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ORIGINAL ARTICLE

Investigating the Effect of Olfactory Deprivation on the bed nucleus of the Stria Terminalis and medial Amygdala Efferents to the Medial Preoptic area in Rats

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ABSTRACT

Most of the researchers believe in the necessity of the olfactory neural circuits in regulating various social activities such as sexual behavior and aggression. Deprivation of sensory stimulation caused a negative effect on the pattern of synaptic connections among dendritic and axonal circuits on neural system. In this study, after nostril obstruction, an injection of Horse Radish Peroxidase (HRP) as tracer was applied into the nucleus of the medial pre-optics area with retrograde method. Subsequently, the number of labeled neurons with HRP was measured at the medial amygdala and Steria terminalis in male and female rats. Additionally, the number of the apoptotic cells in the olfactory bulb was assessed by using the TUNEL test. The results showed that numbers of labeled neurons were significantly reduced in the amygdala nucleus domestic and medium of the stria terminalis as a consequence of olfactory deprivation in male sample rather than female ones. Also, the rate of cell death in the olfactory bulb increased following the induced olfactory deprivation. According on the results of this research, stimulation of the olfactory nerve plays a critical role in the orbit evolution of reproduction in male and female rats.

Keywords: *Olfactory system, Bed Nucleus of Stria terminalis, Medial Amygdala Nucleus, Medial pre-optic nucleus.*

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INTRODUCTION

Chemical sense is one of the primary senses which can be observed from the simplest form of life in bacteria to the most complicated animals like human. During the process of evolution, the neural olfactory epithelium is known as a specialized part of chemical sense for identification of different chemical compounds in the environment [1]. Chemical compounds in the environment, play an important role as a source of information for all the animals and most of the animal behaviors such as feeding, mating and defense which are subjected by this sense. Additionally, most animals regulate their sexual behavior and identify risk factors, using olfaction and identification of chemical compounds found in their individual and social communication environments [2].

Wide range of olfactory information processes by binding of odorents to the olfactory receptors in the olfactory epithelium [3]. Studies have shown that volatile odorents are detected by the main olfactory epithelium and then through the main olfactory bulb are entered into the olfactory cortex and medial amygdala for further analyzing [4]. Moreover, identification of pheromones and non-volatile odorents are placed by receptor neurons in the accessory olfactory Vomeronasal organ [5]. The information received by the vomeronasal organ is transferred to the accessory olfactory bulb and medial amygdala which is known as the first integration of afferent of the main and a accessory olfactory system in the brain in which it is vital in expressing behavioral responses to the chemical-sensing data [6]. Chemical-sensory information is then transmitted into the bed nucleus of the stria terminalis and medial pre-optic nucleus [7, 8].

In several studies, defining the role of trans-synaptic signals in olfactory communication was clearly mentioned and most researchers believe in the pre required and essentiality of this data transmission pathway; Chemicals - sensory, in forming neuronal circuits in setting social activities such as sexual and aggressive behaviors [9-11]. It has been demonstrated that primary sensory stimulation plays a critical role in developing the structure and function of the olfactory system, so that the nervous system during postnatal development and strengthening connections in the brain responds to stimuli in the environment [12-13]. Neural circuits in the brain are specifically sensitive to the stimuli during the critical period of brain development in a way that after this period, the final brain connections are formed and reformation hardly occurs after passing this period. Additionally, in the absence of sensory stimulation or sensory deprivation the development of brain circuits can imply interferes into the normal processes. The deprivation of sensory stimulation during postnatal development can disrupt the pattern of synaptic connections, dendrites and axons in the neural circuits [12-13].

In human, several external factors such as trauma, viral infections, and nasal polyps cause loss of smell sense along with other issues like aging and neurodegenerative disorders such as Alzheimer, Parkinson and multiple sclerosis [2]. Research results have shown that most patients with olfactory disorders are facing serious problems which all significantly reduce the quality of everyday life. These are including mood changes and bad tempers, loss of appetite, difficulty in working, spoiled food eating and often show signs of depression [14]. In contrast, rich environment in sensory stimuli effectively postpones the cognitive and behavior disorders like depression, and act as a vital factor in the protection of neurons in such a neurodegenerative disorders like Parkinson and Schizophrenia disorders [12].

The present research was designed to find the effect of olfactory signals in social life and sexual behavior in mammals. The current report examines the impact of olfactory stimuli on neural circuits involved in smell processing route signals and reproduces the pathway messaging system.

