Original Article

Effects of male pheromones on neuronal morphology in the dentate gyrus of hippocampus of female Mice

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Abstract

***Background & Aims*:** Neurogenesis in the adult mammal brain occurs throughout life. Adult neurogenesis has been clearly demonstrated in the sub granular zone (SGZ) of the dentate gyrus (DG) in the hippocampus. Pheromones that plays an essential role in the development of the central nervous system. The male pheromones are involved in regulating neurogenesis in both the olfactory bulb and hippocampus, which may be important for female reproductive success. The golgi silver impregnation method is a powerful method still routinely used for studying neuronal morphology. The aim of the present study was to investigate the effects of pheromones on the neuronal morphology in the mice hippocampus.

***Materials and Methods*:** Thirty adult NMRI mice 6-8 weeks were used in this study. Adult female animals were divided into four groups. These intact and bilaterally ovariectomized mice and males were placed with and without wire mesh in cage. The animals were sacrificed by cervical dislocation and brain was sectioned (50 μm) and stained with golgi method and observed by microscope.

Results: Neuronal arborization in pheromones group has different with and without pheromones groups. The morphology features of the dentate gyrus neurons of the hippocampus were varied in the exposure to the pheromones as compared to other group.

Conclusion: This study report that the pheromones of males stimulate neurogenesis and neuronal morphology in the hippocampus of female mice.

Key words: Golgi method, Neurogenesis, Hippocampus, Pheromones

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Introduction

Neurogenesis is the process of producing new neurons in the adult brain, begins with the progenitor cell proliferation and reaches its peak by generating new functional nerve cells (2, 1). In particular, the process involves progenitor cell proliferation, cell fate determination, survival, migration and maturation (extension of axons and dendrites and Synaptogenesis) (3). Factors that affect the cell proliferation are those that induce or inhibit mitotic progenitor cells, while the factors affecting the survival of the cell, induce or inhibit the differentiate into mature nerve cells. Eventually, the number of new neurons increases not only by increasing cell proliferation, but also with increased survival of new neurons (4). SGZ is a part of the hippocampus and one of the two main areas of neurogenesis in the adults'

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brain. Structurally, they form a narrow strip of cells that are between the granular cell layer and umbilical (Hilus) of the dentate gyrus. Germ cells are divided in this area and daughter cells create adult granular cells that their axon is spread in the moss fiber direction and reaches Ammon's horn (5). At the same time, growing dendrites move toward the molecular layer. Since the newly generated cells in the hippocampus are distinct locally, no chain migration can be seen in dentate gyrus (6). Preliminary evidences indicate a strong relationship between sex hormones and neurogenesis, arising from observations that show naturally occurring fluctuations in sex hormones can affect adult neurogenesis in several brain regions and in different species (7). Often, male and female adult hippocampal neurogenesis differs in response to circulating steroid hormone levels (4). Pheromones are chemical molecules as signs for communication between members of a species. Pheromones have been used by many species from unicellular organisms to mammals (8). In fact, the role of pheromones is similar to the role of hormones except that hormones can be achieved from the internal endocrine glands while pheromones are the result of external glands. The chemical composition of pheromones and hormones of animals is similar and they are often derived from steroids. Pheromones can affect reproductive behavior on some (especially at mating time). Hormones lead to a harmony and order in the expression of appropriate responses to pheromone messages (9). Pheromones organize social and reproductive behavior in most mammalian species. The effects are concentrated by the vomeronasal and olfactory system (10). Pheromones affect the ratio of LH / FSH and the production of testosterone and estradiol via GnRH and in other words, pheromones fluctuate the hypothalamic- Pituitary- gonadal; As a result, affects subsequent behavior of these changes (11). Male chemical signal plays a role in regulating the menstrual cycle in females in many species. For example, in mice, the male urinary message can cause three physiological responses: accelerating the maturity of young females, facilitating conception and simultaneous female menstrual cycle. The three process are performed through the neuroendocrinology paths, causing rapid changes in the secretion of LH and prolactin (12). Hormone-dependent behaviors can also be influenced by other sensory inputs. The smells that were under exposed in certain social and environmental conditions to mammals will help the mice to change the status of LH hormone by sense of smell (13). Based on the model presented for details of neuroendocrinology systems of mammals, LH is a reliable factor for measurement and comparison of sexual behavior with the sense of smell in humans (14). In addition, the differences in sexual behavior of mammals, sex differences have been observed in brain structures such as the hippocampus and microglia cell function (15). This study aimed to link brain dentate gyrus structures of the hippocampus with the physiological behavior and neurogenesis phenomena. Changes in the morphology of hippocampal neurons represent the relationship and the role of pheromones in the process of maturation and differentiation of neurons.

