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Title: Olfactory Sensory Neurons Facilitate Aerobic Exercise-Induced Spatial Memory Improvement

Running title: The Role of Olfactory Bulb in Exercise

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Abstract

Introduction: The positive effects of exercise on spatial memory and learning have been shown. The olfactory sensory neurons 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, we aimed to evaluate the role of olfactory sensory neurons in mediating the effects of aerobic exercise on memory.

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

Result: Our results showed that destroying olfactory sensory neurons significantly reduces olfactory bulb 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: These findings suggest that the olfactory bulb activity is one of the probable mechanisms for improving spatial memory following exercise.

Keywords: Aerobic exercise, Spatial memory, Olfactory bulb, Local field potentials.

Introduction:

Exercise and physical activity are inescapable necessities for health (Bherer, Erickson, & Liu-Ambrose, 2013). Various studies have shown that physical activity plays an essential role in brain health (Bherer et al., 2013; Di Liegro, Schiera, Proia, & Di Liegro, 2019). In this line, 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, Gharakhanlou, Naghdi, Khodadadi, & Heysiattalab, 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 pathways 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, Zeman, & Jarabek, 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 percent more resistance to the air stream, causing 10 to 20 percent more oxygen uptake and improving the overall pulmonary volume. (Allen, 2017). Also, previous research has shown changes in the brain oscillatory activities and improved cognitive performance after yoga interventions associated with specific breathing exercises (breathing from the nose) (Desai, Tailor, & Bhatt, 2015). Therefore, it seems that 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 an important role in information processing (Kepecs, Uchida, & Mainen, 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 (PFC), and amygdala (Grosmaître, Santarelli, Tan, Luo, & Ma, 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, Ciatipis, Draguhn, Tort, & Brankač, 2014). Also, different brain regions, especially the hippocampus, show reduced activity during intubation due to eliminating nasal airflow for a long time and 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).

Taken together, 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. To this aim, we destroyed the nasal epithelium to investigate the modulatory effect of OSNs on aerobic exercise-induced spatial memory improvement.

Material and methods

Animals

Thirty-two (8 rats in each group) adult male Wistar rats (250-300 g) were obtained from the Pasteur Institute (Tehran, Iran). Rats housed in standard cages in a climate-controlled room ($23 \pm 2^\circ\text{C}$, 50% to 55% humidity) with a 12 h light/12 h dark cycle. Food and water were available enough. The "Ethics Committee approved all animal protocols of Faculty of Medical Sciences, Tarbiat Modares University." (Ethics Code: IR.MODARES.REC.1397.160).

Surgery and LFP recording

We used intraperitoneally injection 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 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 μ m in diameter, A.M. system Inc., USA) were implanted unilaterally into the right hemisphere of the OB (AP: 8.5 mm, ML:-1 mm, DV: -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 (Fig 1) (Dehdar et al., 2019).

One week after surgery, animals were divided into two exercise (ES: exercise saline, EM: exercise methimazole) and two control groups (CS: control saline, CM: control methimazole). At the beginning of each week, one exercise and one control group to destroy the OSNs received 300 mg/kg methimazole (Genter, Deamer, Blake, Wesley, & Levi, 1995), and the other two groups received saline. Also, 30 minutes before methimazole injection, the animals were injected with thyroxine (10 μ g) to prevent the effects of methimazole on metabolism (Bergman & Brittebo, 1999). At the end of each week, LFPs were recorded for 15 minutes. For this purpose, the socket, fixed to the animal's head, was connected to a miniature buffer headstage with high-input impedance (BIODAC-A, TRITA Health Tec., Tehran, Iran), via cables to a main AC coupled amplifier (1000 amplification) and to 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, Barnes behavioral test was performed (Fig 1).

(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 of 15-23 m/min for 15-50 minutes per day and five days per week. The training period was divided into three stages: familiarity, 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 minutes in the first week and reached 18 meters per minute in the second week. In the third and fourth weeks, the time reached 50 minutes, and the speed reached 20 meters per minute. In the fifth and sixth weeks, it maintained with increasing speed to 23 meters per minute. 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 did not have any exercise.

Behavioral tests

Barnes maze is 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 familiarity day, 90 seconds allowed

rats to explore the field freely. After going into the target hole, the rats stayed in the escape box for 30 seconds. 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 70% alcohol solution. Auto Vision software (2015) was used to analyze Barnes' behavioral test.

Cresyl violet histochemistry

To elucidate the effects of methimazole on OSNs destruction, the olfactory bulb 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) then dehydrated in increasing alcohol concentrations. The tissues were cleared in xylene, mounted, and then cover-slipped. All the samples were captured at 40× magnification, and the neuronal count was measured using ImageJ software (number of neurons/field), and statistical comparisons were made among the groups.

