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ORIGINAL RESEARCH

Beneficial Effect of Atomoxetine Treatment on Scopolamine-Induced Cognitive Impairments and Molecular Changes in Rats

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Abstract

Objective: Atomoxetine is a noradrenaline re-uptake inhibitory drug that is currently used in the pharmacological treatment of attention deficit/hyperactivity disorder in children, adolescents, and adults. Based on the findings that noradrenergic system is closely associated with learning and memory processes, dysfunction of this system might contribute to the pathophysiology of cognitive disorders. Therefore, atomoxetine, which alters the noradrenergic neurotransmission in the synaptic cleft, has a potential to modulate learning and memory functions in various areas of the central nervous system. In the present study, it was aimed to investigate the effects of atomoxetine treatment on the cognitive impairments in amnesic animals using behavioral and immunohistochemical methods.

Methods: Male Sprague-Dawley rats (adult, weighing 250-300 g) were used in the experiments. Atomoxetine at doses of 3 or 6 mg/kg (i.p.) was administered to the rats for 14 days. Experimental amnesia was induced in the animals by applying scopolamine (0.5 mg/kg, i.p.) injection. Following the treatment protocol, Morris water maze and passive avoidance tests were conducted to assess spatial and emotional learning and memory parameters of rats, respectively. Piracetam (300 mg/kg daily for 14 days) was used as reference drug in these experiments. In addition, motor performances of the animals were evaluated by activity cage and Rota-rod tests. Subsequent to behavioral tests, rats were euthanized and immunohistochemical analyses were performed to determine the alterations in the brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) levels in the hippocampi of the animals.

Results: In the Morris water maze test, amnesic rats found the hidden platform slower and spent less time in the target quadrant compared to the healthy controls. On the other hand, atomoxetine treatments significantly decreased the escape latencies, and also increased the target quadrant time values of the amnesic animals at both doses. Data obtained from the passive avoidance test showed that second transition latency values of the amnesic rats were significantly shortened with respect to the control animals; while atomoxetine treatment prolonged these shortened values significantly. It was observed that atomoxetine did not alter motor coordination or spontaneous locomotor activities of animals. Moreover, this drug increased the BDNF and NGF levels in the hippocampal sub-regions which were diminished by scopolamine administration.

Conclusion: The obtained findings showed that atomoxetine treatment significantly improved the impaired learning and memory performances of the rats induced by scopolamine administration. Furthermore, anti-amnesic effects of this drug were concomitant with increased neurotrophic factors levels in the hippocampal brain regions. These results suggest a notable therapeutic potential of this drug in the treatment of dementia and several cognitive disorders; however, it should be underlined they are needed to be confirmed by well-designed clinical trials.

Keywords: Atomoxetine, Scopolamine-Induced Amnesia, Morris Water Maze Test, Passive Avoidance Test, Brain-Derived Neurotrophic Factor, Nerve Growth Factor, Hippocampus

INTRODUCTION

Atomoxetine (ATM) is an FDA-approved drug used as an alternative to psychostimulants for over 30 years in the treatment of attention deficit and hyperactivity disorder (ADHD). ATM is known to be effective not only in children but also in adults in relieving symptoms such as attention deficit, hyperactivity and impulsivity (1). The mechanism of action of non-stimulant drug ATM is different from classical psychostimulants used in the treatment of ADHD (2).

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Noradrenergic system has been reported to affect emotional memory through modulation of the amygdala and related mediotemporal structures (3). Moreover, moderate increase in noradrenergic neurotransmission in the prefrontal cortex is known to strengthen working memory. Therefore, a selective noradrenaline reuptake inhibitory drug, ATM has a potential to modulate learning and memory processes by altering noradrenaline levels in various areas of the central nervous system. Indeed, some previous studies have pointed a nootropic efficacy for ATM. For example, acute administration of this drug, specifically at a dose of 3 mg/kg, has been shown to increase cortical acetylcholine levels in rats and enhance their memory parameters in novel object recognition and radial arm maze tests (2). ATM administration is known to improve learning deficits in rats with attention-deficit hyperactivity disorder (4) but cognitive function-improving effects of this drug have also been shown in some other animal models of cognitive impairment such as prenatal stress (5), and traumatic brain injury (6). ATM has been shown to improve memory and other components of executive function also in aged rhesus monkeys (7).

In the literature, it is possible to come across some clinical studies pointing out the effects of ATM on cognitive performance. For example, in a recent phase II study conducted with patients with mild Alzheimer's disease, ATM has been shown to reduce Tau and pTau in patients' cerebrospinal fluids (CSF). It has also been shown to normalize panels of CSF protein biomarkers associated with synaptic function, brain metabolism, and glial immunity, and increase brain activity and metabolism in key temporal lobe circuits. Results of this study have pointed out the potential of ATM to slow the progression of Alzheimer's disease (8). Another clinical trial with perimenopausal and postmenopausal women by Epperson and colleagues have suggested that ATM treatment could result in significant subjective improvement in attention, concentration, and memory performance of these women (9).

Actually, the beneficial effects of ATM on cognitive performance have been associated with increases in central levels of certain neuromediators such as noradrenaline, dopamine (10), acetylcholine (2) and histamine (4,11), which are closely related to learning and memory processes in the brain (3,12,13). Although the effects of ATM on central levels of cognition-related neurotransmitters have been studied previously, the changes this drug causes in brain neurotrophic factor levels are not yet known. In addition, studies investigating the beneficial effect of ATM on amnesic animals are quite limited in the literature. Therefore, we aimed to investigate the anti-amnesic activity potential of subacute ATM treatment using a scopolamine (SCP)-induced amnesia model in rats and to clarify ATM-induced changes in hippocampal levels of some neurotrophic factors, which are important modulators of learning and memory processes.

METHODS

Experimental Animals

Male Sprague-Dawley rats weighing 250–300 g and of the same age were used in the experiments. Rats were housed in well-ventilated rooms at a constant temperature of 24 ± 1 °C under a 12 h light/12 h dark cycle (lights on between 8:00 and 20:00 h) and received standard animal food pellets. All procedures related to animal experiments were approved by the Anadolu University Animal Experiments Local Ethics Committee, Eskişehir, Turkey (Date: 11.05.2022; Protocol No: 2022-16).

Chemicals and Drugs

ATM was purchased from Sigma-Aldrich (St. Louis, MO, USA); piracetam (PRC) (Nootropil®) from UCB Pharma (Istanbul, Turkey); SCP hydrobromide, monobasic potassium phosphate, dibasic sodium phosphate, paraffin, paraformaldehyde, xylene, ethanol from Sigma (St. Louis, MO, USA); rabbit recombinant monoclonal anti-Brain-Derived Neurotrophic Factor (BDNF) antibody (ab108319), rabbit polyclonal anti-Nerve Growth Factor (NGF) antibody (ab216419) from Abcam; Antibody Diluent (003118) and UltraVision Quanto Detection System HRP DAB (TL-060-QHD) from Thermo Scientific (MA, USA); ketamine (Alfamine®) from Atafen (İzmir, Turkey); xylazine (Xylazinbio®) from Bioteva (Ankara, Turkey); physiological saline %0.9 from Polifarma (Tekirdağ, Turkey).

