

## Effects of 17- $\beta$ estradiol on neurogenesis in the hippocampus of ovariectomized mice

Maryam Azari<sup>1</sup>, Mohammad Taghi Ghorbanian<sup>1\*</sup>

Received: 19 May, 2018; Accepted: 11 Aug, 2018

### Abstract

**Background & Aims:** Adult neurogenesis occurs in the two main areas of the brain of most mammalian species in; sub ventricular zone, and the dentate gyrus of the hippocampus. Many factors such as 17- $\beta$  estradiol affect neurogenesis in the hippocampus. The aim of this study was to investigate the effect of exogenous 17- $\beta$  estradiol on neurogenesis in the ovariectomized (OVX) mice.

**Materials & Methods:** NMRI mice were divided into five experimental groups: 1- Sham, 2- Control, 3- Treated with single dose of 17- $\beta$  estradiol two weeks after ovariectomy and euthanized 24 hours later, 4- Treated with single dose of 17- $\beta$  estradiol two weeks after OVX and euthanized 48 hours later, 5- Treated with single dose of Sesame Oil (vehicle) 2 weeks after OVX and euthanized after 24 hours. Animals were perfused transcardially with paraformaldehyde. Brains were removed and its sections for cresyl fast violet staining and Glial fibrillary acidic protein (GFAP) and BrdU immunohistochemistry were prepared. Cells were counted and investigated using light and fluorescent microscopy.

**Results:** Neuronal density and Proliferation of hippocampal progenitor cells in the CA1 region of 17- $\beta$  estradiol -treated mice were significantly increased up to 24 hours. The density of astrocytes in different region of the hippocampus was significantly increased after treatment by 17- $\beta$  estradiol.

**Conclusion:** Cell shape and density of hippocampal CA1 neurons are influenced by 17- $\beta$  estradiol. In addition, density and morphology of glial cells, especially astrocytes in different regions of the hippocampus are affected by 17- $\beta$  estradiol. Thus, 17- $\beta$  estradiol can improve neurogenesis in the CA1 region of the hippocampus.

**Keywords:** 17- $\beta$  estradiol, Ovariectomy, Neurogenesis, Hippocampus, Dentate Gyrus

**Address:** Damghan University, Damghan, Iran. P.O. Box 3671641167

**Tel:** +98-23-35220112

**Email:** ghorbanian@du.ac.ir

### Introduction

Neurogenesis in the adult mammalian is a process, which includes both cell proliferation and survival. The continuous ability of neuron formation was discovered at different areas of the adult brain that accommodate

neural stem cells (NSCs) and are termed neurogenic zones. Neural stem cells are multipotent and self-renewal, producing neurons, astrocytes, and oligodendrocytes in the central nervous system (1). In normal condition, neurogenesis mainly occurs in two

<sup>1</sup> School of Biology and Institute of Biological Sciences, Damghan University, Damghan, Iran

<sup>2</sup> School of Biology and Institute of Biological Sciences, Damghan University, Damghan, Iran (Corresponding author)

distinct areas of the brain called the sub ventricular zone (SVZ) in the anterior part of the brain ventricle and the subgranular zone (SGZ) in the dentate gyrus (DG) in the hippocampus (2-8). In some species, it might occur in other regions, as in CA1 of the hippocampus, neocortex, amygdala, striatum and substantia nigra (9, 10). Previous study has demonstrated that astrocytes function as the progenitors of these new neurons (11). The interaction between astrocytes and NSCs has an important role in neurogenesis (11, 12). Astrocytes in hippocampus produce signals that promote proliferation and differentiation of NSCs. Moreover, factors derived from astrocytes may stimulate synaptogenesis in newly formed neurons (12, 13). Astrocytes play an important role in the generation, migration, integration, and survival of new neurons (14). Many researchers have shown the hippocampal neurogenesis is also modulated by the steroid hormones like testosterone, estrogen, adrenal steroids, and peptide hormones like prolactin. The evidence indicates that fluctuations of ovarian hormones are powerful modulators of neurogenesis in the adult hippocampus (5, 15). Among the factors regulating neurogenesis, estrogen further attention has been paid (3). Estrogen has a direct effect on areas associated with memory and cognition in the brain including dorsal part of the hippocampus, where episodic and spatial memory is formed (14, 15). On the basis of its neuroprotective and neurotrophic effects on neurons *in vivo* and *in vitro*, estradiol may affect proliferation and survival of new cells formed in SGZ and DG. Neurogenesis can be regulated either by estrogen produced in ovaries or other hippocampal neurons (16). In adult female, estrogen, progesterone, and glucocorticoids fluctuate during the estrous cycle, pregnancy and lactation. Exogenous and endogenous estradiol affects cell death in DG. High levels of estradiol has been shown to decrease cell death in the hippocampus of virgin OVX female rats (6).

Estrogen influences neurogenesis, anti-apoptotic activities, reproduction, cell proliferation and survival of newly formed neurons in DG of the hippocampus. These effects depend on time period, dose, and density of estrogen receptors in cells. Alterations in physiological conditions of the body especially hormonal changes following aging deficiencies in learning and memory, changes in neurotransmitters and steroid receptors occurs within the hippocampus (17). As there is much document that estrogen loss induced by menopause can deteriorate the effects of aging on cognitive functions, the clinical trials of hormone substitution therapies and their effectiveness on cognitive symptoms experienced by women (18). Replacement of steroid hormones especially estradiol may minimize these degenerative changes and might be a candidate as a treatment. In this study, we examined the effects of 17- $\beta$  estradiol on the neurogenesis in OVX mouse to evaluate this hypothesis.

