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# Sex-Dependent Effects of Rivastigmine on Melatonin Receptors in Middle-Aged Rats

Nilüfer AKGÜN ÜNAL[1], Elif TÜRKDÖNMEZ AK[2], Ömer ÜNAL[3], Erdal AĞAR[4], Mustafa AYYILDIZ[4]

[1]Department of Biophysics, Faculty of Medicine, Ondokuz Mayis University, Samsun, Türkiye
[2]Department of Physiology, Faculty of Medicine, Ordu University, Ordu, Türkiye
[3]Department of Physiology, Faculty of Medicine, Samsun University, Samsun, Türkiye
[4]Department of Physiology, Faculty of Medicine, Ondokuz Mayis University, Samsun, Türkiye

## ABSTRACT

**Introduction:** Melatonin (5-methoxy-N-acetyltryptamine) is closely linked to the body's natural circadian rhythm. Dementia is significantly more common among older individuals. Rivastigmine (RIVA) functions as a reversible inhibitor of acetylcholinesterase and butyrylcholinesterase, prescribed to help alleviate the symptoms of dementia. The mechanism behind RIVA's neuroendocrine interactions remains unclear despite its efficacy in treating specific symptoms. Our research aimed to investigate the effects of RIVA on memory, anxiety, and the expression of melatonin receptor genes MT1 and MT2 in middle-aged, 13-month-old male and female rats.

**Methods:** Rats were administered intraperitoneal injections of 2 mg/kg RIVA or saline once a day for ten days in succession. Twenty-four hours after the final injection, rats underwent the Y-maze and elevated plus maze tests, after which tissue samples were collected for qPCR analysis.

**Results:** Behavioral tests revealed sex-specific responses; elevated plus maze test results indicated that RIVA did not alter exploratory behavior in female rats but decreased it in male rats compared to controls. At the same time, RIVA treatment resulted in significant decreases of MT1 and MT2 gene expression in the hippocampus of both sexes, but cortical expression showed different patterns in males and system.

**Conclusion:** RIVA treatment in middle-aged rats may cause changes in MT1 and MT2 gene expression concomitant with behavioral alterations. The variation in melatonin receptor expression across sexes indicates a potential sex-dependent impact in aging populations, suggesting an interaction between cholinergic and melatonergic systems.

**Keywords:** Aging, anxiety, cholinergic system, melatonin, memory, rivastigmine, sex

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## INTRODUCTION

Melatonin is an indoleamine with amphipathic characteristics, acting as a strong antioxidant and a regulator of circadian rhythms (1,2). The ageing process is marked by a gradual decrease in physical adaptability and disturbances in the body's natural sleep-wake cycle, resulting in a substantial decrease in the secretion of melatonin (1). The age-related decline in melatonin levels is frequently worsened in neurodegenerative disorders like Alzheimer's disease (AD) (3). Primarily MT1 and MT2 melatonin receptors, which are G-protein coupled receptors, are widely distributed in the central nervous system, including the hippocampus and prefrontal cortex, regions critical for cognitive function (4).

The central cholinergic system, responsible for memory, learning, and neuronal development, undergoes considerable degeneration as individuals age. Standard treatments for dementia include Acetylcholinesterase inhibitors (AChEIs), such as rivastigmine (RIVA), aiming to boost cholinergic transmission (6). RIVA, a reversible cholinesterase inhibitor medication, slows down the metabolism of Acetylcholine (ACh) and butyrylcholinesterase (BuChE). The main effect of RIVA is cholinergic potentiation, but its interactions with other neuroendocrine systems, especially the melatonergic system, need more research.

### Highlights

- **Rivastigmine modulates MT1/MT2 expression with sex-specific differences.**
- **Receptor-level changes align with sex-dependent behavioral variations.**
- **Melatonergic response differences suggest the need for sex-specific pharmacotherapy.**
- **MT1/MT2 plasticity may be pivotal in the cognitive effects of cholinergic treatment.**

**Correspondence Address:** Nilüfer Akgün Ünal, Ondokuz Mayis University, Faculty of Medicine, Department of Biophysics, Samsun, Türkiye • **E-mail:** nilufer.akgununal@omu.edu.tr
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Recent studies suggest a functional interplay between cholinergic signaling and melatonin receptors (7). For instance, cognitive impairment is often linked to abnormalities in both systems, and melatonin has been shown to modulate cholinergic function (8,9). However, there is a gap in the literature regarding how chronic cholinesterase inhibition affects melatonin receptor expression in middle-aged organisms, and whether this effect is sex-dependent. Considering that sex differences play a crucial role in the prevalence and pathology of neurodegenerative diseases, understanding these interactions is vital.

