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The expression level of muscarinic M1 receptor subtypes in different regions of rat brain

Sıçan farklı beyin bölgelerinde M1 muskarinik reseptör alt tipi seviyeleri

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ABSTRACT

Objectives: Post-traumatic stress disorder (PTSD) is characterized by life threatening trauma, overexcitation, flashbacks and nightmares. Research on PTSD is faced with the challenge of understanding how a traumatic experience leads to long lasting detrimental effects on behavior and functions of the brain. Many pharmacological agents are available in the pharmacotherapy of the PTSD where there is no adequate evidence to support the efficacy of any specific agent. It is hypothesized that M_1 muscarinic receptor subtypes might play important role in the recall of negative experience. The aim of this research is to investigate both the behavioral and the molecular efficacy of chronic fluoxetine (FLU) (2.5mg/day; i.p) treatment in PTSD and also the probable effect of pharmacotherapy on M_1 muscarinic receptor subtype expression in rats.

Materials and Methods: For experimental design random selection was performed to all groups; Control, Stress and Treatment groups. The effects of chronic FLU treatment were evaluated in terms of expression levels of the M_1 receptors in the hippocampus and the frontal cortex of the rats' brain.

Results: When the rats were subjected to the trauma reminder on the last day of the experiment (Day 30), the anxiety indexes of the stress group were found to be significantly higher than the control ($P< 0.001$). Moreover, it has been observed that chronic FLU treatment restored the anxiety scores in stress groups by lowering the anxiety indexes ($P< 0.001$).

Conclusion: In this study, it has been indicated that stress induces anxiety like behavior and reduces M_1 expression in the hippocampus and the frontal cortex of the rats' brain. These effects can be prevented by lowering the dose of chronic FLU therapy.

Keywords: Muscarinic receptors, Stress, Cat litter, Fluoxetine, Hippocampus, Frontal cortex

ÖZ

Amaç: Travma sonrası stres bozukluğu (TSSB) yaşamı tehdit eden travma, aşırı uyarılma, flashbackler ve kabuslar ile karakterizedir. TSSB araştırmaları, travmatik bir deneyimin beyinin davranış ve işlevleri üzerinde uzun süreli bozulma etkilerine yol açtığını anlamanın zorluğuyla karşı karşıyadır. Herhangi bir spesifik ajanın etkinliğini destekleyecek uygun kanıt olmadığında, TSSB'nin farmakoterapisinde birçok farmakolojik ajan kullanılmaktadır. Olumsuz bir deneyimin geri çağrılmasında M_1 muskarinik reseptör alt tiplerinin önemli rol oynayabileceği öngörülmektedir. Bu araştırmanın amacı, TSSB'de kronik fluoksetin (FLU) (2.5 mg/gün; i.p) tedavisinin hem davranışsal hem de moleküler etkinliğini ve farmakoterapinin sıçanlarda M_1 muskarinik reseptör alt tipi ekspresyonu üzerine muhtemel etkisini araştırmaktır.

Gereç ve Yöntem: Deney tasarımında tüm gruplar rastgele olarak seçilerek Kontrol, Stres ve Tedavi grupları oluşturulmuştur. Kronik FLU tedavisinin etkileri, sıçan hipokampüs ve frontal korteksinde, M_1 reseptörlerinin ekspresyon seviyeleri açısından değerlendirilmiştir.

Bulgular: Sıçanlar deneyin son gününde (30.Gün) travma hatırlatıcılara maruz kaldıklarında, stres gruplarındaki anksiyete indekslerinin kontrol grubuna göre belirgin bir şekilde arttığı gözlemlenmiştir ($P<0.001$). Ayrıca, kronik FLU tedavisinin stress gruplarında anksiyete değerlerini düşürerek geriye döndürdüğü gözlemlenmiştir ($P<0.001$).

Sonuç: Bu çalışmada, stresin kaygı benzeri davranışları arttırarak sıçan beyin hipokampüs ve frontal korteksinde M_1 ekspresyon seviyesini azalttığı gözlemlenmiştir. Düşük doz kronik FLU tedavisi ile bu etkilerin geri döndürülebileceği öngörülmektedir.