## Materials and Methods

### Experimental Animals:

In this study thirty-five female NMRI mice aged 6-8 months were housed four per cage under conditions of constant temperature ( $23\pm 1^\circ\text{C}$ ) and humidity (50%) in a 12:12 hr light-dark cycle with ad libitum access to food and water. Five groups of OVX female mice were used in our experiment: (1) Control group, OVX with no treatment (control); (2) sham-operated mice with no treatment (sham); (3) OVX with 17- $\beta$  estradiol treatment, euthanized 24 hours later (24h-treatment); (4) OVX with 17- $\beta$  estradiol treatment, euthanized 48 hours later (48h-treatment); (5) OVX with vehicle treatment (vehicle) (19). All procedures were conducted by the Guidelines on Animal Care and approved beforehand by the Institutional Animal Care and Use Committee of Biology Department of Damghan University.

Mice were bilaterally OVX under ketamine (100 mg/kg) and xylazine (10 mg/kg) anesthesia and aseptic

conditions. Briefly, a single midline incision was made in the low abdominal area to expose the ovary; oviducts were bilaterally ligated and ovaries removed. After suturing their muscles and skin, the animals were returned to their home cages to recover for two weeks. The hormone therapy began two weeks after surgery. 17- $\beta$  estradiol (Sigma; 10  $\mu$ g) was dissolved in 0.1 mL of sesame oil and 0.01mL ethanol and administered subcutaneously (19). Two hours after 17- $\beta$  estradiol treatment all groups received 50  $\mu$ g of BrdU intra peritoneally. The mice were perfused transcardially with 4% Paraformaldehyde (PFA) in Phosphate-buffered saline (PBS), and brains were postfixed overnight and embedded in paraffin.

#### **Nissl staining and Immunohistochemistry:**

After fixation and paraffin embedding, five  $\mu$ m coronal sections were prepared with use of the rotary microtome. According to the Paxinos atlas, SN was located at a distance 2.8 to 4.52 mm of bregma. Sections were stained with cresyl fast violet 0.5% and cells were counted under light microscope. Primary antibodies were monoclonal anti-BrdU (Immunohistology Grade) mouse ascites fluid (mouse IgG1 isotype) (1:200; Sigma-Aldrich) 30  $\mu$ L per each sample, for BrdU staining, and monoclonal Anti-Glial Fibrillary Acidic Protein (GFAP) (mouse IgG1 isotype) (1:200; Sigma-Aldrich) 30  $\mu$ L per each sample, for GFAP staining; secondary antibodies were Anti-Mouse IgG(Fab specific) Peroxidase conjugate (1:200 Sigma- A2304) 30  $\mu$ L per each sample, for BrdU staining, and Goat Anti-Mouse IgG affinity purified, Rhodamine conjugate (1:200; Cat No: AP 124 R) 30  $\mu$ L per each sample, for GFAP staining. Fluorescent sections were examined

with a JapanE600 (Nikon Eclipse) fluorescent microscope. Controls included omitting the primary and secondary antibodies.

