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# IMMUNOLOCALISATION OF α AND β OESTROGEN RECEPTORS IN BASOLATERAL AMYGDALA OF RABBIT MALES

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## Abstract

Expression of α (ER-α) and β (ER-ß) oestrogen receptors in neurons of basolateral complex of the amygdala was studied in rabbit males. The examinations were carried out on brains of 12 sexually mature rabbit males weighing 3-4 kg. An immunohistochemical method (IHC) with primary Er-α antibodies, NCL-L6F11 and Er-ß: NCL-ERbeta clone EMR02 was applied. The percentage of immunoreactive neurons was determined. Morphometric and statistical analysis of immunoreactive (ER+) neurons was carried out including the localisation of IHC reaction within the examined neurons. The main place of IHC-ER-α reaction was the cytoplasm of nervous cells, whereas in the examination of IHC-ER-β, the reaction was observed mainly within cell nucleus. The nuclei of basolateral complex are a region of relatively high percentage of ER-β+ neurons and lower percentage of ER-α+ neurons in comparison with other nuclei of the amygdala. Basolateral nucleus was characterised by a higher percentage of ER- β+ neurons in which the reaction was observed in the cytoplasm.

**Key words:** rabbit, amygdala, oestrogen receptor, basolateral.

Oestrogens affect tissues through binding with specific receptors present in target tissues. Oestrogen receptors occur in two main forms: α and β (ER-α and ER-β). They are ligand dependent factors activating transcription. Structurally within oestrogen receptors one can distinguish six functional subunits labelled from A to F. Oestrogen receptors α and β differ from one another within the domains (6, 20). As demonstrated in experimental studies oestrogens play an important role not only in the functioning of the reproductive system but have a significant impact on many other organs and tissues not related to reproduction, including structures and functions of the central nervous system in both females and males. They affect also maturation, proliferation, and lifespan of neurons, and synaptogenesis (1, 13). The amygdala (*corpus amygdaloideum*, CM) and hippocampus are the main centers of the limbic system (16). Functioning of these brain centers is primarily associated with memory and emotions. Through efferent junctions it also affects autonomic nervous system's functions. Changes in neurons of the amydgala and hippocampus according to many scientists accompany such disorders as Alzheimer's disease, schizophrenia, depression (8, 12). The amygdala plays an important role in the regulation of defensive reflexes in humans and animals. It plays a crucial role in anxiety reaction (9, 10).

Within the amygdala one can distinguish phylogenetically younger basolateral complex (BLC), phylogenetically older corticomedial complex (CMC), and so-called other nuclei. Into basolateral complex the following nuclei are included: lateral (LA), basolateral (BL), and basomedial (BM). Morphology and cytoarchitecture of the amydgala in rabbits have been thoroughly described in scientific literature (4, 7, 21). Studies on morphology of the amygdala in both males and females showed that it is also characterised by sexual dimorphism. Anatomical differences within the amygdala of males and females mainly referred to medial nucleus. They concern volume of nerve nuclei, neuron size, and density of dendritic spines (3, 15, 19).

## Material and Methods

The research material consisted of the brain collected from 12 sexually mature rabbit males (*Oryctolagus cuniculus*) of New Zealand breed, weighing 3-4 kg. The material came from a rabbit slaughterhouse. The collected material was divided into smaller fragments and embedded in paraffin blocks according to the rules adopted in immunohistochemical examinations. The material from the right brain hemispheres was used in the research. The histological slides were prepared from the blocks cutting the

hemispheres frontally into 5 μm thick sections. The sections were placed on silanised glasses SuperFrostRPlus (MENZEL-GLASER). The preparations were incubated for 48 h at 4°C with primary monoclonal mouse IgG1 Er-α antibody (NCL-L6F11 clone, Novocastra) diluted 1:40 and monoclonal IgG1 Er-β antibody (NCL-ER-β clone EMR02, Novocastra) diluted 1:50. Visualisation was performed by LAB/LSAB method using system for visualisation LSAB (DAKO). In order to diversify the sections, they were stained with Mayer's haematoxylin. In the places of positive immunohistochemical reaction, final insoluble reaction product of brown colour, indicating the presence of receptors was visible. Control sections were incubated without primary antibody. Finally, the sections were closed in a Canadian balm. The results of immunohistochemical reaction were viewed under a light microscope (OLYMPUS BX40). To determine the structures of the amydgala, atlases and publications describing the structure and cytoarchitecture of rabbit's amydgala were used (4, 5, 7, 21).

