Neurochem Res (2016) 41:1468–1474
DOI 10.1007/s11064-016-1858-4

ORIGINAL PAPER

Chronic (3-Weeks) Treatment of Estrogen (17β-Estradiol) Enhances Working and Reference Memory in Ovariectomized Rats: Role of Acetylcholine

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Received: 2 October 2015 / Revised: 1 February 2016 / Accepted: 2 February 2016 / Published online: 15 February 2016

Abstract Recently there has been a growing interest in the effects of estrogen on cognitive functions. In this study, we aimed to examine 17β-estradiol treatment on working and reference memory in ovariectomized rats. We also examined the changes in the acetylcholine (ACh) levels in the brain areas associated with learning and memory. The study was performed on Sprague–Dawley type 3-month-old female rats. The rats were divided into four groups as control, ovariectomy (OVX), and OVX and estrogen treatment (10 μg/day i.p. 17β-estradiol) groups for 3 (OVX + E3) and 21 days OVX + E21). The rats were trained on eight arm radial maze task with eight arms baited to assess spatial memory, in addition four arms baited to assess both working and reference memory performances. The electron microscope images of the ACh vesicles in the frontal cortex, temporal cortex and hippocampus areas of the brain which are important regions for learning and memory were screened. Results showed that long term 17β-estradiol treatment has positive effects on both reference memory and working memory and that ACh vesicles increased in the examined brain areas, especially in hippocampus. Our results suggest that 3 weeks 17β-estradiol treatment may have an ameliorative effect on the memory through the central cholinergic system.

Keywords Ovariectomy · 17β-estradiol treatment · Learning · Acetylcholine · Rat

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Introduction

Estrogen is known to regulate neuroendocrine functions related to reproduction [1]. Recent studies on estrogen have focused on cognitive functions and the associated neural systems [2]. Gonadal hormones are known to affect cognition [3]. It was noted that the effect of estrogen on cognitive functions such as learning and memory derived from the effect of this hormone on neuromodulators like acetylcholine [4] and catecholamine [5]. Some studies report that estrogen does not affect, or even deteriorate spatial memory [6], others state that estrogen has a favorable effect on short-term or working memory [7]. Data obtained in human studies suggest that estrogen replacement after menopause improved the memory performance [8]. It was reported that estradiol increased activity in basal specific forebrain, hippocampus, and frontal cortex projections, which contain acetylcholine [9]. It has been reported that chronic estrogen replacement to ovariectomized rats improved their performance in spatial and non-spatial memory tests [10, 11]. Young females treated with β-estradiol or high doses of genistein have better performance in spatial memory task than aged animals [12]. Similarly, it was claimed that chronic β-estradiol treatment could impair memory-associated functions [13]. Other studies suggest that hormone treatment does not have any effect on cognitive functions [14]. Results obtained with acute or chronic estrogen administration, as well as results pertaining to types of memory also varies [15]. Although estrogen was demonstrated to affect performance in various measurements of learning and memory functions, the mechanisms underlying this effect are not known.

Cholinergic neurons have estrogen receptors and these neurons end in the frontal cortex and hippocampus are activated by estrogen treatment. Recently structures in the medial temporal lobe, including the hippocampus and connected areas have been proposed to constitute "medial temporal lobe memory system" that is specialized for memory functions in the mammalian brain. The primary functions of these areas are thought to be responsible for storage and recall of facts and events [16]. In a study, the same pattern of impairment in discrimination of learning was observed in humans with temporal cortex lesions and monkeys with hippocampus lesions [17]. Amnesic subjects in this study with lesions limited to the hippocampus were not impaired in any aspect of visual discrimination learning, consistent with the effect of selective hippocampus lesions in monkeys [18].

In the light of these data, we aimed to determine the levels of acetylcholine (ACh) in brain areas such as frontal cortex, temporal cortex and hippocampus and its relation to spatial memory, reference and working memory in rats with acute (3 days) and chronic (3 weeks) 17-beta-estradiol treatment.

