Effects of tacrolimus on c-fos in hippocampus and memory performances in streptozotocin model of Alzheimer's disease of rats

Abstract

Background/aim: Calcineurin, an inhibitor of calcium dependent phosphatase is highly presented in a brain of an Alzheimer's disease. Aging brain gets more sensitive to hyperactivation of calcineurin and this event causes tau neurofibrillary plaque accumulation which is one of the outcomes of this disease. The regions of hippocampus are much effected from the results of this process. Our hypothesis is that a calcineurin inhibitor, tacrolimus, could prevent the accumulation and the decrease of the neuronal cells. Therefore, this immunosuppressive drug could be a candidate for an early treatment of Alzheimer disease.

Materials and Methods: Fifteen male *Wistar albino* rats were divided to three groups; control, Alzheimer and Alzheimer+Tacrolimus. The Alzheimer group received an injection of streptozotocin intracerebroventricularly for the purpose of modelling the disease via generating free radicals leading a cognitive impairment. Alzheimer+Tacrolimus group first received an oral drug, a calcineurin inhibitor for 10 days afterwards prepared for the model as same as the Alzheimer group received. Finally, all groups performed the Morris water maze test for four days then sacrificed. For the aim of counting neurons in the hippocampus stereological methods, as well as for an evaluation of cellular response to stress in dentate gyrus a c-Fos immunohistochemistry was performed.

Results: According to the probe trial of Morris water maze test, the latency time was dramatically higher at both Alzheimer and Alzheimer+Tacrolimus group ($p < 0.01$). We confirmed these results with our stereology data. The results from stereology technique

indicate that there was a neuronal decrease at the hippocampus regions in Alzheimer and Alzheimer+Tacrolimus group. Our outcomes from immunohistochemical data showed a significant increase in the number of c-Fos-positive cells in Alzheimer group when comparing with Alzheimer+Tacrolimus group ($p < 0.001$).

Conclusion: There was none preventive effect for neuronal loss in the hippocampus under the effect of tacrolimus drug according to stereological results. However, tacrolimus administration may have reduced cellular stress and cell damage.

Key words: Alzheimer's disease, c-Fos, Morris water maze test, stereology, tacrolimus

1. Introduction

Alzheimer's disease (AD), the most common dementia in older population is a progressive neurodegenerative age-related disease. The frequency of this disease is 0.1% between 60 and 65 ages and increases logarithmically up to 48% over age of 85. Considering that by 2050, 25% of the world's population will be over 65; AD will be the most important health problem on the top [1]. There is no promising cure yet in fact the main factor that lies under the mechanism to cause the defects is not even known for certain. However, we know that the most important defect of this disease is the debates which appears as a form of amyloid beta (Aβ) plaque accumulation [2]. These plaques later become a slayer for the neuron itself. It is not accurate to label the pathway either as apoptosis or as necrosis which is another fact to discuss [3]. Unless, it is certain that significant amount of neuron dies through this process. Another known defect is the tau neurofibrillary tangles which occur between the neurons and in later on interrupts the communication between each of them. Without communication and nutrition, neurons begin to die and soon cell reduction happens. As time progresses more the Aβ plaques

accumulate more debits surrounds the brain. Patients usually die of aspiration pneumonia about 9 years after disease onset [4].

Different drugs are used for AD treatment, but they can only slow the decline of the diseases' progression. A class of a pharmacotherapy which contains cholinesterase inhibitors such as Tacrine, Rivastigmine and Galantamine are used for AD treatment nowadays [5]. According to the amyloid cascade hypothesis, a mutation in the genes plays a role in amyloid processing. This mutation causes an imbalance between the production of amyloid and the clearance. Eventually, amyloid depositions appear and these depositions induce amyloid plaques and also neurofibrillary tangles [6]. During aging, calcium (Ca2+) dependent protein phosphatase calcineurin (CaN) increases in the hippocampus. CaN which also have known as protein phosphatase 2B (PP2B) is a Ca2+ sensitive serine/threonine phosphatase. CaN is a heteromeric protein which contains two subunits. These subunits are a catalytic subunit and a regulatory subunit (CaNB) [7]. CaN is activated by calmodulin (CAM). By the activation of CaN by CAM, CaN get responsive to some ion channel types causing Ca2+ fluctuation in the brain. Eventually, this distribution can cause several pathogenesis outcomes including tau hyperphosphorylation, synaptic protein loss, neuroinflammation, decreased neurotransmission levels and cell death [8].

