Praca oryginalna *Original paper*

Expression of estrogen receptors α in hippocampal astrocytes of ovariectomized female rabbits

IZABELA KRAKOWSKA, JADWIGA JAWORSKA-ADAMU, LESZEK KRAKOWSKI*

Department of Animal Anatomy and Histology, Faculty of Veterinary Medicine, Agricultural University, 20-033 Lublin, Akademicka 12, Poland
*Department and Clinic of Animal Reproduction, Faculty of Veterinary Medicine, Agricultural University, 20-612 Lublin, Głęboka 12, Poland

Krakowska I., Jaworska-Adamu J., Krakowski L.

Expression of estrogen receptors α in hippocampal astrocytes of ovariectomized female rabbits

Summary

The aim of the study was to trace the ERα immunoreactivity in the hippocampal astrocytes of ovariectomized rabbits with and without the application of 17β-estradiol. The study comprised sexually mature female rabbits that had undergone ovariectomy. The animals were divided into two experimental groups. Group I comprised of the ovariectomized rabbits and group II – the ovariectomized animals treated with 17β-estradiol. The immunocytochemical reaction was conducted with the application of two antibodies against estrogen receptors α. In the ovariectomized rabbits which did not receive 17β-estradiol the astrocytes were characterized by ERα immunoreactivity. Similarly in group II expression of ERα was found in the hippocampus astrocytes following the application of 17β-estradiol. In astrocytes, these receptors are located in the cell body and initial processes and rarely in cell nuclei. The results suggest that astrocytes are the target cells for estrogens, changing their function and modulating hippocampal neuron activity.

Keywords: hippocampial glia, estrogen, steroid receptor

Not only neurons but also astrocytes are the targed cells affected by estrogen. In adult individuals they are involved in balancing the homeostasis of the central nervous system (cns), remodeling synapses and neuroprotection (12, 18-20). Estrogen stimulates these glia cells to secrete neurotrophic factors preventing neuron death (6, 7). Similarly to neurons, astrocytes point to the presence of estrogen receptors (ERs) especially in regions responsible for neuroendocrine mechanisms (2, 11, 18, 21). Other glia cells: oligodendrocytes and microglia do not display ERs immunoreactivity (15). The study revealed through using immunocytochemical methods in the hypothalamus that about half of the astrocytes *in vitro* express ERs. Just as in the case of the neurons, the presence of estrogen receptors α (ERα) and estrogen receptors β (ERβ) in astrocytes was revealed in different areas of the brain, including the hippocampus, using *in vitro* auto radiographic and immunocytochemical methods (10, 11). In hippocampus, in cerebral cortex there occur fewer ERα as well as the dominant ones ERβ (1). Similarly as in neurons, also in astrocytes ERα are localized in the body, nucleus and in initial processes (10, 11). Other authors have discovered ERα only in astrocyte nuclei (22). Much research has been done in different areas on many animal species: guinea pig (8), rat (24, 25), mouse (13), hamster (17), sheep (16), pig (26), cat (23).

The available literature contains a scarcity of data pertaining to ERα in rabbit brains, and mainly concerns neuronal ERα immunoreactivity in the forebrain, including the hippocampus – in which, with the exception of subiculum, no traces of the above have been noted (4, 6). Rabbits as poliestral animals are a very interesting research subject since their *oestrus cycle* is not typical of other animal species. Mature females are in a state of more or less permanent *oestrus*. A very characteristic aspect is the induced ovulation which occurs following copulation. Rabbit females have mature ovarian vesicles in their ovaries, which release estrogens. If fertilization does not occur, the vesicles undergo atresion (9). The aim of the study was to trace the ERα immunoreactivity in the hippocampal astrocytes of the ovariectomized rabbits with and without the application of 17β estradiol.

