine and clonidine + morphine, 3) yohimbine and yohimbine + morphine, 4) yohimbine + clonidine + morphine. The total number of animals in these experiments included 208 adult male Wistar rats, which were divided into 26 groups of 8. Intraperitoneal injection of morphine (4, 5, 6 mg/kg) was done to cause memory impairment. Different doses of α2-adrenergic receptor agonists and antagonists (clonidine and yohimbine, respectively) (1, 2, 4 µg/rat) were injected into the CA1 region of the hippocampus. A shuttle box apparatus was used to examine passive avoidance memory, and the ELISA technique was used to measure the expression levels of factors involved in mitochondrial biogenesis (PGC-1α, NRF-1 and TFAM) in the CA1 region. Then, the groups were examined and compared in terms of memory impairment.

Findings: Injection of the effective dose of morphine (6 mg/kg) impaired passive avoidance memory compared to the saline group (118.88±15.62 vs. 285.13±7.50) ($p<0.001$). Injection of clonidine (4 µg/rat) into the CA1 region increased memory compared to the saline group (291.25±6.86 vs. 230.25±5.64) ($p<0.05$), and its injection before the effective dose of morphine prevented memory impairment by morphine (250.62±13.72 vs. 96.12±14.57) ($p<0.001$). Yohimbine injection (4 µg/rat) resulted in a poor memory compared to saline group (161±19.69 vs. 241±15.20) ($p<0.05$) and its injection before low dose of morphine (4 mg/kg) caused inhibition of memory recall (113.12±13.9 vs. 241.5±21.59) ($p<0.001$). Low dose yohimbine injection (1 µg/rat) caused inhibition of clonidine-induced response while clonidine plus morphine caused inhibition of memory recall. The effective dose of morphine also decreased the expression levels of PGC-1α (118.25±19.85 vs. 185.1±8.8), NRF-1 (63.42±6 vs. 106.62±11.95) and TFAM (19.5±0.89 vs. 37.6±5.44) in the CA1 region compared to the saline group ($p<0.05$). Injection of the effective dose of clonidine before morphine increased the expression of these factors ($p<0.05$), while this increase was inhibited by injection of a low dose of yohimbine.

Conclusion: The results of the study showed that mitochondrial biogenesis and α2-adrenergic receptors in the hippocampal CA1 region may be involved in morphine-induced memory impairment.

Keywords: *Mitochondrial Biogenesis, A2-Adrenergic Receptors, Morphine, Memory Impairment.*

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Introduction

Studies have shown that morphine affects brain circuits involved in memory and learning (1). Morphine's effects on memory are mediated by μ-opioid receptors and their interactions with various brain neurotransmitter systems (2). Findings from studies have shown that the effects of morphine on cognitive processes depend on the dose of the drug, the time of injection, the route of administration, and the model of memory assessment (3, 4). However, there is considerable evidence that injection of morphine, an agonist of μ-opioid receptors, causes memory impairment and amnesia in laboratory animals (5, 6), while injection of an antagonist of these receptors, such as naloxone, prevents the induction of amnesia by morphine (7). The exact mechanism by which morphine causes cognitive impairment is not yet fully understood. Some studies have shown that morphine, by binding to μ-opioid receptors in the presynaptic neuron and blocking voltage-gated calcium channels, reduces the release of excitatory neurotransmitters involved in memory and learning processes, such as glutamate, acetylcholine, and norepinephrine, which are essential for information transmission and memory formation (8). It has also been reported that morphine-induced memory impairment may be due to its inhibitory effects on the activity of the adrenergic system. A study has shown that systemic injection of morphine can impair passive avoidance memory in rats by inhibiting the release of norepinephrine in the prefrontal cortex (9).

Adrenergic pathways, most of which originate in the locus coeruleus, play an important role in many complex brain functions such as learning and memory (10). Norepinephrine released from these neurons exerts its effects through two classes of G protein-coupled receptors, α and β-adrenergic receptors. These receptors are widely distributed in the central nervous system, including the hippocampus (11). α receptors are divided into two subgroups, α1- and α2-adrenergic. α1-adrenergic receptors are mostly postsynaptic, while α2-adrenergic receptors can be present in both postsynaptic and presynaptic hippocampal neurons (12). α2-adrenergic receptors play a very important role in learning and memory. Clonidine, as a specific agonist of these receptors, has various effects on the central and peripheral nervous systems, including blood pressure control, anxiety and tension control, and cognitive processes (13). Yohimbine, as a specific antagonist of α2-adrenergic receptors, can reduce norepinephrine release and sympathetic activity by inhibiting these receptors. It may also increase anxiety by stimulating mood (14).

The presence of mitochondria in neurons is of great importance, because one of the most important and vital factors related to long-term memory is the proper functioning of mitochondria (15). The process of increase in the number of mitochondria is called mitochondrial biogenesis, which is essential for events such as energy production, calcium signaling, and the aging cycle (16). Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is a master regulator of mitochondrial biogenesis, activating transcription factors such as nuclear respiratory factor (NRF) and mitochondrial transcription factor A (MTFA). It has been shown that any defect in mitochondrial biogenesis can lead to neurodegenerative diseases as well as memory and learning disorders (17).

Although the role of mitochondrial biogenesis and α2-adrenergic receptors in cognitive processes is well established, and many studies have reported morphine-induced memory impairment, the role of mitochondrial biogenesis and α2-adrenergic receptors in the CA1 region of the hippocampus in morphine-induced memory impairment has not been investigated so far, and no study has been reported on α2-adrenergic receptors and mitochondrial biogenesis. Therefore, the aim of the present study was to investigate the role of mitochondrial biogenesis and α2-adrenergic receptors in the CA1 region of the hippocampus in morphine-induced memory impairment.

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Methods

After approval by the Ethics Committee of Kharazmi University with the code IR.KHU.REC.1402.011, this experimental study was conducted on male Wistar rats (200-250 g). The animals were kept in a room with a temperature of 22±2°C, humidity of 50±10%, and a 12:12 light–dark cycle, with adequate access to water and food.