Materials and Methods

In this study, 30 mice (NMRI) with an approximate age of 8. 6 weeks (mature) were used. Razi Vaccine and Serum Research Institute have developed the mice and they were transferred to the whereabouts of laboratory animals at Damghan University and were kept in good condition 12 hours of daylight and 12 hours of darkness, at temperature of 24-20 degrees Celsius with enough food and water and were kept available. Work on animal ethics on the agenda of the ethics committee of the Faculty of Biology, University of Damghan have been taken into consideration.

Bilaterally ovariectomized and treatment with 17beta-estradiol:

Diestrous rats phase were selected after determining the estrous cycle (Di- stage have chosen because the current cell composition in this stage is similar to ovariectomized model and the levels of ovarian hormones are at their lowest) and were anesthetized based on the weight by a mixture of anesthetic ketamine (100 mg / kg) and xylazine (10 mg / kg) intraperitoneally and were incubated in the stomach. After shaving with a razor scalpel, a small incision was done on the skin of back midsection and locating the ovaries was performed and the ovaries were removed completely with castrate scissors. The incision was sewn with sutures and penicillin was poured on it. Animal ovariectomy leads to the removal of Gennady hormones and make analyzing the effect of pheromones possible (removing the effects of gonadal hormone).

Castration of male mice:

Based on the weight, the mice were anesthetized with a mixture of ketamine and Xylazine intraperitoneally and were incubated in the back. Hair area under the belly was shaved and disinfected. A small incision was created in the skin and muscle area with a scalpel blade. Testis, epididymis and fat were removed and the incision was sutured. After two weeks of surgery and weight control before and after surgery, the rats were divided randomly into the following groups:

1. Healthy female rats in indirect contact with healthy male rats for 30 days (the pheromone)
2. Healthy female rats in contact with male castrate mice
3. Ovariectomy group in direct contact with healthy male rats
4. Ovariectomy group in indirect contact with healthy male rats

Direct contact: Male and female rats were placed in a cage in a fence-free place.

Indirect contact: Male and female were separated by the fence in a cage.

Mouse brain was placed in a solution of Golgi (a mixture of potassium dichromate 5%, and 5% soluble mercuric chloride and 5 % potassium chromate solution) after extraction in a dark place for a week. After the set time, brains were brought out of solution and were prepared by using a 50-micrometer section Vibratome. The slices were prepared in a solution of 10 %ammonia. Finally, the images were prepared using microscope (Nikon Eclipse, E 600, Japan), and imaging system of a digital camera (Nikon, DXM 120, USA). Evaluation of the tonality and assessment of the extent of frills and splits of neurons was performed by Image j software.

Measurement of the estrogen, progesterone and LH hormones using ELISA:

Blood was measured directly from the heart of mice to measure estrogen. Serum was isolated by centrifugation. Serum samples were kept at -70 before the measurement. Hormone levels in collected serum were measured using the kit to measure the LH hormone (Italy-Diametra-35375) according to the instructions in the microplate spectrophotometer device kit (BioTec-Power Wave XS) at 450 nm. The first six wells are for standard solutions or calibrator and control serum and the serum of tested groups are in the next wells.