Material and methods

Fourteen sexually mature female rabbits of the Medium White Polish breed, 3-4 kg body weight, and 1,5-3 years of age were used for the study. All experiments were conduced with the approval of the Ethics Board, and consistent with regulations concerning animal experiments. The rabbits were subjected to bilateral ovariectomy (OVX) under full general anesthesia. 2 mg/kg of body weight of xylazyne and 0.05 mg/kg i.m. of body weight of atropine was applied as premedication. General anesthesia was conduced with 5% ketamine at a dose

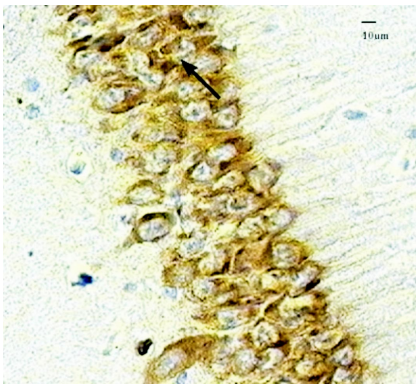

Fig. 1. In OVX rabbits, ERα immunoreactivity in astrocytes (↓) very intense, localized in nucleus, cell cytoplasm and the intitial astrocyte processes, and in other – very weak or absent in CA1

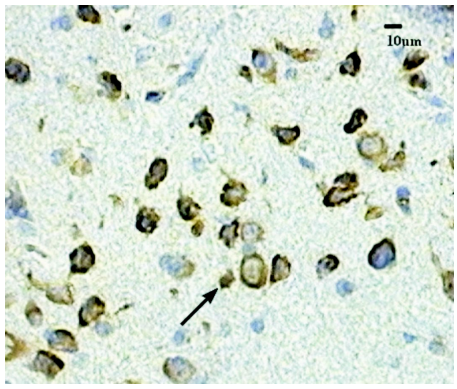

Fig. 2. In OVX rabbits, the very intensive of ERα immunoreactivity in astrocytes (↓) in subiculum

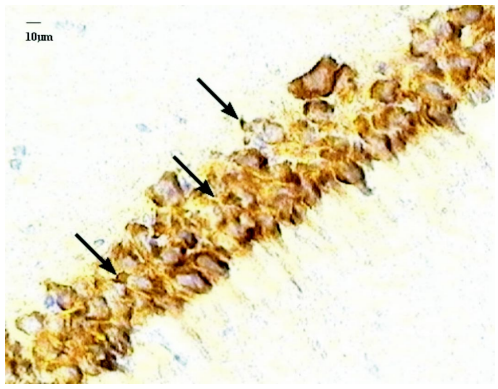

Fig. 3. In OVX+E2 rabbits ERα immunoreactivity in astrocytes (↓) very intesne, localized in nucleus, cell cytoplasm and the initial astrocyte processes, and in other – very weak or absent in area CA3

of 30 mg/kg of body weight. The animals were divided into two experimental groups and kept in controlled conditions with access to water and food *ad libitum.*

The rabbits were divided into two experimental groups. Group I consisted of 6 females OVX which did not receive 17β estradiol (E2) and from which material was taken 4 weeks following OVX. Group II comprised of 8 ovariectomized females which, 4 weeks following OVX, were given E2 (Masalin by Polfa) for 7 days at a daily dose of 5 µg/rabbit i.v. (OVX+E2). 24 h after the last dose of E2 the rabbits underwent euthanasia using morbital. The hippocampus was cut into paraffin slices 4 µm thick, which were deparaffined and then incubated in 3% H_2O_2 at room temperature for 10 minutes in order to block endogen peroxidase. The preparations were rinsed in distilled water and 0.5 M TBS (Tris Buffered Saline). The immunocytochemical reaction to ascertain ERα in astrocytes was conducted using the DAKO LSAB+Kit Peroxidase method. Antibodies were diluted in 0.5 M TBS of pH = 7.6, and the preparations were washed in this following the application of antibodies. The slices were incubated with two monoclonal mouse antibodies anti-ERα: NCL-L-ER-6F11 and NCL-ER-LH2 (Novocastra 1 : 40) for 48 hours at a temperature of 4°C. Next, secondary monoclonal biotylinated anti-mouse IgG (Biotynyled Link IgG, DAKO) was applied for 1 hour at room temperature and then the slices were incubated for 1 hour at room temperature with ready streptovidine-peroxidase comlex developed on rabbit (DAKO). DAB (3,3'-diaminobenzidine tetra hydrochloride, DAKO) was used as the chromogen at room temperature for 10 minutes. Additionally, the slices were stained using Mayer hematoxyline. A final non-soluble product was obtained of varied intensity of brown. The control of the reaction was carried out, omitting the primary antibodies for the antigen or it was replaced by normal rabbit serum. No reaction product was observed. ERα immunoreactive astrocytes were observed and photographed under a Jenaval optic microscope (Zeiss).

