

Expression of α estrogen receptors in the neurons of the hippocampus in male rabbits

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Summary

The subject of the present paper was to show presence of α estrogen receptors in neurons of hippocampal formation in adult male rabbits. Estrogen hormones perform a crucial role in sex differentiation and reproduction, but also influence a number of brain functions not connected with reproduction. Clinical examinations prove estrogens' influence on cognitive processes, memory, mood, emotional behavior, pain sensation, mobility, state of balance. Estrogens have a neuroprotecting action, they influence neurons' length of life and as a result of that, degenerative diseases of the nervous system connected with age. The examinations prove that estrogen substitutive therapy decreases the development rate of Alzheimer and Parkinson's disease, multiple sclerosis, osteoporosis, depression and migraines. It is thought that estrogen administration in the case of these diseases diminishes intensification of symptoms. In relation to this, it is crucial to learn the mechanisms of hormones' activity and determine the localization of their receptors. The obtained results indicate that estrogen hormones can influence cognitive processes, learning and memory. Learning the mechanisms of estrogens' activity and determining which brain structures they influence can be helpful in treating neurodegenerative diseases. In adult rabbit males α estrogen receptors occur in neurons of the hippocampus in cytoplasm as well as cell nucleus. In the examined regions cytoplasmic and cytoplasmic-nuclear reaction predominates.

Keywords: hippocampus, estrogen receptor, rabbit, male

The limbic system is often called the emotional brain because some of its regions are responsible for states of pleasure and negative emotions defined as reward and punishment centers. At present it is assumed that centers of the limbic system are responsible not only for emotional functions but also for functions connected with memory. We divide the hippocampus into the dental gyrus and the hippocampus. There are three cellular layers in dental gyrus: molecular layer, granular cell layer and polymorphic cell layer. In the hippocampus we can observe pyramidal cell layer, stratum oriens, stratum radiatum and stratum lacunosum-moleculare (1). Axons from pyramidal cells of the CA3 region reach pyramidal cells of the CA1 region of the hippocampus. Neurons of the CA1 region send axons and form synaptic junctions with neurons of the intra-olfactory cortex. Information connected with memorizing processes from the whole region of the cerebral cortex is transmitted to the hippocampus through the intra-olfactory cortex. Numerous examinations have proved a close relationship between the hippocampus

and the processes of memorizing and learning (2, 3). All kinds of hippocampal injuries lead to minor or major memory disorders. Both a mechanical injury of the hippocampus and a pharmacological blocking of the N-methyl-D-aspartate (NMDA) receptors prevents the production of LTP (long term potentiation) and disturbs the so-called spatial memory (4, 5, 8). Examinations (6) prove that apart from the activation of NMDA receptors, an increase in the secretion of glutaminic acid by axons of the CA3 region and the depolarization of the postsynaptic membrane of regions from the CA1 are necessary to form LTP. In the central nervous system (CNS), estrogens act through intracellular receptors of neurons and membrane receptors. These hormones can influence neurons directly or through the influence of neuroglia, immunocompetent cells or brain circulation. Recent research proves that estrogens are formed within the brain in neurons and astrocytes. Under the influence of aromatase present in both types of cells, estrogens can be formed from androgens in a process of aromatization, which is a multistage transformation

running only in one direction. In the process of the aromatization of androgens, estrone (E1) and 17 β estradiol (E2) are formed. During brain development sex-dependent neurons are able to bind androgens. In the same developmental period estrogens are formed from androgens under the influence of aromatase. During ontogenesis under the influence of sexual hormones the phenomenon of sexual dimorphism takes place in the regions of brain which are sex-dependent. In the prenatal period estrogens activate the functions of these brain regions influencing sex-dependent behaviour (7, 11, 14). As early as in the developmental period and early postprenatal period estradiol influences the differentiation, development and survival rate of neurons, their migration, an increase in the number of dendritic endings, formation of synaptic junctions as well as cell death. It has been proved that estradiol has efficient neuroprotective activity when it is used in various cases of brain injury such as ischaemia, hypoxia and mechanical injuries. In relation to the neuroprotective activity of estrogens one can conclude that neurons containing estrogen receptors (Ers) are more resistant to injuries and are quicker to regenerate after such injuries. In the case of diseases connected with neuron injuries they undergo neurodegeneration more slowly. Sexual hormones, both estrogens and androgens, do not act directly on tissues dependent on them, but join with the corresponding receptors.