Results

Histological comparison between ovariectomized rats with direct or indirect contact with healthy male mice showed no significant differences. Overall, drawing histology of nerve cells in dentate gyrus shows that the cell splits are high in the area and are drawn to the molecular region. Cells in CA1 have the same situation and their short tributaries are compressed toward the front and high splits are compressed toward the molecular layer. Grainy cell body of neurons in the

area is smaller and has fewer ramifications. Cells in CA1 are well seen by the pyramidal cell body and processes. (Figure 1 and 2). Comparison of the hippocampus images of ovariectomized mice compared to normal mice in terms of number of branches, density of neuron branching shows superiority in healthy female rats. Processes of nerve cells in dentate gyrus are thick and long (Figure 1 and 2). Neuron cell body of pyramid-shaped area are in three forms of pyramid-shaped, triangular and polyhedron. Gear cell density and especially cell body in SGZ being clearly indicates the high density of the cells in healthy mice compared to the ovariectomized group (Figure 1). Comparison of the neural density, frills' connections and tonality in DG in two groups of healthy females indirectly associated with healthy male and also in female healthy associated with the castrate male revealed that DG neuronal density of healthy subjects showed a significant difference compared to healthy female with castrate male. This increase was not statistically significant despite the increase in tonality in female group with direct contact with the healthy male compared to indirect ($P \leq 0.05$). The results of LH hormone assessment are shown in Figure 3.

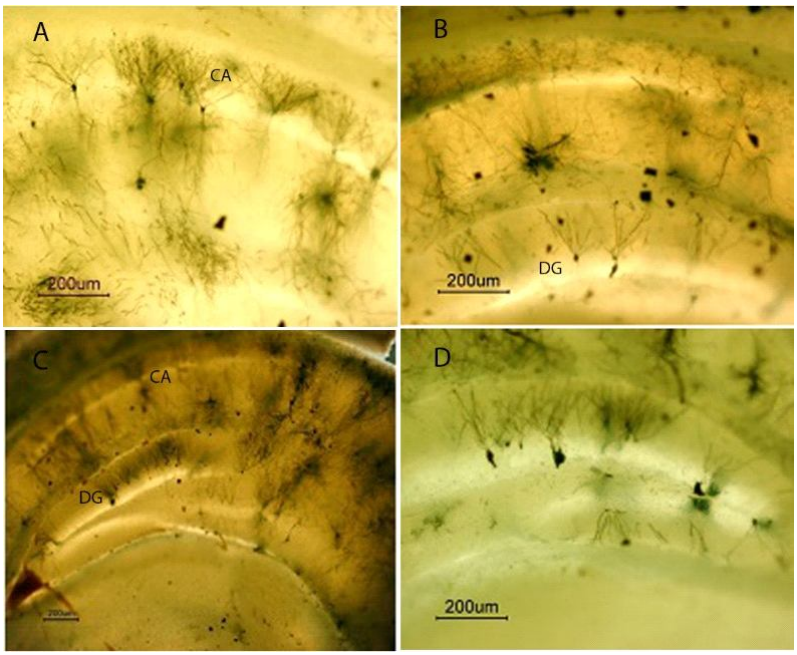

Image 1: microscopic sections of hippocampus stained through Golgi method: A and B: hippocampus section of ovariectomized rat model with indirect contact with male mice. Pictures C and D: ovariectomy female rat hippocampus sections with direct contact with male mice. DG dentate gyrus and CA1 of hippocampal. The difference can be seen in the branching pattern of nerve cells.

