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Expression of α and β oestrogen receptors within the claustrum in rabbit males

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Abstract

The aim of the study was to investigate the presence of α (Erα) and β (Erβ) oestrogen receptors in rabbit male's claustrum, and thus to examine whether oestrogens may influence nerve cells' functions of this structure. The material for the study was obtained from the brains of seven rabbits. To detect the presence of Erα and Erβ, specific monoclonal antibodies were used. The expression of Erα and Erβ was demonstrated in proper claustrum, as well as in the nucleus endopiriformis. For Erβ, the reaction occurred mainly in the cell nucleus, whereas Erα was localised predominantly in the neuron cytoplasm. For both Erα and Erβ, the nuclear and cytoplasmic localisation was observed in a small percentage of the examined cells. The obtained results may indicate oestrogen influence on the brain area and also suggest a further research on oestrogen-dependent pathways in males, which still remain greatly unexplored.

Key words: rabbit male, claustrum, oestrogen receptors.

Introduction

The claustrum is a brain structure located along the sulcus rhinalis, between the putamen and insula, from which it is separated by external and extreme capsules. The brain area of interest consists of dorsal part, considered as proper claustrum and ventral part, actually called nucleus endopiriformis, between which there is a narrowing lying on the level of sulcus rhinalis. Rabbit proper claustrum has a claviform shape, consisting of a large number of multipolar and fusiform cells irregularly shaped. Nucleus endopiriformis, on the other hand, is narrow, triangular with few oval and fusiform cells (1, 3, 5).

The claustrum has many junctions with various areas of the neocortex, except claustrum's ventral part lying near the corpus amygdaloideum, which has junctions with the allocortex. These junctions are bidirectional and are defined as claustro-cortical neural feedback loop. The claustrum is also connected with the limbic system responsible for the processes of memory, striatum, thalamus, and hypothalamus. Due to the multiplicity of junctions, the claustrum can be regarded as the structure controlling activity of different brain areas, as well as a filter which limits the flow of information through these areas (3, 11).

Recent studies indicate the presence of oestrogen receptors (Ers) in the claustrum, that may prove the effect of oestrogens on nerve cells' function in this brain structure (15). Oestrogens belong to a group of steroid hormones, derivatives of cholesterol. Until now, it has been thought that oestrogens are typically female hormones involved in the maturation and functioning of the reproductive system. In male individuals, in which oestrogen concentration is much lower than in females, they were considered a by-product resulting from testosterone synthesis. However, recent studies have proved that oestrogens play an important role in the regulation of many organs' functions also in male organism. Oestrogens are formed by androgen to oestrogen aromatisation by cytochrome P450 aromatase enzyme. This process takes place primarily in the cell nucleus, also in epithelial cells of ducts introducing sperm cells, as well as in sperm, and in a small amount in adrenal cortex (8). Oestrogens through their receptors are responsible for spermatogenesis.

In addition to the function connected with reproduction, oestrogens have a significant impact on proper functioning of nervous system due to the presence of Ers in the brain of females and males. Oestrogen receptors are intracellular receptors present in the cell nucleus, as well as in the cytoplasm. They occur in two forms: α (Erα) and β (Erβ). Both subtypes of the receptors have a similar structure but different localisation: Erβ predominates in the testes, ovaries, cerebral cortex, and cerebellum, whereas Erα is located mainly in the uterus, epididymis, adrenal glands, hypophysis, and hypothalamus (7, 13, 14, 18). The presence of Ers in the brain demonstrates the influence of oestrogen hormones on neuron functions in certain brain areas. Oestrogens can also be synthesised in nerve cells and astrocytes, as it was confirmed in recent studies (21).

Oestrogens affect brain cells and structures both in foetal life and afterwards, through all ontogenetic stages. During embryonic development, oestrogens affect sexual differentiation of certain brain parts, resulting in sexual dimorphism. During postnatal period, oestrogens are responsible for cognitive functions, motor coordination, mental status, pain, and also affect memory processes (2, 7, 12, 14, 19). They also play a protective role against Alzheimer's disease delaying its development through increased secretion of choline acetyltransferase (7, 16).

The functioning of the nervous system depends on steroid hormones, which are synthesised in the brain in a large amount and they affect the growth, maturation, and differentiation of nerve cells. The aim of the study was to demonstrate the localisation and expression of Ers in the claustrum, an important transmitter between many brain structures, in males in which oestrogen's role in the nervous system is still underestimated.