Material and methods

Brains of 10 sexually mature rabbit males (*Oryctolagus cuniculus*), New Zealand breed, of body weight 3-4 kg, were used as the material for the examination. The brains were fixed in buffered formalin pH 7.4 for 24 h. The brains were washed with tap water, dehydrated in alcohols of increasing concentrations, and placed in xylene. In order to label structures of the hippocampus the following atlases were used (9, 16). Cross sections corresponded to brain cross-sections on the levels in relation to bregma point -5.5 mm. In order to carry out immunocytochemical staining hemispheres were cut frontally, 4 μ m thick paraffin sections were made, they were deparaffinized in xylene, hydrated and after that placed in 0.01 M citrate buffer pH = 6.0 and cooked in a microwave in 2 stages for 10 min. in order to unmask antigens. Cold slides were washed with fresh buffer TBS (Tris Buffered Saline) pH = 7.6 for 5 min. Later they were incubated in 3% H₂O₂ at room temperature for 10 min. in order to block endogenous peroxidase. The slides were rinsed in H₂O dest. and 0.5 M TBS.

Immunocytochemical receptor detection was carried out using DAKO LSAB+Kit Peroxidase method. Antibodies were diluted in 0.5 M TBS pH = 7.6 and the slides were washed in it (2 \times for 5 min.) after using antibodies. The sections were incubated with primary monoclonal mice antibody anti-Er α NCL-L-ER-6F11 (NOVACAstra, 1 : 40) for 48 hours at a temperature of 4°C. Next, secondary monoclonal biotinylated anti-mice IgG (Biotynylated link IgG DAKO) was used for an hour at room temperature. The slices were incubated with readymade complex

streptavidine-peroxidase (DAKO) for an hour at room temperature. As a chromogen DAB (3,3-diaminobenzidine tetrahydrochloride, DAKO) was used at room temperature for 10 min. In addition, the slices were stained with Mayer's hematoxylin in order to expose nuclei of nervous cells. In the place of the reaction's occurrence brown staining was obtained. Later, the slices were rinsed, dehydrated, cleared and closed using Canadian balsam. Negative control of reaction specificity was carried out omitting primary antibodies. A reaction product was not observed in the slides. The trials carried out have proved immunohistochemical reactions' specificity. The obtained results underwent examinations in a light microscope.

The examinations were carried out using program cell 1 D (Soft Imaging System). Morphometric examinations concerned neurons of hippocampal formation taking into consideration those regions in which immunocytochemical reaction α estrogen receptor took place. One assessed a number of neurons per area unit of measure in which:

- reaction in the cell nucleus took place (cyt-/nuc+),
- reaction in the cytoplasm took place (cyt+/nuc-),
- reaction in the nucleus and cytoplasm took place (cyt+/nuc+),
- neurons did not show any reaction in the nucleus and cytoplasm (cyt-/nuc-).

The borders of each examined region were determined in which the reaction in neurons was detected. They were labeled as follows: the granular cell layer of the dental gyrus- (dgg), the cell layer of the hippocampus (CA4), the pyramidal cell layer of the hippocampus (CA1 region labeled as CA1), the pyramidal cell layer of the hippocampus (CA2 region-CA2), the pyramidal cell layer of the hippocampus (CA3 region-CA3).

Results and discussion

In adult rabbit males the immunohistochemical reaction showing Er α presence in neurons of the above mentioned regions of the hippocampus was detected. In the granular cell layer of the dental gyrus the reaction in the cytoplasm took place in the majority of neurons. The number of neurons showing the presence of the receptors in more cytoplasm was significantly higher than the number of neurons in which the reaction referred to both the cell nucleus and cytoplasm. In the examined region a small number of neurons did not show any Er α expression at all. Granular cells of the dental gyrus are characterized by the greatest number of neurons with cytoplasmic reaction in comparison to the remaining examined regions (Fig. 5). In this region neurons of pure Er α expression in more cell nucleus were not observed (Fig. 4). In neurons of the CA4 cell layer the reaction was observed in the cytoplasm and main processes: in the cell nucleus the majority of neurons did not show any reaction (Fig. 3). In the CA4 region a large number of neurons do not show Er α expression at all. Quite a large number of nerve cells of this region have got immunostaining both in the nucleus and in cytoplasm. In the CA3 region only in the pyramidal cell layer was Er α expression detected