Materials and Methods

This study was carried out in the Selcuk University Experimental Medicine Research and Application Center and approved by ethics committee of the research center in part that ovariectomy was performed at the mentioned center. The study included 24 adult female rats of Spraque-Dawley strain, which were 3 months old and weighed 200–250 gr. The rats were kept at 18–21 °C (room temperature), under 12 h light/dark cycle (07.00 a.m.–07.00 p.m.) and fed with standard rat pellets.

Experiments for learning were performed at the Istanbul University, Medical Faculty, Department of Physiology. Experimentals groups were divided as follows.

1. *Control group (n = 6):* Animals in this group was not subjected to any procedure.
2. *Ovariectomy group (Ovx) (n = 6):* Ovariectomy performed on animals after general anesthesia.
3. *Ovariectomy + 3 days 17-beta-estradiol group (Ovx-E3) (n = 6)*: The rats were injected with 10 μg/day 17beta-estradiol (in 0.5 ml olive oil) through the intra-peritoneal route for 3 days just after 30 days following ovariectomy.
4. *Ovariectomy + 21 days 17-beta-estradiol (Ovx-E21) (n = 6):* The rats were injected with 10 μg/day 17-beta-estradiol (in 0.5 ml olive oil) through the intra-peritoneal route for 21 days just after 30 days following ovariectomy.

Ovariectomy

Ovariectomy was performed on rats under general anesthesia (60 mg/kg ketamine (Eczacibasi, Turkey) and 5 mg/kg xylazine (Bayer, Germany). The hair on the back of the rats was shaved for ovariectomy. Following appropriate asepsis and antisepsis with betadine, the rats were placed in the ventral position and the skin was incised from one-third upper point of the between medial part of the back and tail. After subcutaneous tissues were separated, peritoneal cavity was accessed through abdomen of back wall muscles. Ovaries were removed together with their adipose tissue. The ovaries were separated from the adipose tissue, and then clamped, ligated and extradited. After controlling the bleeding, other organs were replaced inside the peritoneal cavity and the muscle was sutured with 2/0 chrome catgut and the skin by 2/0 silk [19].

The rats were injected with 10 μg/kg 17β-estradiol (in 0.5 ml olive oil) through the intra-peritoneal route for 3 and 21 days just after 30 days following ovariectomy. This dose was modified from a previous study [20].

Behavioral Testing with Radial Arm Maze (RAM)

Among behavioral tests, one of the most suitable devices for measuring spatial learning and memory is the radial arm maze (RAM) [21]. Briefly RAM consists of eight horizontal arms (57 × 11 cm) placed radials around a central platform above the floor. Automated doors are located at the entrance of each arm. Experimental subjects are placed on a central platform from which they have to collect hidden baits placed at the end of the arms. In the present study, baited and unbaited arms were fixed throughout the tests. The 1st, 3rd, 6th, and 8th arms were baited while the 2nd, 4th, 5th, and 7th arms were unbaited. The animals were trained for adaptation on three consecutive days prior to the beginning of the learning period. During these adaptation sessions, the rats were allowed to explore the baited arms of the maze for 10 min. Baits are scattered on the arms. A rat was placed on the center platform that was closed off by a door. After 20 s the door was opened and rat was allowed to obtain food pellets until all pellets were eaten or for 10 min. Each session lasts all eight arms have been entered (an arm choice was scored if the rat traversed half the length of an arm). On the last day of adaptation (3 days), the sessions ended when all eight arms had been visited. Following adaptation period, the animals were trained one session per day for ten consecutive days. One piece of baits was placed at the end of each arm in a well that hides the food sight, and the animal was allowed to explore the maze freely. 3 days before starting the experiment and during the test, rats were subjected to a food deprivation regimen of 22 h/day with water

ad libitum. Rats were food-restricted but not water to gradually reach 85 % of their free-feeding weigh for the duration of training and testing. After the adaptation period, all arms were baited for the spatial memory test during 10 days (one session/day). After the animals were placed in the center of the maze, the arms that they visited were observed for 10 min. In order to test spatial memory, during their visits to the first seven arms, re-entry into a previously visited arm was scored as an error (the procedure of the 8-arm radial maze depends on the principle of considering re-entry into a previously visited arm an error).

