

EXPERIMENTAL STUDY

Is there a new pathway relationship between melatonin and FEZ1 in experimental rat model of Alzheimer's disease?

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ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disease. This study was performed to determine the possible relationship between melatonin, which is known to play a role in the neuro-protective mechanism in AD, and fasciculation and elongation protein zeta 1 (FEZ1). Thirty male rats were included and separated into 3 groups (n = 10) as vehicle (artificial cerebrospinal fluid), streptozotocin (STZ) and STZ+melatonin (MLT). Two intracerebroventricular (icv) injections of 3 mg/kg STZ were made 48 hours apart. MLT injections were implemented for 14 days (ip; 10mg/kg/day). The Morris Water Maze (MWM) test was performed and rats were sacrificed to assess FEZ1 gene expression and protein levels from the hippocampus tissues and serum levels of noradrenaline (NA), dopamine and serotonin were determined from the blood samples. It was determined that the FEZ1/ β -actin protein ratio in the STZ group was significantly higher than that of the Vehicle group ($p < 0.05$) and in the MLT-administered group, the protein levels were decreased to the levels observed in the Vehicle group. Serum NA levels of STZ and STZ+MLT groups were found to be lower than those in the Vehicle group, while no difference was found regarding dopamine and serotonin levels.

These findings show that reversal of increased FEZ1 levels in AD-induced rats with melatonin administration is the evidence of the effect of melatonin through FEZ1 in AD (Tab. 2, Fig. 5, Ref. 67). Text in PDF www.elis.sk.
KEY WORDS: FEZ1, Alzheimer's disease, melatonin, rat, microtubules, mitochondria.

Introduction

Alzheimer's disease (AD) is the 4th most common cause of death among people over 65 years of age (1). Clinically, AD is characterized with progressive loss of cognitive abilities, followed with dementia and eventually death. Histopathologically, it is characterized with two lesions called neurofibrillary tangles (NFT) and amyloid plaques (AP) (2, 3). In AD, AP and NFT are observed in many regions of the brain, especially in the cortex of frontal, temporal, and parietal lobes, hippocampus and basal forebrain cholinergic nuclei (4). Firstly, they are formed in the transentorhinal cortex and then spread to entorhinal cortex, hippocampus and to cerebral cortex, respectively (5). In AD, loss of synaptic junctions is observed in both neocortex and hippocampus (6).

Fasciculation and elongation protein zeta 1 (FEZ1) is a protein comprised of 392 amino acids in humans and 393 in rats. It is expressed in the brain and is essential for axonal growth and elongation (7). In a study conducted on adult and developing rats, high FEZ1 mRNA expressions were observed in the hippocampal neurons of the rats in both groups (8). Immunohistochemical studies demonstrated that many neurological disorders, including mental disorders, develop due to neocortex dysfunction and these disorders may be related to FEZ1 that is expressed in the neocortex (9). FEZ1 mRNA has been shown to give strong signals in the hippocampus, olfactory bulbs, dentate gyrus and cerebellar cortex regions of the brains of adult rats. FEZ1 mRNA expression being especially intense in the hippocampus is interpreted as an indicator of high neuronal plasticity necessary for memory and learning (10). Although previous studies reported that FEZ1 is associated with neuronal development, neuropathies and viral infections, the findings of recent studies performed using proteomic techniques suggest that FEZ1 may be associated with cell motor proteins and various intercellular events such as signal transduction (11).

The significance of mitochondria in the development of neurodegenerative disorders, including AD, have been demonstrated previously (12). Increasing evidence suggests that Amyloid β ($A\beta$) has a deleterious effect on mitochondrial function in the AD brain (13). Mitochondrial transportation within axons is essential for axonal maintenance and its irregularity may lead to neurodegenerative disorders (14). Recent studies on neuronal axons revealed

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Acknowledgements: This study was supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK) (Project No: 214S410).

an FEZ1-mediated kinesin-based mechanism (15). It has been shown that FEZ1 interacts with kinesin in the axon, thus generating an FEZ1/kinesin complex which regulates the transportation of mitochondria throughout microtubules (16). Moreover, it has also been suggested that FEZ1 is also required for the anterograde transport of mitochondria along the axon in the hippocampal neurons (17). A decrement that may occur in microtubule-dependent transport was reported to possibly induce the proteolytic modification of amyloid precursor protein (APP), which may result in the development of APs and AD (18).