LFP and Statistical analysis

In this study, power spectral density of OB signals was computed using the MATLAB pwelch function. 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, Statistical analysis was performed by GraphPad Prism software (GraphPad Software, USA). The achieved results were compared by t-test, Two-way, and three-way ANOVA, and we were using

Bonferroni for ANOVA post hoc testing. We used Cohen's d to measure effect size. Data are shown as mean \pm SEM, and the $P < 0.05$ was considered statistically significant.

Result

Methimazole injection destroyed the OSNs

An apparent increase in the number of cells was evident. Histological images showed an increased number of neurons in the E_S compared to the C-S group. (Fig 2. a). Also, statistically, the granule cell number increased in the E_S group compared to other groups denoting that exercise can increase the number of neurons ($p= 0.01$, $d= 2.09$). The granule cell population significantly decreased in the C-M group, denoting that methimazole could decrease the number of neurons ($p=0.03$, $d=1.88$) (Fig 2. b). When we eliminated the OSNs, exercise could Compensate for this decrease in the E-M group ($p=0.01$, $d= 2.47$) (Fig 2). The present study results show that exercise increases the number of neurons, and methimazole leads to the destruction of OSNs.

(Figure 2)

Aerobic exercise increases Power spectral density with increased non-invasive stimulation of the OSNs

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 C_M group during seven weeks, and this decrease was significant between weeks one and seven ($p_{\text{delta}}= 0.0025$, $p_{\text{theta}}= 0.24$) (Fig 3. a, b). Comparison between weeks one and seven show that exercise increased the PSD in E_S and E_M groups, while there was no significant difference in PSD between the first and seventh weeks in the C_S group. Additionally, in week seven, a significant difference in PSD was observed between

E-S and E-M groups (Fig 3. c, d). Thus, exercise improved the power of delta and theta frequencies. In comparison, methimazole injection prevented this increase.

(Figure 3)

Exercise increased respiration-olfactory bulb synchronization

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 E_S group ($p= 0.003$, $d=2.29$) (Fig 4, c). Methimazole decreased the cross-correlation in week seven compared to week one ($p= 0.0002$, $d=2.49$) (Fig 4, b). as well, there was a significant difference between saline and methimazole control groups (Fig 4. e). No significant cross-correlation difference was found between weeks one and seven in the C_S group ($p= 0.9$) (Fig 4, a).

Also, representative traces of LFP from OB and breathing showed in four groups. In the C_S group, OB oscillation was coupled with animal respiration, while this coupling was not seen in the C_M group (Fig 4, f, g).

(Figure 4)

Destroyed OSNs impair learning and spatial memory

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 (Fig 5, a, b).

Statistical comparison of 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 C_M group ($p= 0.002$, $d=4.3$). In the E_S compared to the C_S group, there was a significant increase in the goal sector preference (GS/NGS) ($p= 0.005$, $d= 2.14$) (Fig 5, d). Also, In the E_S compared to the E_M group, there was a significant increase in the GS/NGS ($p= 0.0002$, $d= 5.3$).

(Figure 5)

Discussion

Our results indicated that aerobic exercise improves spatial memory, consistent with previous reports (Inoue 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, Laplagne, Leão, & Tort, 2016). We presume that OSNs stimulation enhances during exercise and increases connectivity in the OB and other brain regions improving spatial memory, and 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, Kondo, Kashio, Suzukawa, & Yamasoba, 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 the respiration entrained brain oscillations. 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, Kozma, & Kay, 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 entrainment of OB neuronal activity with respiration is independent of odor perception (Grosmaître et al., 2007; Heck et al., 2019). Our result indicated that with 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, Brankač, & Draguhn, 2018; Tort, Hammer, Zhang, Brankač, & Draguhn, 2021). Also, differences in nasal airflow rate can affect olfactory processing

(Esclassan et al., 2012; Hammer, Schwale, Brankač, Draguhn, & Tort, 2021). Given that nasal airflow increases with respiratory intensity and rate and that aerobic exercise increases these parameters, we suggest that the 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 reported that OB and hippocampus are synchronized in theta oscillation during reversal learning (Macrides, Eichenbaum, & Forbes, 1982). Therefore, OSNs may modulate OB oscillations through nasal respiration which in turn drive 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, Gispen, & Spruijt, 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. Indeed, aerobic exercise can be defined as a non-invasive mechanical stimulation method for the brain to restore cognitive deficits such as memory impairment.

We have faced some limitations to suggest 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 evaluate 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.