Material and Methods

The brains from seven sexually mature rabbit males (Oryctolagus cuniculus) of New Zealand white breed, weighing 3-4 kg, were obtained at rabbit slaughter-house. The brains, immediately after collection, were cut into smaller fragments, fixed in buffered formalin, dehydrated in ethyl alcohol, and then embedded in paraffin blocks according to the methods used in immunohistochemical (IHC) examinations.

The blocks were cut into 5 μm-thick sections, which were placed on silianised glasses SuperFrostPlus. Cross-sections covering the claustrum corresponded to brain cross-sections on the level -1.5 mm in relation to the bregma (6). The sections were incubated for 48 h at 4°C with primary monoclonal mouse IgG1 Erα antibody (NCL-L6F11 clone, Novocastra) diluted 1:40, and monoclonal IgG1 Erβ antibody (NCL-Er_β clone EMR02, Novocastra) diluted 1:50. The positive IHC reaction was demonstrated by the insoluble product of brown staining. To emphasise cell nuclei of neurons, the sections were stained with Mayer's haematoxylin. Control sections were incubated without primary antibody. The final stage was dehydration of the sections in ethyl alcohol, clearing in xylene, and mounting in Canadian balm. The sections were examined under a light microscope and semiquantitative analysis of Ers expression was performed. On the basis of the analysis, the relation of neurons with Ers expression to neurons without IHC reaction was evaluated. Additionally, the percentage of neurons exhibiting different intracellular localisation of the examined receptors was determined by grouping neurons exhibiting cytoplasmic, nuclear, and nucleo-cytoplasmic reactions.

Results were documented using digital camera Colorview IIIu (Soft Imaging System). The obtained images were subjected to semiquantitative analysis using cell D programme (Soft Imaging System). The presence of positive IHC reaction or its lack, as well as intracellular localisation of IHC reaction product were the main differentiation criterion.

Statistical analysis was performed using computer programme Statistica 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* Tukey's test. Probability value $P < 0.05$ was adopted as the boundary of statistical significance of the obtained differences. For each parameter arithmetic mean ($\bar{x}$), standard deviation (SD), and standard error of mean (SEM) were calculated. The presented results were demonstrated in the form of $\bar{x} \pm$ SD.

Results

Immunohistochemical reaction for α and β Ers was observed within neurons in the nucleus and cytoplasm of nerve cells. On the basis of localisation of the reaction, neurons were divided into four groups: 1) IHC-Er reaction was observed within the cytoplasm of neurons (cyt+); 2) IHC-Er reaction was observed within the cytoplasm and nucleus of neurons (cyt+/nuc+); 3) No IHC reaction for Er was observed (cyt-/nuc-); 4) IHC-Er reaction was observed within nucleus of neurons (nuc+).

Oestrogen receptor α (Erα). In proper claustrum, positive IHC reaction was shown in 52% of neurons. Nerve cells constituted the highest percentage, in which the reaction was observed in the cytoplasm. Very few nerve cells showed the nuclear reaction. The positive reaction in both the nucleus and cytoplasm referred to a few percent of neurons. Many nerve cells of claustrum's dorsal part were negative for Erα.

The positive IHC reaction in claustrum's ventral part (nucleus endopiriformis) was demonstrated in 55%

of neurons. The highest percentage was observed, as in proper claustrum, in nerve cells with cytoplasmic reaction and, as compared to proper claustrum, the nuclear reaction was observed in a small percentage of examined cells. The percentage of neurons exhibiting IHC Erα positive reaction in the cell nucleus as well as in cytoplasm was 11. Less than the half of nerve cells demonstrated negative IHC reaction of nucleus endopiriformis (Fig. 1).

Oestrogen receptor β (Erβ). In neurons, proper claustrum positive IHC reaction for the presence of Erβ was demonstrated in 80% of the cells. Neurons in which the reaction was observed only in the cell nucleus, made the highest percentage. A few percent of neurons showed the reaction in the cytoplasm. Eleven percent of neurons revealed the reaction in the nucleus and cytoplasm, while in 20% of the cells no positive IHC Erβ reaction was shown.

