

Research Paper



Investigating Olfactory Sensory Neurons Facilitation For Aerobic Exercise-induced Spatial Memory Improvement

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ABSTRACT

Introduction: The positive effects of exercise on spatial memory and learning have been demonstrated in research. The olfactory sensory neurons (OSNs) respond to mechanical stimulation induced by nasal airflow which is associated with airflow intensity. Accordingly, nasal breathing can modulate brain oscillations in nonolfactory areas, and respiration-entrained oscillations aid the improvement of cognitive abilities. Given that aerobic exercise increases the rate of respiration and intensity of nasal airflow, this study evaluates the role of OSNs in mediating the effects of aerobic exercise on memory.

Methods: We examined spatial memory following exercise in animal models of olfactory sensory neuron impairment (methimazole injection 300 mg/kg/week).

Results: Destroying OSNs significantly reduces olfactory bulb (OB) activity at delta and theta frequency bands as well as its coupling to respiration. More importantly, it abolished the positive effect of exercise on spatial memory ($P < 0.05$).

Conclusion: The OB activity is one of the probable mechanisms for improving spatial memory following exercise.

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Highlights

- Breathing through the nose has many health benefits, particularly in exercise.
- Exercise improved spatial memory and the destruction of nasal airflow stimulates the olfactory sensory neurons (OSNs) has reduced this effect.
- Exercise improves cognitive results by increasing respiration rate and intensity.
- Exercise can non-invasively stimulate the olfactory bulb (OB) in the brain.

Plain Language Summary

Exercise has been shown to improve spatial memory and learning. This study explores how olfactory sensory neurons, which respond to mechanical stimulation from nasal airflow, contribute to this process. When we breathe through our noses, the airflow can influence brain activity in areas not directly related to smell. These respiration-related brain oscillations enhance cognitive functions. Given that aerobic exercise increases breathing rate and nasal airflow intensity, this study aimed to understand the role of olfactory sensory neurons in the memory benefits of exercise. Our findings suggest that the stimulation of the olfactory bulb (OB) during exercise is a key mechanism through which exercise improves spatial memory.

1. Introduction

Exercise and physical activity are inescapable necessities for health (Bherer et al., 2013). Various studies have shown that physical activity plays an essential role in brain health (Bherer et al., 2013; Di Liegro et al., 2019). In the meantime, animal and human studies provide reasonable evidence that exercise improves cognitive performance and reduces the risk of dementia and cognitive impairment (Weuve et al., 2004). For example, our previous studies showed beneficial effects of exercise on A β -induced learning and memory impairment by up-regulation of the PGC-1 α /FNDC5/BDNF pathway and increasing the A β clearance (Azimi et al., 2018; Khodadadi et al., 2018). While evidence supports the positive effects of exercise on cognitive performance, little is known regarding the mechanisms that may mediate this phenomenon.

Exercise increases the expression of neurotrophic factors in the brain and improves cognitive function by promoting neural plasticity, neurogenesis, and angiogenesis (Liu et al., 2019). Recently, it has been suggested that direct nerve stimulation to the brain during exercise may also be related to the beneficial effects of exercise on the brain (Liu et al., 2019). Exercise has been reported to promote rodents' learning and memory performance and reverse memory and spatial learning impairment in old animals (Cefis et al., 2019). Therefore, several path-

ways are involved in these mechanisms, and exercise is a complex neurobiological modulator that involves interactions between the brain and the environment (Liu et al., 2019). However, there are still many barriers and challenges to modulating learning and memory in the exercise research trajectory.

The respiratory system plays a critical role in all types of exercise. Breathing through the nose and mouth during physical activity is controversial in exercise science, and extensive research has been done in this regard (Bennett et al., 2003). Breathing through the nose has many health benefits, particularly during exercise. These include heating, humidifying, and purifying/filtering the air in preparation for delivery to the lungs (Allen, 2017). Nose versus mouth breathing induces 50% more resistance to the air stream, causing 10% to 20% more oxygen uptake and improving the overall pulmonary volume (Allen, 2017). Also, previous research has shown changes in brain oscillatory activities and improved cognitive performance after yoga interventions associated with specific breathing exercises (breathing from the nose) (Desai et al., 2015). Therefore, breathing through the nose during exercise can affect brain function and behavior.