Conclusion

Herein we suggest that 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.

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Figure Legends:

Fig. 1 Experimental design (A) Timeline of the study design. The rats received intraperitoneal injections of saline or methimazole (300 mg/Kg) every week. LFP was recorded at the end of any seven weeks after the last session (five days /week) of exercise. (B) Schematic representation of electrode implantation site on rat head. (C) Histological confirmation of a recording site in OB. R, recovery; E, Exercise; LFP, Local field potential; ISM, injection saline or methimazole; BT, behavioral tests; OB, olfactory bulb.

Fig. 2 Aerobic exercise increases the number of neurons (A) Cresyl violet staining of the olfactory bulb in four groups (Scale bar =20 μ m). Numerous granule cells (\uparrow) can be noticed. (B) Neuronal count of cresyl violet stained tissues. Values are expressed as the mean \pm SEM. Data were analyzed by Two-way ANOVA between four groups, followed by posthoc Tukey's multiple comparison test. n = 4 per group. *, significant difference with C-S group; #, significant difference with E-S group; \$, significant difference with C-M group; C-S, control saline; C-M, control methimazole; E-S, exercise saline; E-M, exercise methimazole.

Fig. 3 Methimazole decreases delta and theta power in OB (a) Power comparison of delta (< 4 Hz) and theta (4-12 Hz) OB frequencies in seven weeks. The power of OB decreased during seven weeks. (b) Statistical comparison of delta and theta frequencies. The Power of OB in delta and theta frequencies significantly decreased in week seven compared to week one. (c, d) Comparison of delta (< 4 Hz) and theta (4-12 Hz) in 4 groups. The exercise increased power in week seven compared to week one, and methimazole prevented this increase. Values are expressed as the mean \pm SEM. Data were analyzed by per t-test in the methimazole group and three-way repeated-measures ANOVA between four groups in weeks one and seven, n = 10 per group. **, p < 0.01; *, significant difference with saline; #, significant difference with week one; \$, significant

difference with the control group; PSD, power spectral density; OB, olfactory bulb, C-S, control saline; C-M, control methimazole; E-S, exercise saline; E-M, exercise methimazole.

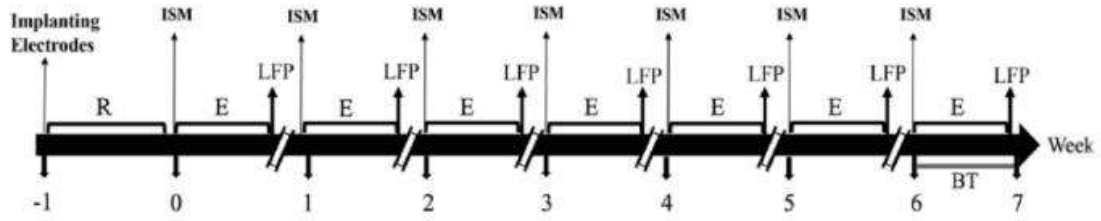
Fig. 4 Exercise improved synchrony between respiration and OB activity (a-d) Maximum respiration-OB cross-correlation values in all groups. Exercise improved the correlation between respiration and OB oscillations. (e) Comparison of cross-correlation values between 4 groups in week 7. (f-i) Representative traces of LFP from OB and breathing in four groups. LFP signals were not coupled with animal respiration in the C_M group. Values are expressed as the mean \pm SEM. Data were analyzed by per t-test, $n = 6$ per group. *, $p < 0.05$; 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.

Fig. 5 destroyed OSNs impair learning, and spatial memory (a, b) Escape latency and latency to first were recorded in the training trial and statistically analyzed. (c) 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) Distance move and mobility were statistically analyzed. All animals had normal locomotor activity. Values expressed as the mean \pm SEM. Data were analyzed by two-way ANOVA, $n = 6$ per group. * significant difference with C-S group; # significant difference with E-S group; \$ significant difference with C-M group; d, Cohen's d; C-S, control saline; C-M, control methimazole; E-S, exercise saline; E-M, exercise methimazole; GS, goal sector; NGS, non-goal sector; Min, minimum; Max, maximum.