Administration of Drugs

The animals were randomly allocated into five experimental groups (n = 8 per group) as a control group receiving physiological saline (Control) and SCP-induced amnesia groups receiving physiological saline (SCP + Control) or ATM (SCP + ATM-3 and SCP + ATM-6) or PRC (SCP + PRC) for each behavioral experiment.

ATM at doses of 3 or 6 mg/kg was administered to the rats intraperitonally (i.p.) (2,14). PRC was given at a dosage of 300 mg/kg orally as a positive control (15). ATM, PRC, and the physiological saline as the control solution were administered once a day for 14 days. Behavioral tests were initiated 30 minutes and 60 minutes after i.p.

administrations and p.o. administrations, respectively. The tests evaluating cognitive functions were conducted on different experimental groups and performed during the day between 9:00 A.M.-4:00 P.M.

Induction of the Experimental Amnesia Model

In the Morris water maze tests, the relevant experimental groups were administered SCP (0.5 mg/kg, i.p.) for the first four days of the experiment. As for the passive avoidance tests, SCP was administered only once to the relevant experimental groups. In order to induce the amnesia model, all the SCP injections were performed 30 minutes before the acquisition trials (16).

Evaluation of Motor Activity

Rats' spontaneous locomotor activity and motor coordination parameters were evaluated using activity cage and Rota-rod tests.

Activity Cage Test

The locomotor activities of the animals in all groups in the experiment were assessed using an activity cage device (Ugo Basile, 7420, Varese, Italy). For 10 minutes, the rats' vertical and horizontal locomotor activity values were recorded (17).

Rota-Rod Test

Before the experiments, the training protocol was applied to all experimental groups; the rats were trained on a Rota-rod apparatus (Ugo Basile, 47700, Varese, Italy) for three consecutive days. During the experiments, the equipment was set at a constant speed of 8 rpm, and the time until the rats fell from the moving rod was recorded as "falling latency" for 10 minutes (18).

Evaluation of the Learning and Memory Parameters

Passive Avoidance Test

The step-through passive avoidance device (Ugo Basile, 7551, Italy) was used to assess fear-related emotional memory, in which subjects had previously learned to avoid an environment presenting an aversive stimulus (19). The animals were placed in the device consisting of a lit white chamber and a dark chamber separated by an automated sliding door with dimensions of 22 x 21 x 22 cm. All two sections of have a conductive grid floor capable of applying electric current to the animal's feet via a shock generator. At the beginning of the experiment, the rats underwent a training trial. They were carefully placed in the illuminated chamber. After 30 seconds, the door separating the compartments was opened, allowing them to enter the dark chamber freely. Following this, an acquisition trial was performed 15 minutes later, and the animals were placed in the brightly illuminated chamber again. After a 30-second acclimation phase, the automated sliding door opened, and the transfer time of the experimental animal to the dark chamber was recorded as the "first transition latency"; however, if a rat did not move across within 5 minutes, it was excluded from the experiment. When the animal entered the dark chamber entirely, the door between the sections closed automatically, and a 0.5 mA electric shock lasting 3 seconds was applied to the animal's paws via the device's conductive grid floor. The rat was then removed from the apparatus and returned to its cage, and the device was carefully cleaned so that no odors that could provide clues remained. The memory trial was conducted 24 hours following the acquisition trial. The memory acquired by the painful aversive stimulus was assessed by measuring the "second transition latency". During this trial, no electric shocks were applied to the subjects and the time until entering the dark section was measured up to 5 minutes.

Morris Water Maze Test

The Morris water maze procedure assessed the animals' spatial learning and memory performances (20). The maze is formed of a large circular tank (150 cm×60 cm). Tank filled with a water depth of 50 cm at a constant temperature of 25±1°C. Throughout the experiment, a disguised cylindrical escape platform was submerged 2 cm below the water's surface in the same target quadrant of the maze. The water was rendered opaque with a non-toxic food dye so that the escape platform would not be visible to the subjects. Fixed visual cues consisting of simple geometric shapes were placed around the maze to help them navigate the hidden escape platform's location in the maze (21,22). In the experiments, animals were subjected to four consecutive trials per day with a 5-minute interval. The rats were released gently into the water from different quadrants each time and allowed 120 seconds to find the platform. When they found it, they were allowed to explore the surroundings for another 20 seconds. The time taken to discover the platform was recorded as "escape latency". For four successive days, the animals were subjected to acquisition trials. On the 5th day, the platform was removed, and time spent in the target quadrant was recorded for 120 seconds (23).

Immunohistochemical Staining

The rats were anesthetized with ketamine/xylazine (100/5 mg/kg, i.p.) (24) 30 minutes after the last ATM administration, once the behavioral tests were finished. Afterwards, rats were transfused transcardially with 0.1 M neutral phosphate-buffered saline (PBS) and 4% paraformaldehyde in PBS. The brain was quickly dissected and cleaned with ice-cold PBS. Following overnight post-fixation in 4% paraformaldehyde in PBS, dehydrated brain tissues were cleared in xylene before being embedded in paraffin blocks. The dorsal hippocampus sectioned at 3 µm thickness (compatible with the coordinates of – 2.52 mm, – 3.00 mm, and – 3.48 mm according to the bregma reference point (25) in the coronal plane for the immunohistochemical staining. After the selected sections were mounted on positively charged slides, they were kept in an oven at 70°C for 30 minutes and deparaffinized. Following deparaffinization, the samples were hydrated with decreasing concentrations of ethanol series, tap water, and distilled water. To avoid antigen masking of brain tissue, the sections were treated under high pressure with citrate solution (pH 6.0). Endogenous peroxidase activity was inhibited with hydrogen peroxide (TA-125-HP) for 30 minutes, and a protein-blocking solution (TA-125-UB) was applied to the slides to prevent non-specific background staining,. Anti-BDNF antibody (1:250) and anti-NGF (1:100) were used to investigate hippocampal levels of neurotrophins. After incubating for 8 hours at room temperature with primary antibodies, the sections were treated with HRP Polymer Quanto (TL-125-QPH), and Ultra V Block (TA-125-UB) for 30 minutes at room temperature. Sections were rinsed again in PBS before being treated with DAB chromogen. The sections were dehydrated, passed through xylene, and coverslipped.