Anahtar kelimeler: Muskarinik reseptör, Stres, Kedi kumu, Fluoksetin, Hippokampüs, Frontal korteks

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Introduction

Post-traumatic stress disorder (PTSD) is characterized by overexcitation, flashbacks and nightmares that leads to

persistent anxiety [1]. The avoidance symptom cluster is characterized by individuals' efforts to avoid and emotionally detach or numb themselves from people, places, things, and activities that remind them of the traumatic event. Furthermore, hyperarousal, is characterized by heightened physiological reactivity as evidenced by exaggerated startle response, difficulty in concentrating, and hypervigilance. Recent studies show that people who have experienced traumatic events try to avoid remembering those events and also the stimuli that trigger the physical symptoms of anxiety [2]. In addition to this, PTSD has always been associated with a triggering etiologic factor, besides this it is considered as the only mental disorder involving an explicit condition [3]. The disease also has social aspects as a result of natural disasters or traumatic effects like terrorist attacks that usually affect a whole society. Therefore, it is really important to determine the underlying pathophysiological mechanisms of the disease in order to be able to develop better and new therapeutic drugs.

The most common types of the muscarinic receptors that are found in the brain are M_1 type receptors and they are localized in the hippocampus, cerebral cortex, sympathetic ganglions and striatum [4,5]. A role for muscarinic receptors has been hypothesized in many neurodegenerative disorders. Although, the human disorder is not identically mimicked in animals, at least the researches can give an idea about experimental stress-induced models of depression. Exposing the animals to emotional or physical stress can lead to a depression-like behavior. In these experimental models, exposure of animals models to a stressor (like a predator threat or a predator scent) is the main aim that is commonly used in order to investigate the mood disorders [6]. In our study, the rats were exposed to a stress for 5-10 min and then anxiety was evaluated by an open field, elevated plus maze (EPM) after several days once the reminder of the trauma was established [7-9].

A variety of pharmacological agents are available in the pharmacotherapy of the PTSD. However, current evidence is not sufficient to support the efficacy of any specific agent in the prevalent symptoms of PTSD [10,11]. In this study, it is hypothesized that M_1 muscarinic receptor subtypes might play an important role in the recall of negative experience. Changes in the expression of the M_1 muscarinic receptor subtypes in the dorsal hippocampus and the frontal cortex of the rats were examined to see whether there is any alterations in the levels of the muscarinic receptors in those locations to indicate the idea that muscarinic receptor subtypes may play an important role in the pathophysiology of PTSD. Normally selective serotonin re-uptake inhibitors (SSRIs) are commonly used for treatment of PTSD and in this study cronic fluoxetine (FLU) was used as a SSRI source. FLU (2.5 mg/kg) was used for the chronic treatment for 30 days to see whether muscarinic receptor subtypes played an important role in the PTSD. The aim of this study was to investigate both the behavioral and the molecular efficacy of chronic FLU treatment in PTSD and also the probable effect of pharmacotherapy on M_1 muscarinic receptor subtype expression in rats.

Materials and Methods

A. Animals and Conditions

Initially, an approval from the institutional ethical committee was obtained before the experimental period (approval no:, 13.2015.Sep; Near East University Animal Experiments Local Ethics Committee). In this study, Wistar Albino female rats (n=32) weighing 220–250g and 8-10 weeks were used. The rats were fed ad libitum with standardized rat chow and water and they were habituated to the housing conditions in the isolation room for 10 days to become adapted to moisture, temperature and light. After adaptation period, the experiments were held under the same conditions during 1 month period. These housing conditions were: Constant room temperature 21±3 °C, humidity ratio 50±5% and 12 h light/dark cycle. As the rat models are nocturnal animals; all experiments were performed in the dark phase of rats' at 10:00 a.m.

B. Drugs and Solutions

Treatment groups received intraperitoneal (i.p) injections of 2.5 mg/kg/day FLU (Eli Lilly, Turkey) that were suspended in physiological saline for a month whereas control groups only received physiological saline injections. The injections were applied 10 min before the test, and continued daily, at the same time of the day.