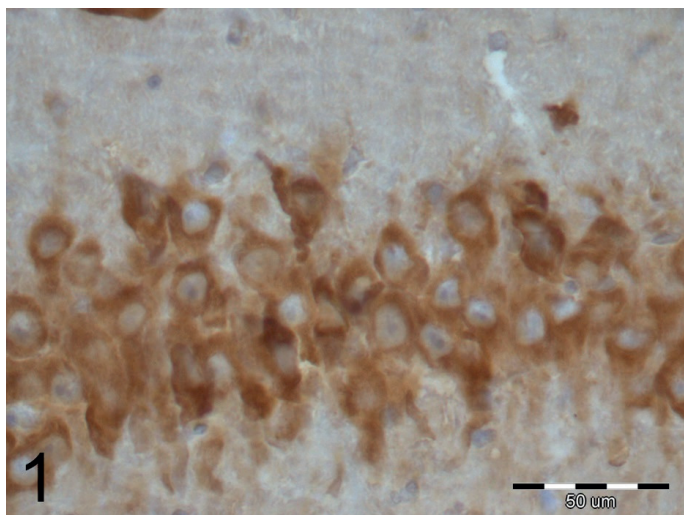


Fig. 1. Immunoreactivity of ERα in neurons in the CA3 sub-field (400 ×)

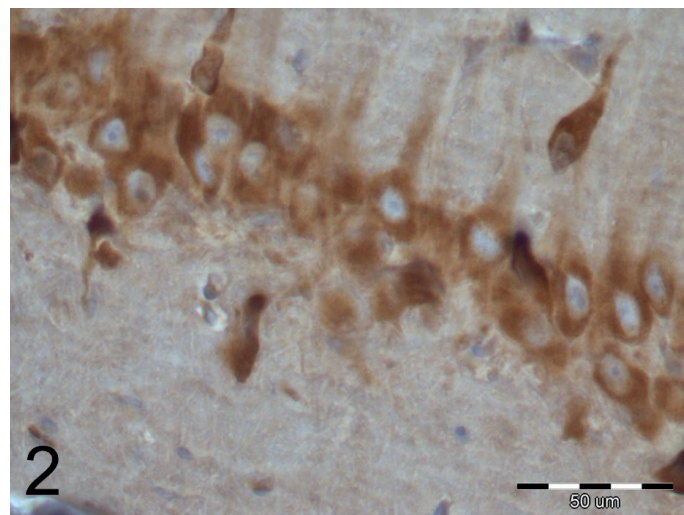


Fig. 2. Immunoreactivity of ERα in neurons in the CA1 sub-field (400 ×)

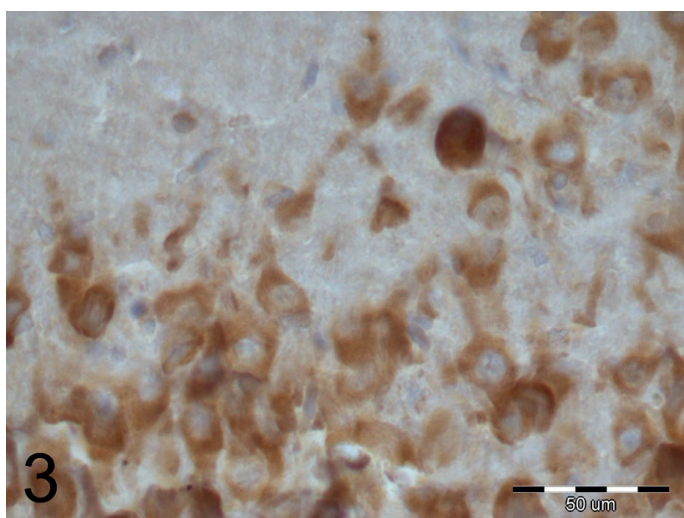


Fig. 3. Immunoreactivity of ERα in neurons in the CA4 sub-field (400 ×)