Immunohistochemical Analyses

Immunolabeled sections were imaged using a light microscope with an integrated camera using Olympus CX31RTSF (Olympus GmbH, Hamburg, Germany). Photomicrographs were taken from all prepared sections using LCmicro (Olympus GmbH, Hamburg, Germany) imaging software. The BDNF and NGF immunoreactivity of the photomicrographs was assessed in the percentage of immunoreactive area in the CA_1, CA_3, and dentate gyrus (DG) subfields of the hippocampus by using the ImageJ 1.50i (National Institutes of Health, Bethesda, MD, USA) image processing and analysis application.

Statistical Analysis

GraphPad Prism ver. 10.1.0 (316) software package (GraphPad Software, La Jolla, CA, USA) was used for the statistical analyses and generating the graphs. In evaluation of the data that required multiple comparisons between the groups, data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Experimental data obtained from the escape latencies measured in the Morris water maze test were assessed using two-way repeated measures ANOVA followed by the Bonferroni multiple comparisons test. The results were expressed as mean ± standard error of the mean (SEM). Differences between data sets were considered significant when $p<0.05$.

RESULTS

Experiments on Motor Performance

The Activity Cage Test

Figures 1A and **1B** show the effects of ATM administration on the "horizontal" [$F(2, 21) = 3.01$; $p>0.05$] and "vertical" [$F(2, 21) = 0.77$; $p>0.05$] activities of the rats. The results show that ATM administration at doses of 3 and 6 mg/kg for 14 days did not cause any significant change in the numbers of vertical and horizontal movements.

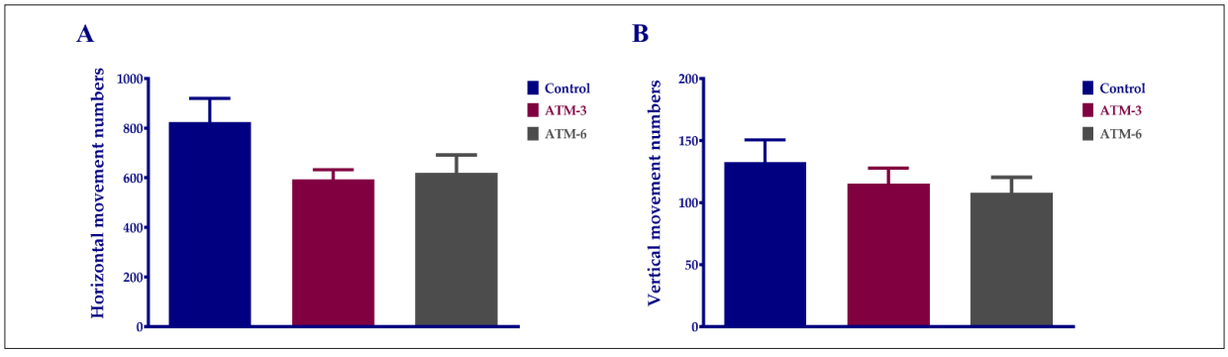

Figure 1. The effects of ATM administrations (3 mg/kg and 6 mg/kg) on the horizontal **(A)** and vertical **(B)** movement numbers measured in the activity cage tests. One way analysis of variance followed by Tukey HSD multiple comparison test, n = 8.

The Rota-Rod Test

Figure 2 shows the effects of ATM administration on the "falling latency" parameters of rats [F (2, 21) = 1.4; p>0.05]. The results show that ATM administration at doses of 3 and 6 mg/kg for 14 days did not cause a significant change in the falling latencies of rats.

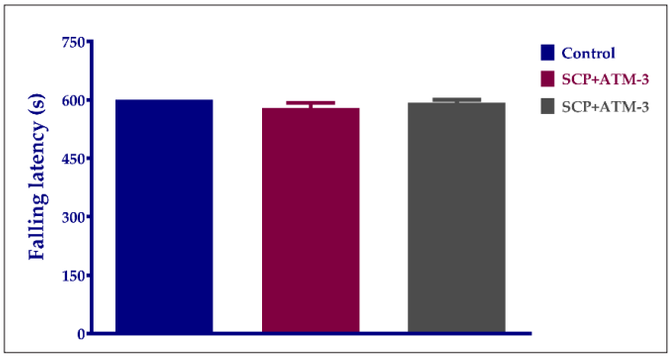

Figure 2. The effects of ATM administrations (3 mg/kg and 6 mg/kg) on the "falling latencies" measured in the Rota-rod tests. One way analysis of variance followed by Tukey HSD multiple comparison test, n = 8.

Learning and Memory Experiments

The Morris Water Maze Test

Figure 3 shows the alteration in the "escape latency" values measured in the Morris water maze test. The two-way repeated measures ANOVA showed that both the treatment factor [F (4, 35) = 18.12; *p<0.001*]**;** and the time factor [F (3,105)=55.74; *p<0.001*] had effect on the escape latency values in amnesic rats. There was no significant interaction between treatment and time [F (12,105)=1.04; *p>0.05*].

The multiple comparisons test following ANOVA showed that the escape latency values measured on the 2nd ($p < 0.05$), 3rd ($p < 0.001$) and 4th ($p < 0.001$) days in the Morris water maze test were significantly higher in rats in the SCP-induced amnesia group than those in the control group; indicating that SCP-administered rats had difficulty in discovering the platform when compared to controls. However, PRC (300 mg/kg) or ATM (3 ve 6 mg/kg) administrations significantly lowered the increased escape latency values in the amnesic rats on days 2, 3, and 4 (**Figure 3**), which indicated that ATM treatment improved the SCP-induced impairments at both doses that was comparable to PRC.

Figure 4 demonstrates the time spent in the "target quadrant" in the Morris water maze tests [F (4,35) = 10.06; $p < 0.001$]. Tukey HSD analyses performed for the multiple comparison showed that rats in the SCP-induced amnesia group spent significantly less time in the target quadrant than those in the control group ($p < 0.001$), indicating that amnesic rats had difficulty in remembering the location of the hidden platform. However, PRC (300 mg/kg) ($p<0.01$), ATM (3 mg/kg) ($p<0.05$) and ATM (6 mg/kg) ($p<0.001$) administrations significantly increased the time spent in the target quadrant compared to the amnesic rats that did not receive treatment, which suggests that ATM treatment was effective in improving the memory performances of rats with SCP-induced amnesia that was comparable to PRC.