Melatonin is a hormone produced by the pineal gland in the brain that regulates the behavioral and physiological circadian rhythms. This hormone varies in response to day and night signals, and melatonin levels always elevate at night (19). It is suggested that irregularity of noradrenergic innervations may be responsible for decreased melatonin levels and loss of melatonin rhythm in AD (20). Melatonin has been reported to play a role in the neuro-protective mechanism in AD by affecting neuronal signals as well as mitochondrial function (21). In addition to the role of FEZ1 in mitochondrial transportation, high neuronal plasticity in memory and learning (10), brings a possible relationship between FEZ1 and melatonin to mind.

Materials and methods

Ethics and animal care

In the present study, 30 male rats weighing 220–280 g were used. Rats were separated into 3 equivalent groups ($n = 10$) as vehicle, Streptozotocin (STZ) and STZ+melatonin (MLT). Throughout the study, animals were kept in an environment with 21 ± 1 °C temperature, 12-hour light/darkness periods, and access to tap water and standard rat pellet diet *ad libitum*. All procedures were performed according to the protocols of the Experimental Animals Ethical Board, Inonu University (Protocol # 2014/A-50)

Intracerebroventricular (icv) STZ injection

Rats were anesthetized under a combination of ketamine and xylazine (22) and placed into stereotactic device (Harvard Apparatus, USA) via their external auditory canal. According to the Paxinos-Watson Stereotaxic Rat Brain Atlas, the coordinates of the right and left lateral ventricles were determined with reference to Bregma point (0.8 mm posterior from Bregma, 1.4 mm lateral from sagittal suture and 4.8 mm vertical from skull) (23). Using Hamilton syringe, 3 mg/kg STZ were administered first to the right lateral ventricle and 48 hours later to the left lateral ventricle as 10 μ l volume per ventricle (24–26).

Intraperitoneal (ip) melatonin injection

Melatonin was dissolved in ethanol prior to administration and adjusted to the appropriate concentration with physiological saline (10 mg/kg). It was ensured that the final concentration of ethanol in this solution did not exceed 0.5 % (27, 28). The first melatonin injection was performed via ip (1 mg/kg/day) injection 1 h before the STZ administration and continued for 14 days (29).

Morris water maze (MWM) Test

Spatial learning and memory of animals were tested utilizing the MWM test (30). The MWM was a black and large circular pool that was 150 cm in diameter, 60 cm in height, and made of stainless steel. The water temperature was maintained at 23 ± 1 °C with an automatic heater (31). A black stable platform with a diameter of 8 cm was placed 1 cm below the surface of the water. To hide the platform, the water was colored with a non-toxic black dye (Mixol concentrate dye colorant 20 ml No: 1 Black).

Data were obtained with a stable camera system connected to a video surveillance system located in the center of the pool that was divided into 4 hypothetical quarters (32). The rats in all groups were given a free-floating swim for platform adaptation. After that, MWM tests were performed 4 times a day for 5 days. Each rat was allowed 90 seconds to find the platform. Rats that found the platform in that time limit were kept on the platform for 30 sec (33).

The time to reach the platform, the distance to find the platform, and the time to stay on the quadrant were measured and recorded. Scores were analyzed using EthoVision XT10 Image Analysis (Noldus Information Tech.) software program.