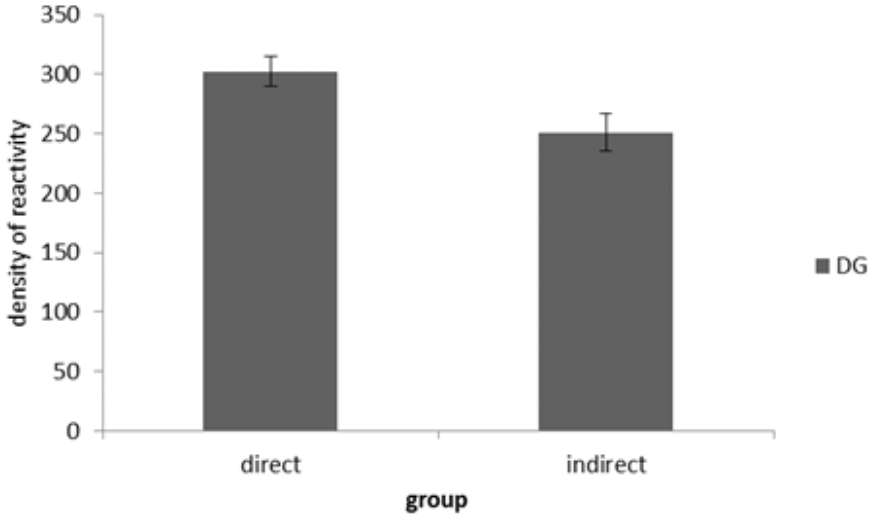

Figure 1: cell density and tonality of the neuronal connections in DG. Despite the increase in tonality in female rats directly contact with the male compared to females indirectly related to male mice (indirect), the increase showed no significant difference ($P \leq 0.05$).

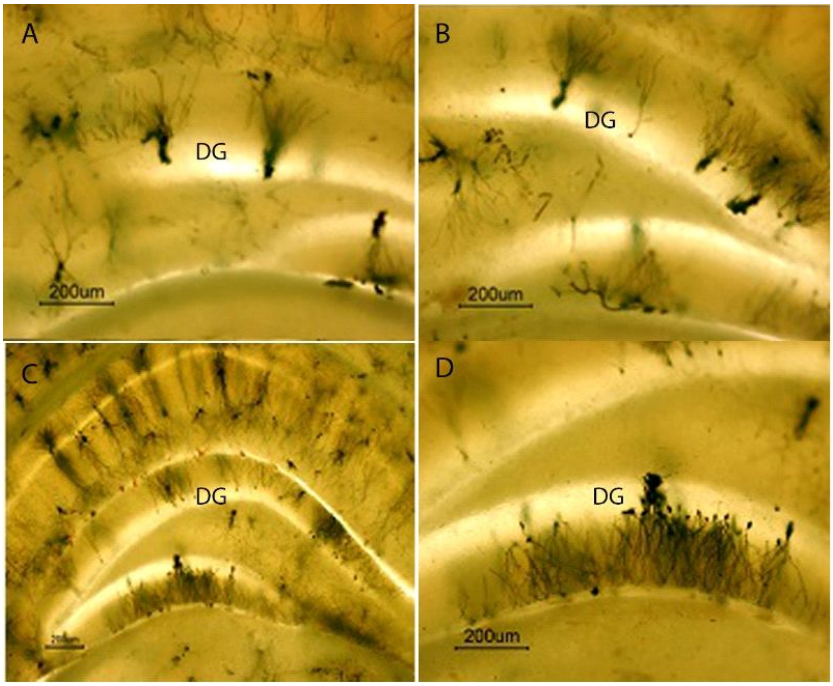

Figure 2: Microscopic sections of hippocampus stained through the Golgi method: A and B: Hippocampus sections of healthy female mice in contact with male mice castrate. Pictures C and D: the hippocampus section of female mice in indirect contact with healthy male mice. Dentate gyrus DG. The difference in the splits and tonality and patterns can be seen.