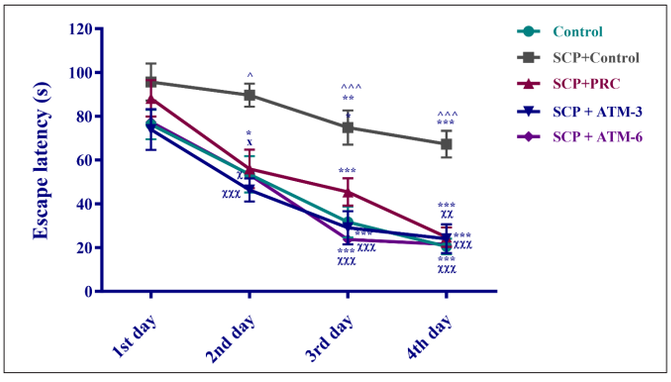

Figure 3. Escape latency values of healthy rats (Control) and control solution (SCP + Control), 200 mg/kg PRC (SCP + PRC), 3 mg/kg ATM (SCP +ATM-3) or 6 mg/kg ATM (SCP + ATM-6) administered amnesic rats in the Morris water maze tests. Significant difference against to the corresponding day of the control group $^{\wedge}p < 0.05$, $^{\wedge\wedge\wedge}p < 0.001$; to the corresponding day of the SCP + control group $^{x}p < 0.05$, $^{xx}p < 0.01$, $^{xxx}p < 0.001$; to the first day of the groups $^{*}p < 0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Two-way repeated variance analysis followed by Bonferroni multiple comparison test, n = 8.

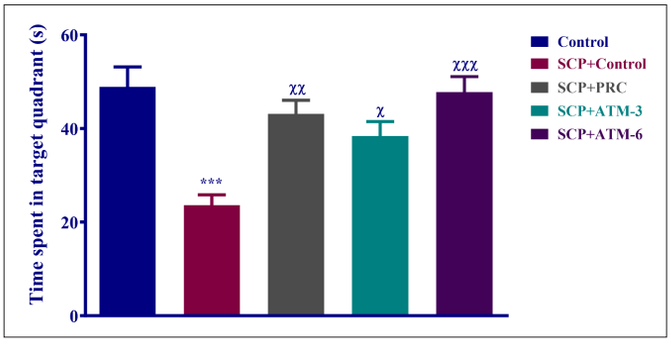

Figure 4. Time spent in the target quadrant values of healthy rats (Control) and control solution (SCP + Control), 200 mg/kg PRC (SCP + PRC), 3 mg/kg ATM (SCP +ATM-3) or 6 mg/kg ATM (SCP + ATM-6) administered amnesic rats in the Morris water maze tests. Significant difference compared to the control group $^{***}p < 0.001$; Significant difference compared to the SCP + control group $^{x}p<0,05$; $^{xx}p<0,01$; $^{xxx}p<0,001$. One way analysis of variance followed by Tukey HSD multiple comparison test, n = 8.

The Passive Avoidance Tests

Figure 5A shows the results of the acquisition trials on the first day [F (4,35)=0.64; p>0.05]. There was no difference in the first transition latency values measured during the acquisition trials between the groups. The results of the memory trials on the second day [F (4,35)=11,81; p<0.001] were presented in **Figure 5B**. Rats receiving SCP had significantly shorter second transition latencies compared to the control group ($p < 0.001$) demonstrating that these animals had difficulties in learning and remembering the electric shock applied in the dark compartment. However, administration of PRC ($p < 0.001$) or ATM ($p < 0.001$) for 14 days significantly prolonged the second transition latency values of the amnesic rats which indicates an improvement in the disrupted cognitive functions.

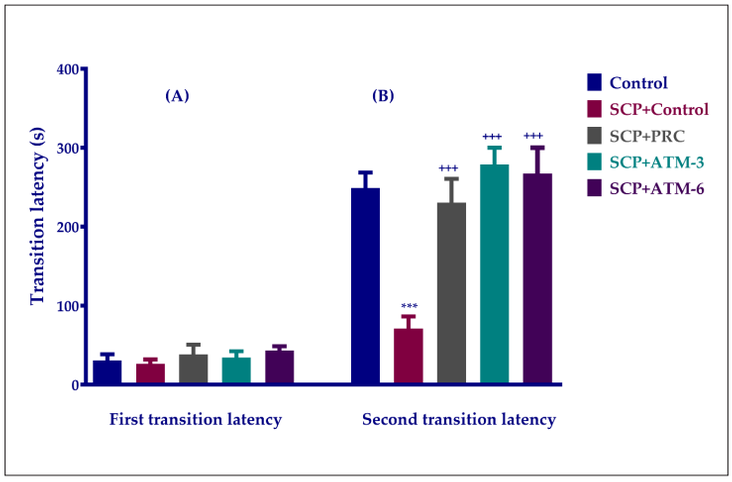

Figure 5. First transition latency **(A)** and second transition latency **(B)** values of healthy rats (Control) and control solution (SCP + Control), 200 mg/kg PRC (SCP + PRC), 3 mg/kg ATM (SCP +ATM-3) or 6 mg/kg ATM (SCP + ATM-6) administered amnesic rats in the passive avoidance tests. Significant differences compared to the control group ***p < 0.001; Significant difference compared to the SCP + control group +++p < 0.001; One way analysis of variance followed by Tukey HSD multiple comparison test, n = 8.

Immunohistochemistry

BDNF Immunoreactivity in the Hippocampal Formation

Photomicrographs presenting the BDNF immunoreactivities in the DG, CA_1 and CA_3 sub-regions of hippocampus are given in Figure 6, Figure 7, Figure 8, respectively. It was observed that BDNF immunoreactivity was decreased by SCP administration; however, 14-day ATM treatment reversed this reduction.

Alterations of BDNF densities in the DG [F (2, 15) = 5.9; $p < 0.05$], CA_1 [F (2, 15) =9.43; $p < 0.005$] and CA_3 [F (2, 15) =6.93; $p < 0.05$] sub-regions of hippocampus were presented in Figures 6D, 7D and 8D, respectively. Statistical analyses showed that BDNF densities in all the sub-regions of the hippocampus were significantly lower in rats with SCP-induced amnesia than those of the control group. On the other hand, ATM treatment significantly increased the BDNF densities in the amnesic rats.

NGF Immunoreactivity in the Hippocampal Formation

Photomicrographs presenting the NGF immunoreactivities in the DG, CA_1 and CA_3 sub-regions of hippocampus are given in Figure 9, Figure 10, Figure 11, respectively. It was observed that NGF immunoreactivity was decreased by SCP administration; however, 14-day ATM treatment reversed this reduction.

Alterations of NGF densities in the DG [F (2, 15) =10.54; $p < 0.01$], CA_1 [F (2, 15) =10.57; $p < 0.01$] and CA_3 [F (2, 15) =25.44; $p < 0.001$] sub-regions of hippocampus were presented in Figures 9D, 10D and 11D, respectively. Statistical analyses showed that NGF densities in all the sub-regions of the hippocampus were significantly lower in rats with SCP-induced amnesia than those of the control group. On the other hand, ATM treatment significantly increased the NGF densities in the amnesic rats.