Terminating the experiment and obtaining blood and tissue samples

Following the final tests, animals were sacrificed via decapitation. The hippocampus region was rapidly separated from the excised brain and maintained at -80 °C until FEZ1 gene expression analysis. FEZ1 gene expressions were determined using real-time PCR (RT-PCR) and the protein levels using Western blot. Serum dopamine, serotonin and noradrenalin (NA) levels were measured from the obtained blood samples.

Total RNA isolation and quantitative real-time PCR

In order to ascertain β -actin and FEZ1 mRNA levels in the brains collected from animals, tissue samples were placed in RNA storage solution and total RNA were purified utilizing High Pure RNA Tissue kit (Roche, USA; lot No: 11596700, ref No: 12033674001). Pure RNA content of the samples was assessed with spectrophotometric analysis.

Tab. 1. β -Actin and FEZ1 specific primer sequences.

Gene	Primer sequence (Forward and Reverse)	Ref. Sequence No	PCR product length
β -Actin	F: 5'CTAAGGCCAACCGTGAAAAG 3' R: 5'GCCTGGATGGCTACGTACA 3'	NM_031144.3	79 bp
FEZ1	F: 5'CTCCAGTGAAGAACCAGTTGC 3' R: 5'GTCAGAGCATCCCAAACCTC 3'	NM_031066.1	76 bp

For cDNA synthesis Transcriptor First strand cDNA Synthesis Kit (Roche, USA, Lot no:10842322, Ref no: 04 896 866 001) was utilized. Real-Time PCR analysis was performed on Light Cycler 96 Real Time- PCR (Roche, , USA) using Fast Start Essential DNA Probes Master kit (Lot no:10519000, Ref no: 06402682001) and Real Time Ready Assay (β -Actin lot no: 90017829, config no: 100081783; Fez1 lot no: 90018146, config no: 100101827”(8 pmol/ μ l) hydrolysis probe primers (Tab. 1).

Serum noradrenalin, dopamine and serotonin analyses

Serum NA, dopamine and serotonin levels were determined using commercial ELISA kits (Cusabio Biotech, China).

Western blot analysis

Samples containing 50 μ g total protein from each tissue lysate were transferred to nitrocellulose membrane after being separated on 6–12 % sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) that were run at 90V on electrophoresis. Following the transfer, the membrane was blotted for 1 hour in a 5 % milk powder solution prepared with TBS (Tris-Buffer-Saline; TBS-T) containing Tween 20 and washed 3 times with TBS/T 10 minutes apart. Then, it was incubated with monoclonal FEZ1 antibody diluted in ratio of 1 : 1000 in 2.5 % milk powder overnight at +4 °C, washed 3 times with TBS/Tween 20 for 10 minutes and treated with horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody in a 2.5 % milk powder solution prepared with TBS/Tween 20 for 1 hour at room temperature. Following the washing of the membrane, it was treated with luminol and peroxide mixed in a 1 : 1 ratio and viewed under UVP ChemiDoc-It2 chemoluminescence. B-actin antibody was used as a loading control and viewed following washing steps after treatment with secondary antibody. Band densities were determined using ImageJ.

Statistical analysis

Data are demonstrated as mean and standard deviation. Statistical analyses were performed using IBM SPSS Statistics, version 22.0 for Windows package program. The normal distribution fitness of the data was assessed by the Shapiro Wilk test. Kruskal–Wallis test or one-way ANOVA was used for the comparison of the data between groups. In multiple comparisons, Tamho test was used for non-homogeneous variances after one-way analysis of variance, and Conover test was used after Kruskal–Wallis test. Values of $p < 0.05$ were considered statistically significant.

Results

MWM test results

Swimming distances: During the 5-day duration, no statistical difference was observed between Vehicle and STZ+MLT groups. STZ group mean swimming distances were found to be significantly higher than those in Vehicle and STZ + MLT groups ($p < 0.05$) (Fig. 1).

Swimming durations: When the swimming times of the 5th day were compared between the groups, it was observed that the

swimming times in STZ group were statistically higher than those in Vehicle and STZ + MLT groups ($p < 0.05$) (Fig. 2).