#### **Cell counting:**

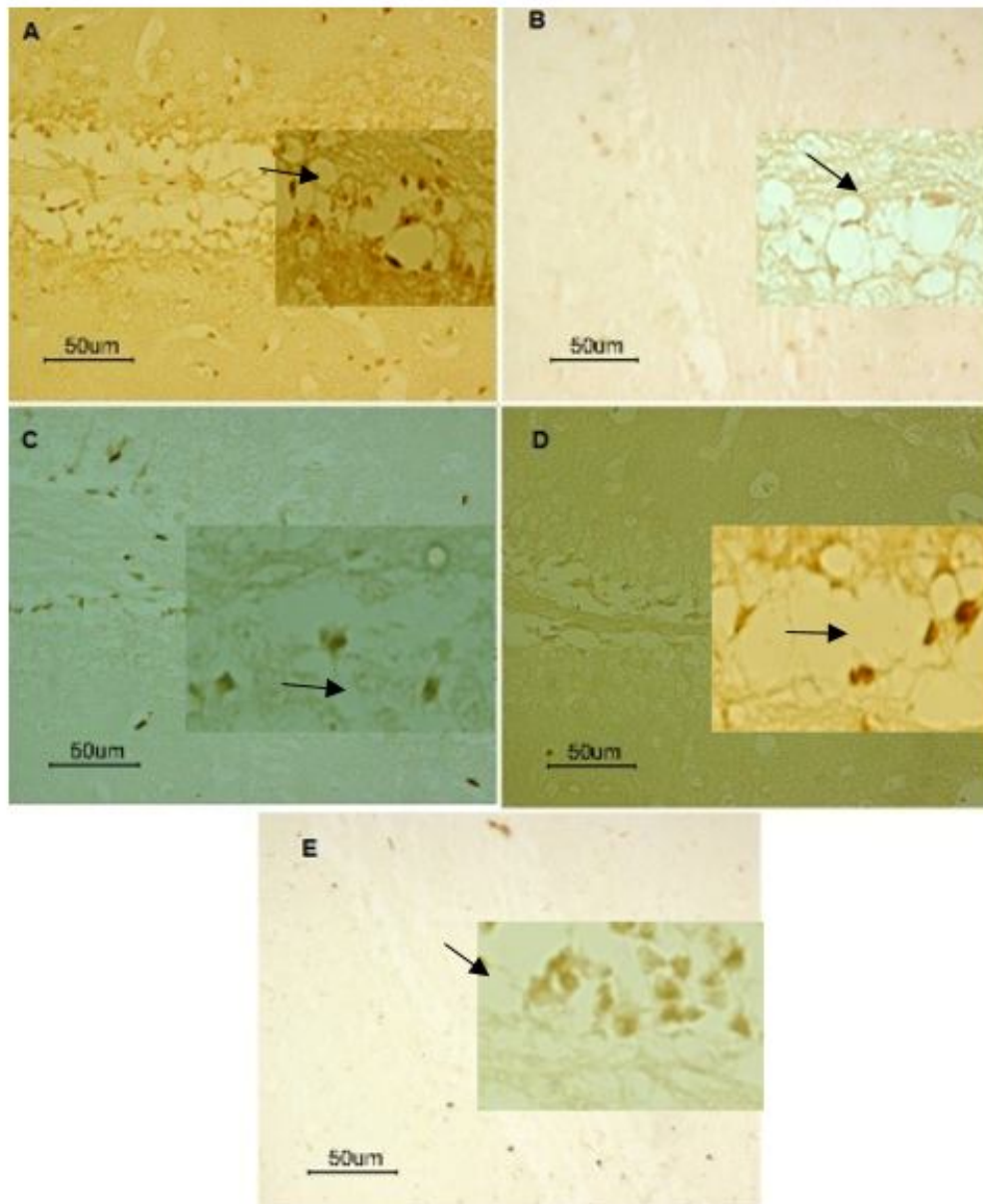
GFAP-positive cells in DG and nissl stained cells in DG, CA1 and CA3 were counted in three 5  $\mu$ m coronal sections per animal (n=5 per group), spaced 100  $\mu$ m apart, by an observer blind to the experimental condition using a Nikon microscope in bright field mode and a 40X objective and Nikon fluorescent microscope for GFAP immunohistochemistry. For the DG, all stained cells, with exception of apoptotic-like cells, within two cell diameters from the inner edge of the granule cell layer (GCL) of the DG were included in the analysis. For CA1 and CA3, cells were counted in five fields, every 400  $\mu$ m<sup>2</sup>. Results were expressed as the average number of cells per section.

#### **Statistical analysis:**

All quantitative data were expressed as mean  $\pm$  SEM. The data were analyzed by a repeated measurement analysis of variance (ANOVA). Cell counting data were analyzed by one-way ANOVA followed by Tukey's post hoc test. P values < 0.05 were considered statistically significant.