Scoring of Behaviour

The first entry into never-baited arms was scored as a reference memory error (RME) and reentry into arms where the food reward had already been eaten was scored as a working memory error (WME) [22, 23].

Tissue Dissection and Processing for Electron Microscopic Evaluation of Acetylcholine Vesicles

After the performance tests were completed, the rats were anesthetized by intraperitoneal (*i.p.*) injection of sodium pentobarbital (60 mg/kg). The chest was surgically opened as fast as possible, a perfusion catheter was inserted into the ascending aorta and right atrium was incised. Perfusion pressure were monitored throughout the procedure and maintained at the pressure of 80-90 mmHg. Primarily 200 ml normal saline was perfused for 2 min followed by 200 ml fixative perfused over a period of 20 min. The fixative used was 0.4 % glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2), then the brain was removed and 5 mm thick pieces were cut and placed in the same fixative to obtain additional hardening. Samples were taken from frontal, temporal cortical areas and hippocampus were immersed in same fixative over night. Next day, after washing with phosphate buffer (pH 7.2), these samples were fixed in 1 % osmium tetroxide, dehydrated in ascending concentration of ethanol then embedded in araldite. 1 μm thick sections were cut from these samples and were stained with toluidine blue and double stained with uranyl acetate and lead citrate, and examined and photographed in the same magnification in a Zeiss EM 10 electron microscope. In order to analyze the data of ACh vesicles statistically, the serial sections were cut from carefully chosen regions of interest. The quantitative results of acetylcholine vesicles were obtained by counting at each terminal using the UTHSCSA Image Tool program. All photographs were analyzed by using NEO Image Analysis program V1.0. Vesicules were marked with the same Image Analysis program. Damaged cells were not evaluated. The marked cells were counted automatically with the same Image Analysis Program [24, 25].

Statistic

Animal's behavioral activities in radial eight arm maze task and results for amount of Acetylcholine vesicle in chosen regions of brain were statistically analyzed by one-way analysis of variance (ANOVA) test. In order to evaluate differences between groups in radial arm maze task, separate repeated measures ANOVA were calculated on number of working memory and number of reference memory errors with group as between-subject factor and days (1–10) as within-subjects factors. All the results are expressed as mean ± SEM. The criterion for statistical significant was ($P < 0.05$) in all statistical evaluation. Significant differences were determined by Tukey's post hoc test.

Results

Error values regarding spatial, reference, and working memory of experiment groups are presented in Fig. 1. Spatial memory error levels were 0.82 ± 0.52, 1.54 ± 1.13, 1.54 ± 1.13, 0.05 ± 0.05 for control, Ovx, Ovx + E3 and Ovx + E21 groups, respectively. Similar parameters of 0.56 ± 0.34, 1.90 ± 0.64, 2.02 ± 1.03 and 0.72 ± 0.34 were obtained for reference memory. Working memory error levels were determined as 0.20 ± 0.15, 0.59 ± 0.32, 0.35 ± 0.24 and 0.08 ± 0.04 for groups. When entries into 8-arm baited radial maze, the first parameter used to assess spatial memory in the first stage of the study, were examined, Ovx group was found to have a