C.Cat Litter Using Predator Scent Test

For experimental design random selection was performed to all groups to induce stress after 10 days of acclimation period.

1. Control group: The rats were exposed to a clean cat litter at the first and the last day of the experiment.

Additionally, during the experimental period (1 month) rats were only receiving physiological saline solution (n=8).

2. Fluoxetine (FLU) alone group: For treatment, continuously FLU injections were applied to rat models for a month. Additionally, rat models were exposed to a clean cat litter (n=8).
3. Stress group: In order to induce a stress condition, the rats were placed on 125 ml of dirty cat litter for 10 min in a cage (30 cm×30 cm×40 cm). The cat litter had been used for 2 days by the same cat and had been sifted for stools (n=8).
4. Stress+fluoxetine (Stress+FLU) group: The rat models were treated with FLU for a month. On the first day of the experiment a dirty cat litter was applied to the rats induced with PTSD model whereas at the last day of the experiment a clean cat litter was used as a trauma reminder (n=8).

For the control group, animal models were placed in an identical cage with clean, unused litter for 10 min [12-15]. The rats were exposed to a clean cat litter as a situational reminder on the last day of the experiment (Day 30). The behavioral experiments were recorded by using a video camera and the anxiety indexes were calculated by using EPM setup on the last day.

D.Elevated Plus Maze Experiments

In this study, EPM were used for experiments where rats were exposed to predator scent test by locating them on an EPM for 5 min. After that, rats were subjected to a clean cat litter, a situational reminder. The height of the EPM was 50 cm from the ground and had two open and closed arms (50 cm × 10 cm). Additionally, those closed arms were surrounded by 10 cm long walls and during experiments, each rat was placed in the central square of the maze facing towards the open arms.

E.Calculation of Anxiety Indexes ($N_{anxiety}$)

The behavioral experiments of the rats were recorded during 5 min using a video camera which was placed on top of the setup. The recordings were used to score the behavioral parameters. The 'arm entry'defined the point when the animal manages to fit inside the EPM with four feet.

This information later was used to score the number of animals that manage to fit fully either into open or enclosed arms and then duration of their stay in each arm was recorded [16]. The anxiety indexes ($N_{anxiety}$) were calculated by using A (cumulative time spent in open arms (s)), B (open arm entries), C (total arm entries) parameters and the below formula:

$$N_{anxiety} = 1 - 1/2\ [(A/300\ s) + (B/C)]$$

F.Tissue Collection and Immunoblotting

All chemicals were purchased from Sigma (St Louis, MO, USA) unless indicated. Following decapitation of rats, firstly brains were removed and then the dorsal hippocampus and the frontal cortex were dissected according to the Rat Brain Atlas [17]. For the separation of the dorsal hippocampus and the frontal cortex, the slides in the anteroposterior planes positioned between 13.20–11.20mm and 7.20–5.70mm, respectively, anterior to the interaural line. Obtained tissues were stored at −80°C for immunoblotting. After homogenizing procedure, the amount of protein contents were determined in each region of the brain by Lowry method [18]. Hundred micrograms of protein was loaded onto 12% sodium dodecyl sulfate-polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes (Schleicher and Schuell, 0.45 mm, Germany). The membranes were blocked with Tris buffered saline (TBS) containing 1% bovine serum albumin at room temperature for 1 h and incubated overnight at 4°C with the primary antibodies against M_1, which was evaluated at 52 kDa (1:200 dilution, Santa Cruz, CA, USA). Results were standardized by using β-actin (Santa Cruz, CA, USA; 1:200 dilution) as the control protein, which was detected at 43 kDa. The densitometric analyses were performed with Bio-Rad Molecular Analyst software (free edition, www.totallab.com).

Statistical Analysis

In order to compare the behavioral effects between the experimental groups and the control group, Unpaired Student's *t*-test was used for statistical analyses. For further evaluation, One-Way analysis of variance (One Way Anova) followed by Tukey's multiple comparison and post hoc test were used for the remaining comparisons. The value for the statistical significance was accepted as $P<0.05$.