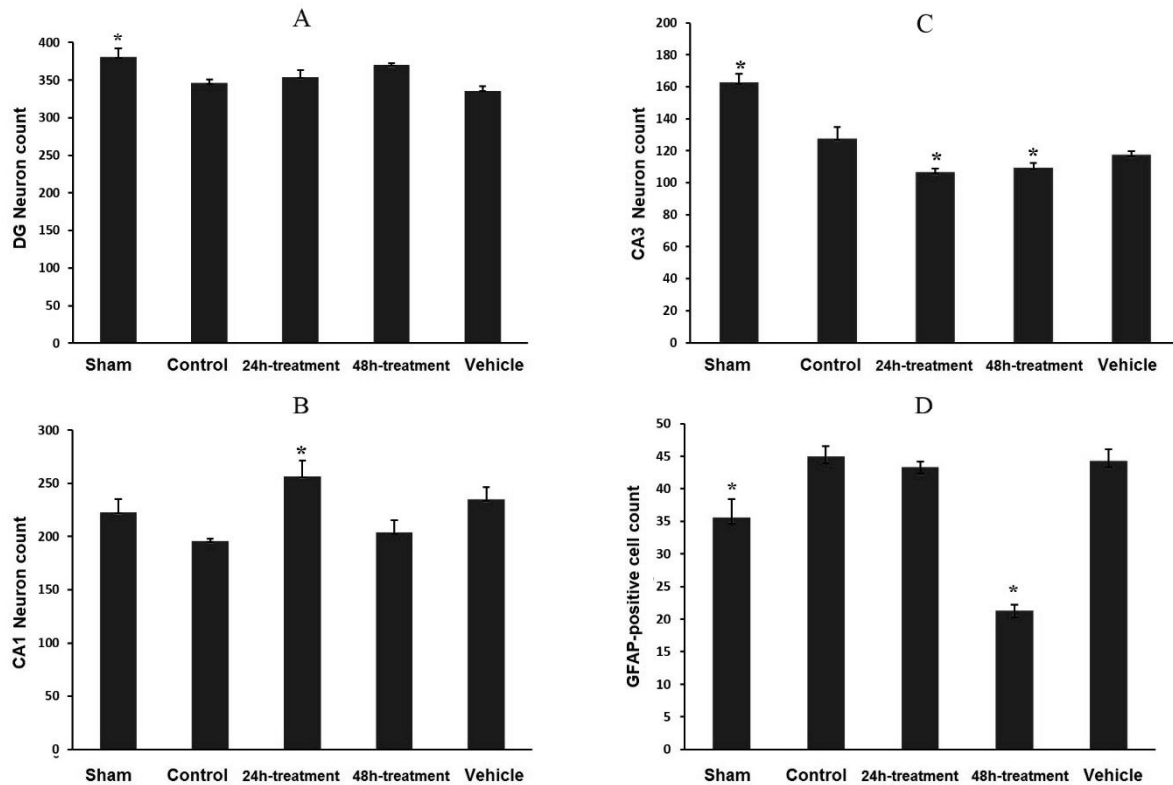
#### **Results**

To determine whether 17- $\beta$  estradiol administration could enhance neurogenesis in the DG of the hippocampus, mice were treated with BrdU, which labels cells that undergo DNA replication in S-phase and therefore reflects the current rate of cell division. As shown in Fig. 1, BrdU -positive cells in the DG in 17- $\beta$  estradiol-treated mice, approved cell division in DG.



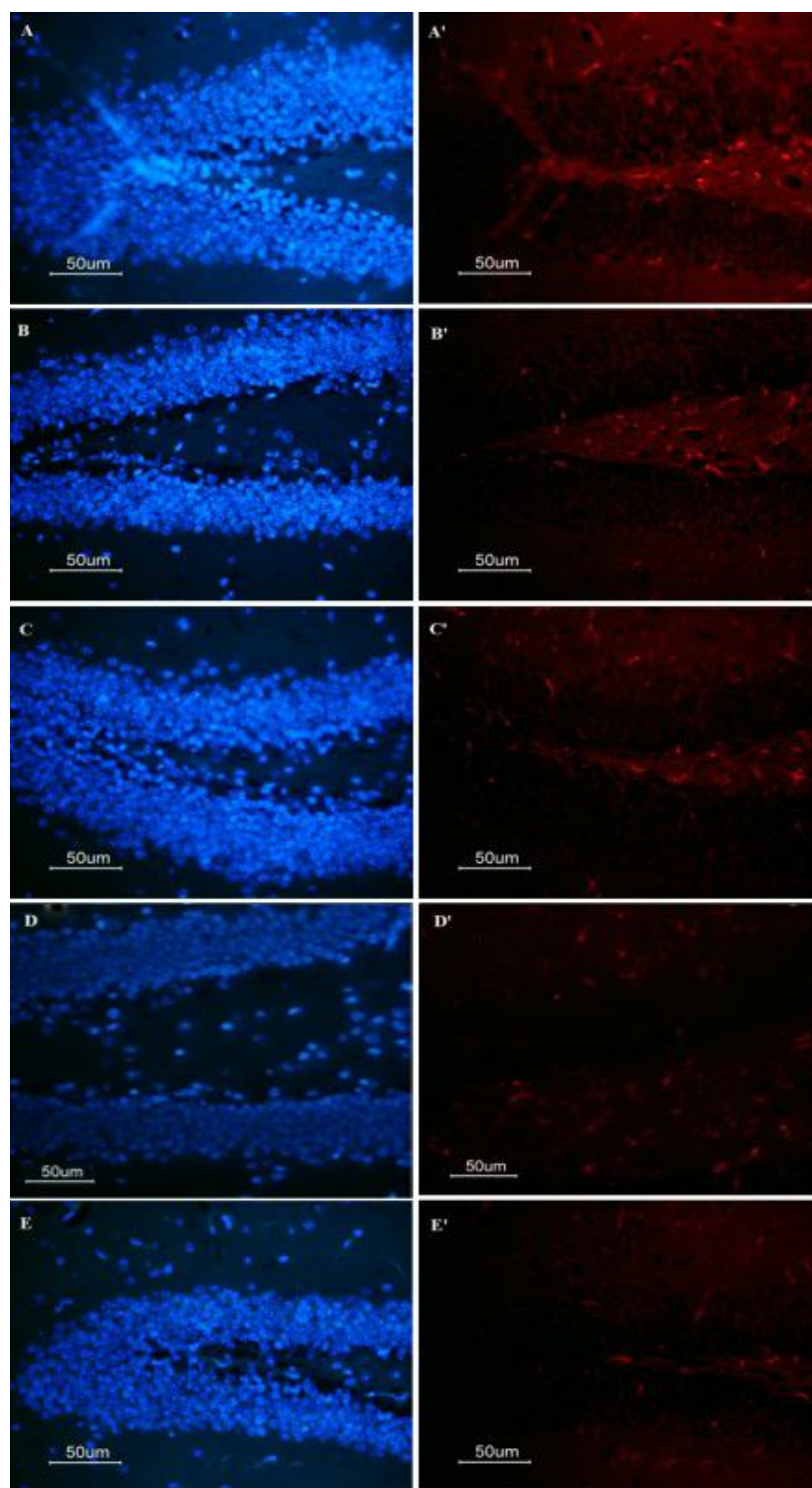
**Fig 1.** BrdU immunohistochemistry showed the presence of labeled cells in the hippocampus. BrdU labeled cells in DG in Sham (A), Control (B), Vehicle (C), 24h-treatment (D), 48h-treatment (E). Immunohistochemistry and detection of proliferating cells by BrdU. Positive cells were shown with arrow.