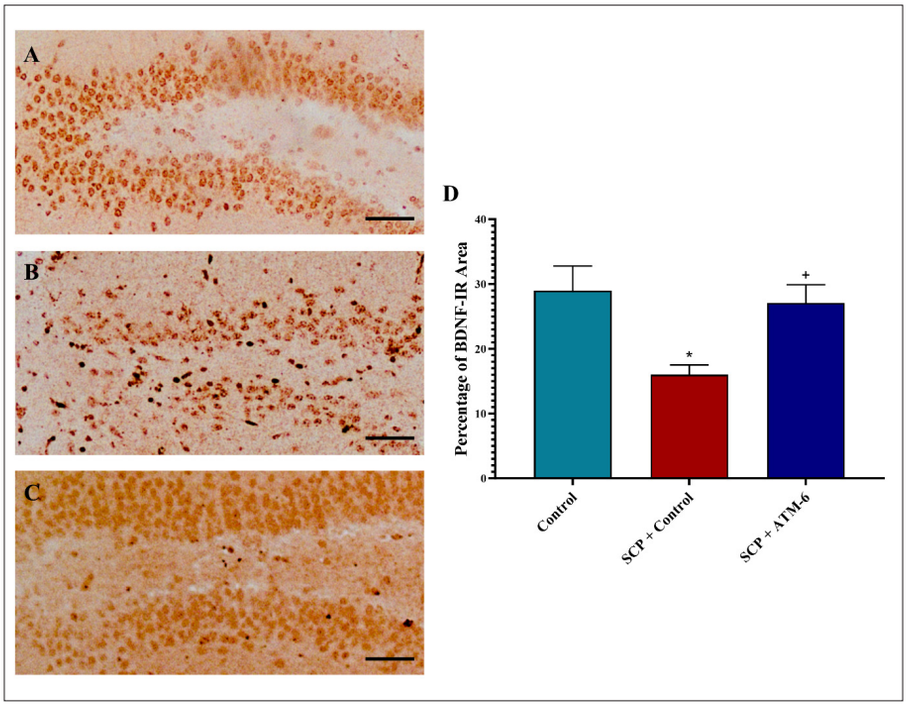

Figure 6. Representative images of BDNF immunoreactivities in hippocampal DG regions of **(A)** healthy rats (Control), **(B)** control solution (SCP + Control), or **(C)** 6 mg/kg ATM (SCP + ATM) administered amnesic rats. (Scale: 25 µm). **(D)** BDNF densities in DG of healthy rats (Control), control solution (SCP + Control), or 6 mg/kg ATM (SCP +ATM) administered amnesic rats. Significant differences compared to the control group $^{*}p < 0.05$; Significant difference compared to the SCP + control group $^{+}p < 0.05$; One way analysis of variance followed by Tukey HSD multiple comparison test, n = 6.

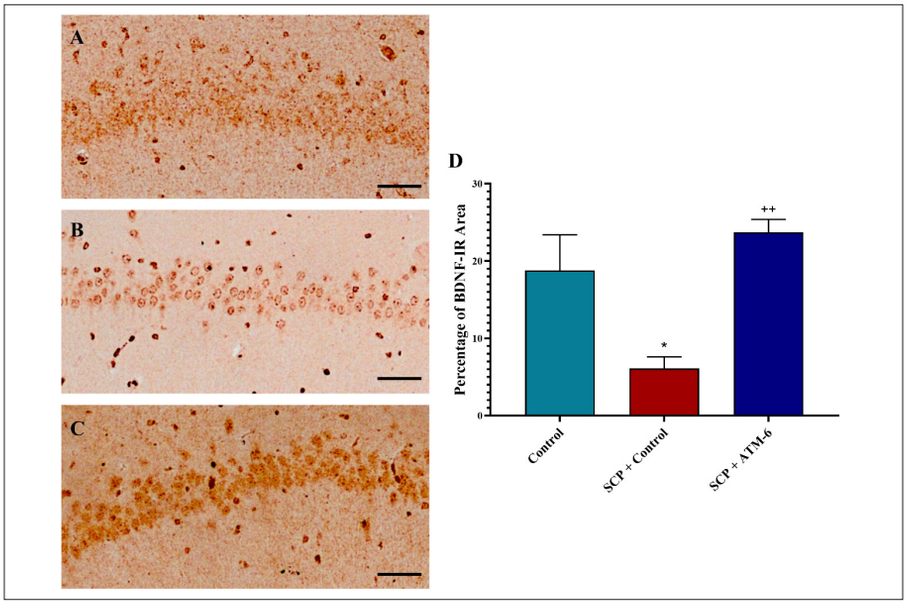

Figure 7. Representative images of BDNF immunoreactivities in hippocampal CA_1 regions of **(A)** healthy rats (Control), **(B)** control solution (SCP + Control), or **(C)** 6 mg/kg ATM (SCP + ATM) administered amnesic rats (Scale: 25 µm). **(D)** BDNF densities in CA_1 of healthy rats (Control), control solution (SCP + Control), or 6 mg/kg ATM (SCP +ATM) administered amnesic rats. Significant differences compared to the control group $^{*}p < 0.05$; Significant difference compared to the SCP + control group $^{++}p < 0.01$; One way analysis of variance followed by Tukey HSD multiple comparison test, n = 6.