Results

A. The Effects of Chronic Fluoxetine Treatment on Behavioral Parameters

When the rats were subjected to the trauma reminder on the last day of the experiment (Day 30), the anxiety indexes of the stress group were found to be significantly higher than the control group (Fig. 1; $P< 0.001$). The mean of the anxiety indexes of the rats for the control group was 0.5751±0.02, FLU alone group was 0.6173±0.02, stress group was 0.9327±0.03, and the stress+FLU treatment group was 0.6734±0.03. Administration of chronic FLU treatment did not appreciably alter anxiety indexes in the control conditions prior to administration of the test. FLU treatment did not have any influence on anxiety indexes of the FLU alone group (Fig. 1). In the stress groups, it has been observed that FLU treatment restored the anxiety scores of rats by showing significantly lower values. As a result, Two-way analysis of variance showed that there was an association with the chronic FLU treatment (df = 3, F = 46.09; $P<0.0001$). Moreover, The *Bonferroni post hoc test* revealed a difference between control and stress groups (t = 4.94, $P< 0.001$).

B. Immunoblotting Analyses

Immunoblotting experiments were performed with 3 rats in each group who had higher anxiety scores. For each rat, the experiments were repeated twice. The frontal cortex and the dorsal hippocampus of the rats were homogenized and immunoblotting were represented as shown in Figs. 2a and 2b.

The mean value for M_1 muscarinic expression level which was found in the frontal cortex for the control group was 1.234±0.05, for the chronic FLU alone group was 1.183±0.05 and for the stress group was 1.060±0.032. However, for the stress+FLU group the mean value was quiet different than the other groups and was 1.313±0.03. This difference was found to be statistically significant ($P = 0.0044$). In frontal cortex, it had been found out that M_1 receptor expression level was increased in the stress+FLU treatment group compared to all groups (Fig. 2a). Two-way analysis of variance indicated that stress+FLU group might play an important role in (df = 3, F = 7.702; $P = 0.0074$) producing a source of interaction. The *Bonferroni post hoc test* revealed a difference in the control group (t = 4.94, $P< 0.05$).

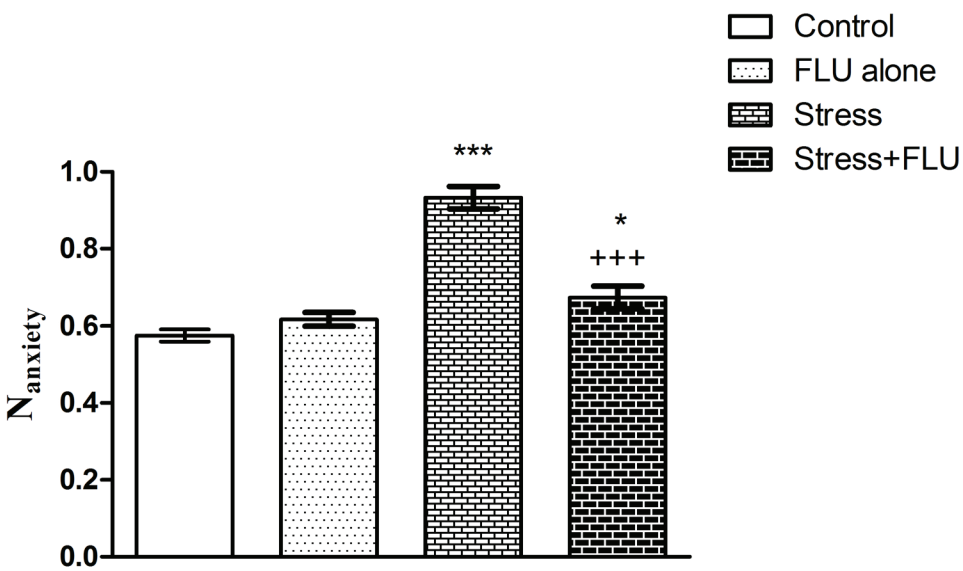

Fig.1. The effects of chronic fluoxetine (FLU) on anxiety indexes in all groups calculated from data obtained using elevated plus maze experiments (n=8 per group).