Medications: Ketamine hydrochloride and xylazine (Alfasan, Netherlands) were used intraperitoneally to anesthetize the animals. Morphine sulfate (Temad Co., Iran) was injected intraperitoneally, and clonidine (specific α2-adrenergic receptor agonist) and yohimbine (specific α2-adrenergic receptor antagonist) (Sigma, UK) were injected into the hippocampal CA1 region. Morphine, clonidine, and yohimbine were dissolved in 0.9% normal saline immediately before injection.

Stereotaxic surgery: Animals were anesthetized via intraperitoneal injection of ketamine (80 mg/kg) and xylazine (20 mg/kg) and then placed in a stereotaxic instrument (Stoelting, USA). After determining the bregma and lambda points, the coordinates of the hippocampal CA1 region for clonidine and yohimbine injection were determined based on the Paxinos atlas as follows: AP= -3.36 mm, ML= ±1.8 mm, and DV= -2.5 mm (18). Guide cannulas (made from a 23-gauge needle and 8 mm long) were placed bilaterally at the designated points and fixed with dental cement. After a one-week recovery period, animals were used for drug injections and behavioral tests. Injections in the CA1 region were performed using a 5 µl Hamilton syringe. An injection cannula (made from a 27-gauge needle and 9 mm long) connected to a Hamilton syringe by a polyethylene tube was inserted into the guide cannula, and 0.5 µl of injection solution was injected into each side in 60 seconds (1 µl per animal). To ensure complete drug diffusion in the CA1 region, the injection cannula was removed 60 seconds after injection. To ensure the correct coordinates of the surgical site and drug injection at the end of the experiments, 1 µl of 1% Methylene Blue Solution was injected bilaterally into the CA1 region. Then, the animal was sacrificed using chloroform, and the brain was removed from the skull and placed in 10% formalin solution for one week. The focal sites (CA1 regions) were confirmed by matching the sections prepared from the brain with the Paxinos atlas. Data from animals whose focal sites were outside the CA1 region were excluded from statistical calculations.

Study groups: Four experimental groups were used in this study. The total number of animals in these experiments included 208 adult male Wistar rats, which were randomly divided into 26 groups of 8:

Experiment 1 (Morphine): In this experiment, four groups of animals were used to investigate the effect of post-training morphine injection on memory impairment. One group received saline immediately after training and three other groups received different doses of morphine (4, 5, 6 mg/kg) intraperitoneally. 24 hours later, they were tested for memory without any injection.

Experiment 2 (Clonidine and Clonidine + Morphine): In this experiment, eight groups of animals were used to investigate the effect of post-training clonidine injection on morphine-induced amnesia. Four groups received clonidine (1, 2, 4 µg/rat) intraperitoneally in the CA1 region immediately after training and saline 5 minutes later. Four other groups received clonidine (1, 2, 4 µg/rat) intraperitoneally after training and 5 minutes later, they received an effective dose of morphine (6 mg/kg) intraperitoneally. All animals were tested for memory 24 hours later without receiving any injections.

Experiment 3 (Yohimbine and Yohimbine + Morphine): In this experiment, eight groups of animals were used to investigate the effect of post-training yohimbine injection on morphine-induced amnesia. Four groups received yohimbine (1, 2, 4 µg/rat) intraperitoneally into the CA1 region immediately after training and saline 5 minutes later. The other four groups received yohimbine (1, 2, 4 µg/rat) intraperitoneally into the CA1 region after training and a low dose of morphine (4 mg/kg) intraperitoneally 5 minutes later. All animals were tested for memory 24 hours later without receiving any injection.

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Fourth experiment (yohimbine + clonidine + morphine): In this experiment, 6 groups of animals were used to investigate the effect of post-training injection of a low dose of yohimbine (1 μg/rat) together with an effective dose of clonidine (4 μg/rat) and also injection of a low dose of yohimbine together with an effective dose of clonidine plus an effective dose of morphine (6 mg/kg). The first group received saline in the CA1 region after training and received saline intraperitoneally 5 minutes later. The second group received saline in the CA1 region after training and an effective dose of morphine intraperitoneally 5 minutes later. The third group received an effective dose of clonidine in the CA1 region after training and saline intraperitoneally 5 minutes later. The fourth group received an effective dose of clonidine in the CA1 region after training and an effective dose of morphine intraperitoneally 5 minutes later. The fifth group, first received a low dose of yohimbine in the CA1 region after training, and 5 minutes later, an effective dose of clonidine in this region, and 5 minutes later, saline was administered intraperitoneally. The sixth group first received a low dose of yohimbine in the CA1 region after training, and 5 minutes later, an effective dose of clonidine in this region, and 5 minutes later, an effective dose of morphine was administered intraperitoneally. 24 hours later, the memory of all groups was tested without any injection. The study protocol is shown in Figure 1.