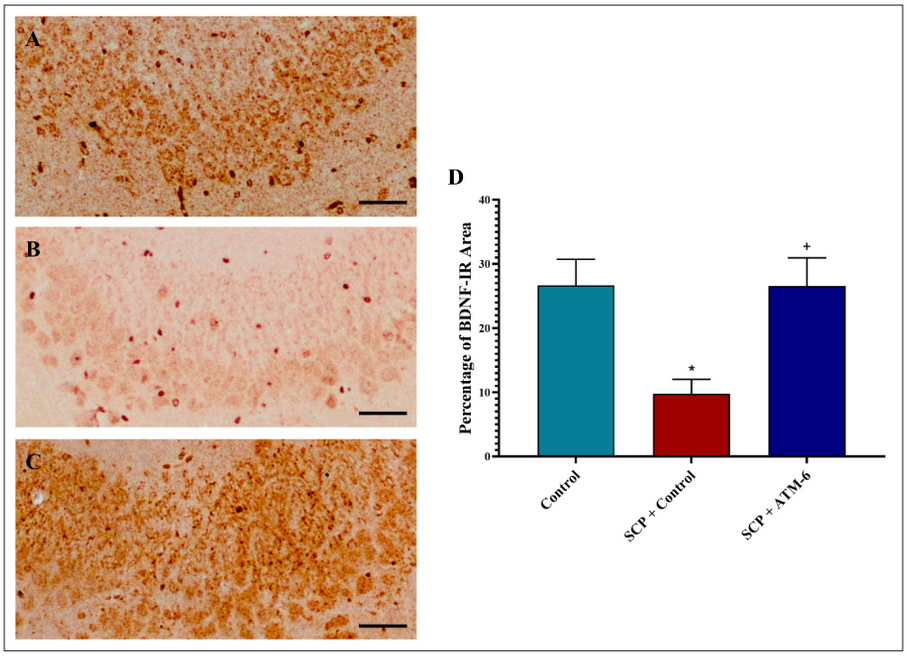

Figure 8. Representative images of BDNF immunoreactivities in hippocampal CA_3 regions of **(A)** healthy rats (Control), **(B)** control solution (SCP + Control), or **(C)** 6 mg/kg ATM (SCP + ATM) administered amnesic rats (Scale: 25 µm). **(D)** BDNF densities in CA_3 of healthy rats (Control), control solution (SCP + Control), or 6 mg/kg ATM (SCP +ATM) administered amnesic rats. Significant differences compared to the control group $^{*}p < 0.05$; Significant difference compared to the SCP + control group $^{+}p < 0.05$; One way analysis of variance followed by Tukey HSD multiple comparison test, n = 6.

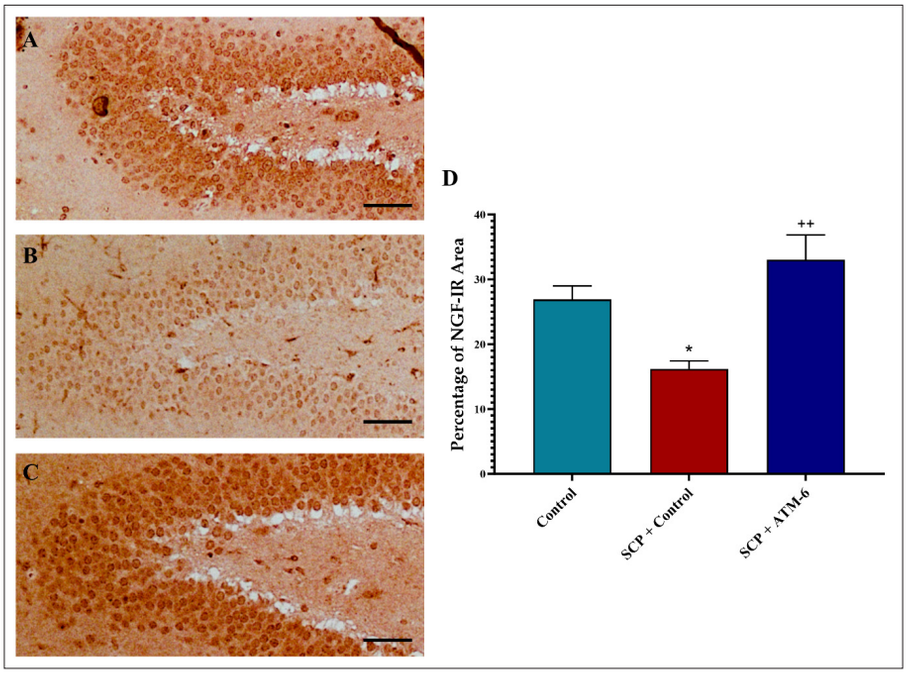

Figure 9. Representative images of NGF immunoreactivities in hippocampal DG regions of **(A)** healthy rats (Control), **(B)** control solution (SCP + Control), or **(C)** 6 mg/kg ATM (SCP + ATM) administered amnesic rats (Scale: 25 µm). **(D)** NGF densities in DG of healthy rats (Control), control solution (SCP + Control), or 6 mg/kg ATM (SCP +ATM) administered amnesic rats. Significant differences compared to the control group $^{*}p < 0.05$; Significant difference compared to the SCP + control group $^{++}p < 0.01$; One way analysis of variance followed by Tukey HSD multiple comparison test, n = 6.

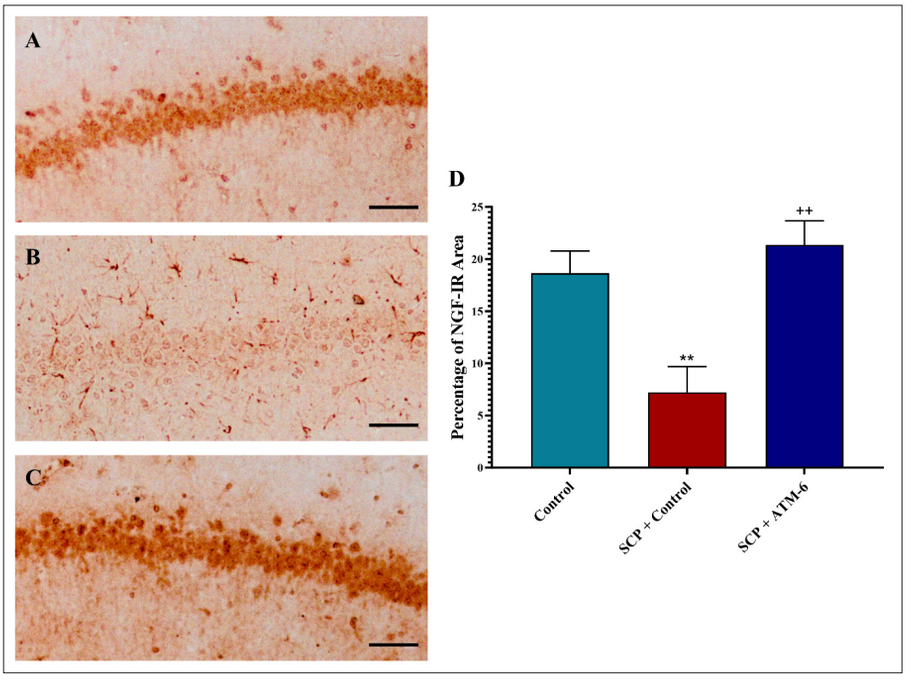

Figure 10. Representative images of NGF immunoreactivities in hippocampal CA_1 regions of **(A)** healthy rats (Control), **(B)** control solution (SCP + Control), or **(C)** 6 mg/kg ATM (SCP + ATM) administered amnesic rats (Scale: 25 µm). **(D)** NGF densities in CA_1 of healthy rats (Control), control solution (SCP + Control), or 6 mg/kg ATM (SCP +ATM) administered amnesic rats. Significant differences compared to the control group $^{**}p < 0.01$; Significant difference compared to the SCP + control group $^{++}p < 0.01$; One way analysis of variance followed by Tukey HSD multiple comparison test, n = 6.