In the nucleus endopiriformis, positive IHC reaction was observed in 77% of nerve cells. Most neurons showed positive reaction in the cell nucleus, as in proper claustrum. A larger number of nerve cells in comparison with dorsal part indicated positive reaction in the cytoplasm, while the reaction in the nucleus and cytoplasm was observed in 19% of neurons. In ventral part of the claustrum, as compared to dorsal part, 23% of nerve cells did not show the positive IHC reaction for Erβ (Fig. 1).

Discussion

Most studies concern the localisation of Ers in females, therefore in this study the impact was put on the expression of the receptors in males. Moreover, not much information concerning the occurrence of Ers in the claustrum contributed to the analysis of Erα and Erβ distribution in that brain structure.

Oestrogens play an important role in the development and functioning of the reproductive system in females and males. They also have a significant impact on proper functioning of circulatory, skeletal, and nervous systems. In the central nervous system (CNS), α and β Ers were observed in the cerebral cortex, hypothalamus, corpus amygdaloideum, hippocampus, cerebellum, and spinal cord (4, 13, 18, 20) in different animal species, among others, in mouse (13), rat (18), sheep (9), monkey (17), rabbit (4), and human (15).

In our study, the presence of α and β Ers was observed in proper claustrum, as well as in nucleus endopiriformis in rabbit males. much higher percentage of neurons showing IHC positive reaction for β oestrogen receptors was demonstrated in proper claustrum when compared to neurons in which IHC reaction was negative. The most intense reaction was observed in the cell nucleus, while cytoplasmic and nucleo-cytoplasmic reactions were relatively weak.