Breathing is one of the primary rhythms generated by the brain and is a critical signal in synchronizing brain activities (Bagur et al., 2021; Chi et al., 2016). Earlier investigations have demonstrated that the respiratory cycle

has a critical role in information processing (Kepecs et al., 2006). Nasal airflow stimulates the olfactory sensory neurons (OSNs) linked to the olfactory bulb (OB) and other brain regions, like the entorhinal cortex, dentate gyrus, and CA1 in the hippocampus, prefrontal cortex, and amygdala (Grosmaître et al., 2007). Local field potentials (LFPs) from the OB exhibit a prominent rhythm entrained by respiration which depends on nasal airflow, called respiration-entrained brain rhythm (Vanderwolf & Szechtman, 1987). Studies show that OB delivers the respiration-entrained brain rhythm to different brain regions (Bagur et al., 2021; Yanovsky et al., 2014). Also, different brain regions, especially the hippocampus, show reduced activity during intubation due to eliminating nasal airflow for a long time, and this might lead to cognitive disorders (Girard et al., 2010).

On the other hand, stimulation of the OSNs using air-puff leads to excitation of the OB, even when the animal cannot breathe intentionally through the nose, which improves working memory performance (Ghazvineh et al., 2021). The OSNs respond to the mechanical stimulation induced by airflow, which correlates with airflow intensity (Grosmaître et al., 2007). Therefore, any changes in peripheral signals cause severe changes in the function of the nervous system, and nasal breathing can improve cognitive functions, specifically learning and memory, through entraining brain oscillations leading to synchronized network activity (Zelano et al., 2016).

Overall, exercise can non-invasively stimulate the OSNs by enhancing the nasal airflow intensity due to increased airflow and respiratory rate. Therefore, we hypothesized that aerobic exercise synchronizes oscillations in different brain regions by stimulating OSNs, which might effectively improve spatial memory during exercise. Accordingly, we destroyed the nasal epithelium to investigate the modulatory effect of OSNs on aerobic exercise-induced spatial memory improvement.

2. Material and Methods

Study animals

A total of 32 (8 rats in each group) adult male Wistar rats (weight=250-300 g) were obtained from the Pasteur Institute (Tehran, Iran). The rats were housed in standard cages in a climate-controlled room (temperature=23±2°C with 50% to 55% humidity) with a 12 h light/dark cycle. Food and water were available sufficiently.

Surgery and LFPs recording

We used intraperitoneal injections of ketamine (100 mg/kg) and xylazine (10 mg/kg) for anesthetization in rats and confirmed anesthesia induction by the absence of the hind limb withdrawal reflex. After confirming the anesthesia, the animal's head was in a flat skull position fixed inside the stereotaxic device (Narishige, Japan), and the scalp was cut along the midline of the skull surface. Then, stainless-steel recording electrodes (127 µm in diameter, A.M. System Inc., USA) were implanted unilaterally into the right hemisphere of the OB (anteroposterior=8.5 mm, mediolateral=-1 mm, dorsoventral=-1.5 mm) (Paxinos & Watson, 2006). Also, we used a stainless-steel screw as a reference and placed it at the right parietal bone. The scalp skin was treated with antibiotics (tetracycline). Small pins were connected to the electrodes and then inserted into a small socket. The whole structure was fixated on the skull with dental acrylic cement (Figure 1) (Dehdar et al., 2019).

One week after surgery, the animals were divided into two exercise (exercise saline [ES] and exercise methimazole [EM]) and two control groups (control saline [CS] and control methimazole [CM]). At the beginning of each week, one exercise and one control group to destroy the OSNs received 300 mg/kg methimazole (Genter et al., 1995), and the other two groups received saline. Also, 30 min before methimazole injection, the animals were injected with thyroxine (10 µg) to prevent the effects of methimazole on metabolism (Bergman & Brittebo, 1999). At the end of each week, LFPs were recorded for 15 min. For this purpose, the socket, fixed to the animal's head, was connected to a miniature buffer head-stage with high-input impedance (BIODAC-A, TRITA Health Tec., Tehran, Iran), via cables to a main AC coupled amplifier (1000 amplification) and the recording system (BIODAC-ESR18622, Health Tec., Tehran, Iran). Spontaneous LFPs of OB were recorded, low-pass filtered<500 Hz, digitized at 1 kHz, and stored for offline analyses with custom-written MATLAB routines (The Mathworks, Inc.). A video tracker was used to record the movement of rats to detect awake immobility periods (Dehdar et al., 2019). Also, after seven weeks of aerobic exercise to measure spatial memory, the Barnes behavioral test was performed (Figure 1).