Tacrolimus (FK506) is an immunosuppressive agent constantly used for treating graft rejection in organ transplantation and also for treating myasthenia gravis, arthritis and atopic dermatitis. As the FK506 binds to FK506-binding protein (FKBP) a complex FK506/FKBP interacts and inhibits CaN's effects [9-11]. There are some studies using tacrolimus for its effect on neurological disease. For example, Pardo et al. [12] reported a study about an administration of FK506 to Huntington's disease (HD) model mice. They

found that after administration of FK506 may be successful in order to induce the phosphorylation of S421 and block polyQ-huntingtin-induced neuronal death. CaN can be a potential use as a therapeutic approach to treat HD. A 5-year follow-up study report based on seronegative myasthenia gravis, a disease occurs in an absence of a seropositive status for anti-acetylcholine receptor (AChR) antibodies, describes a case of juvenile triple-seronegative myasthenia gravis that was successfully managed with tacrolimus therapy [13]. Briefly tacrolimus is widely used in organ transplantation and autoimmune diseases such as myasthenia gravis, inflammatory myopathy, ulcerative colitis and lupus nephritis [14].

In the present study, after an intracerebral ventricular injection of streptozotocin (STZ) (3 mg/kg) on the right brain hemisphere we waited for 14 days to modulate an AD model organization for both Alzheimer and Alzheimer+Tacrolimus group. An immunosuppressive drug Prograf (tacrolimus active ingredient) has given as an oral formulation, suspended in water. Agent was administered orally via gavage at a volume of 0.5 mg/kg per day to the subjects. For demonstrating, the memory spatial behavioral Morris water maze (MWM) test, immunohistochemical analyses and stereology technique were applied for all three groups.

2. Materials and Methods

2.1. Designing the groups

This study was approved by Ondokuz Mayıs University Animal Ethics Committee (No. 2015/05-f-12 dated 20.01.2015). All animals obtained from Ondokuz Mayıs University, Research Center for Experimental Animals (DEHAM). A total of 15 male *Wistar albino* rats (250-300 gr) at 8-12 weeks' old were divided into 3 equal groups as; control, Alzheimer and Alzheimer+Tacrolimus. Rats were kept in standard plastic cages in an air-

conditioned room at 22 ± 1 °C under lighting controls (14-h-light/10-h-dark cycle). At the control group neither surgery nor drug administration was done. The Alzheimer group was consisting of ICV injection of a single STZ dose (3 mg/kg) at the right side of the brain hemisphere including rats for attempting to be an AD model organization [15]. In Alzheimer+Tacrolimus group, the rats received daily 0.5 mg/kg tacrolimus (FK506, Prograf, Astellas Pharmas, US, Inc.) diluted in tap water (0.2 ml) starting 10 days prior to the STZ injection surgery. All groups performed the memory spatial behavioral MWM test at the same time and under same conditions.

2.2. Intracerebral ventricular injection of STZ

STZ (Sigma-Aldrich, S0130 - 50 MG, Inc. Germany) was administered once into the intracerebral ventricular representing by a following rat atlas coordinates from the bregma (posterior, lateral, z axis): 0.8 mm, 1.5 mm, 3.6 mm, respectively [16]. Each 1 µl injection given with Hamilton syringe was administered over 2 minutes at a concentration of 3 mg/kg STZ dissolved in 5 µl citrate buffer (pH: 4.0; 0.05 M) at room temperature. According to the literature, we waited 14 days for the formation of AD rat model organization considering the surgery day as first day [17, 18].