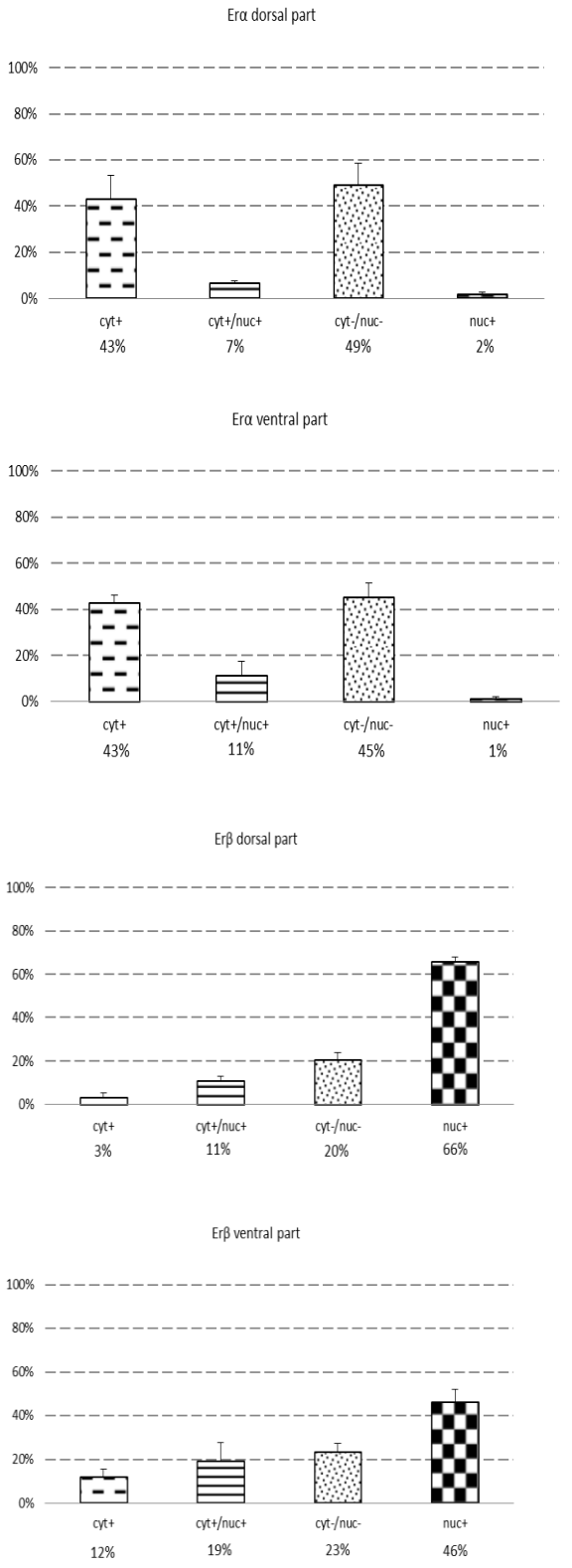
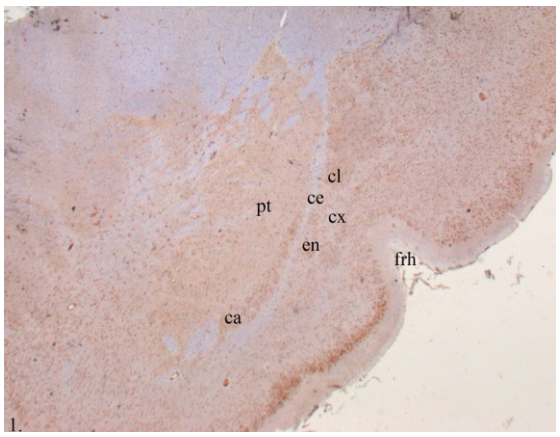
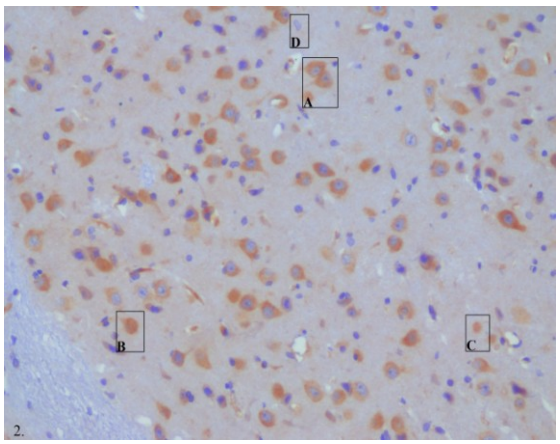
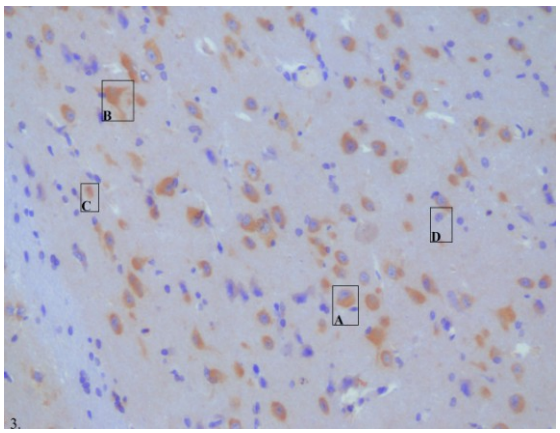
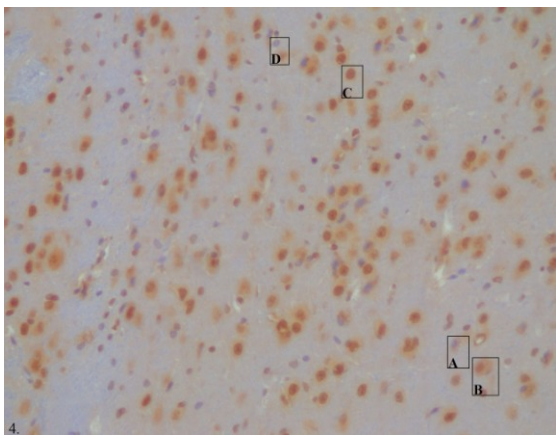


Fig. 1. Immunohistochemical expression of α and β oestrogen receptors in rabbit males' claustrum- dorsal and ventral part (n = 7). Probability value $P < 0.05$ was adopted as the boundary of statistical significance of the obtained differences
cyt+ - positive IHC reaction within the cytoplasm of neurons
cyt+/nuc+ - positive IHC reaction within the cytoplasm as well as nucleus of neurons
nuc+ - positive IHC reaction within nucleus of neurons
cyt-/nuc - no IHC reaction