In summary, the present study aims to investigate the effects of a 10-day RIVA treatment on anxiety, memory, and the gene expression of MT1 and MT2 receptors in the hippocampus and cortex of middle-aged male and female rats. We hypothesized that chronic cholinergic stimulation would differentially alter melatonin receptor expression and behavioral outcomes in a sex-dependent manner.

## METHODS

### Experimental Animals and Groups

Following approval from the Ondokuz Mayıs University Animal Experiments Local Ethics Committee (2024/14), male (n=20) and female (n=20) Wistar rats, aged 13 months, were obtained from Ondokuz Mayıs University Experimental Animal Research Center (OMÜ DEHAM). Considering the lifespan of Wistar rats, 13-month-old animals were classified as middle-aged, representing the transition period to senescence (10). The rats were housed under standard conditions (22±1°C, 12h light/dark cycle) with ad libitum access to food and water. Animals were handled in accordance with the ARRIVE guidelines and institutional regulations.

Forty rats were randomly divided into four groups (n=10/group):

1. Male Control Group (MC): Saline treated.
2. Male RIVA Group (MR): RIVA treated (2 mg/kg).
3. Female Control Group (FC): Saline treated.
4. Female RIVA Group (FR): RIVA treated (2 mg/kg).

### Procedures

Rivastigmine tartrate was dissolved in sterile saline. Rats received intraperitoneal (i. p.) injections of RIVA (2 mg/kg) or saline once daily for 10 consecutive days. Behavioral tests were performed 24 hours after the last injection between 10:00 AM and 02:00 PM.

### Behavior Tests

The Y-maze test was used to assess spatial working memory and exploratory behavior (11). The apparatus consisted of three arms (A, B, and C). Parameters analyzed included the number of alternations, the number of entries into A, B, and the novel arm (C), and the time spent in these arms. The Elevated Plus Maze (EPM) was used to assess anxiety-like behaviors. The time spent in the open and closed arms and the number of entries were recorded during a 5-minute session.

### Gene Expression Analysis (qPCR)

Twenty-four hours after the behavioral tests, rats were anesthetized with Ketamine (90 mg/kg, i. p.; Alfasan, Woerden, The Netherlands) and Xylazine (10 mg/kg, i. p.; Alfasan, Woerden, The Netherlands) and sacrificed by decapitation. The right somatosensory cortex and hippocampus were rapidly dissected. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The purity and concentration of RNA samples were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was carried out using Power SYBR™ Green PCR Master Mix (Applied Biosystems, USA) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) instrument. The cycling conditions were: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A melting curve analysis was performed to verify the specificity of the products. All samples were run in triplicate. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (12), with GAPDH serving as the internal control. The calibrator group was the corresponding saline-treated control group of the same sex (Table 1).

**Table 1.** Primer sequences of target genes

| | |
|---|---|
| **MT1 F** | CCTCTACATCAGCCTCATCTGGCT |
| **MT1 R** | AGCCAGATGAGGCTGATGTAGAGG |
| **MT2 F** | GCCACAGTCTCAAGTATGATAGC |
| **MT2 R** | CCTATCATACTTGAGACTGTGGC |
| **GAPDH F** | ACCACCATGGAGAAGGCTGG |
| **GAPDH R** | CTCAGTGTAGCCCAGGATGC |

### Statistical Methods

Data were analyzed using GraphPad Prism (Version 10). Normality was assessed using the Shapiro-Wilk test. Since the study design involved two factors (Sex and Treatment), data were analyzed using Two-Way ANOVA followed by Tukey's post-hoc test for multiple comparisons. Results are presented as mean ± standard error of mean (SEM). p-value <0.05 was considered statistically significant.

## RESULTS

### Behavioral Findings

In the Y-maze test, RIVA treatment induced sex-specific alterations in exploratory behavior and locomotor activity. Two-way ANOVA revealed significant differences between groups. In male rats, RIVA treatment significantly decreased the total number of arm entries (MC: 9.4±0.96 vs. MR: 4.9±0.85; p<0.0001) and the number of alternations (MC: 5.1±0.84 vs. MR: 1.9±0.46; p<0.0001) compared to controls, indicating reduced locomotor activity and spatial working memory (Figure 1). Furthermore, males treated with RIVA showed a significant reduction in entries into the novel arm (Arm C) (MC: 3.1±0.31 vs. MR: 1.6±0.37; p=0.0003) and Arm B (MC: 2.6±0.37 vs. MR: 1.1±0.23; p<0.0001) (Figure 1). Conversely, female rats exhibited different baseline and treatment-related patterns. Female controls showed higher locomotor activity than male controls (FC: 13.4±0.75 vs. MC: 9.4±0.96). Following RIVA treatment, females also showed a decrease in total arm entries compared to their controls (FC: 13.4±0.75 vs. FR: 8.6±1.14).