MATERIAL AND METHODS

Twenty four Wistar rats pups (1-2 weeks old), weighing approximately 40-30gr were selected that the infancy period was passed. The animals were divided into two groups; experimental and control. Each group consisted of 6 male and 6 female rats. In addition, these sexually naive pups were maintained on a 12/12 hrs light/dark cycle and fed with food and water *ad libitum.* Ethical standards were considered that related to the maintenance of laboratory animals which was approved by the Tehran University of Medical Sciences.

The experimental group, were anesthetized with an injection of ketaminee (40 mg/kg) and xylazin (5mg/kg) intra- peritoneally. Furthermore left and right naris was closed by cauterization, with an electrocautery unit [15]. The animals were cared until they were fully alert and all efforts were made to minimize their suffering and distress. After eight weeks from occlusion of the anterior nasal apertures, apoptotic neurons from olfactory bulb were examined by TUNEL method Roche, Germany [13].

In this study, HRP was used to examine the effects of olfactory deprivation on the efferents of medial amygdala and bed nucleus of stria terminalis nuclei to medial pre-optic area. Two to three days after the stereotaxic injection of HRP into the medial pre-optic area of experimental groups, HRP was absorbed by axonal endings and transferred to perikaryon, animals were anesthetized deeply after an intra-peritoneal injection of ketamine (80mg/kg) and xylazin (15mg/kg) [16-18]. The animals were perfused intracardiacly with fixative solution (glutaraldehyde 1.25% and paraformaldehyde 1% in 0.2 mol buffer phosphate at pH=7.4) followed by 10% sucrose buffer. The brains were removed and cut by a freezing microtome (Cryocut 1800, ELICA) in coronal sections with a thickness of 40 дт and stored in0.1 mol phosphate buffer. The number of labeled neurons and their topography were studied in medial amygdala and bed nucleus of striaterminalis nuclei in both experimental and control groups. Selected slices were traced with reference to the atlas of Paxinos and Watson (1986). Sections were treated with tetra methyl benzidin (Sigma,Mo.,USA) following the procedure of Mesulam *et al.*[19] Sections were then mounted

onto gelatinized slides, airdried and counterstained with neutral red. After assessment of the injection site, slides of each section observed with a light microscope (Optika) and digital photographs were taken. The injection site and retrogradely labeled cells were plotted with the use of a microprojectore. Topographical study on the dispersion of labeled cells with HRP was performed by Adobe Photoshop 7.0 software and optika software. In order to count the labeled neurons in male and female we used six sections for each rat. Most analysis was carried out using statistical software SPSS version 13 (Mann-Whitney and t test).

RESULTS

The labeled neurons were examined after HRP injection into the medial pre optic area and the injection site was confirmed in tissue sections (Fig. 1.)

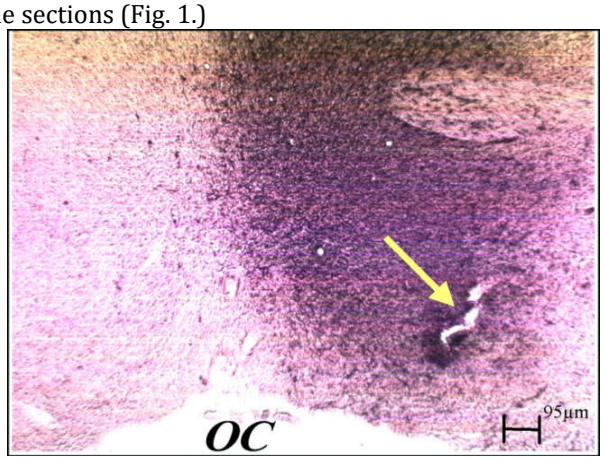

Figure 1: Injection site, scale bar: 95µm (HRP and neutral red staining ×40), **OC**: Optic chiasma

Labeled neurons in medial amygdala and bed nucleus of stria terminalis of male rats

Numbers of labeled neurons were decreased in the medial amygdala and bed nucleus of the stria terminalis of the experimental groups in male rats (Fig. 2, 3). This decrease was statistically significant compared to the control groups ($p<0.05$). After counting the labeled neurons of the medial amygdala in male rats, the total amount was 34.66 (2.65 in the control group and 22. 16+3.12 in experimental group which showed a 36.06% decrease. Furthermore, there has been a 31.84% decrease in labeled cells of bed nucleus (29.83+1.83 in control group and 20.33+1.66 in experimental group).