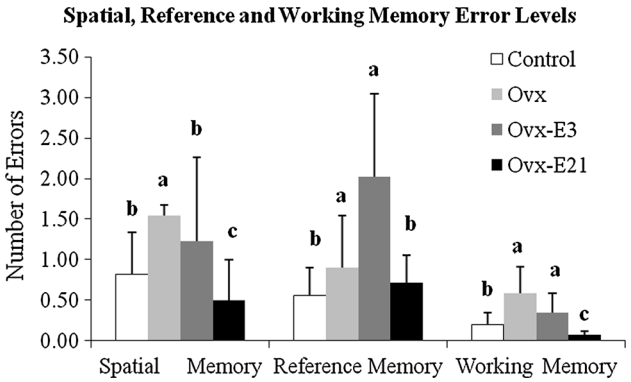

Fig. 1 Spatial, reference and working memory error levels in control (C), ovariectomized (Ovx), ovariectomized + 3 days 17β-estradiol treatment (Ovx + E3) and ovariectomized + 21 days 17β-estradiol treatment groups (Ovx + E21). *$P < 0.05$ (a > b > c). *a* Compared to control and Ovx-E3, Ovx-E21 ($P < 0.05$); *b* compared to Ovx and Ovx-E21 ($P < 0.05$); *c* compared to control, Ovx, Ovx-E3 ($P < 0.05$) for spatial memory. *a* Compared to control and Ovx-E21 for reference memory ($P < 0.05$). *a* Compared to control and Ovx-E21 ($P < 0.05$), *b* compared to Ovx, Ovx-E3, Ovx-E21 ($P < 0.05$); *c* compared to control, Ovx, Ovx-E3 ($P < 0.05$) for working memory

higher number of errors than control and Ovx + 17β-estradiol treatment groups (F (3,12) = 5.07, $P = 0.05$). However, an examination of the effects of short-term (3-day) and long-term 17β-estradiol treatment to Ovx groups revealed that especially 21-day 17β-estradiol treatment significantly reduced the high number of errors caused by Ovx, and even caused fewer errors than those made by the control group [F (3,13) = 6.03, $P = 0.05$]. Reference memory error values with 4 baited arms showed that number of errors increased significantly with Ovx, but then there was a marked improvement after 21-day 17β-estradiol treatment [F (3,17) = 4.25, $P = 0.05$]. Concerning the working memory, it was found that this parameter showed a marked deterioration with an increased number of errors due to Ovx, but was favorably affected by 3-weeks (E21) 17β-estradiol treatment, and culminated in a reduced number of errors [F (3,16) = 4.15, $P = 0.05$]. Still, 3-day 17β-estradiol treatment did not have any significant effect on reference or working memory.

The study also determined acetylcholine (ACh) vesicles in the frontal [Fig. 3(A1)–(A4)]; hippocampus [Fig. 3(B1)–(B4)] and temporal cortex [Fig. 3(C1)–C(4)] areas using electron-microscopy method. ACh vesicle counts in the frontal cortex were 72.5 ± 0.71 in the control group, 30.0 ± 1.41 in the Ovx group, 37.5 ± 1.41 in the acute treatment group (3 days, E3), and 73.0 ± 1.41 in the chronic treatment group (21 days, E21) [Fig. 2, F (3,17) = 5.52, $P = 0.05$]. The number of ACh vesicles in hippocampus were 74.0 ± 3.92, 29.75 ± 8.46, 69.75 ± 5.91 and 212.5 ± 33.04 [Fig. 2, F (3,16) = 4.02, $P = 0.05$] and in the temporal area was recorded to be as 81.0 ± 1.41, 31 ± 1.41, 43 ± 2.82 and 82 ± 4.23, for control, ovx, acute treatment and chronic treatment, respectively [Fig. 2, F (3,20) = 5.47, $P = 0.05$]. It was found that the number of ACh vesicles in the related brain areas reduced significantly after ovariectomy, and that short-term 17β-estradiol treatment failed to restore the decrease in ACh vesicles. However, 3-week 17β-estradiol treatment caused a marked increase in the reduced number of ACh vesicles and produced higher vesicle counts than those in the control group [F (3,16) = 4.02, $P = 0.05$]. An increase of ACh vesicles in hippocampus was higher compared to frontal and temporal cortex (Fig. 2).