Results and discussion

Applying two primary monoclonal antibodies ER--6F11 and ER-LH2 against ERα did not point to any differences in cell distribution, intensity and immunostaining of the receptors and their inner location. In OVX females from group I of the experimental animals, which did not receive E2, the astrocytes were characterized by intense ERα immunoreactivity and only some astrocytes were stained with Mayer hematoxyline (fig. 1 and fig. 2). The results were similar in the second experimental group of OVX + E2 rabbits. A different intensity of colour on ERα of brown astrocytes in CA1, CA3 areas and the hippocampus subiculum region was also observed. The astrocytes were smaller than the neurons, having a star-like shape with a varied intensity of brown colour. The intracellular localization of ERα in astrocytes was described through using immunostaining. Immunoreactivity in all the areas of hippocampus was mainly localized in the cytoplasm of the body cells and in some processes as well as in nuclei of the cell astrocytes. Small astrocytes of intense immunostaining and immunonegative cells were observed in CA1, among intensely immunoreactive ERα neurons. Most of the astrocytes, similarly to neurons in the CA3 area indicated ERα expression. Some astrocytes, however, were immunonegative (fig. 3). In the subiculum, some astrocytes of intense immunocytochemic reaction were spotted as well as other astrocytes not displaying immunostaining.

The results revealed that in OVX rabbit females in all the examined hippocampus areas astrocytes ERα showed immunoreactivity as it was described in rat and rabbit. Intense ERα immunostaining in neurons was displayed in some areas of the forebrain (4, 24). ERα and ERβ exist in both the neurons and astrocytes *in vitro* in different cns areas including the hippocampus of rat (10, 11). ERs were confirmed immunocytochemically *in vivo* in about 50% of the astrocytes of the preoptic area and median eminence in guinea pigs (15). Other authors suggest that ERα do not occur in all parts of the brain, but only in some of its specific areas, including the hippocampus (1). Some studies of ERα in hippocampus neurons of OVX+E2 rabbits showed their absence, and in other areas of the cns exogen estrogen causes the decrease in ERα immunoreactivity (4).

The varied results obtain in this study of intense ERα immunoreactivity in astrocytes and neurons of the hippocampus are difficult to interpret. It is probable that the application of different experimental procedures and of other ERα antibodies (H222 and Zymed) might have

triggered the quite varied results (4). Our studies pointed to the ERα immunoexpression in astrocytes and neurons of rabbit hippocampus. Intracellular localization, on the basis of the ERα immunostaining intensity encompasses the cellular body and the initial processes of the astrocytes and some cell nuclei. This location is similar to the cultured astrocytes of the rat hippocampus described on the basis of immunocytochemic and auto radiographic studies (10, 11). Other authors have also presented nuclear ERα in astrocytes (22). In the astrocytes, just as in the neurons, ERα may show direct influence of the estrogen on glia cells for their proper functioning and they may also regulate neuron-astrocyte interactions. Neurons are as well able to change the ERα expression in astrocytes *in vivo*, since immunoreative astrocytes for ERα are located in neurons and nerve endings (21).