Nissl staining showed a higher number of neurons in DG of a sham with respect to the control group (\*  $P < 0.05$ ). Treatment with 17- $\beta$  estradiol did not affect the number of cells in DG after 24 or 48 hours (Fig. 2 A). On the other hand, the number of neurons in CA1 increased significantly 24 hours after 17- $\beta$  estradiol treatment (\*  $P < 0.05$ ) with respect to the control group (Fig. 2 B).



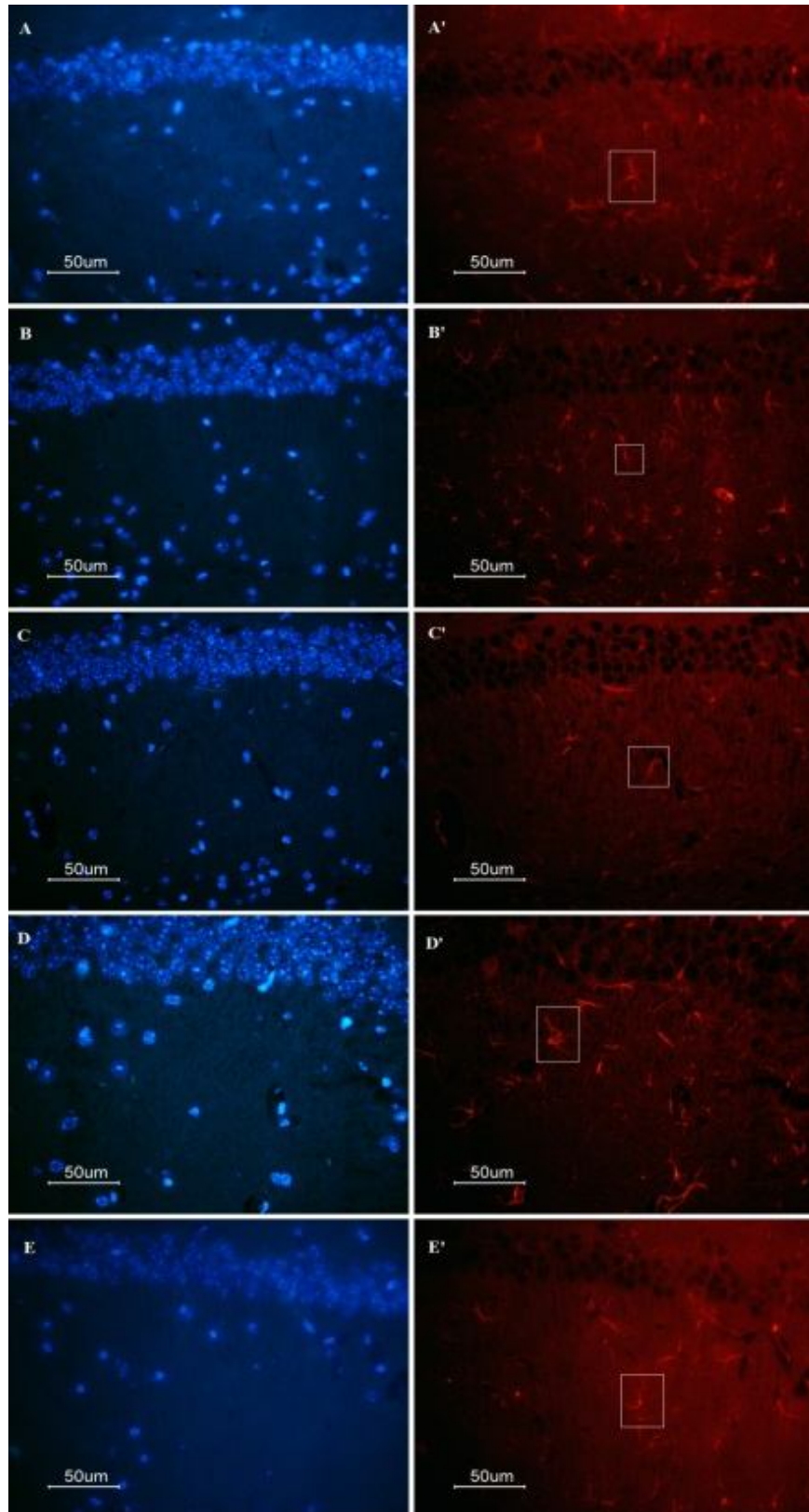
**Fig 2.** **A.** Number of neurons in DG among different groups. Treatment with 17- $\beta$  estradiol did not affect the number of neurons in DG. However, there was a significant increase in the number of neurons in the Sham-operated group, **B.** Number of neurons in CA1 among different groups. Treatment with 17- $\beta$  estradiol significantly increased a number of neurons in CA1 in the 24h-treatment group, **C.** Number of neurons in CA3 among different groups. Treatment with 17- $\beta$  estradiol significantly decreased the number of neurons in CA3 of both 24h-treatment and 48h-treatment groups. There was a significant increase in the number of neurons in the Sham-operated group, **D.** Number of GFAP-positive cells in DG among different groups. Treatment with 17- $\beta$  estradiol significantly decreased the number of GFAP-positive cells in DG of 48h-treatment group. Also, a significant decrease was seen in the sham-operated group (\* $P < 0.05$ ) with respect to the control group.