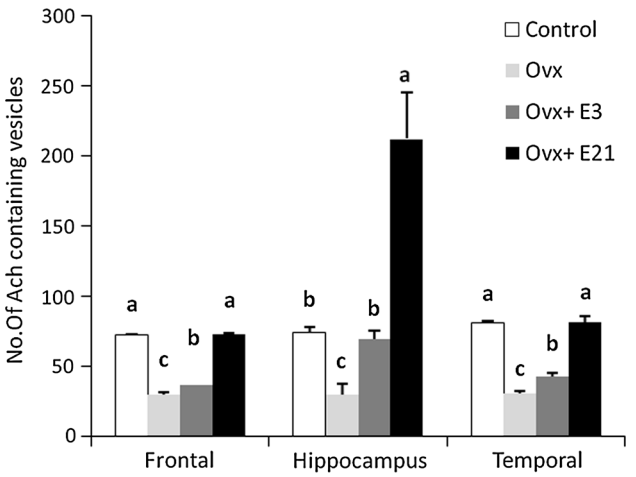

Fig. 2 Ach vesicules numbers in frontal cortex, hippocampal cortex and temporal cortex (*different letters* show statistical significancy; a > b > c) ($P < 0.05$) control, ovariectomy (Ovx), ovariectomy + 17β-estradiol for 3 days (Ovx + E3), ovariectomy + 17β-estradiol for 21 days (Ovx + E21). For frontal cortex: *a* compared to ovariectomy, Ovx-E3 ($P < 0.05$), *b* compared to control, Ovx, Ovx-E21 ($P < 0.05$), *c* compared to control, Ovx-E3 and OVX-E21 ($P < 0.05$). For hippocampus: *a* compared to control, Ovx, Ovx-E3 ($P < 0.05$), *b* compared to Ovx and OVX-E21 ($P < 0.05$), *c* compared to control, Ovx-E3 and OVX-E21 ($P < 0.05$). For temporal cortex: *a* compared to Ovx, Ovx-E3 ($P < 0.05$), *b* compared to control, Ovx and OVX-E21 ($P < 0.05$), *c* compared to control, Ovx-E3 and OVX-E21 ($P < 0.05$)

Discussion

An overall assessment of study results indicates that chronic (3 weeks) 17β-estradiol treatment improved the performance of both spatial reference and working memory. However, 3-day 17β-estradiol administration was not found to have any significant effect on memory performance. Besides, 21-day 17β-estradiol treatment to ovariectomized rats was found to markedly prevent the decline in acetylcholine levels in frontal and temporal cortex and hippocampus.

Reference memory is taken to represent task-learning independent from the experimental procedure (location of food and spatial conditions), while working memory is assumed to reflect storage of experiment-dependent knowledge (of previously visited arms). The results of previous studies on this topic are contradictory. This contradiction is attributed to differences in the animals' age, the type of the task, environmental conditions, animal type, and hormone administration dose and duration. However, the ameliorative effect of estrogen administration to ovariectomized rats is clear [26]. In our study, all studied memory types (spatial, reference, and working memory) were impaired in experimental animals which were deprived of estrogen by ovariectomy. Besides, short-term 17β-estradiol treatment did not produce any effect on the memory types studied and the error values in the relevant group were closer to those found in the ovariectomized group. However, significant improvements in the related memory types were obtained in the third study group (ovariectomy + 3-week 17β-estradiol treatment) whose memory performance was better even than that of the control group. Previous studies reported that estrogen produced positive effects on different types of memory. For