Treadmill exercise

The rats were placed on the rodent treadmill (Tajhiz Gostar Omid Iranian, Tehran, Iran) for moderate regular exercise. Endurance training instructions included seven weeks of progressive running on a treadmill at a speed

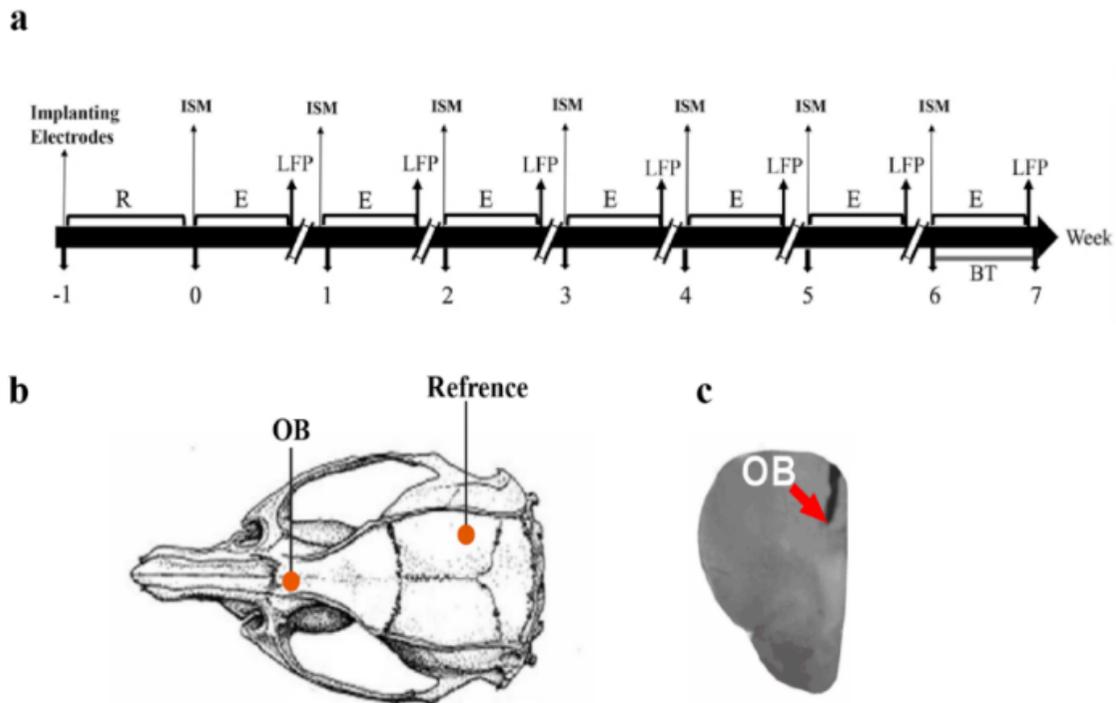


Figure 1. Experimental design

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A) The timeline of the study design (the rats received intraperitoneal injections of saline or methimazole (300 mg/kg) every week and LFP was recorded at the end of any seven weeks after the last session (five days/week) of exercise). B) A schematic representation of the electrode implantation site on the rat head, C) The histological confirmation of a recording site in OB

Abbreviations: R: Recovery; E: Exercise; LFP: Local field potential; ISM: Injection saline or methimazole; BT: Behavioral tests; OB: Olfactory bulb.

of 15-23 m/min for 15-50 min per day and five days per week. The training period was divided into three stages as follows: Familiarization, overload, and maintenance. In the familiarization phase, the rats were forced to run at the speed of 10-15 m/min for 15 min for five days. The animals that could not run were left out of the study. In the overload phase, the time increased to 30 min in the first week and reached 18 m/min in the second week. In the third and fourth weeks, the time reached 50 min, and the speed reached 20 m/min. In the fifth and sixth weeks, it maintained with increasing speed to 23 m/min. This protocol was a moderated exercise, and it is expressed that oxygen consumption in this situation is about 55%-65% for the rats (Chae et al., 2011). The angle of inclination was 0%. The rats in the control groups received no exercise.

Behavioral tests

The Barnes maze was used to measure spatial memory in mice and rats. This maze is made of a circular plate made of black plexiglas, which is 92 cm in diameter. Around this maze, at a distance of 2 cm from the edge

of the maze, 18 holes with a diameter of 8 cm have been installed. A black plexiglas escape box was located under one of the holes (target hole). On the familiarity day, the rats were allowed for 90 s to explore the field freely. After going into the target hole, the rats stayed in the escape box for 30 s. If they did not find the target hole, the experimenter would guide them to the target hole. The training phase included 4 trials per day for four consecutive days. One day after the last training session memory test was taken, and the time spent around the target hole was measured. After each trial to remove odor effects, the maze was carefully cleaned with a 70% alcohol solution. The Auto Vision software, version 2015 was used to analyze the Barnes behavioral test.

Cresyl violet histochemistry

To elucidate the effects of methimazole on OSN destruction, the OB sections of the rats were stained with cresyl violet. In summary, the sections were mounted on gelatin-coated microscopy slides and were counterstained with 0.1% cresyl violet (Merck, Germany), and then dehydrated in increasing alcohol concentrations.