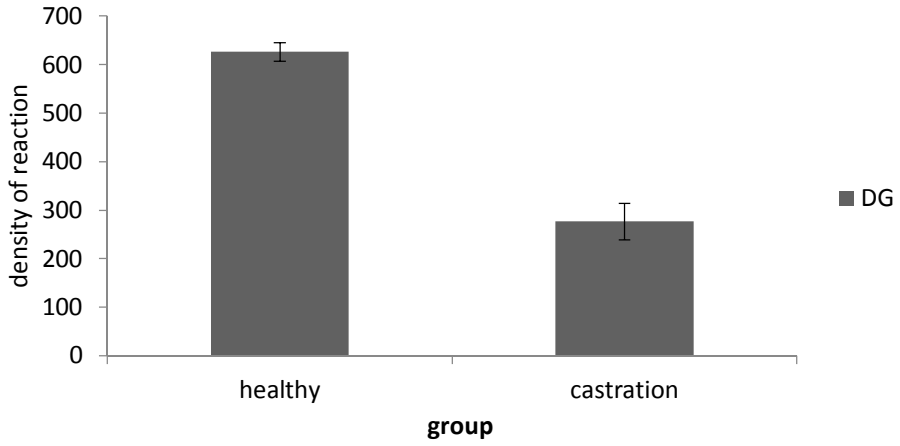

Figure 2: the density of neurons and neuronal connections in the DG tonality area in two groups of healthy mice with directly exposed to normal male mice (Healthy) and indirectly exposed to male castrate mice (Castration). DG tonality of the control group showed a significant increase compared to neutered male (P≤ 0.05).

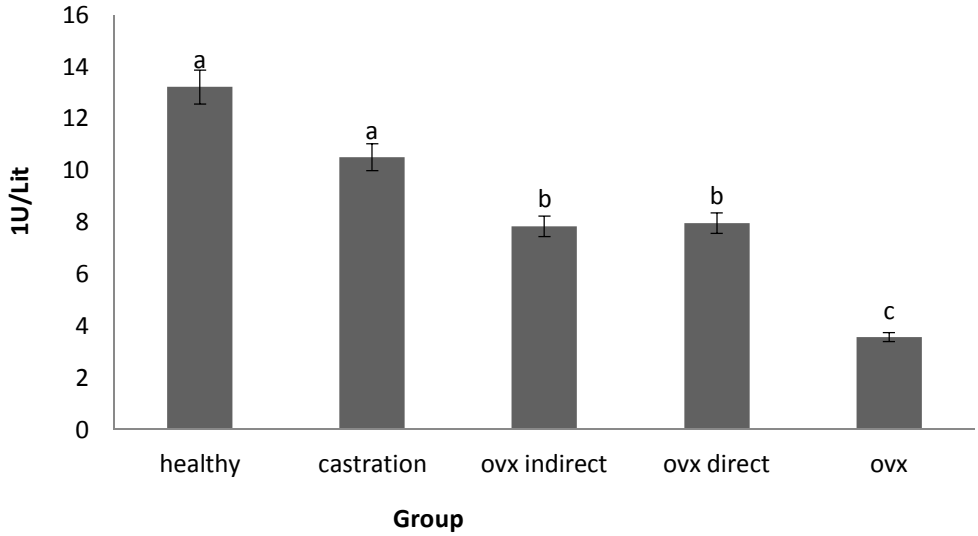

Figure 3: Comparison of LH concentrations in groups: Healthy female-healthy male groups, castration: female male castration, the ovariectomy- Healthy male (ovx indirect), the ovariectomy-healthy male (ovx direct) and ovariectomy (ovx). LH concentration in healthy male-healthy female (indirect) showed a significant increase in comparison to all groups. LH concentration in Ovariectomy-healthy male group compared to ovariectomy group showed a significant increase. Column A with each other and column b with column c are significant (P≤ 0.05).

Discussion

As can be seen in the microscopic sections stained with the Golgi technique, the increased cell density in the dentate gyrus (DG) in ovariectomized rats with direct contact with male mice is negligible compared with indirect. In another part of this study, the comparison if the effect of pheromones in the induction of neurogenesis in the hippocampus was performed by exposing adult female mice in a cage separated by a veil from healthy male and also in female mice in the presence of castrate male mice. Comparison of neuronal density, split redundancies and tonality in DG in two groups showed that the neuronal density in female rats in cage of healthy male mice was more than castrate male. Female sex pheromones directly effect on testosterone levels and thus on the reproduction activity of male rats. Sexual behavior of male mice requires adequate amounts of testosterone in the brain of animals. Males need both the testosterone produced by the testes and chemical-sensory input to olfactory bulb. Castrated males or males without olfactory bulb has no sexual attraction to females (1). There are testosterone and sex hormones of progesterone and estradiol in rat urine. The three hormones associated with sexual messages and create sexual attractiveness in reproductive behavior (16).