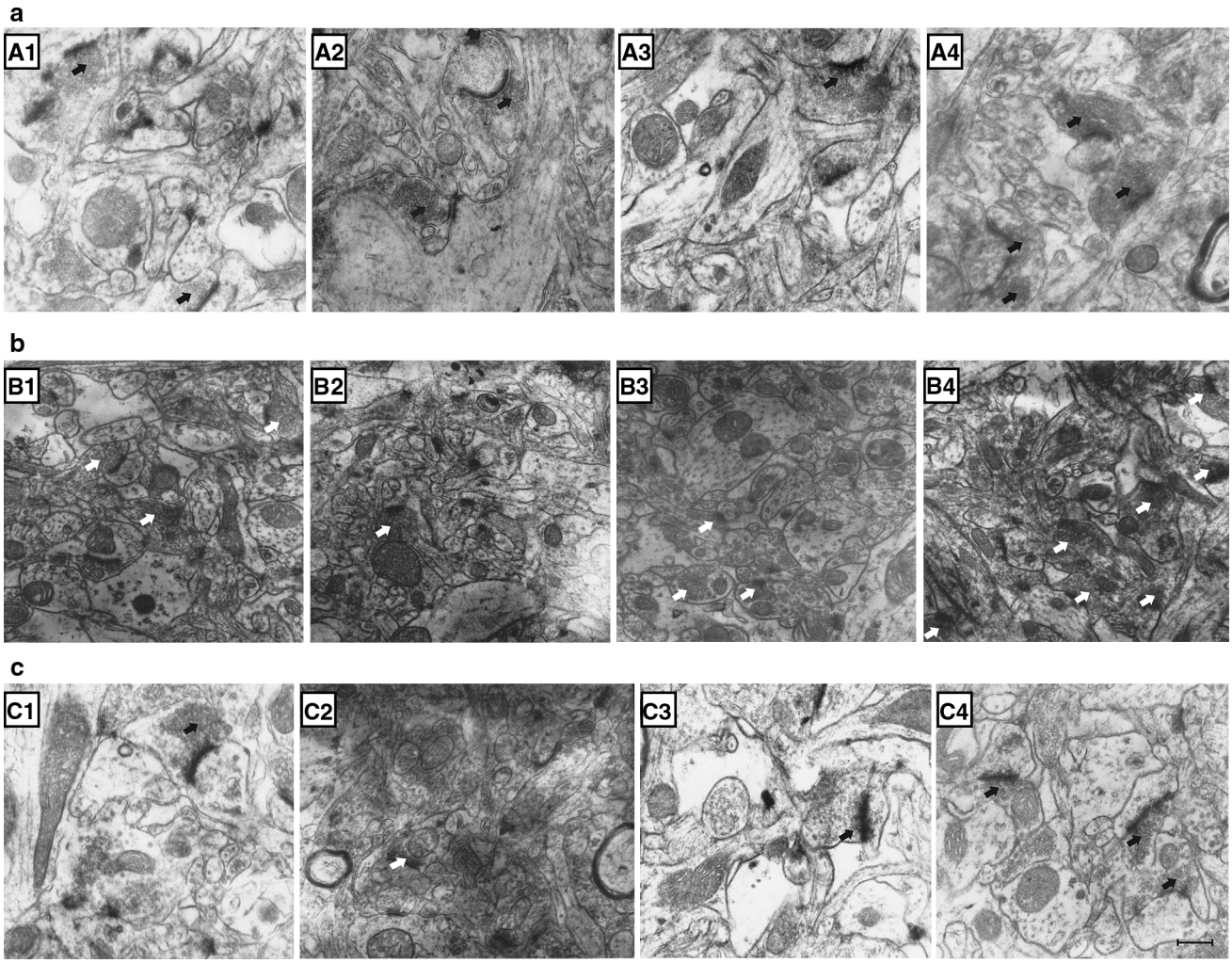

Fig. 3 Representatives electronmicrographs showing density of acetylcholine vesicles in the presynaptic structures of frontal cortex (**a**), hippocampal cortex (**b**), temporal cortex (**c**) of animals from control, ovariectomy, Ovx + E3, Ovx + E21. *Arrows* show acetylcholine vesicles, *bar* represents 1 cm = 0.3 µ. The 21 days 17β-estradiol-treated rats showed obvious increases in the density of acetylcholine vesicles. Control Frontal Cortex ACh Vesicles (*A1*). Ovx-Frontal Cortex ACh Vesicles (*A2*). Ovx-E3 Frontal Cortex ACh Vesicles (*A3*). 6Ovx-E21 Frontal Cortex ACh Vesicles (*A4*). Control Hippocampal Cortex ACh Vesicles (*B1*). Ovx- Hippocampal Cortex ACh Vesicles (*B2*). Ovx-E3 Hippocampal Cortex ACh Vesicles (*B3*). Ovx-E21 Hippocampal Cortex ACh Vesicles (*B4*). Control Temporal Cortex ACh Vesicles (*C1*). Ovx-Temporal Cortex ACh Vesicles (*C2*). Ovx-E3 Temporal Cortex ACh Vesicles (*C3*). Ovx-E21 Temporal Cortex ACh Vesicles (*C4*)

instance, estrogen was found to improve reference memory [27]. Likewise, estrogen was found to offset the impairment caused by neurotoxin in the working memory [28]. The mechanisms of these effects have been explained in several ways. The effects of estradiol on learning and memory depend on a variety of factors. The dose and route of administration of estrogen are both important [29, 30]. In our study 3-day administration of 10 µg/kg β-estradiol did not exhibit any effect on the parameters associated with working memory, while 21-day administration of the same dose significantly prevented the impairments found in the studied memory types after ovariectomy. These results are consistent with those of the studies cited above.

