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Comparison of localisation of α- and β-oestrogen receptors in posterior part of the piriform cortex in rabbit males

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Abstract

The aim of the study was to compare the immunoreactivity and distribution of two kinds of oestrogen receptors within the nerve tissue of the posterior part of the piriform cortex, as well as their localisation within nerve cells. The studied material consisted of 12 brains of sexually mature rabbit males weighing 3-4 kg. In order to prove the presence of α and β receptors, Er-α and Er-β antibodies were used. The examinations showed the presence of Er-α and E-rβ receptors in all layers of the piriform cortex, however, mainly in the IInd and IIIrd layer. The reaction occurred in the nucleus and cytoplasm of neurons. The obtained results can imply the influence of oestrogen on the functions of the examined brain area in rabbits, which in human is responsible for perception and categorisation of odour.

Key words: rabbit male, oestrogen receptor, piriform cortex.

Introduction

Piriform cortex (PC) is the main part of the olfactory cortex. It obtains direct signals from the olfactory bulb (5, 6, 17) and processes them in different way in anterior and posterior part of the piriform cortex (10, 23). The piriform cortex communicates with brain structures, such as the olfactory bulb, enthorinal cortex, amygdaloid cortex, hypothalamus, basal forebrain, brainstem, olfactory tubercle, olfactory peduncle, superior colliculus thalamus, frontal cortex, and with the hippocampus through the enthorinal cortex. The cortex sometimes is called primary olfactory cortex. It is composed of three layers. Nervous fibres dominate in the superficial layer I. Layer II - the middle one, has the most densely arranged neurons, layer III - the deepest, contains neurons, which are loosely arranged. Neurons in layer II are divided into lying superficially pyramidal neurons, identified as SP (superficial pyramidal) and cells identified as SL (semilunar cells) responsible for odour (2, 23). These cells differ from each other by having different sensitivity threshold. In layer III the nerve cells defined as DP (deep pyramidal cells) are present (2, 23).

The olfactory cortex plays a role in the processes involved in memory, emotions, and stress-related responses. Neurons of the olfactory cortex are in most part GABA-ergic interneurons and projection neurons. The examinations carried out on mice demonstrated the presence of GAD65-EGFP neurons in the posterior part of the piriform cortex (29). The examinations have proven the presence of this subtype of neurons in all layers of the posterior piriform cortex - I, II, and III, but the vast majority occur in layer II. Morphologically and also immunohistochemically, the PC is divided by the anterior commissure into anterior and posterior part

(16). The examinations carried out on rats (18) have proven that there are neuronal junctions between the olfactory cortex and amygdala nuclei; it concerns basal nucleus, lateral nucleus, and accessory basal nucleus.

Steroid hormones including oestrogens play an important role in protecting cells of the central nervous system, and thus proper functioning of its structures. It has been demonstrated that they have an influence on cognitive functions, memory, mood, emotional behaviour, pain, and balance (19). Oestrogen, and also progesteron have receptors located in neurons of different brain structures already in the prenatal period (17, 24, 25). In adolescence, the presence of oestrogen receptors in brain neurons was also revealed (22). Oestrogens have an influence on the function of neurons and their interactions by regulating the formation of junctions between them (26). Oestrogen hormones act on nerve tissue by means of receptors. The examinations performed on rabbits have proven that oestrogen receptors occur in structures of the amygdala in rabbit males (15). It refers to α- and β-oestrogen receptors. Therefore, the aim of the study was to verify whether oestrogen receptors are localised in the male piriform cortex of rabbits.