Photographic documentation was performed using a digital camera COLORVIEW IIIu (soft Imaging System). The obtained images, saved in TIF format, underwent morphometric analysis using cell D programme (Soft Imaging System). The main differentiation criterion was the presence of the reaction or its lack, as well as intracellular localisation of immunohistochemical reaction product. Statistical analysis was carried out using computer programme Stasistica version 6.0PL. The obtained results were analysed using one-way analysis of variance (ANOVA). Statistical significance of differences between the analysed results was determined using post-hoc test of Tukey. Probability value $P<0.05$ was adopted as the boundary of statistical significance of the obtained differences and correlations. For each parameter arithmetic mean ($\bar{}$) was calculated as well as standard deviation (SD) and standard error of mean SEM); the presented results were shown in the form of $\bar{}$±SD.

## Results

IHC reaction for the presence of α and β oestrogen receptors was observed within neurons both in the nucleus and cytoplasm of nervous cells. On the basis of localisation of the reaction, the examined neurons were divided into four groups: 1. IHC–ER reaction was observed within the cytoplasm of neurons. 2. IHC-ER reaction was observed within nucleus of neuron. 3. IHC–ER reaction was observed within the cytoplasm as well as nucleus of neuron. 4.

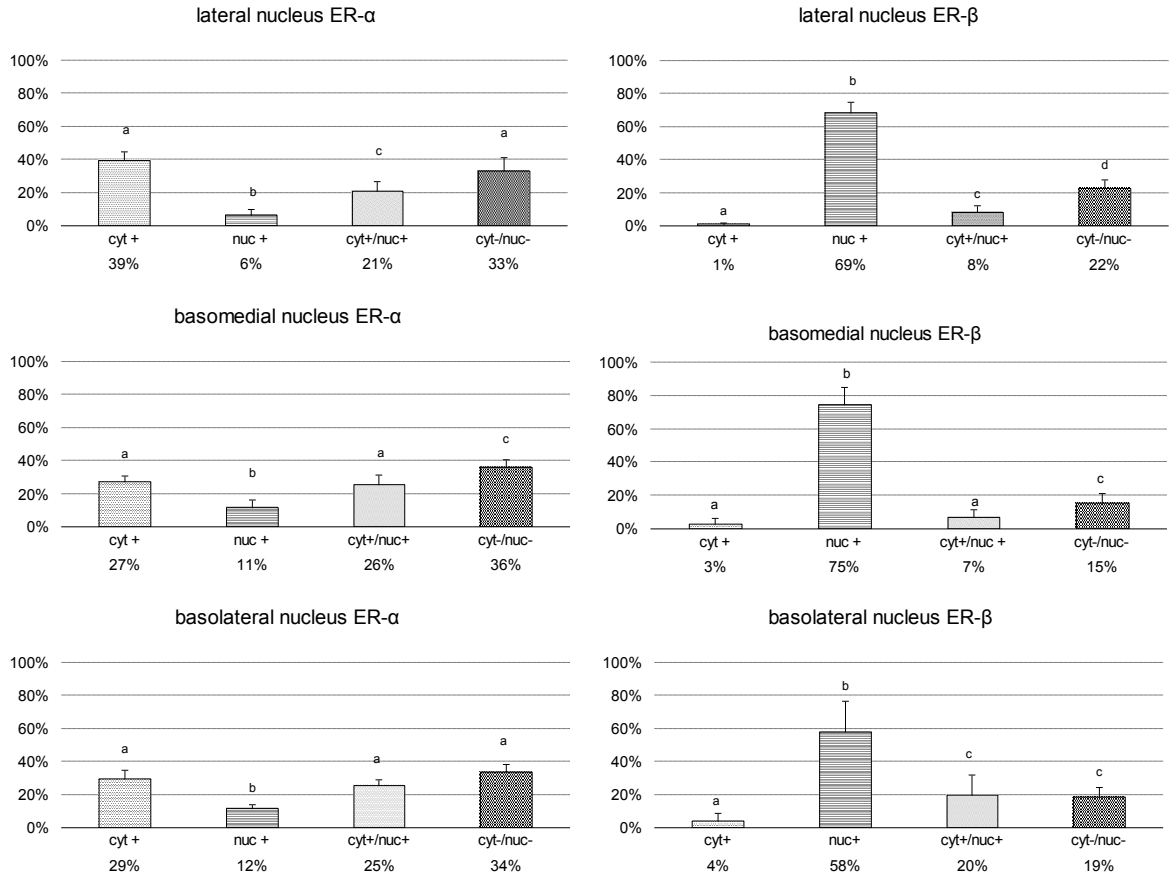



**Fig. 1**. Immunohistochemical expression of α and β oestrogen receptors in basolateral nuclei of the amygdala in rabbit male's brain. Statistically significant differences were marked with different letter symbols for the value of probability $P\leq0.05$.