The mechanism of estrogen reaction with neurons is well known and in these cells ERs are located in the cell nucleus. Neuronally uptake E2 joins some specific nuclear receptors and DNA for transcription and an increase of proteins production, follows which in consequence modulates their functions (8, 13, 16, 17, 23, 26). Intense ERα immunostaining has also been described in the cytoplasm of the neurons of the hippocampus (21). Cytoplasmic ERα localization points at their synthesis in the plasmatic reticulum and from there, their transport to cell nucleus. Recently, these receptors were also localized in the cell membrane, connected with G proteins. These findings suggest that cytoplasmic ERα may also be transported to the cell membrane. Further studies, however, are needed to be able to find an explanation for this (4, 5). In the neurons, E2 binding with ERs influences the creation of axodendric synapses of the hippocampus and the release of neurotransmitters. It has been suggested that cytoplasmic ERα may be activated not only with the ligand – estrogen but also with neurotransmitters or growth factors with the aim of nonspecific action of the cytoplasm and transcription in the nucleus (3). As many different studies have shown, estrogen influences the disconnection of axosomatic synapses of the hypothalamus. This process is participated by the active processes of the astrocytes (12). Applying E2 to the median raphe of the OVX rats causes a 47% increase of dendrite endings at the 16% decrease of GFAP density of the astrocytes of CA3 and CA1 area of the hippocampus. The results of these studies suggest that estrogen administered to the other area of cns exerts a substantial influence on hippocampus structures. In culturing of neurons of this area, the addition of E2 causes a decrease in γ-amino butyric acid (GABA). As a result of the transient inhibition of GABA synthesis, an increase in the number of axodendric synapses occurs in the hippocampus (24). During proestrus and estrus in rats, the hippocampus indicated a 25%value of marked astrocytes for ERα location in the vicinity of neurons and synapses. (21). Not all astrocytes may be directly influenced by estrogens. The results of the study proved that ERs may influence glia cells, despite the fact that the rabbits do not have typical estral cycles.