The results of the Elevated Plus Maze (EPM) test are presented in Figure 2. Rivastigmine treatment significantly decreased the total number of entries into arms in males (MC: 6.0±0.79 vs. MR: 3.0±0.42; p=0.0041), further confirming the reduction in locomotor activity observed in the Y-maze. Specifically, RIVA-treated male rats showed significantly less entry into the open arm (MC: 3.4±0.40 vs. MR: 1.8±0.20; p=0.0017) (Figure 2). Regarding sex differences in anxiety-like behaviors, female controls spent significantly less time in open arms and considerably more time in closed arms (FC: 3.93±0.19 sec vs. MC: 1.7±0.24 sec) compared to male controls (p=0.0003). This pattern reflects a significantly higher baseline anxiety-like behavior in females compared to males (Figure 2). Rivastigmine treatment did not significantly alter the time spent in open or closed arms in males compared to their controls (MR Open: 3.31±0.47 sec; MR Closed: 1.69±0.47 sec).

### Gene Expression Findings

qPCR analysis demonstrated that RIVA significantly altered melatonin receptor expression levels in a region- and sex-dependent manner (Figure 3). In the hippocampus, MT1 receptor expression was significantly downregulated in the male RIVA group compared to controls (MC: 1.007±0.127 vs. MR: 0.110±0.003; p<0.001). Regarding MT2 receptors in the hippocampus, RIVA treatment led to a decrease in males (MC: 1.022±0.229 vs. MR: 0.617±0.029; p<0.05). In females, MT2 expression levels were decreased in the treatment group compared to the control group (FC: 0.309±0.056 vs. FR: 0.021±0.006). In the cortex, a distinct sex-dependent pattern was observed. MT1 expression was significantly decreased in RIVA-treated males (MC: 1.003±0.087 vs. MR: 0.091±0.023; p<0.001), whereas it was increased in RIVA-treated females (FC: 1.023±0.252 vs. FR: 1.372±0.114; p<0.05). For MT2 receptors in the cortex, RIVA treatment caused a significant downregulation in both males (MC: 1.001±0.050 vs. MR: 0.065±0.011; p<0.001) and females (FC: 1.014±0.184 vs. FR: 0.287±0.031; p<0.05) compared to their respective saline-treated controls (Figure 3).

## DISCUSSION

This study examined the impact of RIVA on behavioral traits and melatonin receptor expression in middle-aged rats. A 10-day intraperitoneal regimen of RIVA, an acetylcholinesterase inhibitor, produced varying effects on exploratory and cognitive behavior in the Y-maze, and these effects differed between male and female rats, as shown in Figure 1.

Research indicates that female rodents tend to be more exploratory in new or mildly challenging environments, a notion backed by the