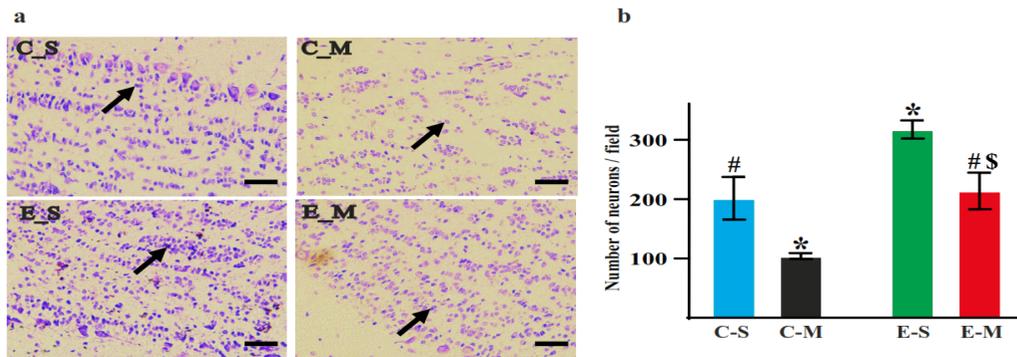


Figure 2. Aerobic exercise increasing the number of neurons

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A) Cresyl violet staining of the OB in four groups (scale bar=20 μ m) (numerous granule cells (\dagger) can be observed), B) Neuronal count of cresyl violet stained tissues.

Notes: Values are expressed as Mean \pm SEM. The data were analyzed by the two-way ANOVA, followed by Tukey post hoc test; *Significant compared to the C-S group, #Significant compared to the E-S group, \$Significant compared to the C-M group.

Abbreviations: C-S: Control saline; C-M: Control methimazole; E-S: Exercise saline; E-M: Exercise methimazole.

The tissues were cleared in xylene, mounted, and then cover-slipped. All the samples were captured at 40 \times magnification, and the neuronal count was measured using the ImageJ software version 1.53e (number of neurons/field), and statistical comparisons were made among the groups.

LFPs and statistical analysis

In this study, the power spectral density of OB signals was computed using the “pwelch” function in MATLAB software, version R2020a. We computed cross-correlation coefficient values for the filtered signals by MATLAB “xcorr” function, with the “coeff” option to normalize values between 1 and -1. For this purpose, respiration and OB signals were bandpass filtered in the respiratory frequencies range. Also, the statistical analysis was performed by GraphPad Prism software, version 8.4.3. (GraphPad Software, USA). The achieved results were compared by t-test, two-way, and three-way analysis of variance. Meanwhile, we used the Bonferroni test for the analysis of variance post hoc testing. We used the Cohen d test to measure effect size. The data are shown as Mean \pm SEM Also, $P < 0.05$ were considered statistically significant.

3. Results

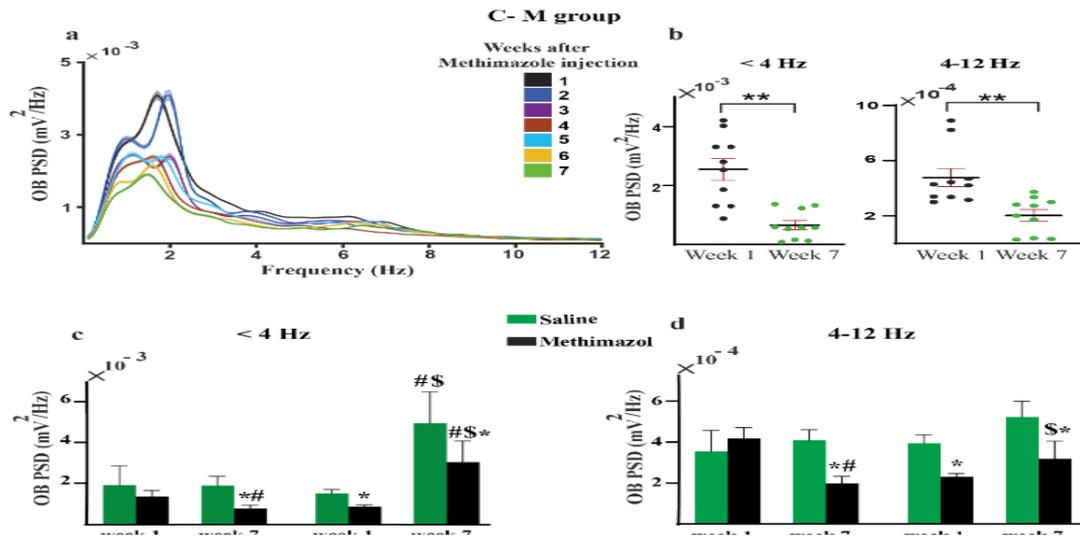
Destruction of the OSNs by methimazole injection

An apparent increase in the number of cells was evident. Histological images showed an increased number of neurons in the ES group compared to the CS group

(Figure 2a). Also, statistically, the granule cell number increased in the ES group compared to other groups, indicating that exercise can increase the number of neurons ($P=0.01$, $d=2.09$). The granule cell population significantly decreased in the CM group, showing that methimazole could decrease the number of neurons ($P=0.03$, $d=1.88$) (Figure 2b). When we eliminated the OSNs, exercise could compensate for this decrease in the EM group ($P=0.01$, $d=2.47$) (Figure 2). The results show that exercise increases the number of neurons, and methimazole leads to the destruction of OSNs.