Time to stay in the quadrant: Compared to Vehicle and STZ + MLT groups, a statistically significant decrease was observed

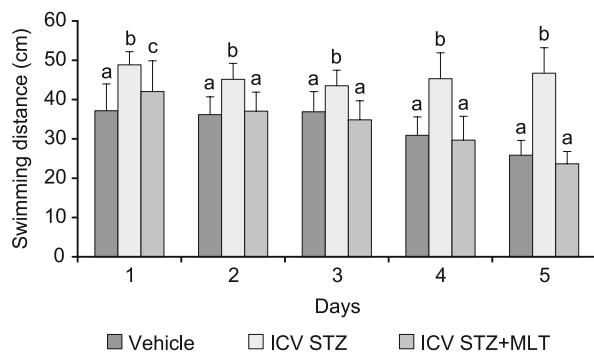


Fig. 1. Mean swimming distances of the rats in 5-day swimming test. Comparisons were performed within each day in each group, not between days. Groups with different letters in graphics indicate that they are statistically different from each other ($p < 0.05$).

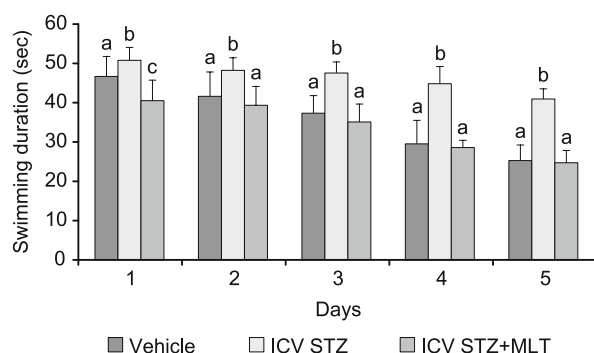


Fig. 2. Mean swimming times of the rats in 5-day swimming test. Comparisons were performed within each day in each group, not between days. Groups with different letters in graphics indicate that they are statistically different from each other ($p < 0.05$).

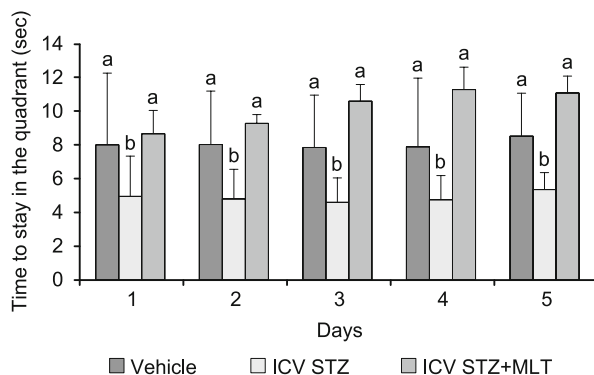


Fig. 3. Mean time to stay in the quadrant of the rats in 5-day swimming test. Comparisons were performed within each day in each group, not between days. Groups with different letters in graphics indicate that they are statistically different from each other ($p < 0.05$).

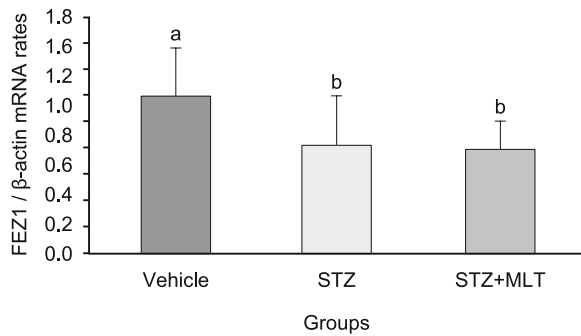


Fig. 4. FEZ1/β-actin mRNA ratios in all groups. There was no significant difference between the groups with the same letters on columns, whereas groups with different letters showed statistically significant difference ($p < 0.05$).