2.3. Stereology technique application

The brain samples were removed from each animal after the standard perfusion protocol using saline and 4% formaldehyde via the vascular system. The obtained samples were kept 10 days in 10% formaldehyde for fixation and then tissue processing steps were performed. After processing steps the samples were embedded in paraffin blocks and sections were obtained with the thickness of 7 microns (µ) with a 1 / 7 sampling using a microtome (Leica RT 2255, Germany, Nussloch) and finally were stained with Cresyl

violet dye (Figure 1). The cross-sectional areas were sampled and imaged using an Olympus BX43F (Japan, Tokyo) microscope and physical dissector method was applied. 'Stereology entails statistical sampling principles and stochastic geometric theory to provide efficient tools for estimation of volume, surface area, length, and number of objects in a 3-D structure by sampling in 2-D sections' [19]. Physical dissector method is one of the stereology methods allowing researchers to count the cells in certain areas using an unbiased counting frame. The counts obtained from the sections are the object density (Nv). We reached the reference volume as a result of multiplying the area calculations made by the computer with the section thickness. The total cell number (N) was obtained as a result of multiplying the object density and the reference volume (Vref). The formula was as given as; $N = Nv \times Vref$ [20]. These results gave us quantitative information about the whole structure of the related hippocampus neuron cell amount. Here we used the physical dissector in conjunction with Cresyl violet staining to estimate the neuronal cell numbers situated in CA1, CA2, CA3 and dentate gyrus (DG) regions of the rat hippocampus to discover the changes in mean neuron number. Statistical signification was determined by one-way analysis of variance followed by ANOVA comparison test.

2.4. Immunohistochemical detection of c-Fos in DG region

C-Fos belongs to a family of transcription factors and undergoes posttranslational modifications, plays an important role in cell proliferation, differentiation and it is required for memory recall and encoding [21]. It is involved in cellular responses to stress, cell damage and death; induced in also stress, mitogenic growth factors, cytokines and neurotransmitters. C-Fos expression were found in the spinal cord, dorsal root ganglion, in brain areas and finally association between the spinal cord and brain areas [22]. In our

study, immunohistochemistry was performed for evaluation of c-Fos-positive cells in the DG of the hippocampus according to a described method. While the sections obtained from the samples at the same time 5 μm slices obtained from each animal for immunohistochemical analyses in poly-lysine glass slides. After the deparaffinization and rehydration processes, the staining was performed using HRP/AEC detection IHC kit specific for rabbit and mouse (Abcam, UK). All steps were performed according to manufacturer's instructions. Prior to immunostaining, sections were incubated at 650W for 10 min in citrate buffer (pH = 6.0) for epitope recovery. Subsequently, immunohistochemical staining was performed using primary mouse monoclonal antibody specific to rabbit anti c-Fos (Abcam) diluted 1:500 in antibody diluent (specimens were incubated overnight at 4 °C). The sections were washed three times for 2 min. with phosphate buffer (pH = 7.4) and incubated with a peroxidase labeled dextran polymer conjugated to goat anti-mouse and anti-rabbit immunoglobulins at room temperature. Color was developed using a liquid 3-Amino-9-ethylcarbazole (AEC) system. Mayer's hematoxylin was used as a counterstain, and Kaiser's glycerol gelatin (Merck AG) was used for mounting coverslips. The stained sections were analyzed by the light microscopy. The tissues were examined for antibody attachment to cellular and matrix components. The numbers of c-Fos-positive cells in DG of the hippocampus were counted hemi-laterally using a light microscope (Leica) and the results were expressed as the number of cells per square millimeter (mm^2).

2.5. MWM test for hippocampal dependent learning

The MWM test is used for many years in the field of neuroscience, developed by Richard G. Morris in 1981 for behavioral neuroscience [23]. This experiment gives researchers an evidence about the subjects' spatial memory beside long term spatial memory when

studying neurocognitive diseases such as AD and traumatic brain injuries [24]. There are several ways to study this method such as monitoring the environment with a camera and also measuring the period at the same time or using technical apparatus for obtaining prepared data conclusions for example coordinates. Animals are placed in a pool filled with tap water and colored with white powder to form the opaque view so that they cannot be able to see the platform. The animal's mission is to find the hidden platform with the skills that they have learned from the previous test (pretests). If the animal has have learned it will be able to find the platform quickly as possible. The numerical value is going to be the record of the latency time which the animals will spend to find the hidden platform in the pool. At our study, we used a camera and a timer for recording the latency time and we wrote down the results for each animal.