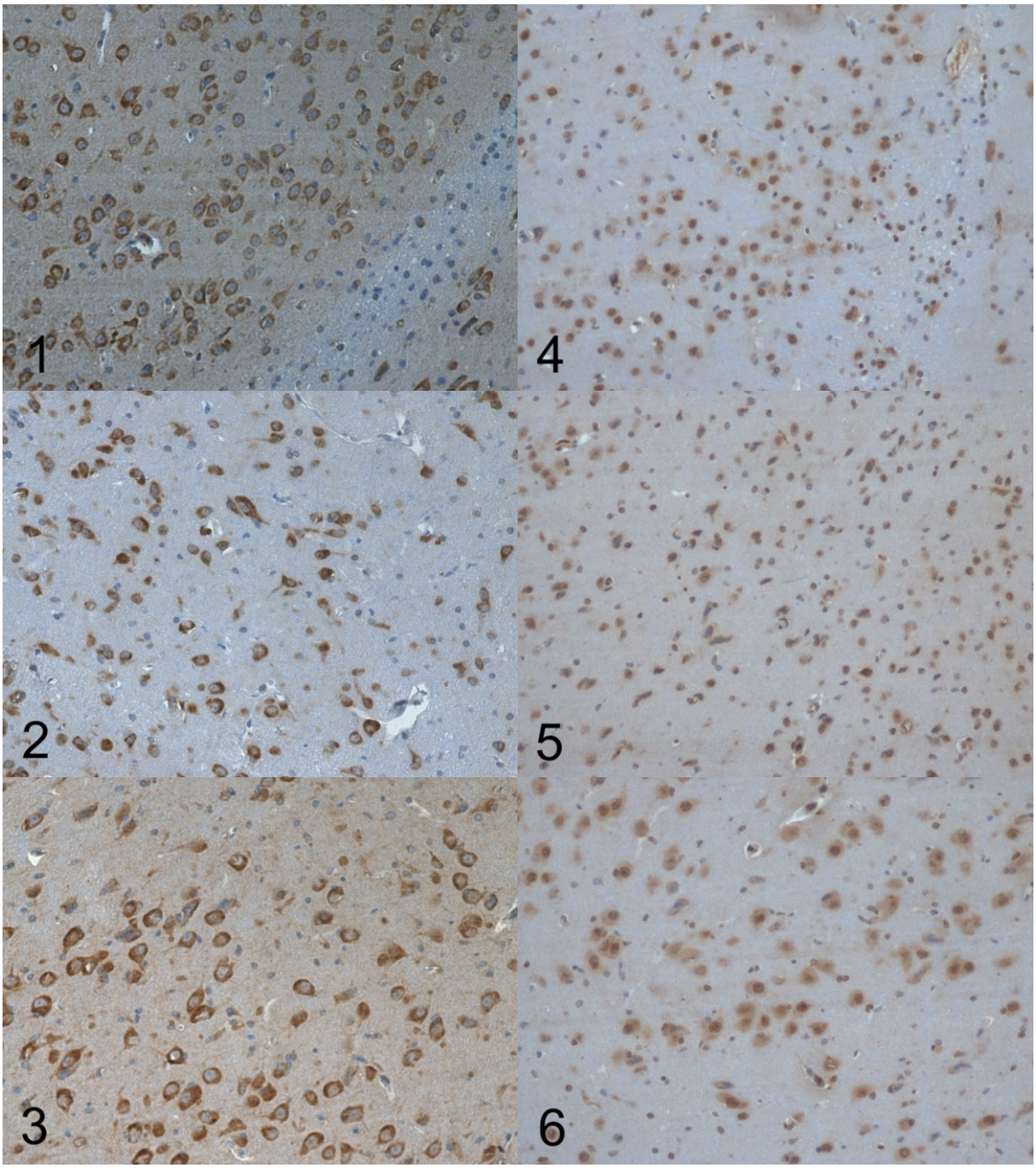



**Fig. 2.** Expression of oestrogen receptors in immunohistochemical examinations: 1. ER-α in lateral nucleus, 2. ER-α in basomedial nucleus, 3. ER-α in basolateral nucleus, 4. ER-ß in lateral nucleus, 5. ER-ß in basomedial nucleus, 6. ER-ß in basolateral nucleus. About 200x

No IHC reaction for ER was observed. In the majority of the immunoreactive neurons in the region of all examined nervous nuclei, the product of IHC reaction for presence of ER-α receptors was observed in the cytoplasm of nervous cells, whereas the presence of ER-β was observed mainly within neuron nuclei. Detailed results of reaction localisation in neurons of individual nuclei of basolateral complex of the amygdala were demonstrated in a form of charts. (Figs 1-6). In nuclei of basolateral complex, a higher percentage of neurons in which IHC-ER-β reaction was demonstrated in relation to other amygdala nuclei was observed. At the same time, in relation to the remaining nuclei of the complex, this region was characterised by a lower percentage of neurons in which IHC-ER-α reaction was observed. In individual nuclei of the complex, ER-α expression was similar, the main reaction place was the cytoplasm of the examined neurons. In basolateral nucleus there was a significantly higher percentage of neurons in which cytoplasmatic reaction for ER-β was observed (Figs 1, 2).