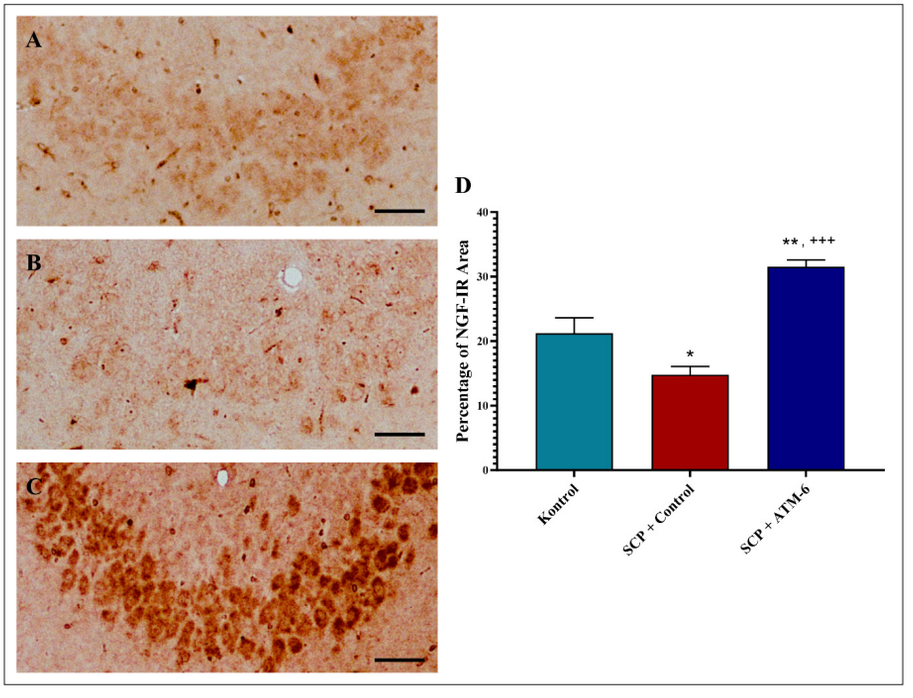

Figure 11. Representative images of NGF immunoreactivities in hippocampal CA_3 regions of **(A)** healthy rats (Control), **(B)** control solution (SCP + Control), or **(C)** 6 mg/kg ATM (SCP + ATM) administered amnesic rats (Scale: 25 µm). **(D)** NGF densities in CA_3 of healthy rats (Control), control solution (SCP + Control), or 6 mg/kg ATM (SCP +ATM) administered amnesic rats. Significant differences compared to the control group $^{*}p < 0.05$, $^{**}p < 0.01$; Significant difference compared to the SCP + control group $^{+++}p < 0.001$; One way analysis of variance followed by Tukey HSD multiple comparison test, n = 6.

DISCUSSION

In this study, effect of ATM treatment on the emotional and spatial learning and memory parameters in rats with SCP-induced amnesia was investigated, and BDNF and NGF levels in the hippocampi of amnesic animals were evaluated. In order to measure the cognitive performances of animals, Morris water maze and passive avoidance tests were used. Before these experiments, activity cage and Rota-rod tests were applied for eliminating the possibility that the results of learning and memory trials were not affected by any disruptions in the animals' motor performances. Findings from both activity cage and Rota-rod tests revealed that ATM did not cause any significant changes in the spontaneous locomotor activities or motor coordinations of rats at both doses.

In the present study, SCP, a muscarinic receptor antagonist, was used to induce experimental model of amnesia. SCP is frequently preferred in nootropic and anti-amnesic drug research studies since it causes reversible impairments in processes like as attention, information processing and new knowledge acquirement in both rodents and humans (26,27,28). It has been reported that this agent affects learning and memory processes, and causes deteriorations in short-term memory formation (29).

In this study, the results of the Morris water maze and passive avoidance tests revealed that the cognitive performances of SCP-treated animals were impaired compared to healthy rats, indicating the development of experimental amnesia model. On the other hand, it was determined that 14-day-ATM treatment at doses of 3 mg/kg and 6 mg/kg significantly improved the cognitive performances of amnesic rats. The passive avoidance task assesses fear-conditioned emotional memory; while Morris water maze test evaluates spatial learning and memory parameters (19,27,30). The anti-amnesic efficacy of ATM assessed in both of these tests was comparable to that of the reference drug, PRC.

ATM has been already known to improve cognitive performance by increasing attention in ADHD, which is the main indication of this drug (4,31). On the other hand, there are some studies referring to ATM's beneficial effects on cognitive processes in the absence of ADHD in the literature. For example, in a study of Tzavara et al., it was reported that ATM administered orally to rats at doses of 1 mg/kg and 3 mg/kg significantly increased the memory performances of the animals in object recognition and radial arm maze tests. This procognitive activity has been associated with its cortical procholinergic activity profile alongside of its effect on catecholaminergic neurotransmission increment (2). In another study conducted by Yuce et al., memory performances of Balb-c mice receiving ATM (5 mg/kg) have not changed in the passive avoidance test; however, drug caused a significant improvement in the second transition latency values in mice priorly received SCP. In the study, it has been suggested that this curative effect of the drug on impaired memory functions could be related to its noradrenaline reuptake inhibitory property (32). Moreover, in a recent study by El Beltagy et al., administration of 30 mg/kg daily dose of ATM for 5 consecutive days has enhanced the cognitive performance of rats in the novel location recognition test, which has been associated with an increase in cell proliferation in the subgranular region of the DG in the hippocampus (33).

In another study conducted by Foster et al. which investigates the possible benefits of ATM in the rehabilitation of stroke patients, it has been reported that a single orally-administered 40 mg dose of ATM has improved motor memory in stroke patients (34). In a similar study conducted by Sczesny-Kaiser et al., it was suggested that the combination of ATM treatment with 10 Hz repetitive transcranial magnetic stimulation had a synergistic effect on motor learning in healthy people (35). All these aforementioned information supports the findings of this study in the Morris water maze and passive avoidance tests.

Following the observation of ATM's anti-amnesic efficacy, we have investigated the putative molecular mechanisms underlying this effect. For this purpose, the hippocampus was examined which is an essential brain area with higher functions in the acquisition and consolidation of explicit memories (36,37).

Neurotrophins are a family of small proteins which play substantial role in neuronal development, functioning, and survival, and synaptic plasticity in the brain. They belong to a class of growth factors and take part in the pathophysiology of neurodegenerative and neuropsychiatric diseases (38,39). BDNF, NGF, neurotrotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) are the four known members of the neurotrophin family. They have distinctive regulatory roles in the survival, differentiation and maintenance of neurons in both the central and peripheral nervous system (40).

One of the most widely distributed and well investigated neurotrophins in the mammalian brain is BDNF. It

involves in a variety of neurophysiological processes including regulation of neuronal and glial development, neuroprotection, modulation of transmission and short – and long-lasting interactions in synapses (41,42).