Statistical analysis

The normality test was determined by Kolmogorov-Smirnov test ($p < 0.05$). The mean ± standard deviation value was used for variables suitable for normal distribution. The presented data were tested for significance in repeated measures one-way ANOVA, using the Tukey's test (post hoc test) for multiple comparisons. The t test was used if the distribution of the variable was normal, otherwise Mann–Whitney U test was performed. Significant results were marked according to conventional critical P value: $p < 0.05$.

3. Results

3.1. Number of neurons in CA1, CA2, CA3 and DG

According to the results from physical dissection method using the formula to find the mean number, the number of the neurons belongs to all of the hippocampus regions decreased when comparing control group with Alzheimer group and Alzheimer group with Alzheimer+Tacrolimus group ($p < 0.01$). There were no significant differences when

comparing Alzheimer with Alzheimer+Tacrolimus group with each other at any part of the hippocampus region ($p > 0.05$). The most dramatic neuronal decrease was seen at region CA2 and DG in Alzheimer group when it was compared with control group ($p < 0.01$). Interestingly, we found neuronal increase at Alzheimer+Tacrolimus group for the DG region when comparing with Alzheimer group ($p > 0.05$). Also, there was a neuronal increase in region CA1 and CA3 for Alzheimer+Tacrolimus group when comparing with Alzheimer group, but it was not statistically significant ($p > 0.05$) (Figure 2).

3.2. Output of the MWM test

MWM test results were correspond to the neuronal loss outputs. The control group had shown successful performance at swimming to find the hidden platform which was hidden with powder inside the pool filled with tap water however the Alzheimer and Alzheimer+Tacrolimus groups were not successful as the control according to their probe test results ($p < 0.01$) (Figure 3).

3.3. Expression of c-Fos in the DG

In this study, we hypothesized that tacrolimus might ameliorate Alzheimer-derived spatial learning and memory impairments by enhancing hippocampal c-Fos expression. To evaluate this hypothesis, we immune-stained for c-Fos and counted the number of c-Fos-positive DG cells. The numbers of c-Fos-positive cells in the DG were 30.74 ± 5.39 for the control group (Figure 4a), 163.37 ± 2.49 for the Alzheimer group (Figure 4b) and finally 130.48 ± 10.4 for the Alzheimer+Tacrolimus group (Figure 4c). We observed a significant increase in the number of c-Fos-positive cells in Alzheimer group when comparing with Alzheimer+Tacrolimus group ($p < 0.001$) (Figure 4).

4. Discussion

AD is an age related progressive neurodegenerative disease that is associated with neurobehavioral deterioration and is characterized by dementia and the loss of neuronal cells in the brain. Diminished cerebral energy metabolism results with the accumulation of Aβ peptide that is related with progressive neuronal degeneration and death of neurons in several brain regions especially in hippocampus. Damage in hippocampus area is widely shown to be related with memory functioning as well as spatial and contextual learning ability [25]. The presence of Aβ accumulation or tau neurofibrillary lesions in AD is less important when compared to neuronal loss. It is accepted that the dementia in AD is mainly correlated with neuronal loss [26]. In the light of this information we performed a stereological evaluation that gives us qualitative information about the mean neuron number of the hippocampal regions. Our results showed that significantly neuronal loss was observed especially in CA1 and CA3 regions in STZ injected groups. However, there was no significant difference between Alzheimer and Alzheimer+Tacrolimus. groups. Our opinion is that the dose of the drug we used was insufficient or the application period was not enough to maintain the effect of the active ingredient. We proffer different doses of tacrolimus and prolonged period for new researches. Stereological results of the CA2 region revealed significant difference between control and Alzheimer+Tacrolimus groups. Although the STZ injection significantly decreased the mean neuron number in CA2 region besides there was no difference between the STZ injected groups. This is probably due to the fact that the loss of neurons in the CA2 region is because of negligible size. Similar to our result, Padurariu et al. [27] investigated the neuronal loss in CA2 region and they did not find any significant difference. Outcomes from our MWM test confirmed the decreased number values of the neurons in the hippocampus regions. The finding of the hidden platform