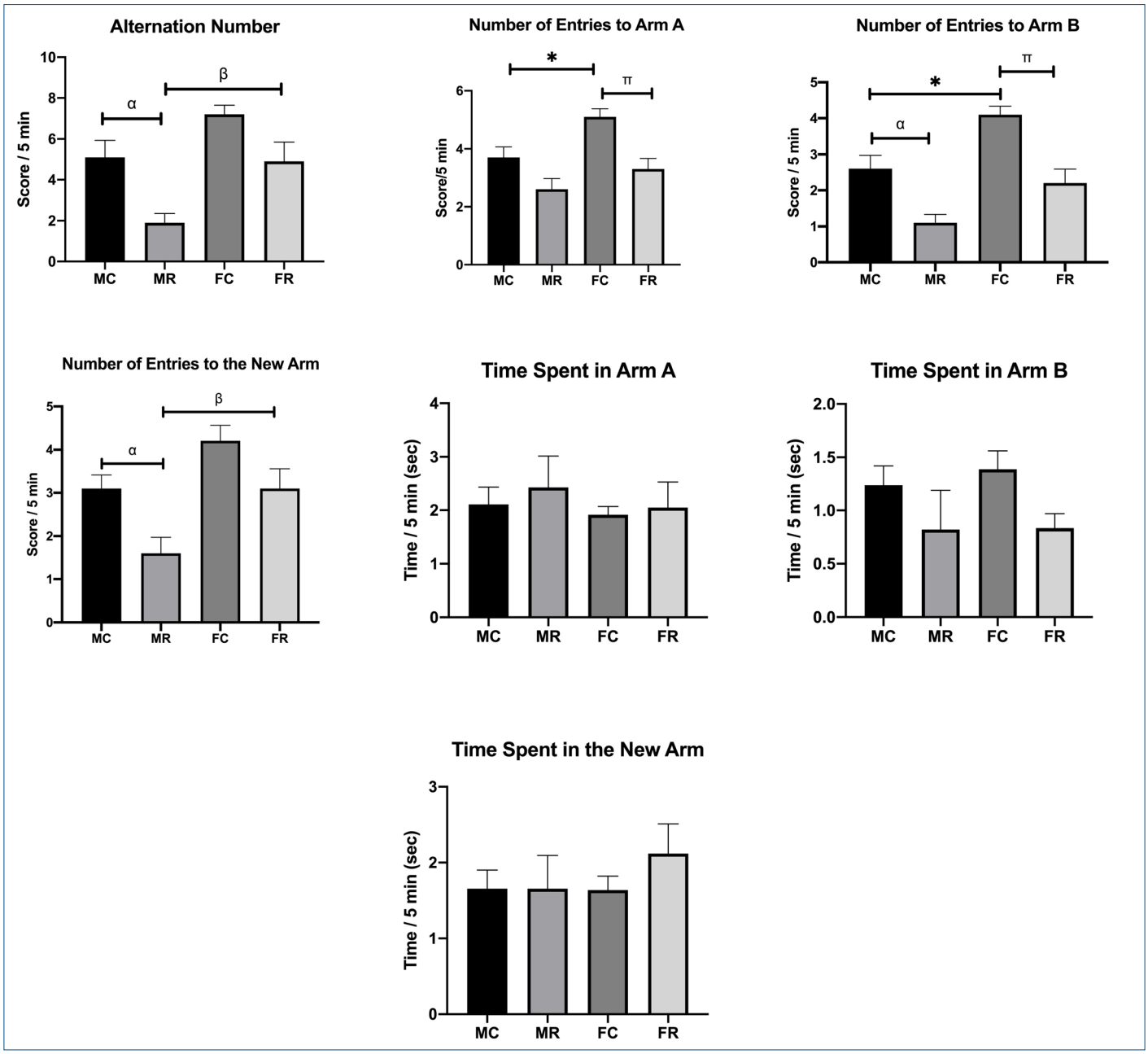


**Figure 1.** Y maze test results for all groups. Values are shown as mean ± SEM. Effects of 2 mg/kg RIVA treatment for ten days on Y-maze test parameters in male RIVA (MR) (n=10); male control (MC) (n=10); female RIVA (FR) (n=10); and female control (FC) (n=10) groups Wistar rats (α: MC and MR groups; β: MR and FR groups; *: MC and FC groups; π: FC and FR groups indicate statistical significance at the p<0.05 level)

observation that control female rats entered arms A and B significantly more times than their male counterparts ($p<0.05$). Evidence suggests that estrogen has a significant impact on both hippocampal and cholinergic function, which may result in enhanced cognitive flexibility and exploration capabilities (13). Studies have shown that estrogen can improve exploratory behavior and spatial learning, potentially by increasing the likelihood that females will investigate more than one path in a maze (14). One possible explanation for the drop in entries among females in Arm A and in both sexes in Arm B is the rise in cholinergic transmission that was already being stimulated by estrogen following RIVA administration in females, as illustrated in Figure 1. In essence, given that females already possess high levels of cholinergic transmission, the additional increase reduces the variability or spontaneity of exploratory behaviour (15).

In males, the number of entries in arm B was reduced compared to controls, suggesting that these abilities are inherently weaker in males compared to females. Research suggests that cholinesterase inhibitors can have far-reaching impacts on behaviour, sometimes leading to reduced activity when the cholinergic system is overwhelmed beyond its optimal capacity. In male subjects, a decline in novel arm entries, arm C performance, and overall alternation scores –which are indicative of spatial working memory– following RIVA (Figure 1) seems to occur due to an over-modulation of cholinergic neurotransmission, thereby disrupting the balance necessary for peak exploration and cognitive functioning. Y-maze alternation behavior is commonly used to evaluate working memory and cognitive flexibility, revealing that males often lack sufficient cholinergic neurotransmission, which limits their performance to that seen in females, whose baseline cholinergic modulation differs naturally. Research has shown that there is differential sensitivity, based on studies that demonstrate sex-specific reactions to cholinergic substances. Okada et al. found that overstimulation of the cholinergic system occasionally results in impaired memory performance in men as noted in (16). The arm entry times indicate that neither the motivation to investigate nor the rats' overall motor abilities were influenced by the treatment. The alteration in arm entry and alternation behaviors is mainly caused by cognitive or cholinergic modulation, rather than a general sedative or motor effect (17).