Increase in power spectral density with increased non-invasive stimulation of the OSNs by aerobic exercise

Power spectral density (PSD) analysis of LFPs showed a significant decrease in the mean delta (<4 Hz) and theta (4–12 Hz) power of OB in the CM group during seven weeks, and this decrease was significant between weeks one and seven ($P_{\text{delta}}=0.0025$ and $P_{\text{theta}}=0.24$) (Figures 3a and 3b). Comparison between weeks one and seven show that exercise increased the PSD in the ES and EM groups, while there was no significant difference in PSD between the first and seventh weeks in the CS group. Additionally, in week seven, a significant difference in PSD was observed between the ES and EM groups (Figures 3c and 3d). Thus, exercise improved the power of delta and theta frequencies. In comparison, methimazole injection prevented this increase.



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Figure 3. a) The power comparison of delta (<4 Hz) theta (4-12 Hz) and OB frequencies in seven weeks (the power of OB decreased during a seven-week period), b) The statistical comparison of delta and theta frequencies (the power of OB in delta and theta frequencies significantly decreased in the 7th week compared to the first week, c, d) A comparison of delta (<4 Hz) and theta (4-12 Hz) in 4 groups (the exercise increased power in the 7th week compared to the 1st week, and methimazole prevented this increase

Notes: Values are expressed as the Mean±SEM. The data were analyzed by t-test in the methimazole group and three-way repeated-measures ANOVA in weeks one and seven; **P<0.01, *Significant difference with saline, #Significant difference with week one, \$Significant difference with the control group. Abbreviations: PSD: Power spectral density; OB: Olfactory bulb; C-S: Control saline; C-M: Control methimazole; E-S: Exercise saline; E-M: Exercise methimazole.

Increase in respiration-OB synchronization by exercise

For the cross-correlation analysis to further explore the impact of exercise on the relationship between respiration and OB activity, we calculated cross-correlation by computing the mean correlation of respiration and OB LFPs.

The mean correlation for the respiratory-OB filtered signals was significantly increased in the ES group (P=0.003, d=2.29) (Figure 4c). Methimazole decreased the cross-correlation in week seven compared to week one (P=0.0002, d=2.49) (Figure 4b). Meanwhile, there was a significant difference between the saline and methimazole control groups (Figure 4e). No significant cross-correlation difference was found between weeks one and seven in the CS group (P=0.9) (Figure 4a).

Also, representative traces of LFP from OB and breathing were shown in four groups. In the CS group, OB oscillation was coupled with animal respiration, while this coupling was not seen in the CM group (Figures 4f and 4g)

Destroyed OSNs impair learning and spatial memory

The Barnes behavioral test results in the training trial showed a significant difference in escape latency and latency to first between groups (a, b). Also, a significant difference in escape latency was observed between days one and seven in the exercise groups (Figures 5a and 5b).

Statistical comparison of the Barnes behavioral test showed that the ratio of hole exploration frequency in the goal sector (GS) to hole exploration frequency in the non-goal sector (NGS) decreased in the CM group (P=0.002, d=4.3). In the ES group compared to the CS group, there was a significant increase in the goal sector preference (GS/NGS) (P=0.005, d=2.14) (Figure 5d). Also, In the ES group compared to the EM group, there was a significant increase in the GS/NGS (P=0.0002, d=5.3).