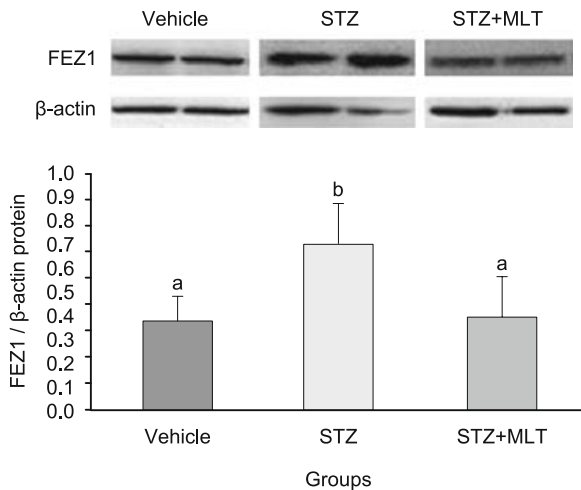


Fig. 5. FEZ1/β-actin protein ratios in all groups. There was no significant difference between the groups with the same letters on columns, whereas groups with different letters showed statistically significant difference ($p < 0.05$).

in terms of time in the quadrant in STZ group ($p < 0.05$). Moreover, when the groups were compared for the 5th day time to stay in the quadrant, it was determined that the values in Vehicle and STZ + MLT groups were statistically higher than those in STZ group ($p < 0.05$) (Fig. 3).

Real time PCR findings

The FEZ1/β-actin mRNA ratios in STZ and STZ + MLT groups were found statistically significantly lower than that in the Vehicle group ($p < 0.05$). The hippocampus FEZ1/β-actin mRNA ratios in the Vehicle group were statistically higher than those in other groups ($p < 0.05$) (Fig. 4).

Western Blot Analysis findings

Statistical analysis showed that the hippocampal FEZ1/β-actin protein ratios of the STZ group were significantly higher than

Tab. 2. Mean serum NA, dopamine and serotonin levels among the groups.

Groups	n	Noradrenalin (pg/ml)	Dopamine (ng/ml)	Serotonin (ng/ml)
Vehicle	10	1364.09±403.74 ^a	0.16±0.04 ^a	25.18±20.95 ^a
STZ	10	712.1±189.52 ^b	0.15±0.04 ^a	28.17±12.15 ^a
STZ+MLT	10	700.83±409.44 ^b	0.09±0.03 ^a	25.93±14.42 ^a

Values are demonstrated as mean±standard deviation. Values of $p < 0.05$ were considered statistically significant. In each column, comparisons were performed among groups, in which groups with different letters were statistically significantly different from each other ($p < 0.05$).

those in the Vehicle group ($p < 0.05$), whereas the protein levels decreased in the MLT group to the levels observed in the Vehicle group ($p < 0.05$). Moreover, it was observed that FEZ1/β-actin protein ratios in STZ + MLT group decreased to levels similar to those in the Vehicle group (Fig. 5).

Serum dopamine, serotonin and NA findings

The serum NA, dopamine and serotonin levels of the animals are given in Table 2. When all groups were compared, it was determined that the serum NA levels in STZ and STZ + MLT groups were statistically lower than those in the Vehicle group ($p < 0.05$). The differences in serum dopamine and serotonin levels between the groups were not significant.

Discussion

Melatonin is suggested to increase neuronal learning and memory-related activities and may become a potential therapeutic approach to AD-like neurodegeneration (34). It also has the ability to act as a cytoskeletal modulator and affect microfilaments and microtubules (35). Melatonin has been reported to play a role in the neuroprotective mechanism of AD by affecting mitochondrial functions as well as neuronal signals (21). It has been suggested that the reduction in melatonin production in elderly people is one of the main factors involved in the development of age-related neurodegenerative diseases such as AD. In one study, it was indicated that melatonin might be useful as an antioxidant therapy agent against oxidative stress, which is observed at the early pathogenetic events of AD, only at this stage of the disease (36).