Gonadotropin hormone and luteinizing hormone (LH) play a role in adult neurogenesis. During the estrous cycle of female mice, LH change plays a major role in pregnancy and also involves in the sexual behavior of male and female mice. Exposure to male pheromones or subcutaneous injections of high doses of LH to ovariectomy mice increases cell proliferation and immature nerve cells in both DG and SVZ. Interestingly, low doses of LH increase the number of BrdU-labeled cells in DG. In addition, an increase in neurogenesis of the hippocampus after exposure to male pheromones in mice lacking LH receptor was evident. The results pointed out the role of LH in adult neurogenesis in the dentate gyrus (8). As can be seen from LH measurement comparison chart, neuronal density chart and staining intensity, the groups of healthy female with healthy male had higher plasma LH level compared to female in the vicinity of the castrate male. It imparts the effect of pheromone secretion of male mice on hormone neural axis, followed by a significant increase in female rat hippocampus dentate gyrus neurons splits. Two studies suggested some chemicals in the urine of male mice (such as 3 and 4 dihydroexbroicomin, heptanone and Octanon) which were effective on physiological behavior of mice as pheromone (15, 17). Mak et al. have shown that pheromones stimulate neurogenesis formation in mice brain. Exposing mice to the urine of male mice causes increased cell proliferation in the adult-born SVZ and the dentate gyrus. Male pheromone is responsible for the formation of chemical sensory stimuli to increase proliferation in the female hippocampus and SVZ. In fact, exposure to the smell of male castrate blocks the increase of mitotic activity in both adult female mouse germs. In addition, male pheromones create a certain sensory stimulus that stimulates proliferation in SVZ of adult females and the dentate gyrus. The destruction of the olfactory epithelium destroys pheromone-stimuli neurogenesis in SVZ and dentate gyrus which expresses that the olfactory system is a main course and transmission of sensory stimuli is responsible for the increased proliferation in the SVZ and the dentate gyrus. Exposure to male pheromones, not only promotes the proliferation of new neurons, but also leads to an integration of a high rate of mature neurons in the olfactory bulb and the hippocampus (18).

It was shown in another study that long-term exposure to male mice increases maternal behavior in mice. Ovariectomy can prevent this effect. This indicates that the presence of ovarian hormones is required (19). An exposure to male mice induces an increase in the number of BrdU-labeled cells in the SVZ

of female mice. This effect can be avoided with ovariectomized and restore by treatment with estradiol. This evidences indicate that exposure to male and circulating levels of estrogen are associated with cell proliferation (19). The ability of female sex hormones to protect nerve cells is in the relationship with the ability to increase BDNF levels. As a result, sex hormones affect cell proliferation and survival by affecting neurotrophic factors which can be named as a factor causing neurons (20). According to the results of hormonal assessment and the significant correlation of female sex hormone levels in the female rats of different groups with neuron tonality of dentate gyrus of hippocampus, the results of present study are reinforced through the results of Golgi method and histological study. Studies show that male pheromones and sense of touch can increase the number of GnRH cells. This increase LH and therefore sexual behavior (21). Nerve cells in the olfactory bulb expressing gonadotropin-releasing hormone, receive messages from pheromones and send to the hypothalamus which leads to the release of LH from the anterior pituitary. Understanding the relationship between pheromones and increased neurogenesis, it is clear that endocrine interactions are likely to be involved in mediating neurogenesis. The olfactory bulb and hippocampus neurogenesis are evolutionarily conserved and a number of environmental and physiological factors increase the neurogenesis in the adult mammalian brain. Evidence suggests that pheromones and hormones mediators can be involved in the neural ductility and neurogenesis in the adult central nervous system (21). Also, one of the stages in the process of neurogenesis mature nerve cells including the development of axons, dendrites and synaptogenesis (1) is shown in this study, using the Golgi method and changes in nerve cell connections.

Pheromones stimulates neurogenesis in the brain stems of female rats. Pheromone of healthy male mice increases the level of the LH hormone in female mice followed by cell proliferation in the dentate gyrus.

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