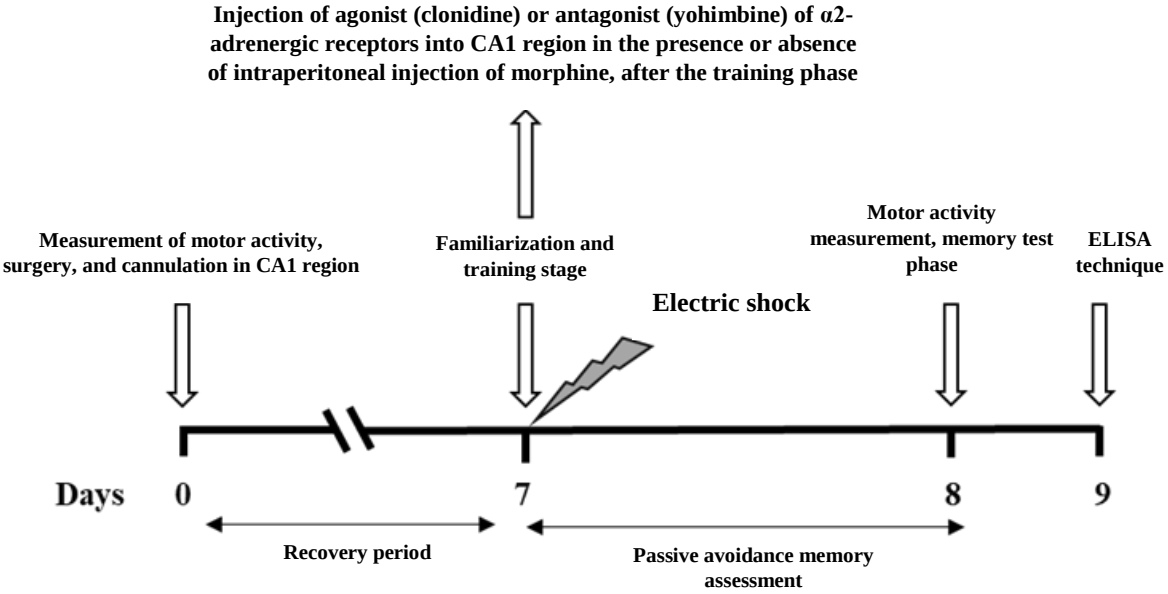

Figure 1. Drug injection protocol and timing of behavioral and molecular assessments

Motor activity assessment: The Open Field device was used for this purpose. This device consists of a transparent, compressed plastic box measuring 40×40×40 cm, equipped with infrared sensors at a distance of 2.5 cm from the bottom edge of the box. All animals were placed in the device for 5 minutes before surgery and drug injection (day 0) and before the memory test (day 8), and then their horizontal motor activities (number of passes in front of the infrared sensors) were recorded by the device during this time and used as an index of motor activity (19).

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Passive Avoidance Step-Down Apparatus (shuttle box): This device is a Plexiglas box with two dark and light sections, each measuring 20×20×30 cm, connected by a guillotine door measuring 9×7 cm. The bottom of the dark section is equipped with steel rods with a diameter of 3 mm and a spacing of 1 cm, which are connected to the stimulator by a communication cable. This device releases an electric current into the steel rods and causes an electric shock to the animal's hands and feet.

Passive avoidance memory assessment: There are several behavioral models for assessing learning and memory in laboratory animals. The passive avoidance learning model is widely used in pharmacological studies to investigate long-term avoidance memory, in which the hippocampus plays an important role. In this learning model, the animal learns to suppress its innate desire (entering the dark area) to avoid harmful events (electric shocks to the hands and feet) (20). Passive avoidance memory assessment was performed in 3 stages and on 2 consecutive days:

1. Familiarization stage: In this stage, each animal was placed inside the light section with its back to the door. After 10 seconds, the door was removed to allow the animal to enter the dark section. As soon as the animal entered this section, the door was closed so that the animal could move freely there for 30 seconds and familiarize itself with the device.

2. Training stage: This stage was performed 30 minutes after stage 1. First, the animal's palms, feet, and tail were lightly wetted (this procedure does not cause any stress to the animal and is only to prevent damage to the animal's hands, feet, and tail when applying the electric shock) and then it was placed in the light section with its back to the door. After 10 seconds, the door was removed and the animal was allowed to enter the dark section. As soon as the animal entered the dark section, the door was closed and an electric current of 1 mA and a frequency of 50 Hz was passed through the animal's legs for 2 seconds. 20 seconds after the shock was applied, the door was removed to allow the animal to enter the light section. If the animal remained in the light section for 120 seconds after entering the light section and did not enter the dark section, it indicated that the rat had learned. In this case, the animal was transferred to its cage after receiving saline or drug (post-training treatment).

3. Test phase or memory recall: This phase was performed 24 hours after the training phase and without injecting any drug to measure passive avoidance memory. For this purpose, each rat was placed individually in the light section of the device and after 10 seconds, the guillotine door was opened. The delay time for the animal to enter the dark section (step-through latency) was recorded. In this phase, no electric shock was applied to the animal and the maximum time for the rat to stop in the light section was 300 seconds. In this test, an increase in the delay time in entering the dark section indicates an increase in memory and a decrease in the delay indicates weakening of memory (21).

ELISA technique: The expression level of factors involved in mitochondrial biogenesis (PGC-1α, NRF-1 and TFAM) at the protein level was evaluated using the ELISA technique, which has high sensitivity, accuracy and specificity for this purpose. Initially, the animals were deeply anesthetized with CO2 and were beheaded with a guillotine in the shortest possible time. After removing the hippocampus, the CA1 regions were carefully separated and stored in a -80 °C freezer until the ELISA experiments were performed. The dilution buffer available in the kits was used to lyse the tissue samples. For 100 mg of tissue, 500 µl of buffer was added and the tissue was homogenized. Then, the resulting solution was centrifuged at 12,000 rpm for 5 minutes at 4 °C and the supernatant was used as a sample. According to the manufacturer's instructions for each kit (PGC-1α: #MBS2706379, NRF-1: #ABIN6958273, and TFAM: #MBS1600609), the specified amount of the diluted solution in the kit was added to all wells of the plates. Then, a specified amount of sample and standard was added to each well and incubated for a specific time and temperature according to the instructions of the relevant kit. Then, the plates were emptied and the plates were washed 4 times, each time with a specific amount of washing solution. Then, a specified amount of PGC-1α, NRF-

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1, or TFAM conjugate solution was added to each well according to the instructions of the relevant kit and incubated for a specific time and temperature. Then, the plates were emptied and washed 4 times each time with a specific amount of washing solution in the kit. In the next step, a specified amount of substrate solution was added to each well and incubated for a specific time and temperature in the dark according to the instructions of the relevant kit. In the final step, a specified amount of stop solution was added to each well. Using an ELISA reader set at 450 nm, the absorbance of samples and standards was read, and the data was then entered into Excel, and based on the line slope and lambda width, the concentration of factors involved in mitochondrial biogenesis in each well was calculated and expressed as pg/mg (22). This technique and the analysis of the resulting data were performed by a researcher who was unaware of the type of division of the study groups.