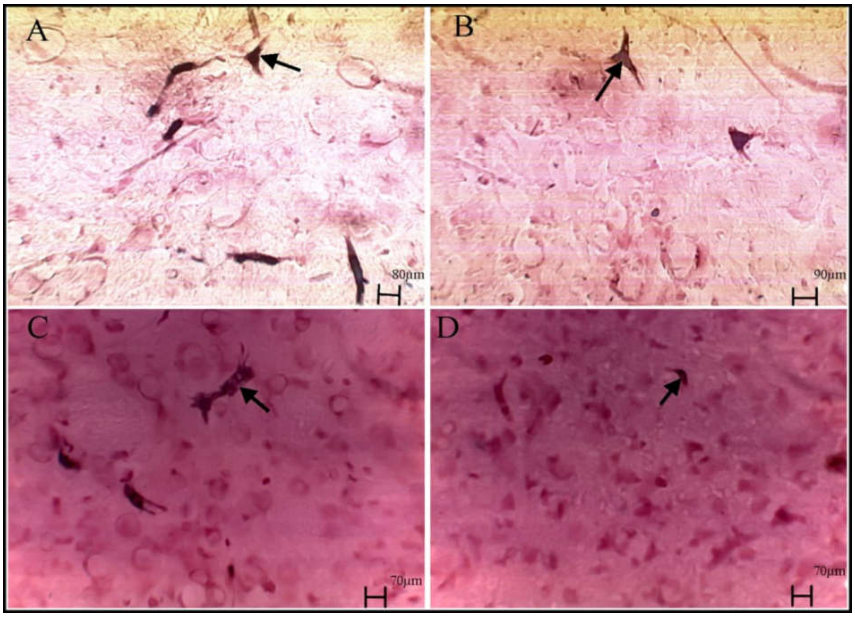

Figure 2: Comparison of labeled neurons in **A**) control and **B)** experimental male rats in medial amygdala and comparison of labeled neurons in **C)** control and **D**) experimental male rats in bed nucleus of stria terminalis (HRP and neutral red staining× 400).

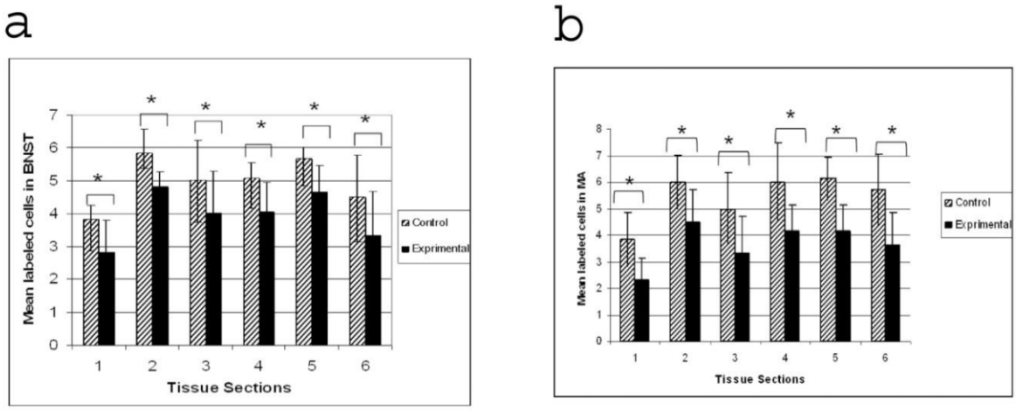

Figure 3: Comparison of labeled neurons in control and experimental male rats in **a)** bed nocleius of and **b)** medial amygdala.

Labeled neurons in medial amygdala and bed nucleus of stria terminalis of female rats

In control groups of female rats the total number of labeled neurons in medial amygdala and bed nucleus were 28.83+1.16 and 41.4+2.07, respectively. On the other hand, in experimental groups these total numbers of labeled neurons were 22.83+0.75 and 29.33+1.86 in medial amygdala and bed nucleus respectively. Subsequently, a 20.81% reduction was detected in medial amygdala and a 29.27% decrease in bed nucleus (Fig.4, 5).