Female rats exhibit a greater tendency to explore the Y-maze due to estrogen's increased effect on both the cholinergic system and spatial memory pathways. In female subjects, the enhancement of cholinergic function caused by RIVA goes beyond what is considered optimal, leading to a reduction in exploratory entries, as seen in arm A where the probability of the observed difference is less than 0.05 (Figure 1). In contrast, males, who have lower baseline cholinergic transmission than females, show a decline in exploratory behavior and working memory performance, particularly in arm B entries and switching to new areas, due to abnormal cholinergic signaling modulation. Studies suggest that estrogen and acetylcholine have a considerable impact on the regulation of exploratory behavior and memory (18–20). Joue et al. (2024) investigated the effects of estrogen on spatial behavior (21), and Mesulam (2004) provided insights into the impact of cholinesterase inhibitors and the importance of optimal cholinergic levels for cognitive function (22). The combined findings suggest that natural sex variations and the use of medication to modify the cholinergic system can interact to yield different outcomes in tasks assessing spatial memory and exploratory behavior.

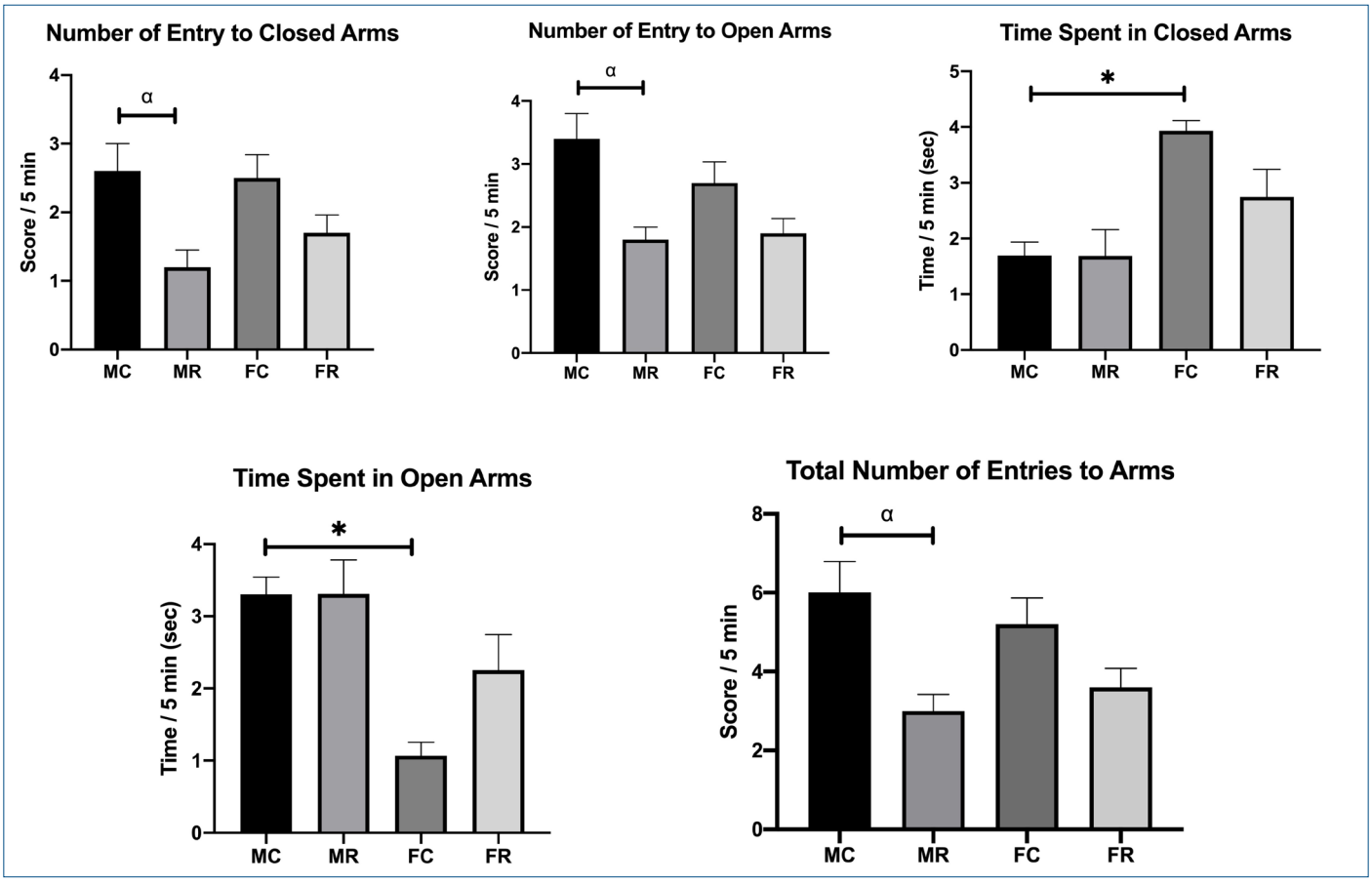


**Figure 2.** Elevated plus test results for all groups. Values are shown as mean ± SEM. Effect of ten days 2 mg/kg RIVA injection on elevated plus test parameters in male RIVA (MR) (n=10); male control (MC) (n=10); female RIVA (FR) (n=10); and female control (FC) (n=10) groups Wistar rats (α: MC and MR groups; *: MC and FC indicate statistical significance at the $p<0.05$ level).

The results of the elevated plus maze test show that there are sex differences in anxiety-like behaviors and that RIVA affects these behaviors by regulating the cholinergic system. In male rats treated with RIVA, there was a significant decrease in the total number of arm entries ($p<0.05$), as seen in Figure 2. The drug seems to have a sedative or inhibitory effect on male movement or exploratory drive as a result of over-stimulation of the cholinergic system, which could be due to excessive stimulation. Rivastigmine increases the availability of acetylcholine by preventing its breakdown. Optimal