4. Discussion

Our results indicated that aerobic exercise improves spatial memory, consistent with previous reports (Inoue

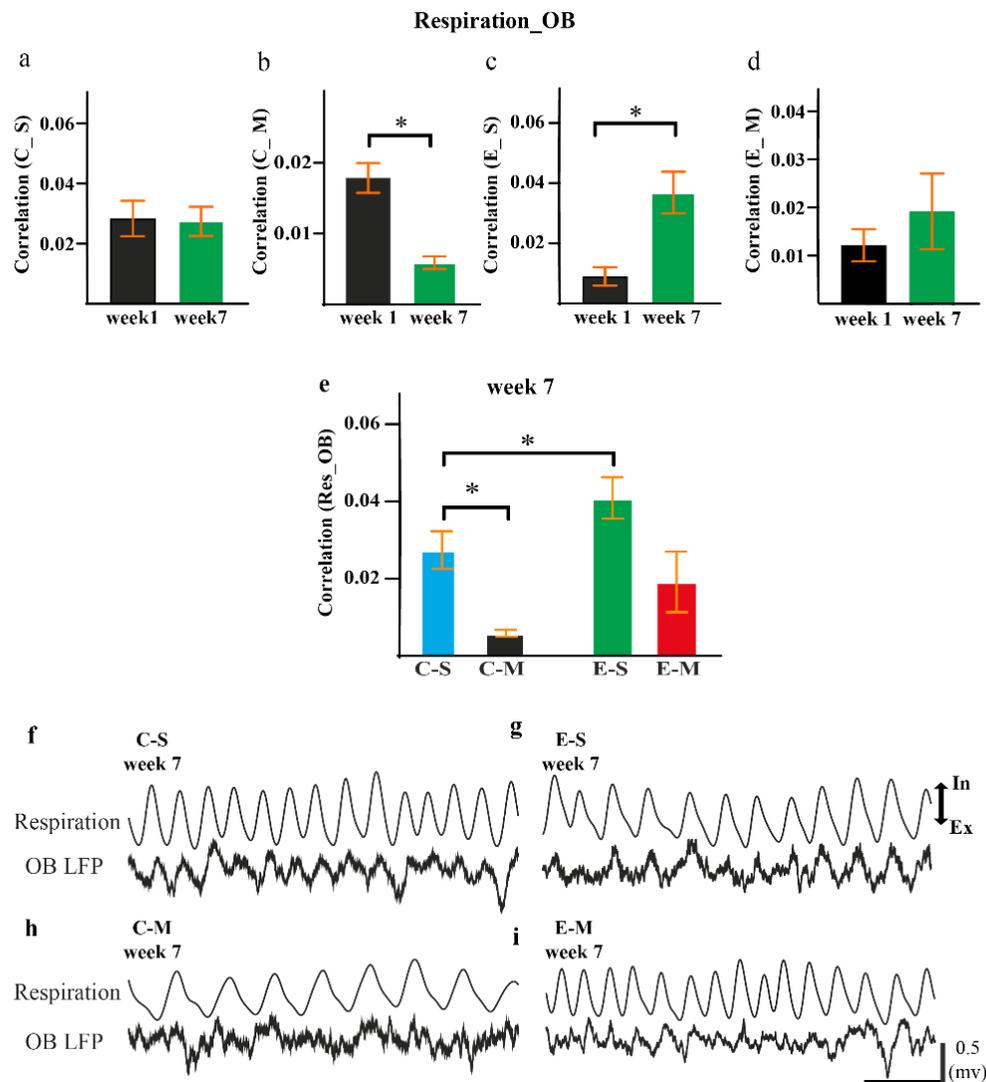


Figure 4. a, b, c, d) The maximum respiration-OB cross-correlation values in four groups (exercise improved the correlation between respiration and OB oscillations), e) Comparison of cross-correlation values between the four groups in week 7, f, g, h, i) The representative traces of LFP from OB and breathing in four groups

Notes: Values are expressed as Mean±SEM. The data were analyzed by t-test; *P<0.05.

Abbreviations: OB: Olfactory bulb; LFP: Local field potential; C-S: Control saline; C-M: Control methimazole; E-S: Exercise saline; E-M: Exercise methimazole; In: Inhalation; Ex: Exhalation.

et al., 2015; Uysal et al., 2015; Wang & Wang, 2016). Stimulation of the OSNs during nasal respiration generated brain oscillations. OSNs are shown to respond to the chemical stimulus (odor) and are activated by nasal airflow (Lockmann et al., 2016). We presume that OSN stimulation enhances during exercise and increases connectivity in the OB and other brain regions improving spatial memory, and can be introduced as a probable mechanism. Therefore, we have used the methimazole injury model to investigate this mechanism. Methimazole is an olfactotoxic drug and disrupts the OSNs all

over the epithelium by activating the apoptotic cascade (Sakamoto et al., 2007) without disturbing breathing rates during normal behaviors (Moberly et al., 2018). Our results indicated that the administration of methimazole reduces the number of neurons in the olfactory epithelium and the OB. Also, impairment of the OSNs is associated with decreased activity of OB at delta and theta frequency bands, as well as the coupling and synchronization between respiration and OB oscillations. Therefore, these results show that methimazole could destroy OSNs and reduce respiration-entrained brain oscillations.