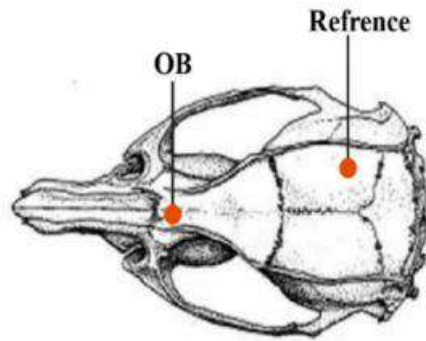
Figures:

Fig. 1

a



b

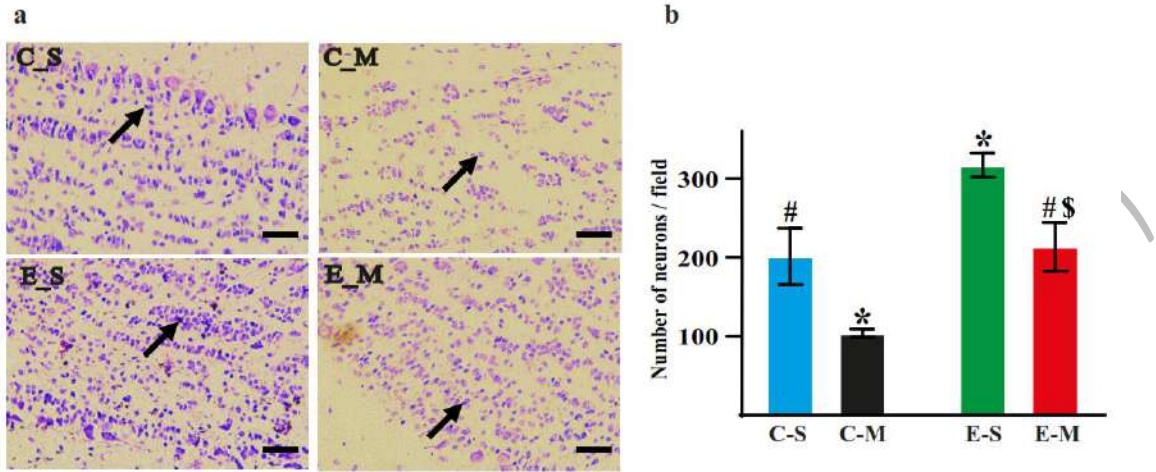


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Fig. 2