cholinergic activity is required for normal exploratory behavior, but overstimulation can lead to either behavioural inhibition or anxiety-like states. Studies that have shown cholinergic overstimulation are in agreement that it can lower overall activity levels (23). Studies suggest that cholinesterase inhibitors may decrease overall alertness or cause motor slowing, a symptom observed with other substances affecting the cholinergic system (24). A decline in overall activity could be responsible for the lower total number of entries into the maze (Figure 2).

Female rats spent less time in the open arms and more time in the closed arms than males ($p<0.05$), indicating higher anxiety-like levels in females under standard conditions. Studies have shown that variations in estrogen levels can influence anxiety-like behaviors. Research has shown that estrogen has anxiolytic effects, yet other studies suggest that females exhibit increased anxiety-like behaviors in the elevated plus maze under certain conditions or at distinct stages of the estrous cycle (25). Strain variations and test conditions can lead to different anxiety-like responses in males and females. Numerous studies have documented a pattern, in which females tend to select the closed arms, indicating a more cautious or risk-averse approach to coping with stress. RIVA's impact is more noticeable in males, particularly in terms of decreased arm entries ($p<0.05$), although its effects can differ depending on individual anxiety levels within each sex. Reduced general activity in male rats, involving entries into both open and closed arms, suggests a direct depressant effect on exploratory behaviors resulting from excessive stimulation of the cholinergic system. Female subjects showed higher baseline anxiety levels, indicated by increased time spent in the closed arm and reduced time spent in the open arm of an anxiety task (Figure 2), which was found to be influenced by changes in cholinergic activity. In RIVA, a female's primary behavior is primarily due to her naturally anxious state, potentially linked to hormonal effects and differing receptor sensitivities (26).

This study of cholinergic systems in aging and dementia underscores the fine balance necessary in cholinergic neurotransmission processes.