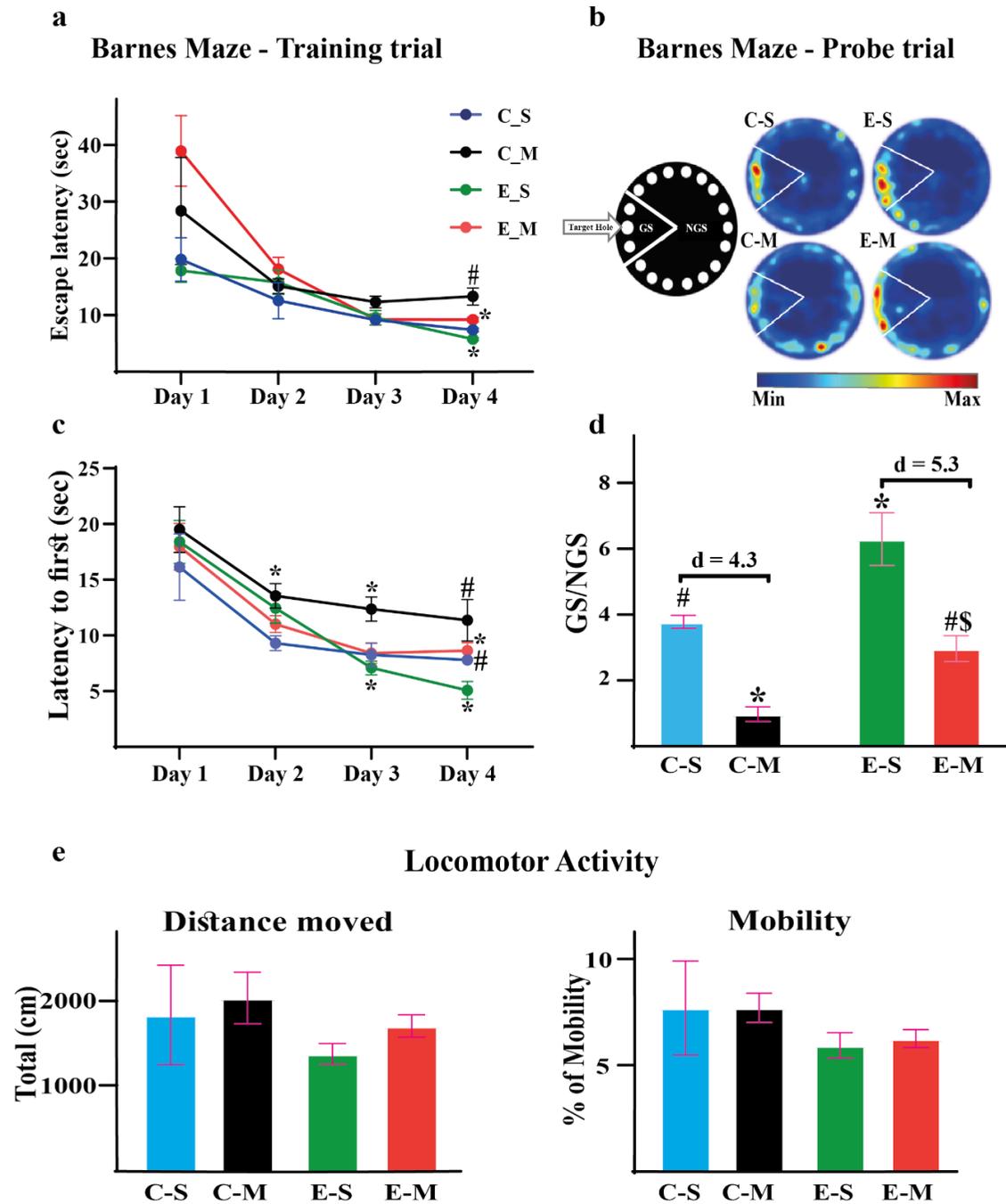


Figure 5. a, b) The escape latency and the latency to first recorded in the training trial, c) The schematic representation of the Barnes maze and Barnes maze heat maps in the four-group probe trial (the color scale represents the time of visits to the maze (min and max), d) The ratio of GS to NGS explorations, e) The total distance and percentage of mobility in for groups

Notes: All animals had normal locomotor activity. Values are expressed as Mean±SEM. The data were analyzed by two-way ANOVA. *Significant compared to the CS group, #Significant compared to the ES group, \$Significant compared to the CM group.

Abbreviations: d: Cohen d; C-S: Control saline; C-M: Control methimazole; E-S: Exercise saline; E-M: Exercise methimazole; G-S: Goal sector; NGS: Non-goal sector.

tions. On the other hand, exercise increased the number of OB neurons and the synchrony between respiration and OB oscillations. To the best of our knowledge, this is the first report of improved spatial memory with aerobic exercise by modulating OB through stimulating OSNs.