Interestingly, in CA3, the number of neurons significantly decreased 24 and 48 hours after 17- $\beta$  estradiol treatment (\* $P < 0.05$ ) with respect to the control group. However, cell counts in CA3 of the sham group revealed a higher number of neurons (\* $P < 0.05$ ) with respect to the control group (Fig. 2 C). GFAP immunohistochemistry showed an increase in GFAP-positive cells in DG 24 hours after 17- $\beta$  estradiol treatment in compared with 48 hours group (\* $P < 0.05$ ) with respect to the control group (Fig. 2 D). Morphologically, astrocytes in DG were star-shaped with short extensions traversing into granular cell layer. These cells were more granular and had more extensions in CA1 of 17- $\beta$  estradiol-treated mice (Fig. 3 & 4).

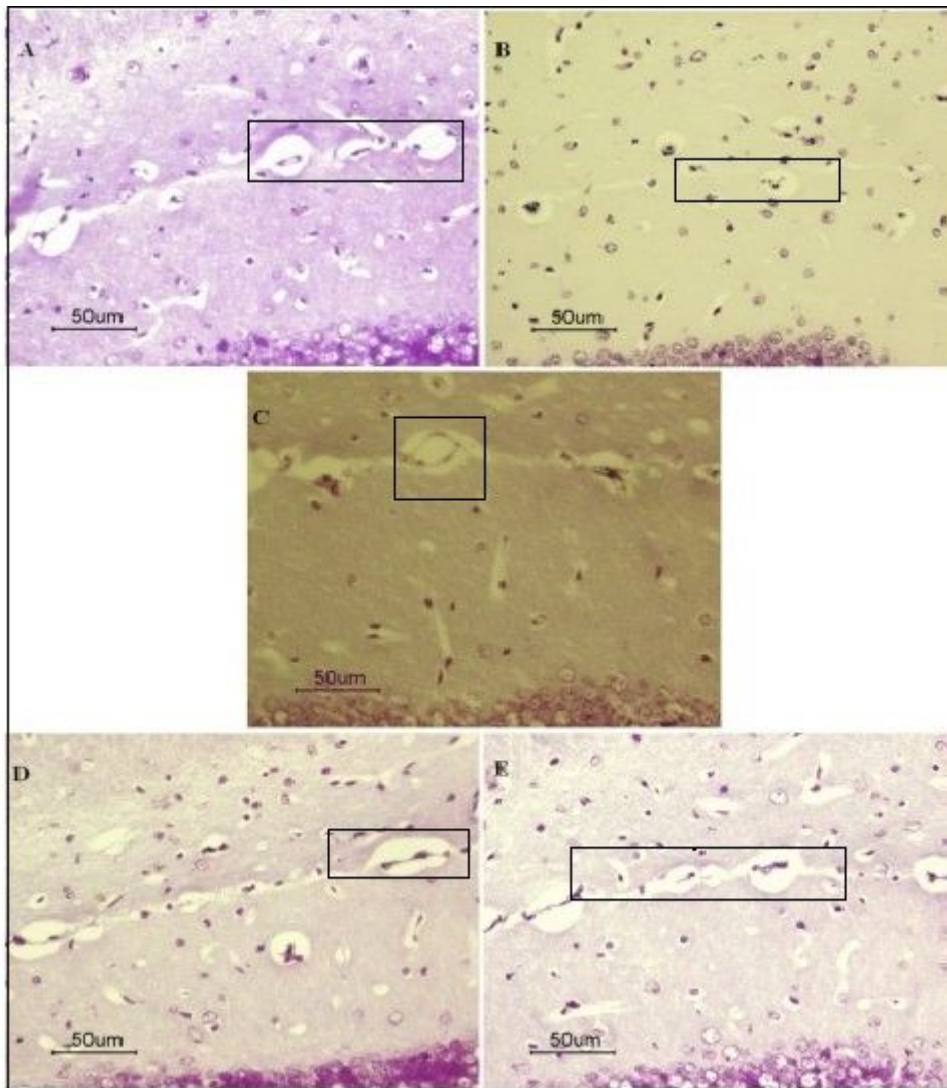


**Fig 3.** GFAP Immunohistochemistry of DG in different groups. DAB staining of nuclei (left column) and GFAP-positive cell morphology in DG (right column) are shown in the Sham (A, A'), Control (B, B'), Vehicle (C, C'), 24h-treatment (D, D') and 48h-treatment (E, E') groups.

Studying the histology of the hippocampus revealed that hilus diameter might decrease upon ovariectomy. Apparently, according to the results obtained from Nissl staining, vessel diameter in the hippocampus of 17- $\beta$  estradiol-treated mice might increase (Fig. 8).



**Fig 4.** GFAP Immunohistochemistry of CA1 in different groups. DAB staining of nuclei (left column) and GFAP-positive cell morphology in CA1 (right column) are shown in the Sham (A, A'), Control (B, B'), 24h-treatment (C, C'), 48h-treatment (D, D') and Vehicle (E, E') groups.