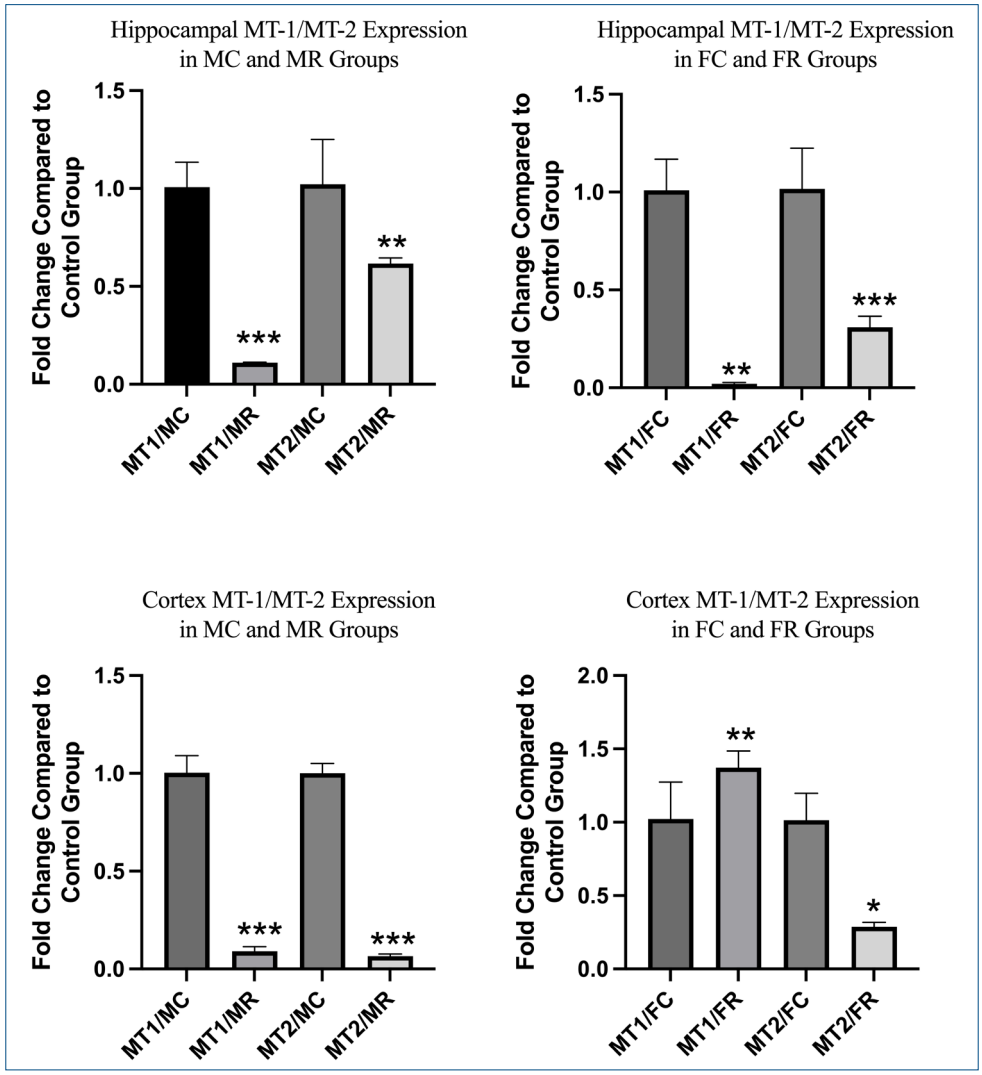


**Figure 3.** Rivastigmine or saline treatment on mRNA expression in the right hippocampus and cortex tissues of each gender rats. The mRNA levels of a MT1 and MT2 were quantified by relative Ct (ΔCt) method and normalized against GAPDH gene ΔCt and presented as relative expressions in comparison to the saline-treated control groups. * $p<0.05$ ** $p<0.01$ *** $p<0.001$. Values are shown as mean ± standard error.

The overactivation seen in the use of cholinesterase inhibitors in certain situations results in decreased exploratory actions and altered levels of anxiety (22). Research on estrogen's impact in rodent anxiety models, as outlined in Walf and Frye (2007) shows that hormonal influences can result in shifts in anxiety-like behaviors (25), a notion supported by the differences found between male and female rats in the current study. Research on sex differences in response to cholinesterase inhibitors has found that females may display varying behavioral responses, possibly due to disparities in receptor density or sensitivity (26). According to study employing the elevated plus maze, elevated anxiety-like behavior in rodents typically correlates with fewer open arm entries and longer durations spent in the closed arms (27). Regarding the specific effects observed, RIVA decreases exploratory behavior in males, as indicated by a lower number of entries overall. This could be indicative of cholinergic overstimulation, which results in reduced overall activity. Data showed that females spent more time in enclosed spaces and less time in open areas, suggesting heightened anxiety-like behaviors. Estrogen and varying levels of sensitivity to cholinergic receptors may be factors in this influence. Previous studies suggest that females exhibit distinct responses to cholinergic modulation compared to males, although the precise impact of RIVA on females in the elevated plus maze remains uncertain beyond baseline variations (28). Considering these findings in their entirety, it can be deduced that both pharmacological manipulation through RIVA and natural sex disparities influenced by hormonal factors significantly contribute to the behaviours observed in the elevated plus maze.