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Fig. 3

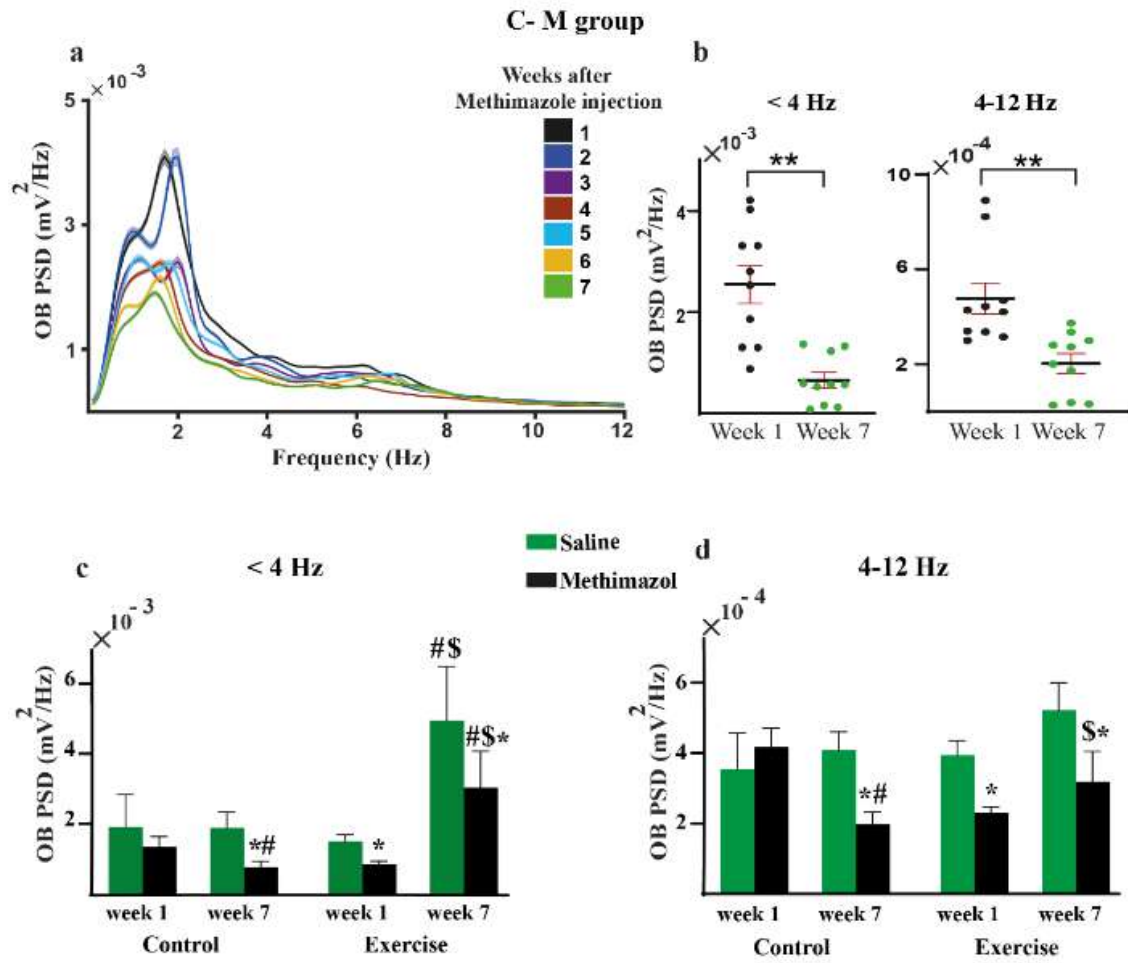


Fig. 4

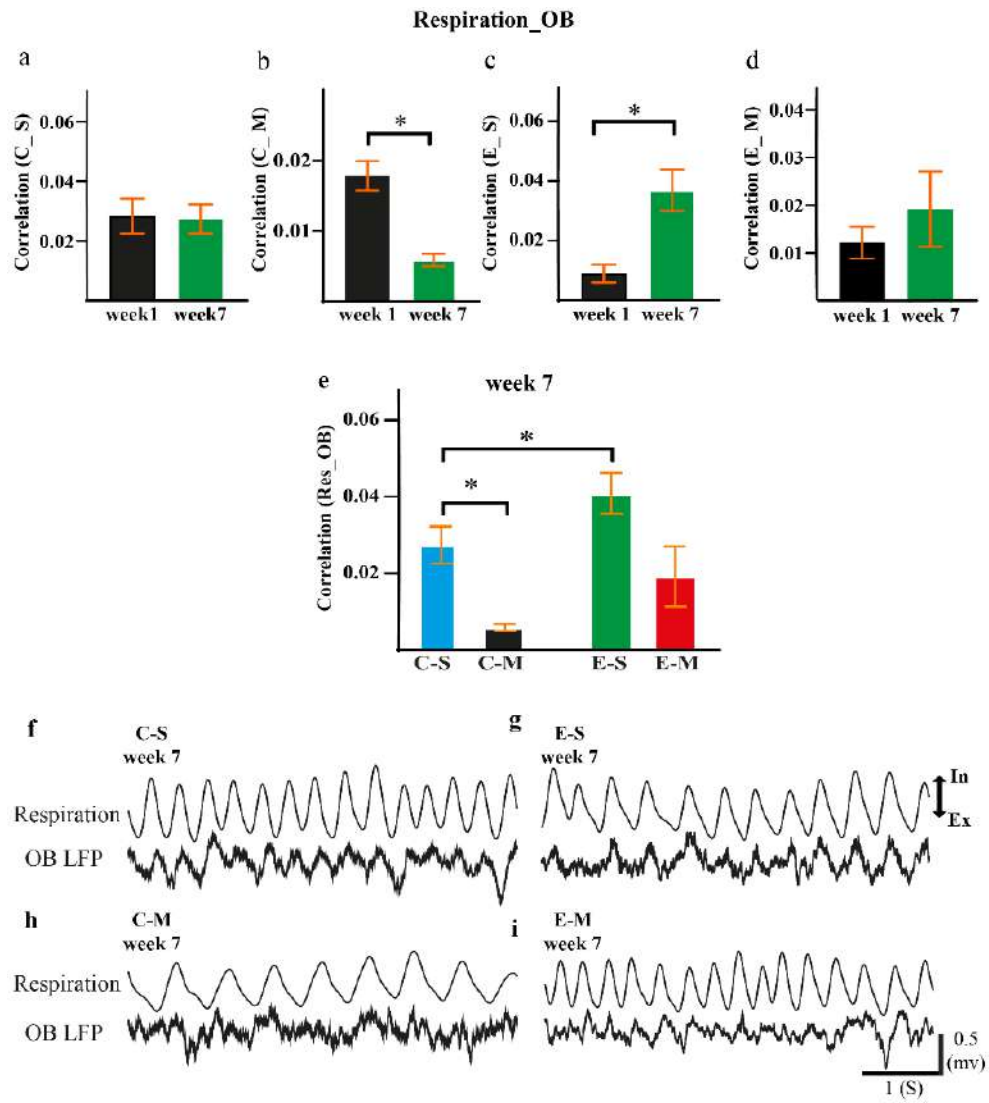


Fig. 5