References

1. *Adams M. M., Fink S. E., Shah R. A., Janssen W. G. M., Hayashi S., Milner T. A., McEwen B. S., Morrison J. H.*: Estrogen and aging affect the sub cellular distribution of estrogen receptor α in the hippocampus of female rats. J. Neurosci. 2002, 22, 3608-3614.
2. *Azcoitia I., Sierra A., Garcia-Segura L. M.*: Localization of estrogen receptor β – immunoreactivity in astrocytes of the adult rat brain. Glia 1999, 26, 260-267.
3. *Blaustein J. D.*: Estrogen receptors in neurons: new subcellular locations and functional implications. Endocrinol J. 1994, 2, 249-258.
4. *Caba M., Beyer C., Gonzáles-Mariscal G., Morrell J. I.*: Immunocytochemical detection of estrogen receptor - α in the female rabbit forebrain: topography and regulation by estradiol. Cell Neuroendocrinol. 2003, 77, 208-222.
5. *Caldwell J. D.*: A sexual arousability model involving steroid effects at the plasma membrane. Neurosci. Behav. Rev. 2002, 26, 13-30.
6. *Cybulska R., Jaworska-Adamu J., Wawrzyniak-Gacek A.*: Funkcje astrocytów w patogenezie niektórych chorób ośrodkowego układu nerwowego. Medycyna Wet. 1994, 50, 447-449 (in Polish).
7. *Dhandapani K. M., Brann D. W.*: Estrogen-astrocyte interactions: implications for neuroprotection. BMC Neurosci. 2002, 3, 6.
8. *Don Carlos L. L., Monroy E., Morrell J. I.*: Distribution of estrogen receptor--immunoreactive cells in the forebrain of the female guinea pig. J. Comp. Neurol. 1991, 305, 591-612.
9. *Harkness J. E., Wagner J. E.*: The Biology and Medicine of Rabbits and Rodents. Lea and Fibiger, Philadelphia 1977.
10. *Hősli E., Jurasin K., Rűhl W., Lűthy R., Hősli L.*: Colocalization of androgen, estrogen and cholinergic receptors on cultured astrocytes of the rat central nervous system. Int. J. Devl. Neurosci. 2001, 19, 11-19.
11. *Hősli E., Rűhl W., Hősli L.*: Histochemical and electrophysiological evidence for estrogen receptors on cultured astrocytes: colocalization with cholinergic receptors. Int. J. Devl. Neurosci. 2000, 18, 101-111.
12. *Jaworska-Adamu J., Cybulska R., Kalinowska M.*: Changes of the morphology of astrocytes in periaqueductal gray matter and arcuate nucleus during the estrous cycle in rats. Medycyna Wet. 2004, 60, 1114-1117. (in Polish).
13. *Koch M., Ehret G.*: Immunocytochemical localization and quantitation of estrogen – binding cells in the male and female (virgin, pregnant, lacting) mouse brain. Brain Res. 1989, 489, 101-112.
14. *Krakowska I., Jaworska-Adamu J.*: Ekspresja receptorów estrogenowych α w zakręcie przyhipokampowym samic królików po 17β estradiolu. Medycyna Wet. 2006, 62, 1059-1060 (in Polish).
15. *Langub M. C., Watson R. E.*: Estrogen receptor-immunoreactive glia, endothelia and ependyma in guinea pig preoptic area and median eminence: electron microscopy. Endocrinol. 1992, 130, 364-372.
16. *Lehman M. N., Ebling F., Moenter S. M., Karsch F. J.*: Distribution of estrogen receptor-immunoreactive cells in the sheep brain. Endocrinol. 1993, 133, 876--886.
17. *Li H. H., Blaustein J. D., DeVries G. G., Wade G. N.*: Estrogen-receptor immunoreactivity in hamster brain: preoptic area, hypothalamus and amygdala. Brain Res. 1993, 631, 304-312.
18. *Luquin S., Naftolin F., Garcia-Segura L. M.*: Natural fluctuation and gonadal hormone regulation of astrocytes immunoreactivity in dentate gyrus. J. Neurobiol. 1993, 24, 913-924.
19. *Matsumoto A., Arai Y.*: Sexual dimorphism in „wiring pattern" in the hypothalamic arcutate nucleus and its modification by neonatal hormonal environment. Brain Res. 1980, 190, 238-242.
20. *Meshul Ch. K., Seil F. J., Herndon R. M.*: Astrocytes play a role in regulation of synaptic density. Brain Res. 1987, 402, 139-145.
21. *Milner T. A., McEwen B. S., Hayashi S., Li C. J., Reagan L. P., Alves S. E.*: Ultrastructural evidence that hippocampal alpha receptors are located at extranuclear sites. J. Comp. Neurol. 2001, 429, 355-371.
22. *Mitra S. W., Hoskin E., Yudkovitz J., Pear L., Wilkinson H. A., Hayashi S., Pfaff D. W., Ogawa S., Rohrer S. P., Schaeffer J. M., McEwen B. S., Alves S. E.*: Immunolocalization of estrogen receptor β in the mouse brain comparison with estrogen receptor α. Endocrinol. 2003, 144, 2055-2067.
23. *Rees H. D., Switz G. M., Michael R. P.*: The estrogen-sensitive neural system in the brain of female cats. J. Comp. Neurol. 1980, 193, 789-804.
24. *Rune G. M., Wehrenberg U., Prange-Kiel J., Zhou L., Adelmann G., Frotscher M.*: Estrogen up-regulates estrogen receptor α and synaptophysin in slice cultures of rat hippocampus. Neurosci. 2002, 1, 167-172.
25. *Shughrue P. J., Lane M. V., Merchenthaler I.*: Comparative distribution of ER-α and -β messenger RNA in the rat central nervous system. J. Comp. Neurol. 1997, 388, 507-525.
26. *VanLeewen F. W., Chouham S., Axelson J. F., Swaab D. F., VanEerdenburg F. J. C. M.*: Sex differences in the distribution of estrogen receptors in the septal area and hypothalamus of the domestic pig (Sus scrofa). Neurosci. 1995, 64, 261--275.

Author's adress: Dr Izabela Krakowska, PhD, Departament of Animal Anatomy and Histology, Faculty of Veterinary Medicine, Agricultural University, 20-033 Lublin, Akademicka 12, Poland; e-mail: izabela.krakowska @ar.lublin.pl