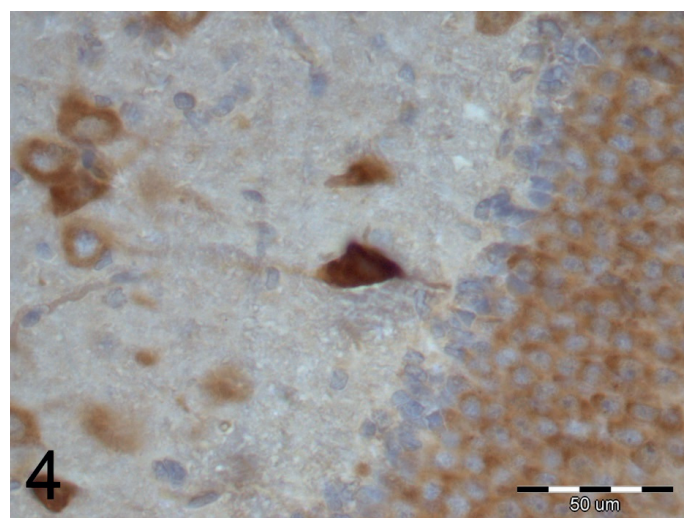


Fig. 4. Immunoreactivity of ERα in neurons in hilus of the dentate gyrus and in the granular cell layer (400 ×)

(Fig. 1). In this region there is a significant dominance of the reaction in the nucleus and in the cytoplasm of pyramidal neurons over the reaction in the more cytoplasm. A small number of neurons did not show ERα presence. The pyramidal cell layer of the CA2 region has α estrogen receptors which are mainly localized in the cytoplasm and in nucleus. In the pyramidal cell layer of the CA1 region one noticed, neurons showing a reaction in the cytoplasm, as numerous as in the previously examined region, a small difference was observed calculating the number of neurons showing immunocytochemical reaction in the nucleus and cytoplasm (Fig. 2).

In the past years estrogens have been perceived as sexual hormones performing a crucial role only in the regulation of physiological processes connected with reproduction. Relatively recently the influence of estrogens on the CNS has been noticed. Taking into consideration functions of the hippocampal formation, examinations of this structure have been started from the perspective of the presence of estrogen receptors.

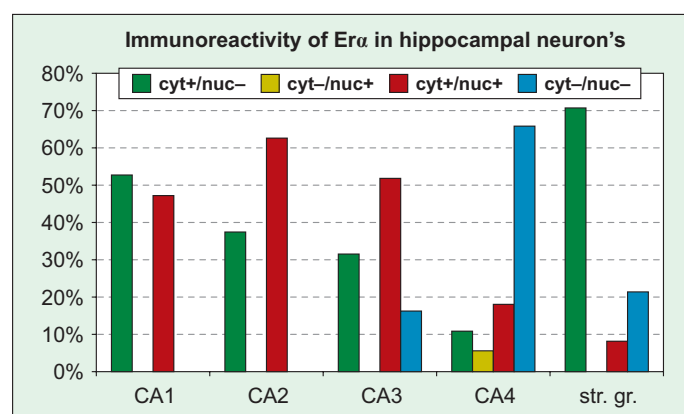


Fig. 5. Expression and localization of ERα in hippocampal areas of the rabbit male

Explanations: cyt+/nuc- positive IHC reaction within the cytoplasm of neurons; cyt+/nuc+ positive IHC reaction within the cytoplasm as well as nucleus of neurons; cyt-/nuc+ positive IHC reaction within nucleus of neurons; cyt-/nuc- no IHC reaction

The occurrence of Er both α and β in pyramidal neurons of the hippocampus and granular cells of the dental

gyrus is particularly crucial. Estrogens influence functions of the hippocampus through estrogen receptors in these cells; these refer to learning and memorizing in particular. Estrogens influence a number of synaptic endings, the synthesis level and the release of neurotransmitters as well as enzymatic activity. Our own examinations prove the theory of the presence of estrogen receptors in neurons of the hippocampus in male rabbits.