*, $P<0.05$; ***, $P<0.001$ shows the difference between the stress groups and the control group. +++, $P<0.001$ represents the variations in treatments within the stress groups.

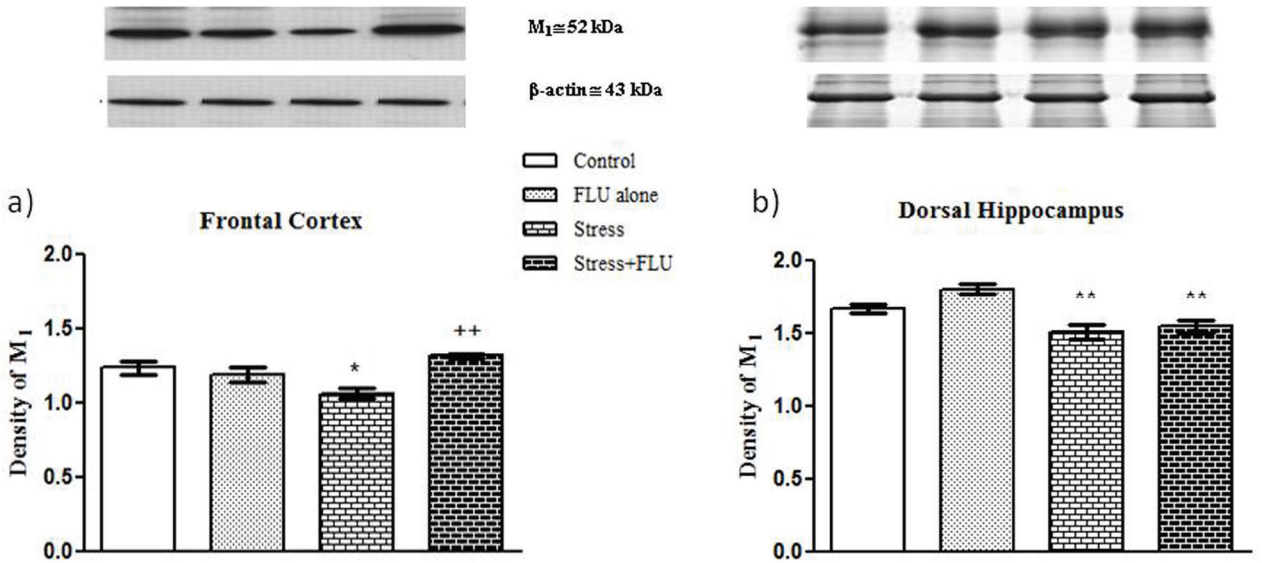

Fig.2. The expression levels of M_1 receptors in both (a) frontal cortex and (b) dorsal hippocampus after predator scent test (PST). Images above (a) and (b) are the representation of the membrane images as a result of the Western Blotting (n=3, each of them was duplicated). The M_1 and β-actin were observed at ≅ 52 and 43 kDa, respectively.

*, $P<0.05$; **, $P<0.01$ shows the difference between stress groups and the control group

++, $P<0.01$ represents the variations in treatments within the stress groups.

The mean values of M_1 muscarinic expression level in the dorsal hippocampus for the control group was 1.670±0.03, for the FLU alone group was 1.799±0.04, for the stress group was 1.508±0.048, and for the stress+FLU group was 1.544±0.05. In the dorsal hippocampus, the level of M_1 receptor expression was increased in the FLU alone group when compared to stress and stress+FLU groups. Two-way analysis of variance indicated that stress+FLU (df = 3, F = 9.621; $P = 0.0036$) produced a source of interaction. The *Bonferroni post hoc test revealed* a difference in FLU alone group (t = 3.57, $P < 0.05$). FLU treatment was found to be effective in restoring the changes in the expression of M_1 receptor of the dorsal hippocampus (Fig. 2b).