Research on melatonin receptor expression changes following RIVA treatment suggests complex relationships and sex-specific results between the cholinergic system and melatonin pathways (Figure 3). Expression of MT-1 and MT-2 receptors was decreased in the hippocampus in both sexes. RIVA-enhanced cholinergic activity leads to a reduction in melatonin receptor expression in the hippocampus, with significant differences found between males and females. Research has shown that interaction between acetylcholine and melatonin signalling pathways contributes to both memory formation and hippocampal plasticity (7). The hippocampus, a brain structure that closely interacts with other involved brain systems, is crucial to both spatial memory and cognitive function. Reduced expression of both MT-1 ($p<0.001$) and MT-2 ($p<0.001$) receptors in cortical tissue in males mirrors the pattern seen in the hippocampus, suggesting a consistent cholinergic influence in brain regions of males. The distinct response seen in cortex tissue in females, marked by increased levels of MT-1 ($p<0.01$) and decreased levels of MT-2 ($p<0.05$), may be associated with sex-specific differences, the influence of hormones on receptor expression, particularly estrogen, and varying basal cholinergic transmission in males and females.

Differences in melatonin receptor regulation between sexes suggest that melatonin may enhance cholinergic function and vice versa (29). The observed differential regulation probably serves as a compensatory mechanism to guarantee that the optimal signalling balance is preserved. The unique female cortex pattern, marked by increased levels of MT-1, suggests that estrogen could influence how cholinergic enhancement affects melatonin receptor expression. Studies have shown that estrogen can affect both cholinergic and melatonergic pathways (8). The downregulation of melatonin receptors in the hippocampus is consistent, whereas this consistency does not apply to the cortex, suggesting that these receptors are regulated differently across various brain regions, which is likely due to their distinct functional roles (30). The findings from these results may help explain why the effectiveness of cholinesterase inhibitors differs between males and females in actual clinical settings. Understanding the interactions involved in conditions like Alzheimer's disease, particularly when both the cholinergic and melatonergic system are affected, is also necessary for optimising treatments. The differential regulation of MT-1 and MT-2 expression in the female cortex suggests that distinct mechanisms control these receptor subtypes, which could be regulated by different transcription factors or elements that respond differently to cholinergic stimulation. These studies' outcomes improve our understanding of how a cholinergic increase resulting from RIVA treatment affects melatonin receptor expression in a pattern that differs by sex and anatomical site. Research suggests a complex interlinking of the cholinergic and melatonergic system, and this interlinking may have substantial implications for grasping sex-related disparities in cognitive capacity and treatment results.

This study has certain limitations. First, the estrous cycle of the female rats was not monitored. Given that estrogen levels fluctuate during the cycle and can influence cholinergic sensitivity, this is a factor that should be controlled in future studies. Second, while 13-month-old rats represent a middle-aged population, further studies with senescent rats are needed to fully model geriatric dementia. The varying responses seen in the female cortex emphasize the significance of taking sex into account as a biological factor in research and clinical settings. Future studies could concentrate on comprehending the molecular processes underlying these sex-specific variations and their implications for cognitive performance and anxiety-like behaviors.

As a result, the present study demonstrates that sub-chronic rivastigmine (RIVA) treatment modulates behavioral parameters and melatonin receptor expression in a sex-dependent manner in middle-aged rats. Our findings indicate that RIVA treatment significantly reduces exploratory activity and locomotor behavior, particularly in males, while highlighting distinct anxiety-like behavioral patterns between sexes. At the molecular level, RIVA administration led to a consistent downregulation of MT1 and MT2 receptors in the hippocampus of both sexes. However, a striking sex-specific divergence was observed in the cortex, where females exhibited an upregulation of MT1 receptors contrast to the downregulation seen in males.

To our knowledge, this is one of the studies to provide preclinical evidence of a region– and sex-specific interaction between the cholinergic system and melatonergic receptors following cholinesterase inhibitor treatment. These results suggest that the therapeutic effects and potential side effects of cholinomimetic drugs may vary significantly between males and females due to differential regulation of neuroendocrine pathways. Consequently, sex should be considered a critical biological variable in the development of pharmacological strategies for neurodegenerative diseases. Future studies incorporating senescent models and estrous cycle monitoring are warranted to further elucidate the mechanisms underlying these complex neuroendocrine interactions.

**Ethics Committee Approval:** The study protocol was approved by Ondokuz Mayıs University Animal Experiments Local Ethics Committee (2024-14). This research was conducted on animals (rats).

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Concept- NAÜ, ETA, ÖÜ; Design- NAÜ, MA, ÖÜ; Supervision- MA,EA, NAÜ; Resource- MA, EA, ETA; Materials- NAÜ, ÖÜ, MA; Data Collection and/or Processing- NAÜ, ETA, ÖÜ; Analysis and/or Interpretation- NAÜ, ETA, ÖÜ; Literature Search- NAÜ, ÖÜ, MA; Writing- NAÜ, MA, ÖÜ; Critical Reviews- MA, EA, NAÜ.

**Conflict of Interest:** The authors declared that there is no conflict of interest.

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