Extremely significant is the fact that $Er\alpha$ in rabbit males is especially located in the cytoplasm of hippocampus neurons or in cytoplasm and nucleus. Reaction exclusively in the nucleus is present in 5%-6% of neurons only in CA4 area. The greatest number of neurons – approximately 70% $Er\alpha$ – is located in cytoplasm in the granular layer of the dental gyrus. Over 50% of $Er\alpha$ is located in neurons of CA1, while over 30% is located in CA3 and CA4 areas. Over 60% of $Er\alpha$ in the CA2 area is located in cytoplasm and nucleus, over 40% $Er\alpha$ in CA1 is located in cytoplasm and nucleus and over 50% is located in the CA3 area. It can be concluded that estrogens can directly influence synaptic junctions between axonal endings of neurons of the CA3 region and dendrites of neurons of the CA1 region. Through an influence on the production of neurotransmitter, their release, formation of synapses in the CA3 region, estrogens can influence the functioning of neurons of the CA1 region. It has been proved (8, 10) that estrogen receptors occurring in the cytoplasm of neurons and receptors occurring in dendrite processes play a crucial role in the formation of synapse and the release of neurotransmitters. Cytoplasmic estrogen receptors can be activated independently from ligand through neurotransmitters or growth factors. Examinations carried out on rats referred to the hippocampus and corpus amygdaloideum. In these structures the authors determined $Er\beta$ occurrence mainly in the cytoplasm of neurons. The localization in the nucleus of nerve cells pertained more to the corpus amygdaloideum than the hippocampus. In the examined structure one described the distribution of α estrogen receptors, which were present both in the nucleus of neurons as well as outside of nuclei. There were no significant differences in rat males and females. The reaction in the hippocampus concerned the pyramidal layer. The cells of the CA3 region reacted the most intensively after the use of antibodies for both α and β estrogen receptors. Similar results referring to localization of estrogen receptors in rat females and males were obtained in cells of the cerebral cortex (12). The examinations carried out by other researchers prove that localizations of $Er\alpha$ and $Er\beta$ do not show any significant differences in rat males and females. In one of the few examinations carried out on rabbit brains (15) the occurrence of many α estrogen receptors only in the subiculum was described. In none of the hippocampus CA regions and in the dental gyrus was the occurrence of α estrogen receptors observed. $Er\alpha$ was present in other brain regions of rabbit females

such as the corpus amygdaloideum, area praeoptica medialis, nucleus infundibularis, or nucleus X. The examinations did not show $Er\alpha$ presence in neurons of nucleus ventro-medialis in the hypothalamus. There are also examinations in rat females and males in the areas of the praeoptica, neocortex, spinal cord and hippocampus. Tissue cultures came from the prenatal and postnatal period. Both in the cerebral cortex and in the hippocampus the occurrence of estrogen receptors in the cell nucleus, cytoplasm and processes of nervous cells were observed. Differences in the occurrence of estrogen receptors in males and females in the examined structures were not detected; only in area praeoptica a difference in density of occurring receptors in relation to sex was detected. In females there were a greater number of receptors in this structure than in males (17). In examinations of other authors (18) the level of aromatase in certain life periods in rat females and males was determined. The highest level was in male and female individuals on the 7th day of postnatal life, later it was decreasing and for the subsequent weeks of their lives it did not change (13). $Er\alpha$ occur in granular cells of the dental gyrus in adult rats and do not show any difference in relation to sex.

In our own examinations we also observed $Er\alpha$ occurrence in rabbit males in granular cells of the dental gyrus. Here the receptors occur at a high density and the reaction takes place in the cytoplasm and nucleus of neurons. The localization of α estrogen receptors in pyramidal cells of the hippocampus occurs in cell bodies and main processes. Nuclei were more intensively stained than the cytoplasm, but the reaction occurred more often in cytoplasm and processes of neurons. In the hippocampus, a smaller number of $Er\beta$ than of $Er\alpha$ was revealed (17). $Er\beta$ were localized mainly in the cytoplasm and main processes and in a smaller number in the nucleus of nerve cells; at the same time nuclei were stained more intensively than the cytoplasm and processes. There were no differences in relation to sex of the examined animals. In the hippocampus there are insignificant differences connected with sex. Among others things the differences concern a greater number of synapses of mossy fibers than in females. The differences also refer to a number of dendritic endings and branches of pyramidal neurons of the hippocampus CA3 region (19). In mice and rats there are differences connected with sex in the number of granular neurons of the dental gyrus (13). The difference also concerns the width of the dental gyrus; in males it is a wider band and asymmetrical. Testosterone administration to rat females in the neonatal period makes their dental gyrus similar to this structure in males. Testosterone administration during this period improves rat spatial memory in Morris' test. These differences take shape during the developmental period, because there are $Er\alpha$ in the rat hippocampus and they are present even in the prenatal development. The occurrence of these receptors is

connected with the presence of aromatizing enzyme, which transforms testosterone into estradiol. Results obtained in the research indicate the presence of ER α in the hippocampus in male rabbits. The location of ER α in neurons of the hippocampus in all described areas was confirmed.

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