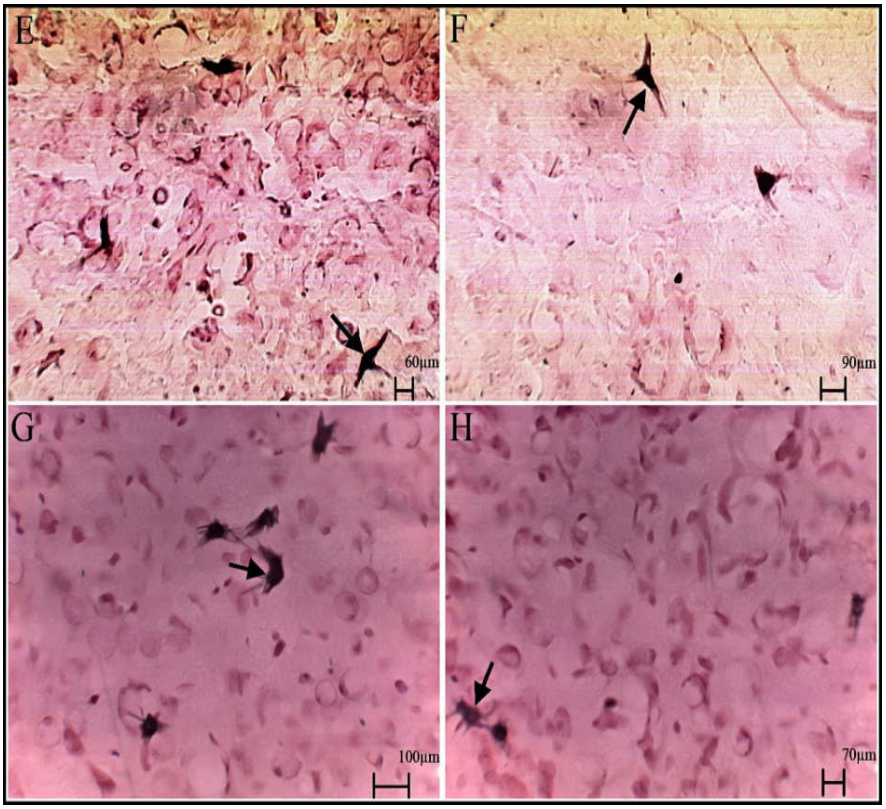

Figure 4: Comparison of labeled neurons in **E)** control and **F)** experimental female rats in medial amygdala and comparison of labeled neurons in **G)** control and **H)** experimental male rats in bed nucleus of stria terminalis (HRP and neutral red staining× 400).

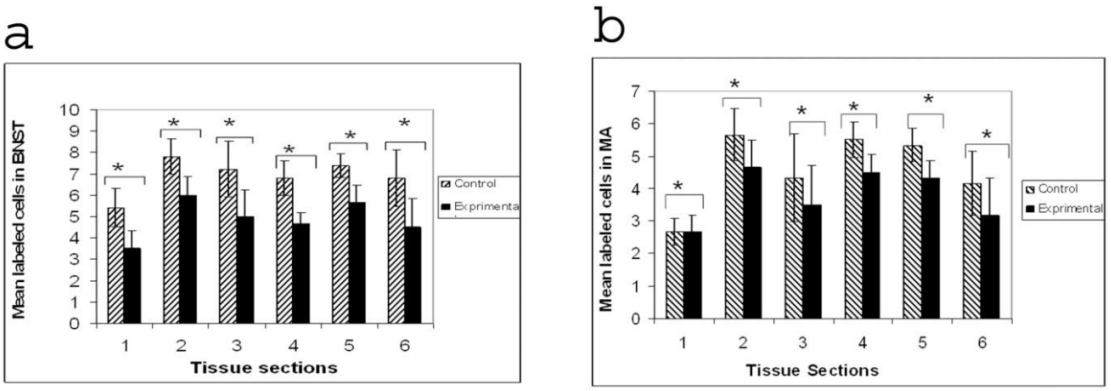

Figure 5: Comparison of labeled neurons in control and experimental female rats in **a)** bed nucleus of steriaterminalis and **b)** medial amygdala

Evaluation of apoptosis in the olfactory bulb

The TUNEL kit as a confirmed staining technique was used to characterize apoptotic neurons in the olfactory bulb according to previous literature [20]. The study of neurons after the TUNEL reaction and the comparison view of typical alive and apoptotic neurons were based on the specific characteristics, including a reduction in cell size, chromatin condensation, DNA fragmentation and the apoptotic bodies.