In the present study, learning experiments also included an assessment of the effect of acetylcholine. The number of acetylcholine vesicles in the examined brain areas (frontal, temporal cortices and hippocampus) decreased markedly due to ovariectomy, and 3-week 17β-estradiol treatment significantly restored these decreases. Estrogen was shown in various studies to be able to modulate neurotransmitters including monoaminergic, glutamatergic, and peptidergic systems. It was established in a study that acute estrogen administration prevented, while chronic estrogen improved learning and memory in a radial maze task and that chronic estrogen produced this effect by modulating monoaminergic and amino acid transmitters not in the hippocampus, but in

the frontal cortex and basal forebrain [31]. In another study where learning, memory, and a number of neurotransmitters in the hippocampus in ovariectomized rats were examined, it was found that the activity of one of these neurotransmitters, namely choline acetyltransferase, in the hippocampus decreased with ovariectomy and then increased with estrogen treatment [32]. Estrogen deficiency is known to reduce the brain blood flow and neurotransmitters or neurons. Estrogen replacement, on the other hand, was shown to modulate both structural and electrical activity in the hippocampus. Since it is known that physiological levels of estrogen can be modulated by prefrontal cortex and hippocampus to recover spatial working memory, recent studies have concentrated on hippocampus estrogen receptors. Estrogen replacement in ovariectomized rats was demonstrated to elevate choline acetyltransferase enzyme levels in the hippocampus and basal forebrain nucleus and to increase the high-affinity transportation of precursor choline [33].Similarly, Takuma et al. [34] reported that estrogen replacement prevents ovariectomy (Ovx) and chronic restraint stress (CS)-induced morphological and behavioral changes. Sex steroid hormones improve performance in some cognitive task by regulating cholinergic function [35]. This activation is occurring via activation of the estrogen receptor GPR30. Estrogen treatment can improve learning on specific task by activating GPR30 and enhancing ACh release in association with food reward [36]. However, different effects may arise from different types of estrogen. To cite one example, it was reported in a previous study that estron (E1) and estradiol produced different effects on choline acetyltransferase immunoreactivity (ChAT-IR) [37]. In the concerned study, E2 increased, but E1 did not cause any change in ChAT-IR in the basal forebrain areas. In our study, β-estradiol was used and this preparation caused a significant increase in acetylcholine levels, parallel to the study cited above.

Acetylcholine is co-present with an increase in working memory [7].The improvement brought about by estrogen in the working memory was reported to result not from modulation of the enzymes associated with acetylcholine synthesis, but from increased hippocampal synaptic plasticity [38]. The fact that estradiol improved the cognitive functions associated with the prefrontal cortex also lends support to our study results [39]. Partial loss of cholinergic neurons was shown to be able to reduce the effect of estrogen on cognitive performance [40]. The results of the concerned study also support our findings. According to previous results, it seems that (3-week) estrogen treatment following ovariectomy produces a positive effect on learning by enhancing cholinergic neurotransmission. Evidence from pharmacologic, electro physiologic, and behavioral studies unequivocally demonstrates that cholinergic hippocampus neurons are critical for the performance of tasks with significant spatial working memory components [41–43]. This study supports previous findings by demonstrating both an increase in the release of hippocampus ACh, and an improvement in spatial working memory, as consequences of sustained treatment with estradiol.

The can be said that limitation of study that firstly the potential effects of 17β-estradiol treatment were nor determined in control animals. Secondly, intact rats that received no procedure were evaluated for experiments.

However, the results of the study indicate that 3-week 17β-estradiol treatment has ameliorative effects on spatial, reference, and working memory in ovariectomized rats and increases acetylcholine vesicles in the examined brain areas. It is suggested that 17β-estradiol improves this effect on memory through the central cholinergic system.

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