BDNF affects synaptic physiology at different aspects. It preserves and repairs existing synapses while also stimulating the formation of new ones (43). BDNF regulation of synaptic plasticity suggests its crucial role in improving cognitive functions such as learning, memory and higher thinking (44).

In the hippocampus and some other brain areas, BDNF has been demonstrated to play a critical function as a mediator of activity-induced long-term potentiation (LTP) (41). A reduction of hippocampal BDNF levels has been reported to impair LTP and decrease the number of synapses which results in deficits in the hippocampus-dependent memory formation and consolidation (43,45). Direct injection of BDNF antisense oligonucleotide into the DG or 7-day continuous administration of BDNF antibody to the brain were reported to impair memory retention in inhibitory avoidance learning tasks (44) and increase the escape latencies in place navigation tests (46), respectively. In another study, BDNF mutant mice had significantly impaired performance compared to wild-type when subjected to Morris water maze task (47). It was also stated that the BDNF levels was reduced in patients with mild cognitive impairment (48,49).

The decrement in the expression of BDNF and cyclic adenosine monophosphate response element-binding protein (CREB) has been revealed as one of the mechanisms in SCP-induced cholinergic dysfunction in the central nervous system (50,51). In a recent study, SCP-induced memory impairment in rats has been accompanied by decreased hippocampal BDNF levels (52). Also, Ishola et al. has shown the diminishing effect of SCP on BDNF levels in the prefrontal cortex and hippocampus of mice (28). In another study, influence of Vitamin D treatment on learning and memory deficits has been investigated and it was found that SCP injection reduced the hippocampal BDNF levels accompanied by impaired memory performance while Vitamin D administration ameliorated this effect (53).

In this study, it was observed that BDNF immunoreactivity in the hippocampal CA_1, CA_3 and DG regions was decreased by SCP administration, as expected. Moreover, ATM treatment significantly enhanced the decreased BDNF levels in these hippocampal regions of the amnesic rats.

Norepinephrine mediates cGMP-dependent increases in BDNF and Akt levels (54,55). It was shown that BDNF levels in cultured hippocampal neurons were elevated by norepinephrine administration (56,57). In a recent study, Wang et al. (58) suggested that norepinephrine activation of β-adrenoceptors in the DG is related with spatial learning and memory improvement in chronic restraint stress administered rats and they also pointed out the involvement of CREB-BDNF signaling pathway.

As a noradrenaline reuptake inhibitor, ATM has been investigated for its effects on BDNF levels in a few studies. Injection of ATM (1 mg/kg) for two weeks during adolescence was shown to decrease CREB and ERK phosphorylation in the orbitofrontal cortex. BDNF mRNA expression was also decreased in the orbitofrontal cortex; while there was no significant difference in the medial prefrontal cortex or *nucleus accumbens* in the *ex vivo* analyses of brain tissues (59). Lee et al. (60) has investigated the effects of ATM and methylphenidate on cell proliferation and neuronal differentiation in the DG and reported that, unlike methylphenidate, ATM treatment at doses of 4, 8 and 16 mg/kg did not increase BDNF levels in the hippocampus of the adolescent mice. On the contrary, in the study of Fumagalli et al. (61) on spontaneously hypertensive rats, it was reported that sub-chronic administration ATM (3 mg/kg, twice a day) increased BDNF expression and signaling in hippocampus, frontal cortex and prefrontal cortex. In our study, cognitive enhancer properties of ATM in SCP-induced amnesia were accompanied by the increment of BDNF levels in the hippocampal regions.

Together with BDNF, NGF is another typical neurotrophin that regulates the maintenance of viability and functioning of neurons, apoptosis, neuronal growth, and differentiation via Trk and p75 neurotrophin receptors. In addition, NGF is the neurotrophic factor that is most similar to BDNF among neurotrophins in terms of amino acid sequence. It has long been stated that these neurotrophins can be important prognostic and diagnostic markers for neurodegenerative diseases (62,63,64).

NGF is produced and released by hippocampal neurons throughout life that provides a potential mechanism for modulating cholinergic inputs and, thereby, hippocampal plasticity (65). NGF also facilitates hippocampal LTP and enhances memory formation (66). It has been shown that chronic i.c.v. administration of NGF in the rat hippocampus induces synaptogenesis by increasing synaptophysin synthesis and improving cognitive functions (67).

There is also a relationship between NGF signaling and aberrant amyloid precursor protein processing. Chronic NGF deprivation leads to Alzheimer-like pathologies, such as Aβ formation and deposition, synaptic dysfunction, and cognitive impairments (68,69). AD11 mouse, a transgenic animal model that expresses anti-NGF antibodies, displays a prominent neurodegenerative phenotype including neuronal loss, cholinergic deficit, and tau hyperphosphorylation (68).

The literature has reported that SCP-induced cognitive impairment is accompanied by decreases in NGF protein levels in both the hippocampus and cortex of mice (70). Some studies report that SCP significantly reduces NGF mRNA expression levels in the hippocampus and frontal cortex (71) and cerebral cortex (72).

Similar to previous studies, SCP administrations effectively reduced NGF levels in the dorsal hippocampus's DG, CA_1, and CA_3 sub-regions of rats in our study. On the other hand, it was determined that SCP administrations could not reduce NGF levels in the mentioned areas in rats treated with ATM for 14 days, and even there was a significant difference in NGF levels in the ATM-treated group compared to the control group in the CA_3 sub-region.

The results of this study clarified that subacute ATM treatments ameliorated SCP-induced cognitive decline in rats. It has been shown that these beneficial effects of subacute ATM administrations occur without causing any deterioration in rats' locomotor activities and motor coordination. It was also concluded that, in the hippocampus, a brain region closely associated with learning and memory processes, ATM treatment exhibited curative effects on the regression caused by SCP application in the levels of BDNF and NGF in rats, which are known to be associated with essential functions such as neuronal survival, growth, differentiation, and synaptogenesis.

In the continuation of this research, more detailed studies are needed to be performed for enlightening the mechanisms underlying these favorable effects. Evaluating the possible efficacy of this drug on cognitive impairments on the levels of other neurotrophins, such as NT-3 and NT-4, in brain regions related to learning and memory, such as the hippocampus and frontal cortex, would also make significant contributions to the literature. In addition, morphometric assessments regarding the density and number of dendritic spines in relevant brain regions depending on ATM treatment, as well as studies on the levels of synaptic proteins such as synaptophysin and post synaptic density (PSD)-95, which are known to be indicators of synaptic integration, could be further planned. Moreover, well-designed clinical studies on patients with cognitive dysfunction should be performed to confirm the anti-amnesic effect of ATM and its nootropic efficacy potential in the treatment of dementia.

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