Data analysis: The Kolmogorov-Smirnov test was used to check the normality of the data distribution. One- or two-way ANOVA was used to determine the significant difference between the tested groups, and the Tukey post hoc test was used to identify the groups with significant differences. Graph Pad Prism version 8 (Graph Pad Software, SA) was used to draw graphs and SPSS version 22 (IBM, SPSS, Armonk, NY, USA) was used for statistical analysis, and the results were presented as mean±standard deviation, and $p<0.05$ was considered significant.

Results

A tissue section of the CA1 region of the hippocampus showing the correct placement of the cannulas compared to the schematic diagram taken from the Paxinos atlas is presented in Figure 2. It should be noted that only data from animals whose surgical location was correct compared to the Paxinos atlas were used in the statistical analysis.

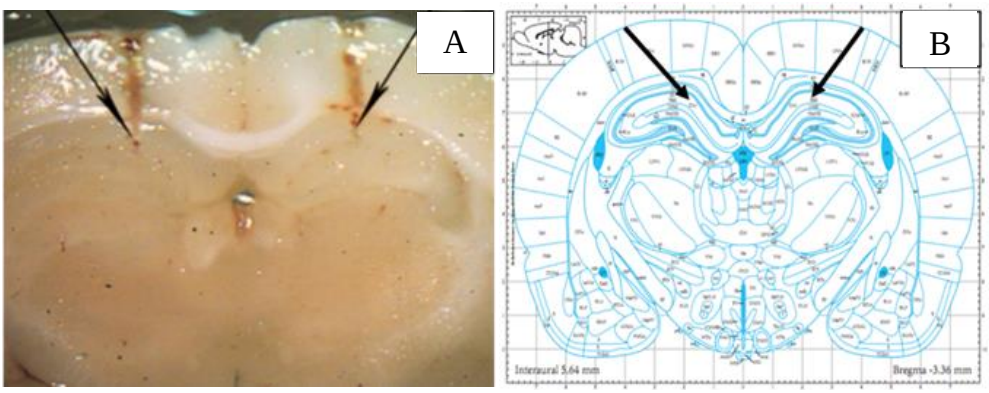

Figure 2. (A) Tissue cross-section of the surgical and drug injection site in the CA1 region and (B) schematic diagram taken from the Paxinos Atlas in which the CA1 region is marked.

In this study, the results of the Open Field test were analyzed using two-way analysis of variance and Tukey's post hoc test, and there was no significant difference in the locomotor activity of the animals in the different groups compared to the saline/saline group on day 0 (before surgery and drug injection) and day 8 (before memory testing) (Chart 1). Each experimental group consisted of 8 rats.

One-way analysis of variance and Tukey's post hoc test showed that morphine injection at a dose of 6 mg/kg caused a significant decrease in the initial latency to enter the dark section in the memory recall phase compared to the saline group (118.88±15.62 vs. 285.13±7.50) ($p<0.001$) (Chart 2). Each experimental group consisted of 8 rats.

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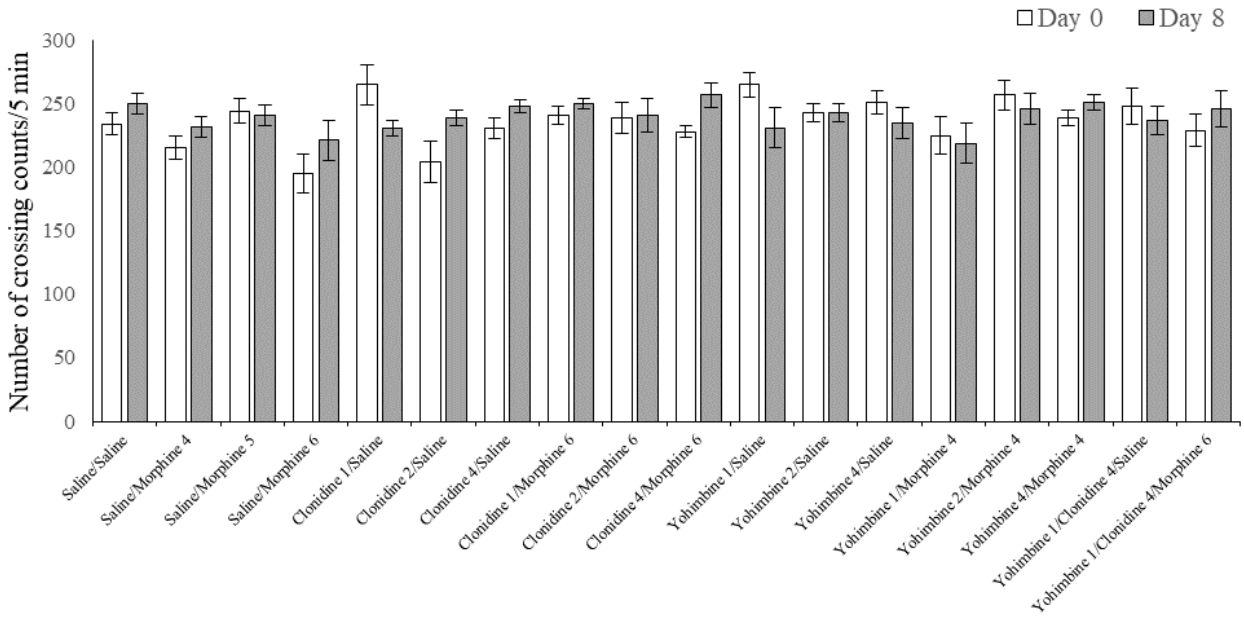