Material and Methods

The material for the study consisted of 12 adult rabbit male brains. Animals were 2 years of age, weighing 3-4 kg. The material was obtained from a rabbit slaughterhouse. The brains were collected immediately after the slaughter. Histological slides were prepared cutting hemispheres in frontal plane into 4 μm thick sections. In order to determine location of the piriform cortex, atlas describing the structure of rabbit brain was used (7). Crossections showing the piriform cortex were at 0.5 mm in relation to the bregma. Oestrogen receptors (α and β) were detected by immunohistochemical method using Dako LSAB+Kit Peroxidase. The slices were incubated with monoclonal mouse ER-α and ER-β antibodies, afterwards anti-mouse monoclonal biotynylated antibody IgG was used. Then, the slices were incubated with streptavidine/peroxidase complex. Diaminobenzidine (DAB) was used as a chromogen. For visualisation of cell nuclei, Mayer's haematoxylin was applied. In the places on positive immunohistochemical reaction, final insoluble reaction product of brown colour, indicating the presence of receptors, was visible. Negative control of immunohistochemical reaction specificity was performed omitting incubation stage with primary antibody. The results were observed under a light microscope.

Statistical analysis was carried out 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* 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 ($\tilde{x}$) was calculated as well as standard deviation (SD) and standard error of mean SEM; the presented results were shown in the form of $\tilde{x}$ ± SD.

Results

The immunohistochemical examinations showed that neurons of the piriform cortex contained immunoreactive neurons with both oestrogen α- and β-receptors (Figs 1-3). The examinations revealed that α (Er-α) immunoreactive oestrogen neurons demonstrated mainly cytoplasmatic reaction (Fig. 4). On the other hand, β (Er-β) immunoreactive oestrogen neurons showed both nuclear and cytoplasmic reaction. In immunohistochemical examination of β oestrogen receptors, two main parts of posterior piriform cortex (PPC) were distinguished - dorsal posterior piriform cortex - dPPC and ventral posterior piriform cortex-vPPC.

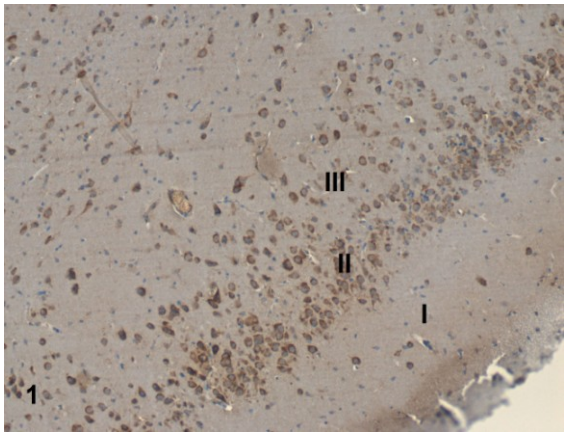


Fig. 1. Immunodetection of oestrogen receptors in posterior part of piriform cortex in rabbit male. Er-α in I, II, and III layer (100x)

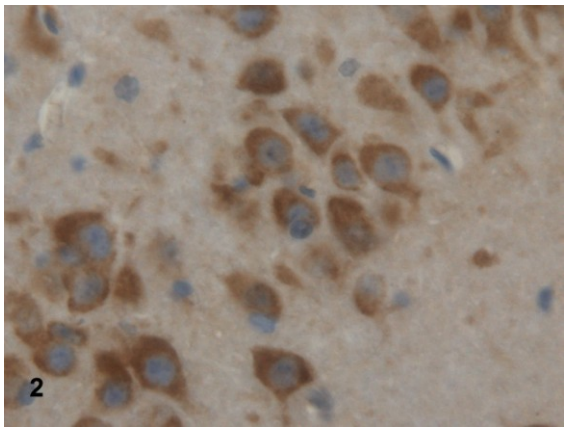


Fig. 2. Immunodetection of oestrogen receptors in posterior part of the piriform cortex in rabbit male. Er-α in II layer (600x)

In immunoreactivity examinations of neurons Er-β in vPPC, both nuclear and cytoplasmic receptors were detected in most neurons, but in dPPC a number of neurons with only cytoplasmic reaction significantly increased. The difference between dPPC and vPPC was visible generally in layer II (Fig. 5).