The apoptotic neurons can be detected by the piknotic and dark nucleus with a white halo around the cells. The nucleus of the apoptosis cells can be distinguished from the typical nucleus of the neurons, due to the presence of DNA fragments and the reaction of these components from the 3 OH terminals with Terminal deoxynucleotidyl transferase (TDT) of the enzyme solution in the TUNEL kits. After TUNEL staining, the average number of labeled neurons was 3.25±1.28 and 18.5±2.07 for control and experimental group sections, respectively in which this difference was statistically significant (P=0. 0001, see Fig. 6).

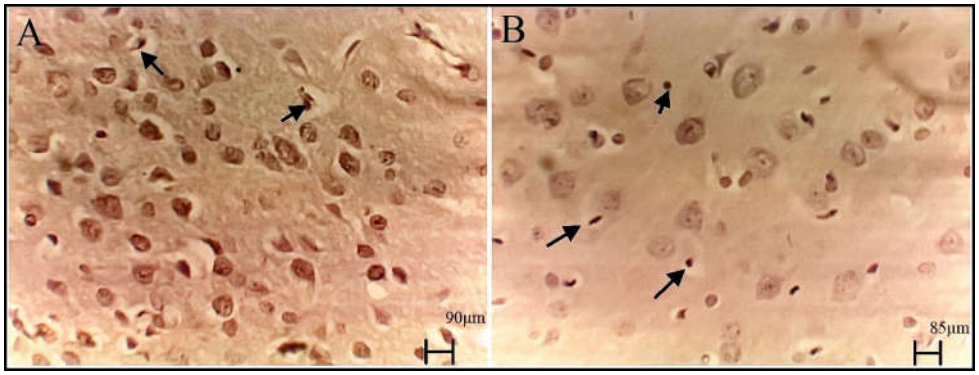

Figure 6: Comparison of Apoptotic neurons in **A)** control Scale bar 90µm and **B)** experimental groups Scale bar 85µm (TUNEL, ×400).

DISCUSSION

For most of the male and female mammals, olfactory signals play important roles in sexual and social behaviors [21]. Nasal cavity has two olfactory organs; the olfactory epithelium for general olfactory sensing and vomeronasal organ for receiving pheromones [5, 22]. Messages received by the olfactory epithelium are passed *via* the olfactory bulb to the olfactory tubercle, piriformis and other areas of the cerebral cortex. On the other hand, pheromones information which is received by a subsidiary organ of the olfactory bulb is passed through the medial amygdale [5, 23]. Consequently, studies have shown that the interaction between the main and accessory olfactory systems occur at the level of the amygdale [5, 23].

Transmission of olfactory information to the central nervous system requires the existence of healthy neural circuits that controls these components [5]. In correlation to our previous researches [16-18] in labeling and tracing neurons' activity, the present study examined the neurons activity using HRP injection into the medial pre-optic area. The results reveal that the number of labeled neurons is significantly decreased at the medial amygdala and bed nucleus of striaterminalis as a result of bilateral occlusion of the anterior nasal apertures and creating olfactory deprivation in both sexes of the experimental groups.

This reduction in the number of labeled neurons at the medial amygdala and bed nucleus of striaterminalis can be either resulted from the activity plunge of neurons in the medial amygdala or the reduction of neurons activities and less stimuli transmission in olfactory bulb. Few researches are conducted on the effects of olfactory deprivation on the central processing pathway of olfactory signals, including the olfactory cortex and amygdala sectors. However, few researches have shown that following the surgical removal of the olfactory bulb, piriformis cortex neurons in which receive signals from the olfactory, have faced apoptosis due to the absence of afferent stimulations [24-25].

In similar researches an increase rate of cell apoptosis was observed by using a one-sided obstruction in the nostrils and olfactory deprivation with TUNEL staining in the cortex piriformis, although the effect was less than the bulbectomy method [26]. These results suggested that the survival rate of neurons in periformis cortex is directly related to stimuli of this sector, which is received from the olfactory bulb. Additionally, removal of the olfactory epithelium and vomeronasal organ has a negative impact on neuron activities in the medial amygdala which results in the reduction of the medial pre optic nucleus projection activity [23, 26].