Chart 1. Effect of surgery and drug injection on animal locomotor activity

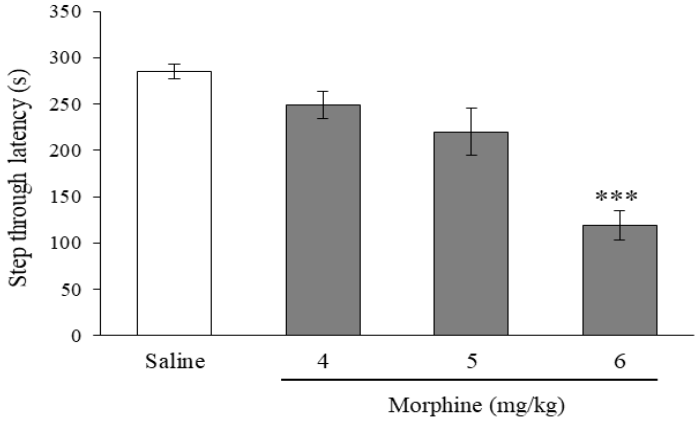

Chart 2. Effects of post-training morphine injection on passive avoidance memory. Animals received saline or various doses of morphine immediately after training and were tested for memory 24 hours later without any injection. Significant differences between groups were determined by Tukey's post-hoc test. Each experimental group consisted of 8 rats (p<0.001* compared to saline group).**

Two-way ANOVA revealed a significant difference between the groups that received clonidine (1, 2, 4 µg/rat) with saline after training and the groups that received the same amounts of clonidine with an effective dose of morphine (6 mg/kg). One-way ANOVA followed by Tukey's post-hoc test showed that animals that received 4 µg/rat of clonidine with saline immediately after training showed a significant increase in memory recall on the test day compared to the saline/saline group (291.25±6.86 vs. 230.25±5.64) ($p<0.05$) (left panel of Chart 3). In addition, Tukey's post hoc test showed that injection of 4 µg/rat of clonidine after training and before injection of the effective dose of morphine significantly increased the latency of the animal to enter the dark section of the apparatus compared to the saline/morphine group (250.62±13.72 vs. 96.12±14.57) ($p<0.001$) (right side of Chart 3). Each experimental group consisted of 8 rats.

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Two-way ANOVA revealed a significant difference between the groups that received yohimbine (1, 2, 4 µg/rat) plus saline after training and the groups that received the same doses of yohimbine plus a low dose of morphine (4 mg/kg). One-way ANOVA followed by Tukey's post hoc test showed that animals that received 4 µg/rat of yohimbine plus saline immediately after training showed a significant decrease in memory recall on the test day compared to the saline/saline group (161±19.69 vs. 241±15.20) ($p<0.05$) (left panel of Chart 4). In addition, Tukey's post hoc test showed that injection of 2 and 4 µg/rat yohimbine doses after training and before injection of low dose morphine significantly reduced the latency of the animal to enter the dark section of the apparatus compared to the saline/morphine group (157.5±20.51 vs. 241.5±21.59 and 113.12±13.90 vs. 241.5±21.59, respectively) ($p<0.05$ and $p<0.001$) (right side of Chart 4). Each experimental group consisted of 8 rats.

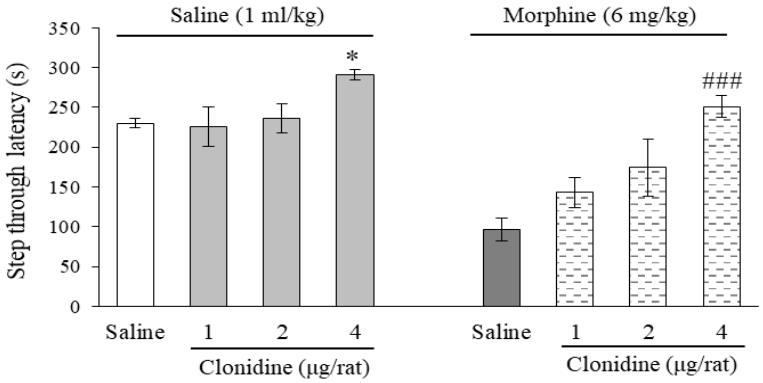

Chart 3. Effect of post-training injection of different doses of clonidine on passive avoidance memory in the presence or absence of morphine. Immediately after training, animals received different doses of clonidine in the CA1 region and saline or an effective dose of morphine intraperitoneally and 24 hours later, they were tested for memory without any injection. Significant differences between groups were determined by Tukey's post-hoc test. Each experimental group consisted of 8 rats (*p<0.05 compared to saline/saline group, ###p<0.001 compared to saline/morphine group).

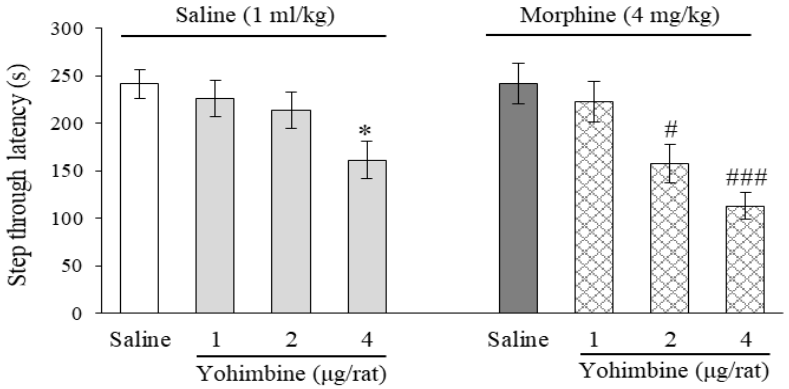

Chart 4. Effects of post-training injections of different doses of yohimbine on passive avoidance memory in the presence or absence of morphine. Immediately after training, animals received different doses of yohimbine in the CA1 region and saline or a low dose of morphine intraperitoneally and were tested for memory 24 hours later without any injections. Significant differences between groups were determined by Tukey's post-hoc test. Each experimental group consisted of 8 rats (*p<0.05 compared to saline/saline group, #p<0.05 and ###p<0.001 compared to saline/morphine group).