Melatonin has been reported to play a role in the neuroprotective mechanism of AD by affecting mitochondrial functions in addition to neuronal signals. Moreover, it has been suggested that the loss of general neuronal plasticity in aging can be prevented with melatonin. The fact that FEZ1 regulates mitochondrial transport and has high neuronal plasticity for long-term memory and learning suggests that there is a possible relationship between melatonin and FEZ1.

In our study, the statistical difference observed between STZ and STZ + MLT groups in the MWM test is the evidence that melatonin administration, which was initiated prior to the injections, may have a protective/inhibitory function. Recent studies have suggested that melatonin reduces Alzheimer-like tau hyper-

phosphorylation, and plays a role in anti-inflammatory processes and cholinergic neuron protection (37). In another study on experimental models of Alzheimer's, melatonin was demonstrated to have many beneficial effects such as inhibiting A β production and preventing amyloid fibril formation as well as ameliorating anti-oxidative damage, anti-apoptosis and cognitive functions by inhibiting tau protein hyperphosphorylation (38). In a study conducted on transgenic mice that received exogenous melatonin, the expected time-dependent increase in A β was partially inhibited and the mortality rate of treated transgenic mice was decreased (28).

Neurons are able to communicate through synaptic transmission (39) by mitochondrial movement in the axon, microtubule-dependent transport (dynein and kinesin) and transport of various intracellular cargos (synaptic vesicles, mitochondria, cytoskeletal elements and mRNAs) (40–42). In the absence of these intracellular cargoes, autoinhibition of kinesin1 prevents unnecessary ATP hydrolysis and provides rapid and specific control of both temporal and spatial motor activities. It has been reported that the interaction between two binding partners contributes to the auto-inhibition of kinesin1 (14). In one study it was suggested that Jun N-terminal kinase (JNK)-interaction protein 1 (JIP1) cargo protein is not sufficient to activate Kinesin1, and the second binding partner is FEZ1. In the same study, it was shown that the binding of JIP1 and FEZ1 to Kinesin is sufficient to activate microtubule binding and motor motility (43).

In our study, a statistically significant increase in FEZ1 protein levels was observed in the STZ group. Tau hyperphosphorylation in the pathology of AD involves impairment in the binding ability of microtubules and microtubule degradation, which interrupts the axonal transport of the vesicles and inhibit synaptic function. Thus, hyperphosphorylated tau proteins accumulate in the axoplasm. This increase in FEZ1 may indicate that it may be due to an interruption in axonal transport or an accumulation of the FEZ1 protein. In addition, the fact that FEZ1 levels in the melatonin-injected group are similar to those in the control group or lower suggests that melatonin may have positive effects on the pathophysiology of AD and maintaining normal FEZ1 levels. The observation that FEZ1 protein levels in STZ group are statistically higher than those in the Vehicle group is very important in terms of indicating a relationship between AD and FEZ1. Furthermore, the similarity of FEZ1 levels between STZ + MLT and Vehicle groups is the evidence that melatonin may have a protective/preventive (and possibly also inhibitory) role in AD.

It was found that the hippocampal FEZ1 mRNA levels in the STZ group rats were statistically lower than those in the control group ($p < 0.05$), and the MLT application did not change the decreased gene expression in the STZ group. When protein expressions were examined, the FEZ1 protein levels in the STZ group was statistically higher than those in the control group ($p < 0.05$), while MLT application significantly decreased FEZ1 protein levels in the STZ group ($p < 0.05$). This inconsistency between the mRNA and protein levels has also been reported many times previously. In these studies, it was stated that there was a very weak correlation between mRNA and protein levels and it was deter-

mined that the gene expression levels mostly do not reflect the protein levels (44–46).