took longer for the STZ injected animals. There was a significant difference when comparing control and Alzheimer groups with each other. This finding is accordance to the hypothesis that IVC injection of STZ causes neuronal decrease and this leads damage to the spatial memory. There are several studies using MWM test for evaluating the impairment of spatial memory. For example, Bu and Zu [28] have shown the effects of β-amyloid on memory functions by using MWM test. According to their data the escape latency among the control and the treated groups showed significant differences. C-Fos is an immediate early gene that its expression is used as a marker for changes in metabolic activity of neurons. It's up-regulation in the hippocampus is known to be related with increased spatial memorial activity [29]. We observed a significant increase in the number of c-Fos-positive cells in DG region for Alzheimer group when compared to the Alzheimer+Tacrolimus group. This result supports the outcome from the DG region results from the stereology method for Alzheimer and Alzheimer+Tacrolimus groups. So, we think that due to the cell proliferation and differentiation there was a neuronal activity at the Alzheimer+Tacrolimus group which was led by tacrolimus which is a great advancement for our hypothesis. In fact, the expression of the c-Fos in the hippocampus was positively correlated to the spatial recognition score obtained from the MWM test. Consequently, AD led to increase in expression of c-Fos in the DG and that possible treatment with tacrolimus resulted in the alteration of the expression of c-Fos. C-Fos expression following brain injuries has been considered related with regeneration level of the neurons. Similar methods for our study, Tsai et. al., [30] applied MWM test to examine the results of the c-Fos expression in rat brains after a transient focal ischemia; because memory impairment is a result of a brain ischemia. Else, Beauquis et al. [31] used a transgenic PDAPP-J20 mouse animal model for AD. They observed that without

any amyloid plaques were present and low cerebral levels of amyloid peptides were detectable, there were structural, morphological, and cellular alterations in the hippocampus. In addition, their reports suggest that the transgenic group showed a high number of c-Fos-positive nuclei in central and basolateral amygdala.

5. Conclusion

We demonstrate that an ICV injection of STZ (3 mg/kg) on the right brain hemisphere leads rats to be an AD model suffering from neuronal loss, decrease in neuronal activity and spatial memory impairment ($p < 0.01$; One-way ANOVA). ICV injection of the STZ leads apoptosis and necrosis in the hippocampal brain regions CA1, CA2 and CA3. We tested the probable protecting effects of tacrolimus which was given orally before the STZ injection on hippocampus. The STZ injection caused significant neuronal loss in hippocampal regions. However, we found that c-Fos expression was decreased in the hippocampus after tacrolimus application, suggesting that tacrolimus may have had reducing the negative effect of STZ injection that leads to cellular stress and cell damage. However, our opinion is that the dosage was not enough to inhibit the effects of STZ on the CA1, CA2 and CA3 regions of hippocampus.

Based on these results tacrolimus may be an effective agent on cell proliferation in experimental Alzheimer disease model. In order to a new study, for the propose of the prevention of the neuronal loss in CA1, CA2, CA3 the dosage or/and the application time of tacrolimus may be renewable.