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Chart 5 shows the inhibitory effect of low dose yohimbine (1 µg/rat) on the response induced by clonidine (4 µg/rat) and clonidine plus effective dose of morphine (6 mg/kg) on memory recall. Tukey's post hoc test showed that animals that received low dose yohimbine 5 min before effective dose of clonidine showed a significant decrease in memory recall on the test day compared to the group receiving clonidine/saline (199.13±16.17 vs. 294.25±6.15) ($p<0.01$). Moreover, low dose yohimbine injection prevented the inhibitory effect of clonidine (4 µg/rat) on amnesia induced by morphine injection (6 mg/kg) and induced amnesia (149.37±18.21 vs. 254.13±17.78) ($p<0.001$). Each experimental group consisted of 8 rats.

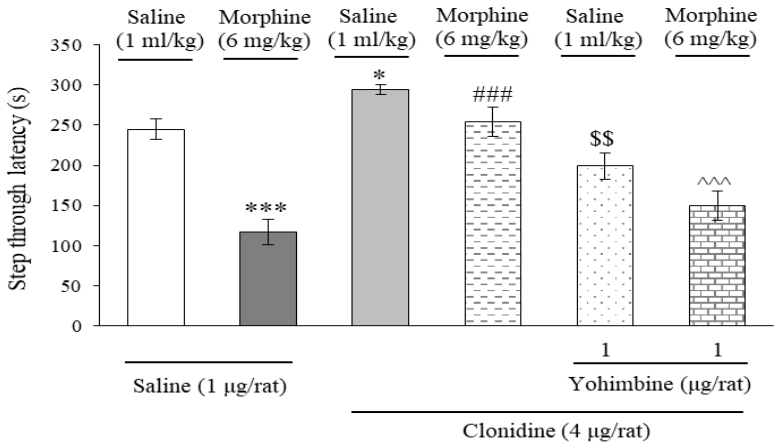

Chart 5. Inhibitory effect of yohimbine on the response induced by clonidine and clonidine plus morphine on passive avoidance memory. Significant differences between groups were determined by Tukey's post hoc test. Each experimental group consisted of 8 rats (*p<0.05 and ***p<0.001 compared to saline/saline group, ###p<0.001 compared to saline/morphine group, $$p<0.01 compared to clonidine/saline group, ^^^p<0.001 compared to clonidine/morphine group).

Chart 6 shows the effects of post-training injection of the effective dose of morphine (6 mg/kg), the effective dose of clonidine (4 µg/rat) alone and in combination with the effective dose of morphine, the low dose of yohimbine (1 µg/rat) plus the effective dose of clonidine, and the low dose of yohimbine plus the effective dose of clonidine plus the effective dose of morphine on the expression of factors involved in mitochondrial biogenesis. Statistical analysis showed that the effective dose of morphine caused a decrease in the expression levels of PGC-1α, NRF-1, and TFAM proteins compared to the saline/saline group (118.25±19.85 vs. 185.1±8.8, 63.42±6 vs. 106.62±11.95, and 19.5±0.89 vs. 37.6±5.44, respectively ($p<0.05$) (Chart 6a, 6b, and 6c). Injection of an effective dose of clonidine after training led to an increase in the expression levels of factors involved in mitochondrial biogenesis, but these increases were not statistically significant. However, injection of an effective dose of clonidine before injection of an effective dose of morphine increased the expression levels of PGC-1α and TFAM proteins compared to the saline/morphine group (180.19±15.29 vs. 118.25±19.85 and 39.23±3.97 vs. 19.50±0.89, respectively) ($p<0.05$) (Chart 6a and 6c). It should be noted that the increase in the expression levels of NRF-1 protein compared to the saline/morphine group was not statistically significant (Chart 6b). Injection of low dose yohimbine before injection of effective dose clonidine caused a decrease in the expression level of PGC-1α and TFAM proteins compared to the clonidine/saline group (128.11±11.21 vs. 212.9±15.16 and 24.98±3.07 vs. 43.15±1.83, respectively) ($p<0.01$ and $p<0.05$) (Chart 6a and 6c). However, the decrease in the

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expression level of NRF-1 protein compared to the clonidine/saline group was not statistically significant (Chart 6b). Moreover, injection of low dose yohimbine together with effective dose clonidine plus effective dose morphine caused a decrease in the expression level of PGC-1α protein compared to the clonidine/morphine group (91.48±9.71 vs. 180.19±15.29) ($p<0.01$) (Chart 6a). However, the decrease in the expression levels of NRF-1 and TFAM proteins compared to the clonidine/morphine group was not statistically significant (Chart 6b and 6c). Each experimental group consisted of 4 rats.