Translational efficiency indicates the number of concluded protein molecule production per mRNA at a certain time period. It can be measured in ribosomes by the ribosomal density, which indicates the specific enrichment of individual mRNA species. Different translational efficiencies for mRNA molecules can directly affect the mRNA-protein association (47). Different mRNA and protein levels observed by us were probably owing to post-transcriptional mechanisms. Furthermore, decreased mRNA levels in the STZ group may be due to down-regulation or non-specific loss of RNA (48). The increase in FEZ1 protein in the STZ group can be explained differently, for instance, it may be related to the ribosomal density in the setting (49) or it may be a compensating mechanism against AD in the brain. In fact, in a study on microglia and astrocytes in neurons, in which the potential function of FEZ1 as a natural marker of neuronal susceptibility to HIV-1 infection was investigated, the level of FEZ1 protein was found elevated. Researchers have interpreted this as a neuroprotective effect of the FEZ1 protein (50). We also think that the increase in FEZ1 levels observed by us in the STZ group may be due to a neuroprotective mechanism.

It has been suggested that cholinergic insufficiencies in AD are accompanied with alterations in the neurotransmitter systems, including NA, dopamine and serotonin, and that other neurotransmitter systems are involved in the pathogenesis of the disease (51, 52). Most studies have shown that the delivery of serotonin plays an inhibitory role on dopaminergic activity (53–55). However, some studies suggest the opposite (56, 57). This may be partially due to the subtypes and activities of the serotonin receptors. For example, while 5HT_{2C} agonists inhibit dopaminergic effects (58), 5-HT_{1B} and 5-HT₃ agonists increase dopamine release (59, 60). Dopamine D₂ receptors are expressed in specific regions of the hippocampal complex (hippocampal areas, entorhinal cortex, perirhinal cortex). In one study, AD was reported to be associated with reduced expression of the dopamine D₂ receptor, however, it was observed that the effects were different regarding the area and rostrocaudal axis in the hippocampal complex. It has been demonstrated that there was no decrease in the dopamine D₂ receptor expression in the entorhinal cortex, which is affected by AD. Regions with loss of D₂ receptors have been found to be typically lacking the neuritic plaques, neurofibrillary tangles, or significant neuronal loss (61).

Similar with other studies, in the presented study, no statistically significant difference was observed between STZ, Vehicle and STZ + MLT groups in terms of dopamine levels. In a study on dopaminergic, noradrenergic, and serotonergic neurons in the frontal and temporal cortex following the death of subjects with AD, which was compared with the control group, a decrease was demonstrated in terms of NA and serotonin levels, while no decrease was observed in dopamine levels (62).

Locus coeruleus is a core of noradrenergic neurons in over 90 % of central nervous system (CNS) that is isolated from the pons (63, 64). The entorhinal cortex receives many noradrenergic projections from locus coeruleus (65). It has been suggested that

a deficiency in the noradrenergic system, which is largely caused by cells from locus coeruleus in the brain, plays an important role in the development of neurodegenerative diseases such as AD and Parkinson's disease (66). In one study, no alterations were observed in dopamine levels (67), while loss of noradrenergic neurons in locus coeruleus accompanied a similar decrease in NA levels in temporal cortex (67).

In the presented study, no statistically significant difference was found between the groups in terms of dopamine and serotonin levels, while the decrease in NA levels was statistically significant, aside from the Vehicle group. It was observed that exogenous melatonin administration is not effective in elevating NA, dopamine or serotonin levels. This finding confirms the notion that the entorhinal cortex affected by AD receives a large amount of noradrenergic projections from locus coeruleus and an insufficient noradrenergic system may play an important role in the development of neurodegenerative diseases such as AD and Parkinson's disease (65, 66).

In this study, no statistically significant difference was observed between the groups in terms of dopamine and serotonin levels and it was determined that the application of exogenous melatonin was not effective in altering NA, dopamine and serotonin levels.

Consequently, the reversal of elevated FEZ1 levels in AD-induced rats with melatonin administration suggests that the effect of melatonin may be via FEZ1 in AD.

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Received September 9, 2018.

Accepted October 10, 2018,