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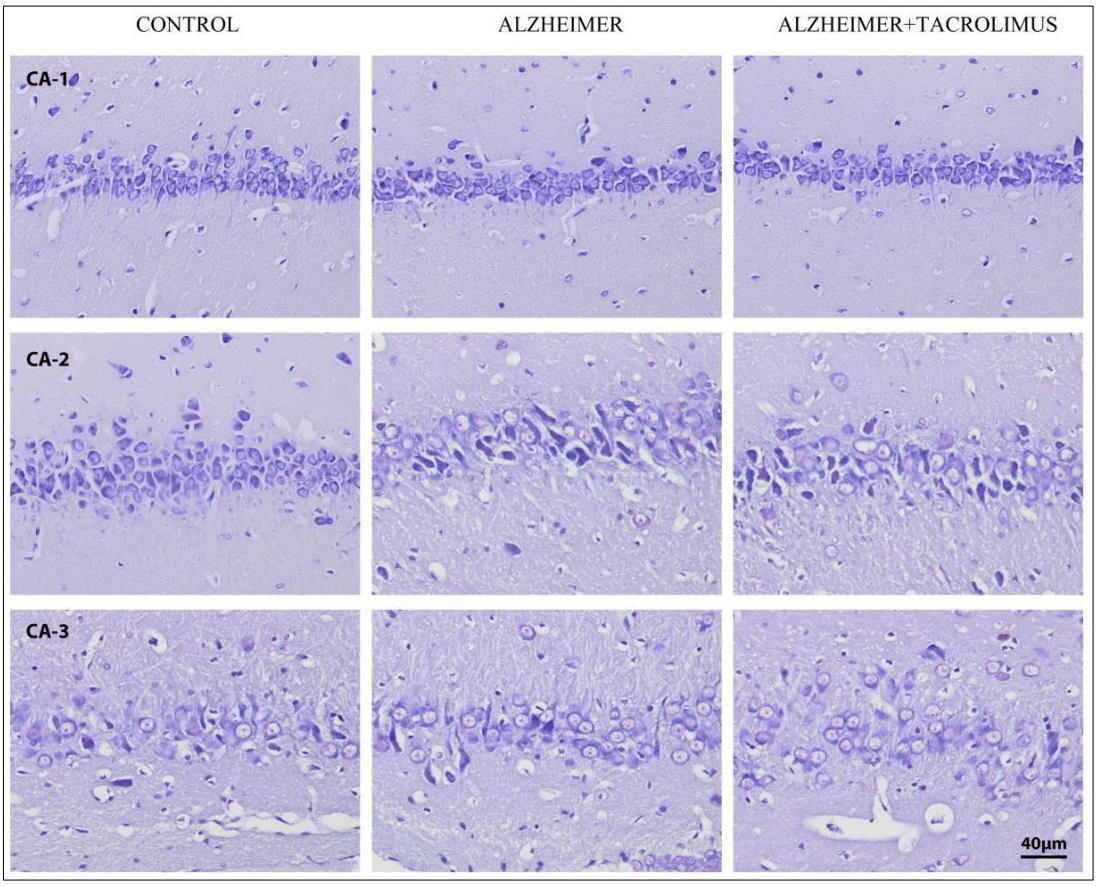

Figure 1. Coronal sections obtained from hippocampus stained with Cresyl violet are seen in this figure. All of the groups represent their own CA1, CA2, CA3 regions. Scale bar represents 40 µm.