## Discussion

In the studies on distribution of β-oestrogen receptors in the brain of rat males exposed to ovariectomy, immunoreactive neurons of different brain regions were observed, among others in amygdala. Within amygdale, the presence of ER- β was observed in neurons of medial nucleus and cortical nuclei (23). Expression of β oestrogen receptors was also compared in females and males. In rat's brain, the regions characterised by high immunoreactivity in both females and males were distinguished. Reaction of receptors within neurons was observed in the cytoplasm and cell nucleus. The differences between males and females referred to such structures as the nucleus *mammilaris medialis, locus ceruleus, nucleus reticularis pontis*. In these brain regions the receptors were seen only in males. Only in females β-oestrogen receptors were present in the nucleus *vestibularis superior* (24). One also distinguished brain regions; in which immunoreactive neurons were observed in both sexes; however, an increase in reaction in females and males was different (24). The differences were also observed in intracellular receptor's location. In the amygdala, the reaction only in cell nuclei of neurons was observed (24). Other studies have compared expression of α- and β-oestrogen receptors in individuals of the same gender. The examination results have proved uneven distribution of isoforms of oestrogen receptor in the brain of ovariectomised mouse and rats females (14, 22). The carried out examinations have revealed brain regions in which high percentage of immunoreactive neurons of both isoforms of oestrogen receptor *e.g.* bed nucleus of the *stria terminalis* and nucleus medialis amygdala was observed (14). Additionally, regions in which predominated individual isoforms were observed. In the amygdala of ovariectomised mice, immunohisto-chemical reaction for the presence of both isoforms of oestrogen receptor was demonstrated in all regions; however, the medial nucleus was the place in which abundant reaction for α and β oestrogen receptors was observed. Stronger reaction for the presence of α oestrogen receptors was shown in the amygdalo-hippocampal area (14).

Immunohistochemical examinations for the presence of α-oestrogen receptors were also carried out in ovariectomised rabbit females. In the amygdale, reaction of strong density was observed in the medial amygdaloid nucleus, basal nuclei, and cortical nuclei. Weak reaction was also observed in the central amydgaloid nucleus (2). There are no data on the expression of the examined receptors in other regions of the amygdala. In the same examinations, the comparison of α-oestrogen receptor expression in ovariectomised and intact females revealed that the higher percentage of neurons in which IHC reaction was observed occurred in ovariectomised females. Oestradiol administration caused in most of the examined structures, except *infundibulum* nucleus and ventral part of nucleus X, a decrease in the percentage of neurons in which the reaction was observed (2). In immunoreactive neurons, the product of immunohistochemical reaction was observed in both cell nucleus and cytoplasm, but the nuclei of neurons were the main place of reaction. In our studies, expression of α- and β-oestrogen receptors was demonstrated in all regions of the amygdala. Detailed analysis of the intracellular distribution of reaction made it possible to isolate basolateral nucleus as a region of different sensitivity to endogenous level of oestrogen in rabbit males, due to higher percentage of neurons in which IHC reaction for the presence of ER-β in cytoplasm was observed. Experimental studies have confirmed that localisation of receptor in the cell is variable.

Translocation of oestrogen receptors from the cytoplasm to cell nucleus after exposure to the hormone was found (11, 17, 18). Dynamics of distribution of oestrogen receptors were observed after tissue exposure to ligand which, with reference to the obtained results of our examinations, indicates active physiological processes occurring in neurons of the amygdala with oestrogens' participation. Presence of receptors in neurons can be interpreted in the way that IHC positive neurons are the target of the hormone. However, a different localisation of receptors inside nervous cells proves their different sensitivity to endogenous hormone level. The results obtained in this study suggest that in rabbit males, oestrogens play a significant role in functioning of this brain region through binding with specific receptors present in neurons of the amydgala. At the same time, higher percentage of neurons in which IHC reaction for the ER-β was observed, in comparison with the percentage of neurons in which IHC reaction for the ER-α was noted within nuclei of basolateral complex, suggest that oestrogens in rabbit males within lateral nucleus and basal nuclei more intensely affect the functions of neurons through binding with β-oestrogen receptor.

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