**Fig 5.** Blood vessels in the molecular layer of the hippocampus in Sham (A), Control (B), Vehicle (C), 24h-treatment (D) and 48h-treatment (E). It indicates changes in blood vessels in the hippocampus.

## Discussion

This study shows that 17- $\beta$  estradiol treatment mainly affects neurogenesis in CA1 after 24 hours, while it does not influence this process in CA3 or DG. In this study the OVX mice was treated with single dose of 17- $\beta$  estradiol two weeks after that.

A single injection of 0.3  $\mu$ g or 10  $\mu$ g of estradiol restores the ovariectomy-induced reduction in cell proliferation, while doses of 1  $\mu$ g or 50  $\mu$ g are ineffective at restoring cell proliferation levels. The length of hormone exposure is also critical as it

influences the outcome of estrogen exposure on cell proliferation in the dentate gyrus of female rodents (20).

Many factors change neurogenesis in the hippocampus, however the focus of this study will be on 17- $\beta$  estradiol that affect hippocampal neurogenesis, in the OVX mice. Many neurons undergo cell death approximately two weeks after differentiation, although estradiol treatment may either increase or inhibit their survival (6). Treatment with 17- $\beta$  estradiol significantly increased a number of neurons in CA1 in the 24h-treatment. In rats, CA1 is the main responder to estrogen



and in mice its effects are seen throughout the hippocampus, with more morphological changes in CA1 (15). Therefore, estrogen treatment response may vary among species. Treatment with acute dose of estradiol increases cell division 4 hours after administration, but due to hypothalamic-pituitary axis activation and secretion of adrenal steroids, this effect will disappear after 48 hours (6). We observed that a short-term treatment (24 hours) with estradiol has significant effects on neurogenesis. Estradiol has rapid actions through receptors that in many cases are posttranslationally modified forms of the classical estrogen receptors (21). Recent evidence suggests that aging perhaps accompanied by a loss of estrogen sensitivity in the rat hippocampus (15). The menopausal transition is accompanied by changes in ovarian hormonal levels. Women undergoing the menopausal transition often report memory problems, among different symptoms (22). These results indicated a increase in GFAP-positive cells in hippocampus, 24 hours after 17- $\beta$  estradiol treatment in compared with 48 hours group. The primary precursors in the neurogenic region of the adult brain have been identified recently as having the characteristics of astrocytes and expressing GFAP (23). Adult NSCs located in these specific niches and astrocytes promote neuronal fate commitment and proliferation of SGZ NSCs (24). Following damage, cell division was increased and administration of estradiol increased progenitor cell division after 24 hours. Number of GFAP-positive cells in hippocampus of female mice fluctuates during estrous cycle. In proestrous, in which estrogen concentration is high, GFAP-positive cells are more abundant (25). Astrocytes possess estrogen receptors and their response is mainly seen through their morphological alterations. These changes may affect synaptic communication regulation (26, 27). Estrogen receptors are present in smooth muscles of vessels. By stimulation of prostacyclin and nitric oxide production, and also inhibition of

vasoconstrictor formation, estradiol acts as a vasodilator in human and laboratory animals (28). Estrogens are powerful regulators of vasomotor tone, that stimulates formation of the vasodilator nitric oxide (NO) in endothelial cells and 17 $\beta$ -estradiol-dependent activation of endothelial nitric oxide formation and subsequent vasodilation (29).

In summary, exogenous 17- $\beta$  estradiol treatment increases the number of neurons in CA1 and progenitor cell division in the hippocampus after 24 hours. GFAP-positive cells also increase in the hippocampus of OVX female mice following 17- $\beta$  estradiol treatment.

### Conflict of interest disclosure

The authors declare there are no conflicts of interest

### Acknowledgments

The authors express their appreciation to the biological school of Damghan University for supporting this research.

### References

1. Gheusi G, Ortega-Perez I, Murray K, Lledo PM, A niche for adult neurogenesis in social behavior. *Behav Brain Res* 2009; 200(2): 315-22.
2. Brock O, Keller M, Veyrac A, Douhard Q, Bakker J. Short term treatment with estradiol decreases the rate of newly generated cells in the subventricular zone and main olfactory bulb of adult female mice. *Neuroscience* 2010; 166(2): 368-76.
3. Chiba S, Suzuki M, Yamanouchi K, Nishihara M. Involvement of granulin in estrogen-induced neurogenesis in the adult rat hippocampus. *J Reproduc Develop* 2007; 53(2): 297-307.
4. Kordower JH, Chen E-Y, Morrison JH. Long-term gonadal hormone treatment and endogenous neurogenesis in the dentate gyrus of the adult female monkey. *Experimen Neurol* 2010; 224(1): 252-7.
5. Lieberwirth C, Pan Y, Liu Y, Zhang Z, Wang Z. Hippocampal adult neurogenesis: Its regulation and