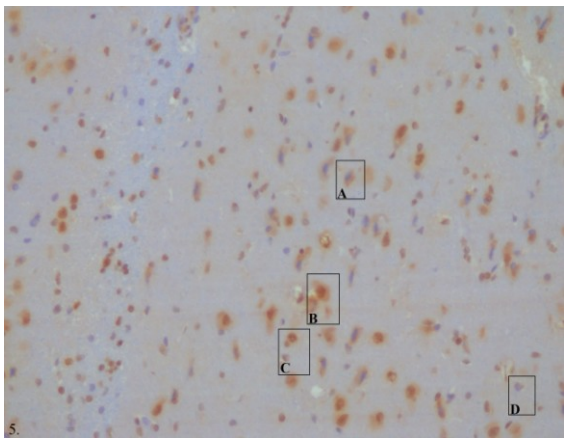


Fig. 2. Expression of oestrogen receptors in immunohistochemical examinations
1. Rabbit's claustrum, cl- claustrum dorsal part; en-claustrum ventral part; cx- extreme capsule; ce- external capsule; pt-putamen; ca- amygdala; frh- rhinal sulcus.
2. Erα in claustrum dorsal part
3. Erα in claustrum ventral part.
4. Erβ in claustrum dorsal part.
5. Erβ in claustrum ventral part. Magnification 100x (1), 200x (2-5).
A. positive IHC reaction within the cytoplasm of neurons (cyt+);
B. positive IHC reaction within the cytoplasm as well as nucleus of neurons (cyt+/nuc+);
C. positive IHC reaction within nucleus of neurons (nuc+);
D. no IHC reaction (cyt-/nuc-)

On the other hand, the expression of α Ers was completely different – a positive reaction was mainly observed in the cytoplasm, while nucleo-cytoplasmic and nuclear reaction was weak. In ventral part of the claustrum *i.e.* in rabbit nucleus endopiriformis, just as in proper claustrum, more neurons showed Erβ positive reaction. Most Ers β were observed in the cell nucleus. However, Ers α were localised mainly in the cytoplasm and in trace amounts in the nucleus.

The examinations carried out up till now have shown that not in all animal species both types of Ers occurred in the claustrum. Mitra *et al.* (13) determined α and β receptors in different brain areas in mouse. The distribution of Erα and Erβ nuclear forms in mouse brain was similar, although the difference was clearly visible in the intensity of the two subtypes depending on the brain structure. In the forebrain the highest concentration of Erβ receptors was observed in cells of vertical and horizontal limbs, medial amygdale, and hypothalamus. In the claustrum, Erβ expression was determined on the cellular level with high to moderate degree, but there were no Erα receptors. Perez *et al.* (17) demonstrated the presence of α scattered receptors in the cerebral cortex (cortical layers I and II) and in the claustrum of female and male rhesus monkeys. The largest cluster of Erα, however, was observed in the amygdala. In our examinations, the presence of α and β receptors was demonstrated in rabbit claustrum as in human brain (15). Oestrogen receptors α predominated in the hypothalamus and amygdala *i.e.* in brain areas responsible for associative and emotional memory

processes. On the other hand, Erβ seems to dominate in hippocampal formation and enthorinal cortex playing an important role in declarative memory.

Zhang *et al.* (20) described the presence of β oestrogen receptors in brain neurons of rats (both females and males) mostly in the cell nucleus. The authors demonstrated that Erβ also occurred in the cytoplasm and nerve cell processes. A very high level of Erβ was shown in anterior olfactory nucleus, vertical limb of the diagonal band, red nucleus, cerebral cortex, Purkinje cells, locus ceruleus, and motor trigeminal nucleus in both male and female rats. An average number of Ers β occurred in *substantia nigra*, medial septum, lateral amygdaloid nucleus, and central gray. Weak IHC reaction was observed in amygdaloid complex and other subregions of hypothalamus.

As regards the presence of Ers in the corpus amygdaloideum, Lonc (10) found numerous Ers located in basolateral complex in rabbit males. Most β Ers were localised in cell nuclei, whereas α receptors occur predominantly in the cytoplasm and also simultaneously in the nucleus and cytoplasm.

The claustrum has diversified bilateral junctions with the cerebral cortex, hippocampus, and limbic system, *i.e.* the brain areas responsible for the processes connected with memory. The presence of Ers was demonstrated in all mentioned brain structures, whereas oestrogen activity in the CNS indicates positive intensification and natural functions of individual areas, *e.g.* in case of damage or neurodegenerative diseases. The presence of α and β Ers in nerve cells of the claustrum may prove a considerable impact of oestrogens on proper functioning of this brain area.

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