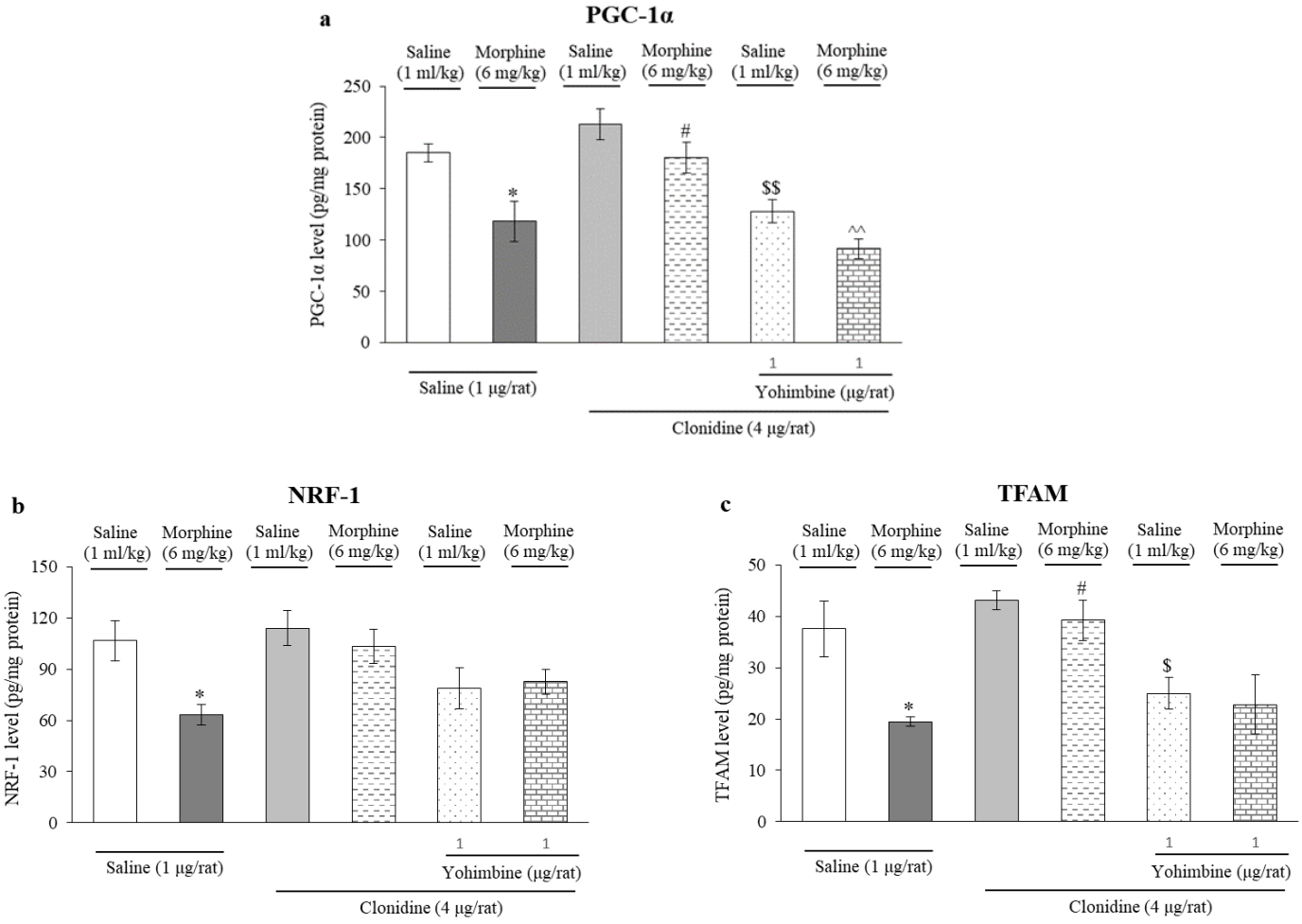

Chart 6. The effect of injection of effective dose of morphine (6 mg/kg), effective dose of clonidine (4 µg/rat) alone and also in combination with effective dose of morphine, low dose of yohimbine (1 µg/rat) with effective dose of clonidine and also low dose of yohimbine with effective dose of clonidine plus effective dose of morphine on expression of factors involved in mitochondrial biogenesis. Tukey's post hoc test was used to identify groups with significant differences. (*p<0.05 compared to saline/saline group, #p<0.05 compared to saline/morphine group, $p<0.05 and $$p<0.01 compared to clonidine/saline group, ^^p<0.01 compared to clonidine/morphine group, each experimental group consisted of 4 rats).

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Discussion

The findings of the present study showed the involvement of mitochondrial biogenesis and α2-adrenergic receptors in morphine-induced memory impairment, such that stimulation of α2-adrenergic receptors in the hippocampal CA1 region by agonists of these receptors and increased expression of factors involved in mitochondrial biogenesis reduced morphine-induced memory impairment in rats. There are many studies regarding the involvement of morphine and the opioid system in cognitive processes. Most of these studies show that morphine leads to memory impairment. On the other hand, the effect of morphine on memory facilitation has also been reported in some studies. These results indicate that the different effects of morphine on learning and memory may depend on the method of drug administration, drug dose, and model of memory examination in animals (23, 24). The results of the present study showed that post-training injection of 6 mg/kg morphine impaired passive avoidance memory in rats, which is called morphine-induced amnesia. In support of our findings, it has been reported that systemic injection of morphine impairs memory consolidation (25, 26). In other studies, it has been observed that injection of morphine into the amygdala and ventral tegmental area of rats impairs passive avoidance memory (27, 28). Other studies have also shown that morphine impairs learning and spatial memory in rats, while injection of naloxone facilitates learning and spatial memory (29, 30). The mechanism by which morphine causes cognitive impairment is not fully understood. Some studies have suggested that morphine-induced memory impairment may be mediated by induction of apoptosis, neuroinflammation, and oxidative stress in the hippocampus (31). In addition, it has been reported that morphine can impair learning and memory by reducing the release of important neurotransmitters involved in cognitive processes, such as glutamate, acetylcholine, and norepinephrine (8, 9).

The involvement of the hippocampal adrenergic system in many brain functions such as learning and memory has been shown by many studies (32). It has been reported that activation of α or β-adrenergic receptors in different parts of the brain, including the hippocampus, enhances memory in rats, and inhibition of these receptors leads to memory impairment (33, 34). The results obtained in this study also showed that post-training injection of clonidine (4 μg/rat), an agonist of α2-adrenergic receptors, into the CA1 region led to an increase in passive avoidance memory in rats. Post-training injection of yohimbine (4 μg/rat), an antagonist of α2-adrenergic receptors, into the CA1 region reduced passive avoidance memory. In agreement with our findings, some studies have shown that systemic or intracerebral injection of α2-adrenergic receptor agonists enhances memory in rats, and injection of α2-adrenergic receptor antagonists impairs memory (35, 36). One study showed that pre-test injection of clonidine into the CA1 region of rats improved scopolamine-induced passive avoidance memory impairment, but yohimbine injection exacerbated scopolamine-induced memory impairment (37). In addition, another study showed that post-training injection of clonidine into the CA1 region improved sleep deprivation-induced memory impairment in rats (38). Studies on rats with genetic mutations in α2-adrenergic receptors also revealed a role for these receptors in enhancing memory and cognitive functions (39).