During nasal breathing, OSNs fire rhythmically with respiration, and this activity is transported to OB cells (Heck et al., 2019). These OB oscillations generated during nasal respiration synchronize neural activities of the brain networks (Singer, 1993). Respiration-entrained brain oscillations emerge due to the phase-locking between olfactory neural oscillations and respiration cycles (Gretenkord et al., 2019). OSNs are sensitive to odors and mechanical stimulation from nasal airflow, and the entrainment of OB neuronal activity with respiration is independent of odor perception (Grosmaître et al., 2007; Heck et al., 2019). Our result indicated that by destroying the OSNs (like breathing through mouth), the coupling between respiration and OB oscillations, and therefore, respiration-entrained brain oscillations were reduced, which was associated with a reduction in memory improvement induced by exercise. Nasal respiration modulates cognitive function while memory encoding and retrieval remain unchanged during mouth breathing (Zelano et al., 2016). Also, our previous study showed that stimulation of the OSNs using air-puff leads to excitation of the OB in conditions where the animal cannot breathe intentionally through the nose, and it can improve working memory performance (Ghazvineh et al., 2021). In addition, rhythmic nasal air puffing promotes brain activity and improves network connectivity in comatose patients (Salimi et al., 2022).

Besides, nasal breathing can modulate brain oscillations in nonolfactory areas, and respiration-entrained oscillations aid the integration of information and improve cognitive abilities (Zelano et al., 2016). Preceding works revealed that changes in breathing frequency could influence nonolfactory information processing (Tort et al., 2018; Tort et al., 2021). Also, differences in nasal airflow rate can affect olfactory processing (Esclassan et al., 2012; Hammer et al., 2021). Given that nasal airflow increases with respiratory intensity and rate and that aerobic exercise increases these parameters, we suggest that nasal airflow might have a role in memory improvements following aerobic exercise. The destruction of OSNs reverses this effect.

Previous evidence demonstrated that OB activity entrains regional oscillations in widespread brain areas, showing higher synchrony during cognitive performance (Tort et al., 2018). For instance, it has been re-

ported that OB and hippocampus are synchronized in theta oscillation during reversal learning (Macrides et al., 1982). Therefore, OSNs may modulate OB oscillations through nasal respiration which in turn drives the changes throughout other brain regions. Also, the coupling between OB oscillations and other brain areas is correlated with cognitive functions (Salimi et al., 2019), and this coupling is crucial for information processing during the expression of behaviors (Fell & Axmacher, 2011). Eliminating the OB leads to cognitive impairments in attention, reference memory, delayed matching, reversal memory, and working memory (van Rijzingen et al., 1995). Therefore, respiration is a crucial neurobiological modulator in organizing cortical activity during memory processes in brain regions like the hippocampus and medial prefrontal cortex (Heck et al., 2019).

Aerobic exercise improved spatial memory; however, destroying OSNs reduced this effect which we believe might be due to the presence of respiration-entrained brain oscillation. This partial compensation in memory impairment is due to several parallel biological pathways recruited by exercise in affecting brain function. The present study results showed that nasal breathing improves spatial memory both at rest and aerobic exercise, and it was more significant following aerobic exercise. Additionally, spatial memory decreases when we eliminate the OSNs and respiration-entrained brain oscillation, like oral breathing. Therefore, our results suggest that nasal breathing is essential for cognitive function in rest and exercise. Aerobic exercise can be defined as a non-invasive mechanical stimulation method for the brain to restore cognitive deficits such as memory impairment.

5. Conclusion

The OB pathway is a probable mechanism for mediating the effects of exercise on spatial memory. These results provide new insight regarding the effect of OB activity on remote brain areas associated with cognition and behavior following exercise. However, the relative contribution of exercise to this mechanism is currently unclear, and further studies are needed.

Study limitations

This study faced some limitations in suggesting the entire mechanism. First, in this study, exercise had a compensatory effect when we destroyed the OSNs. It might be due to the role of other pathways in improving memory, such as increased neurogenesis and blood flow distribution in the olfactory epithelium, which we did not

investigate in this study and should be evaluated in subsequent studies. Second, our study was limited to evaluating the long-term effects of exercise; however, it would be helpful for future studies that evaluate this pathway in short-term exercise. Third, since our recordings were not taken during exercise, we could not evaluate respiration intensity in this study, which needs further experiments and might provide interesting results.

Ethical Considerations

Compliance with ethical guidelines

The Ethics Committee of the Faculty of Medical Sciences, [Tarbiat Modares University](#) approved all animal protocols (Code: IR.MODARES.REC.1397.160).

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Authors' contributions

All authors contributed equally in preparing all parts of the research.

Conflict of interest

The authors declared no conflict of interest.

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