Discussion

It is known that the role of cholinergic mechanisms in stress has not been studied fully. Although PTSD has gained considerable attention in recent decades, it has not always been clear what constitutes adaptive versus maladaptive responses in traumatic stress. Therefore, understanding susceptibility factors that encourage persistent and invasive traumatic memory expression, as well as more global somatic PTSD symptoms, is of great scientific and practical value [19].

In this study, in a rat model of PTSD, the effects of chronic FLU treatment were evaluated in terms of expression levels of the M_1 muscarinic receptors in the dorsal hippocampus and the frontal cortex of the rats brain. Muscarinic receptors are known to play major role in learning, storing memory and in posture control where the blockage of the muscarinic receptors leads to memory loss [20,21]. Therefore, it is really important to understand the underlying mechanism of the cholinergic muscarinic receptors in PTSD.

However, because of the species variation there are no animal models that can identically mimic the mood disorders that exist in humans. According to Cohen et al., studies in rat model of PTSD show that predetor scent test is one of the main test that is used very often to induce stress [22]. Therefore, in this study, for a stressor (predator scent) a dirty cat litter was used and a clean cat litter was used as a situational reminder. Like Cohen et al.[22], also in this study it had been found out that the anxiety index was significantly higher in the stress group. Our data also demonstrated that the cat litter test was a reproducible experiment where the rats were subjected to a dirty cat litter had higher anxiety

indexes when subjected to the clean cat litter, a situational reminder.

In studies where SSRI used as a treatment, it had been found out that there were unwanted, anxiogenic-like effects. Therefore, when those effects were considered, in our study lower dose (2.5 mg) and chronic FLU was used instead of high and acute FLU. As a result, anxiogenic-like effects were eliminated and lower dose and chronic FLU treatment was found to be effective in decreasing the anxiety state.

Many studies reveal that integrating cognitive and neurochemical responses are formed in the hippocampus as a result of stress. This shows that the hippocampal cholinergic system plays a major role in the integration of anxiety and the memory. In addition to this, the cortex and the hippocampus are known to be involved in learning and memory functions [23]. Therefore, in this study the frontal cortex and dorsal hippocampus were studied in order to observe the effects of stress in rats. However, in this study there was no experiment carried out on the learning-memory functions of the rats. For future researches, we thought that carrying out experiments on the learning-memory functions would be important in order to eliminate the limitations of this study.

In the hippocampus it had been observed that there was a decrease in the levels of M_1 receptors as a result of the chronic FLU treatment in the stress groups. Many studies have confirmed that blockade of M_1 receptors, in the dorsal hippocampus lower the anxiety [24,25]. Although, FLU were found to be effective in decreasing the anxiety state, FLU at 10 mg/kg was found to be ineffective. Moreover, 10 mg/kg FLU dose was shown to increase the freezing time [26]. In literature, it was shown that administration of chronic FLU at a 5 mg/kg/ i.p reduced freezing time whereas acute FLU (1 mg/kg) produced the adverse effects [27]. Therefore, doses between 1-5mg/kg were thought to be the ideal dose for the treatment. The down regulation of M_1 receptors may be counted as a natural consequence of the plasticity that was accomplished with effective pharmacotherapy. However, it should not be forgotten that there are different types of muscarinic receptors and cholinergic systems that M_1 type receptors are associated. Therefore, in order to have a certain conclusion, the interaction of M_1 type receptor with those receptors and systems should be studied as well.

Our data also revealed that the frontal cortex and the dorsal hippocampus recovered similarly by having similar results with the control group. However, there were variations in recovering levels of both locations.

Conclusion

In our study, it has been revealed that stress induces anxiety like behavior and reduces M_1 expression in the dorsal hippocampus and these effects can be prevented by lowering the dose of chronical FLU therapy. Our study suggests that, future experimental study design should focus on alterations in anxiety states. Furthermore, in order to understand the underlying mechanisms of neurodegenerative diseases which are induced by stress; the relationship with other cholinergic receptors should be studied in more details. Additionally, in stress related studies, in order to analyse the hippocampal atrophy and amygdalar hypertrophy in terms of histological and morphological parameters, the number of studies on postmortem neuropathology on humans should be increased.

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