According to the similar result that was obtained in this study, it can be concluded that reducing the numbers of labeled neurons in medial amygdala is resulted by decreasing the activity of afferents in olfactory bulb or increasing the rate of cell death in this region. Since the efferent is projected from the main and accessory olfactory bulb to amygdala [26-27], studying the effect of olfactory deprivation on olfactory bulb is helpful which it has the significant role in the stimulation of neurons in medial amygdala. Hence, in the present study that was conducted by using the TUNEL test on the neurons of the olfactory bulb, it was observed that apoptotic neurons in the olfactory bulb are increased eight weeks after the obstruction of the olfactory signals pathway. The size of olfactory bulb as a high plasticity structure depends on the level of activity of afferents [28], and deprivation of olfactory stimulation reduces survival and cell proliferation in the olfactory sensory neurons [29]. The research results have shown that the size of the olfactory bulbs in animals that had olfactory deprivation is smaller than the olfactory bulbs of those animals that received olfactory stimulation [30]. This reduction in the size of the olfactory bulb has been caused by cell death in granular and glomerular region, while the number of mitral cells did not change [13, 31].

Other researches in this filed have also shown the reduction in the rate of cell proliferation in olfactory bulb as a consequent of sensory deprivation. In addition the number of piknotic cells increased within four weeks after nostril obstruction that showed higher levels of cell death, especially among granular cells of the olfactory bulb [32].

The result of the present study also confirms the result of the studies that have been conducted by Fisk in 2001 and Wilson in 2003. In these studies, the rate of apoptosis was assessed using TUNEL test, and it was observed that the nasal airflow obstruction leads to the gradual increase in the labeled cells after about twenty days. This effect on the mitral and granular cells are seen till 60th day and increases with aging. The results of this research proved that the area of dead cells in the olfactory bulb depends on the level of activity at this organ in which the opening of the airflow path flips the trend reduction of the number of dead cells compared to before [26, 33].

Recent studies emphasize that the population of olfactory sensory neurons in animals is moderated by the stimulus substances in the environment [29]. Following the unilateral obstruction of nasal cavities, the airflow to the olfactory epithelium in the same direction is decreased and subsequently, the amount of stimulation applied to the olfactory epithelium was fall down, though the rate is not zero yet. Some sensory stimulation reach olfactory epithelium through the nasal pharyngeal path and via integration with breathing air [25, 34], however, by reduction of incoming sensory stimulation followed by unilateral obstruction of the nasal cavities, the rate of cell proliferation in the respiratory and olfactory epithelium reduced [24, 34] and atrophy of the olfactory nerve and bowman's glands in the blocked direction was also observed [35] .

Bi-directional and lateral connections between mitral and granule cells in the olfactory bulb are shaped by olfactory experiences. Moreover, an environment that is rich in sensory stimulation increases the number of inhibitory neurons. Olfactory deprivation reduces the development of mitral cells, numbers of inhibitory neurons and ultimately, weakness the power of distinguishing different odors [36]. In order to confirm this fact, Hind & McNally had shown that the cell-dendric synapses between mitral and granular cells of rats increase dramatically since 3rd month till 27th month and olfactory deprivation interferes with the formation and maturation of these synapses, so that, the number of mitral cells is not affected but their cell body changes. Additionally, it is proved that the number of dead cells following sensory deprivation, are significantly increased and / or their proliferation decreases. As a result, the number of synapses between mitral and granular cells in the cell body of mitral cells decreases [35].

As the olfactory deprivation can resulted in undermining the structure of the olfactory bulb, olfactory synapses and its efferent, it can also has a similar effects on prirformis cortex and its afferents to the medial amygdala which causes the reduction of neuronal activities in this area and consequently, reduces their ability to receive and transmit impulses to other areas.

In this study, it is also shown that the reduction in the number of labeled neurons in the medial amygdala and the bed nucleus of stria terminalis was observed mostly in male samples rather than female ones. Based on the conducted studies, it can be concluded that estrogen plays a critical role in the defense of neurons in female sample, and as it is observed, brain damages resulting from ischemia in female rats are significantly less than male rats [37].

The research has also shown that estrogen affects the olfactory performance, so that, olfactory dysfunction resulting from neurogenerative disease in females following the reduction of the levels of estrogen during menopause is increased. On the other hand by replacing estrogen, the incidence of such disorders and dysfunctions can be decreased, subsequently, olfactory performance can be improved.

The results of the research shows that estrogen increases the proliferation of basal cells in the olfactory epithelium, and subsequently, helps to distinguish them from the olfactory sensory neurons. The reduction of the amount of estrogen following ovarianectomy causes degeneration of the olfactory epithelium. Moreover, estrogens enhances synaptic connections and formation of dendrites and in the early stages of development and growth, it guarantees neuronal survival and persistence.

In summary, based on the results of this study, stimulation of the olfactory nerves plays a key role in the evolution of reproductive neuronal circuits in male and female rats.

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