- potential role in spatial learning and memory. *Brain Res* 2016 Aug 1;1644:127-40.
6. Pawluski JL, Brummelte S, Barha CK, Crozier TM, Galea LA. Effects of steroid hormones on neurogenesis in the hippocampus of the adult female rodent during the estrous cycle, pregnancy, lactation and aging. *Front Neuroendocrinol* 2009; 30(3): 343-57.
  7. Seki T, Jianfei Lu, Anatol Manaenko, Qin Hu. Targeting Adult Neurogenesis for Poststroke Therapy. *Stem Cells Int* 2017; 2017: 5868632.
  8. Zhao C, Deng W, Gage FH. Mechanisms and functional implications of adult neurogenesis. *Cell* 2008; 132(4): 645-60.
  9. Galea LA. Gonadal hormone modulation of neurogenesis in the dentate gyrus of adult male and female rodents. *Brain Res Rev* 2008; 57(2): 332-41.
  10. Taupin P. Neurogenesis in the adult central nervous system. *Comptesrendusbiologies* 2006; 329(7): 465-75.
  11. Seri B, Garcia-Verdugo JM, Collado-Morente L, McEwen BS, Alvarez-Buylla A. Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus. *J Comparative Neurol* 2004; 478(4): 359-78.
  12. Song H, Stevens CF, Gage FH. Astroglia induce neurogenesis from adult neural stem cells. *Nature* 2002; 417(6884): 39-44.
  13. Song HJ, Stevens CF, Gage FH. Neural stem cells from adult hippocampus develop essential properties of functional CNS neurons. *Nature Neuroscience* 2002; 5(5): 438-45.
  14. Archana Gengatharan, Rodrigo R. Bammann, Armen Saghatelian. The Role of Astrocytes in the Generation, Migration, and Integration of New Neurons in the Adult Olfactory Bulb. *Front Neurosci* 2016; 10: 149.
  15. Maya Opendak, Brandy A. Briones, Elizabeth Gould. Social behavior, hormones and adult neurogenesis. *Front Neuroendocrinol* 2016; 41: 71-86.
  14. Isgor C, Watson SJ. Estrogen receptor alpha and beta mRNA expressions by proliferating and differentiating cells in the adult rat dentate gyrus and subventricular zone. *Neuroscience* 2005; 134(3): 847-56.
  15. Spencer JL, Waters EM, Romeo RD, Wood GE, Milner TA, McEwen BS. Uncovering the mechanisms of estrogen effects on hippocampal function. *Front Neuroendocrinol* 2008; 29(2): 219-37.
  16. Fester L, Ribeiro-Gouveia V, Prange-Kiel J, von Schassen C, Bottner M, Jarry H, Rune GM. Proliferation and apoptosis of hippocampal granule cells require local oestrogen synthesis. *J Neurochemistry* 2006; 97(4): 1136-44.
  17. Saravia F, Beauquis J, Pietranera L, De Nicola AF. Neuroprotective effects of estradiol in hippocampal neurons and glia of middle age mice. *Psychoneuroendocrinology* 2007; 32(5): 480-92.
  18. Yuko Hara, Elizabeth M. Waters, Bruce S. McEwen, and John H. Morrison. Estrogen Effects on Cognitive and Synaptic Health Over the Lifecourse. *Physiol Rev* 2015; 95(3): 785-807.
  19. Brock O, Keller M, Veyrac A, Douhard Q, Bakker J. Short term treatment with estradiol decreases the rate of newly generated cells in the subventricular zone and main olfactory bulb of adult female mice. *Neuroscience*. 2010 17;166(2):368-76.
  20. Mahmoud R, Wainwright SR, Galea LA. Sex hormones and adult hippocampal neurogenesis: Regulation, implications, and potential mechanisms. *Front Neuroendocrinol* 2016;41:129-52.
  21. Yuko Hara, Elizabeth M. Waters, Bruce S. McEwen, and John H. Morrison. Estrogen Effects on Cognitive and Synaptic Health Over the Lifecourse. *Physiol Rev* 2015; 95(3): 785-807.
  22. Yuko Hara, Elizabeth M. Waters, Bruce S. McEwen, and John H. Morrison. Estrogen Effects on Cognitive and Synaptic Health Over the Lifecourse. *Physiol Rev* 2015; 95(3): 785-807.
  23. Seri B, Garcia-Verdugo JM, McEwen BS, Alvarez-Buylla A. Astrocytes give rise to new neurons in the adult

- mammalian hippocampus. *J Neurosci* 2001; 21(18):7153-60.
24. Falk S, Götz M. Glial control of neurogenesis. *Curr Opin Neurobiol* 2017; 47:188-95.
25. Arias C, Zepeda A, Hernandez-Ortega K, Leal-Galicia P, Lojero C, Camacho-Arroyo I. 2009. Sex and estrous cycle-dependent differences in glial fibrillary acidic protein immunoreactivity in the adult rat hippocampus. *Hormones and behavior* 2009; 55(1): 257-63.
26. de Lacalle S. Estrogen effects on neuronal morphology. *Endocrine* 2006; 29(2): 185-90.
27. Xian Liu and Haifei Shi. Regulation of Estrogen Receptor  $\alpha$  Expression in the Hypothalamus by Sex Steroids: Implication in the Regulation of Energy Homeostasis. *Int J Endocrinol* 2015; 2015: 949085.
28. Tostes R C, Nigro D, Fortes Z B, Carvalho M H. Effects of estrogen on the vascular system. *Braz J Med Biol Res* 2003; 36(9): 1143-58.
29. Fredette NC, Meyer MR, Prossnitz ER. Role of GPER in estrogen-dependent nitric oxide formation and vasodilation. *J Steroid Biochem Mol Biol* 2018; 176:65-72.