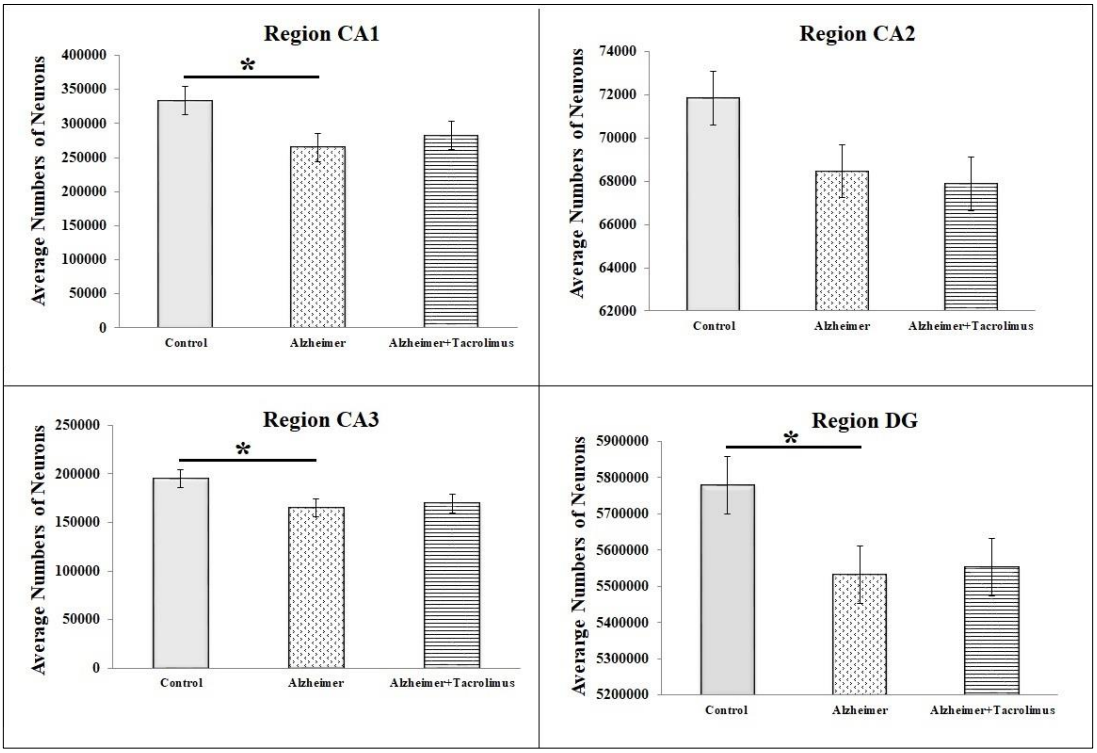

Figure 2. This figure is a sum of the tables of four different hippocampal regions for the mean numbers of neurons (±SEM). The tables show the mean numbers of the neurons from the hippocampus regions. There is a significant difference for CA1, CA3 and DG regions between control and Alzheimer group ($^{*}p < 0.01$). There is no significant difference when comparing Alzheimer with Alzheimer+Tacrolimus group with each other at any part of the hippocampus region ($p > 0.05$).