The results of the present study also showed that post-training injection of clonidine (4 μg/rat) before injection of an effective dose of morphine (6 mg/kg) prevented morphine-induced memory impairment and amnesia. However, post-training injection of yohimbine (4 μg/rat) before injection of a low dose of morphine (4 mg/kg) resulted in memory impairment in rats. In other words, yohimbine enhanced the effect of morphine in causing amnesia. In the present study, injection of a low dose of yohimbine (1 μg/rat), which had no effect on memory alone, before injection of an effective dose of clonidine (4 μg/rat) prevented the effect of clonidine in enhancing memory. In addition, injection of a low dose of yohimbine had an inhibitory effect on the response induced by clonidine together with morphine. In other words, injection of a low dose of yohimbine prevented the effect of clonidine in improving morphine-induced amnesia. Although the

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interaction between the adrenergic and opioidergic systems in processes such as anxiety, learning, and memory has been reported by other researchers (40, 41), the relationship between α2-adrenergic and μ-opioid receptors in the hippocampal CA1 region has not been investigated so far. In line with our findings, a study has shown that injection of atenolol, an antagonist of β1-adrenergic receptors, into the prefrontal cortex of rats causes amnesia, and co-injection of atenolol with morphine exacerbates amnesia in rats, suggesting the involvement of β1-adrenergic receptors in morphine-induced amnesia (42). Another study has also shown that injection of morphine together with propranolol, a β2-adrenergic receptor antagonist, causes impairment of spatial memory in monkeys as well as rats (43, 44). Another study also showed an interaction between morphine and the noradrenergic modulation of basolateral amygdala neuronal activity in anxiety and memory in the elevated plus maze test (45). In this study, it is possible that high-dose clonidine injection reduced morphine-induced memory impairment by stimulating postsynaptic α2-adrenergic receptors and increasing norepinephrine release in the CA1 region, or that the increase in norepinephrine release may have indirectly reduced morphine-induced memory impairment by stimulating the release of other neurotransmitters involved in learning and memory, such as glutamate. However, further studies, especially in the molecular part, are needed to clarify the exact mechanism(s) involved in the interaction between α2-adrenergic receptors and the opioid system. It should be noted that in this study, the results of examining the animals' motor activities confirmed that the data from behavioral tests were solely related to the effects of drug treatment and were not due to the presence of motor disorders in the animals.

The results of the present study also showed that morphine injection (6 mg/kg) resulted in a decrease in the expression of factors involved in mitochondrial biogenesis (PGC-1α, NRF-1, and TFAM) in the CA1 region of rats, indicating that morphine reduced mitochondrial biogenesis. In line with our findings, studies have shown that morphine exposure induces apoptosis and mitochondrial dysfunction in the rat brain (46, 47). In addition, a recent study has shown that long-term morphine injection induces anxiety-like behaviors and increases the expression levels of factors involved in mitochondrial biogenesis such as PGC-1α, TFAM, and NRF-1 in the basolateral amygdala of rats. In addition, this study suggested that excessive mitochondrial biogenesis beyond what is physiologically necessary may lead to increased levels of reactive oxygen species, which in turn may influence anxiety-like behaviors (48). The differences in the results of this study and ours may be due to the dose of morphine administered and the duration of its administration. Given the results of our study and the findings of previous studies, it is likely that morphine exerts at least part of its effects on memory impairment by affecting mitochondrial biogenesis, as many studies have shown that any disruption in mitochondrial biogenesis can lead to cognitive impairment (48). Therefore, finding therapeutic strategies that can reduce the impairment in mitochondrial biogenesis can prevent the occurrence of cognitive disorders caused by morphine to a great extent. In the present study, it was observed that the injection of clonidine with morphine increased the expression of factors involved in mitochondrial biogenesis compared to the group receiving morphine. However, the injection of yohimbine with clonidine or with clonidine plus morphine prevented the increase in the expression of the aforementioned factors. Therefore, it is possible that in this study, clonidine was able to prevent the occurrence of cognitive disorders caused by morphine by increasing the expression of factors involved in mitochondrial biogenesis. Studies on the effect of adrenergic receptor agonists and antagonists on mitochondrial biogenesis in the central nervous system are very few. In one study, it was reported that injection of formoterol (a β2-adrenergic receptor agonist) improved spinal cord injury in mice by increasing PGC-1α and TFAM expression (49).

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Another study also showed that formoterol increased mtDNA copy number, increased PGC1α expression, and genes involved in the mitochondrial electron transport chain in the kidney and heart of mice (50). It was also reported that injection of midodrine (a non-selective α1-adrenergic receptor agonist) increased PGC1α expression in C2C12 myoblasts and HL1 cardiomyocytes, thereby enhancing mitochondrial biogenesis in vitro (51).

The present study investigated the role of mitochondrial biogenesis and α2-adrenergic receptors in morphine-induced memory impairment. Therefore, one of the limitations of this study is that further molecular studies are needed to better understand the interaction between the α2-adrenergic system and mitochondrial biogenesis. Histological studies to count the number of mitochondria and to examine the expression of factors involved in mitochondrial biogenesis at the mRNA level by Real-time PCR and at the protein level by Western blot are recommended for future research.

Overall, the results of this study showed that stimulation of α2-adrenergic receptors by agonists of these receptors and increased expression of factors involved in mitochondrial biogenesis reduced morphine-induced memory impairment. Accordingly, it seems that α2-adrenergic receptors in the CA1 region of the hippocampus and mitochondrial biogenesis may be involved in the memory-degrading effects of morphine.

Conflict of interest: The authors declare that there are no conflicts of interest.

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