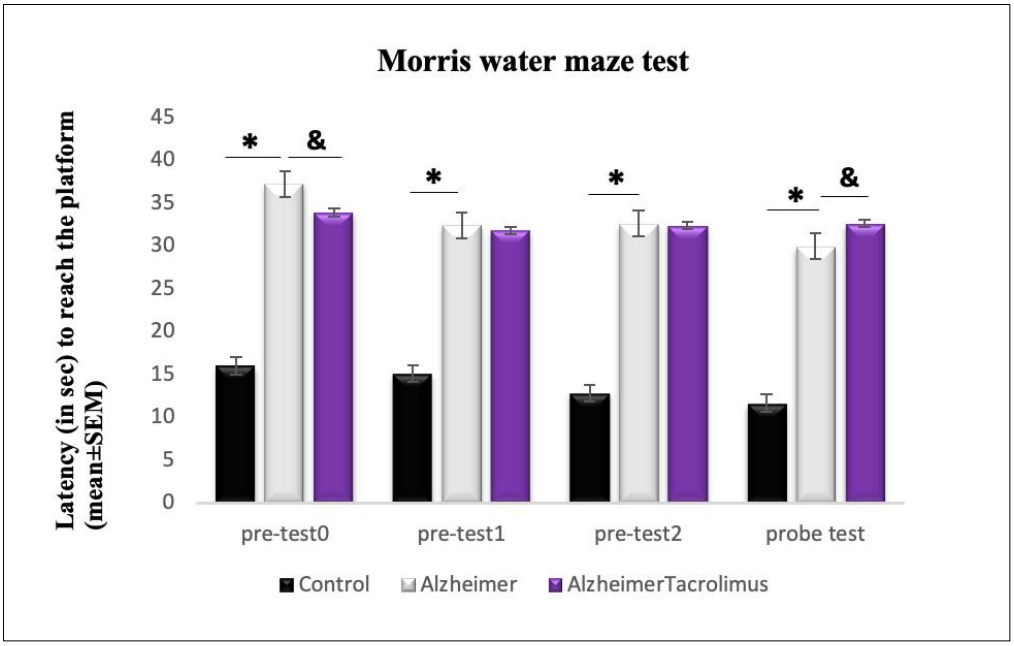

Figure 3. Mean values of the MWM test results are shown in the graphic (±SEM). There is a significant difference between control and Alzheimer group ($^{*}p < 0.01$) and a significant difference between Alzheimer and Alzheimer+Tacrolimus group ($^{\&}p < 0.01$) when comparing for pre-test 0; a significant difference between control and Alzheimer group for both pre-test 1 and pre-test 2 ($^{*}p < 0.01$) is also present. For probe test, there is a significant difference between control and Alzheimer group ($^{*}p < 0.01$) and between Alzheimer and Alzheimer+Tacrolimus group ($^{\&}p < 0.01$).

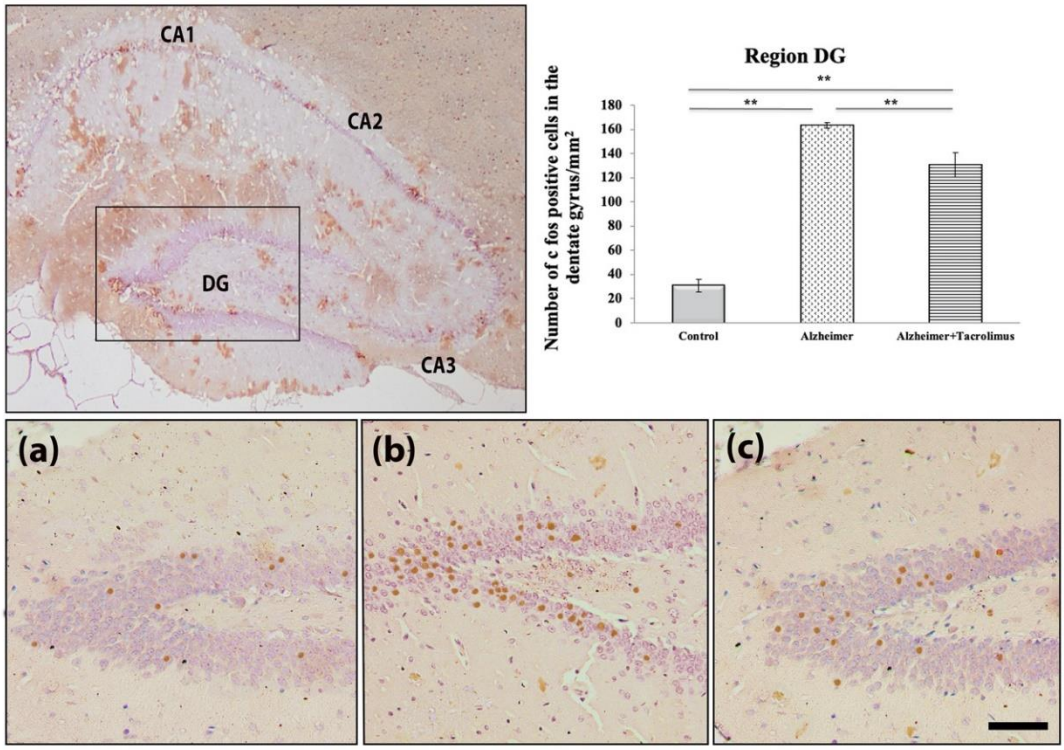

Figure 4. Effects of tacrolimus treatment on c-Fos expression in the DG. The graph indicates the mean number of c-Fos-positive cells in the DG ($^{**}p < 0.01$); the data are represented as the mean ± SEM. The photomicrographs demonstrate c-Fos-positive cells in DG regions of control (a), Alzheimer (b) and Alzheimer+Tacrolimus (c) group. Scale bar represents 50 μm.