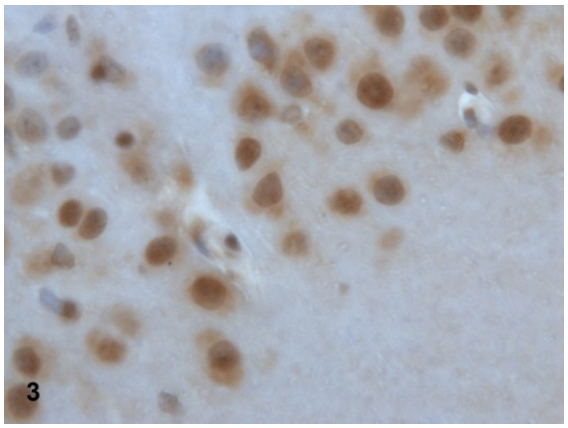


Fig. 3. Immunodetection of oestrogen receptors in posterior part of piriform cortex in rabbit male. Er-β in II layer (600x)

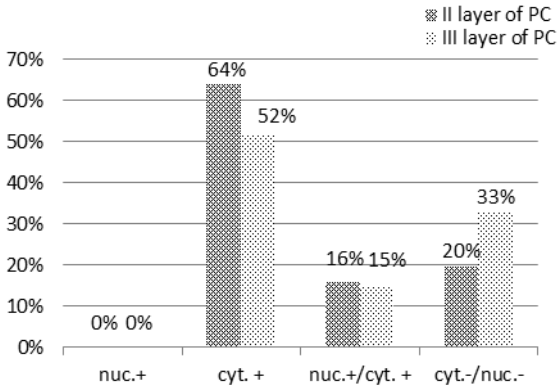


Fig. 4. Immunochemical expression of oestrogen Er-α receptors in posterior part of the piriform cortex in rabbit male

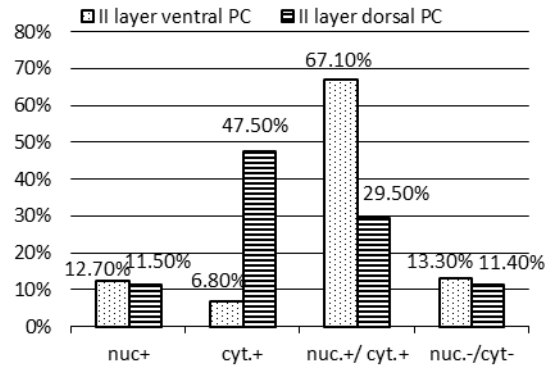


Fig. 5. Immunochemical expression of oestrogen Er-β receptors in II layer of posterior part of the piriform cortex in rabbit male

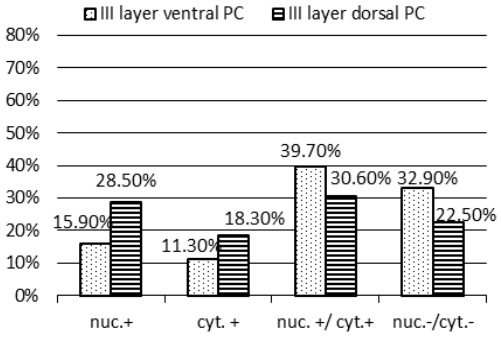


Fig. 6. Immunochemical expression of oestrogen Er-β receptor in III layer of posterior part of the piriform cortex in rabbit male

The reaction confirming the presence of Er-α receptors was observed in layers II and III (Fig. 4). In the examined material there was no difference in the neuron localisation showing the reaction which confirms the presence of Er-α. Therefore, there was no division of PPC into its dorsal and ventral part for these receptors. In layer II, the reaction in the cytoplasm prevailed (Fig. 4). Er-α was shown in the nuclei as well as cytoplasm of neurons in both layers II and III (Figs 1, 2, 4).

In the experiments referring to Er-β receptors, the most positive response was observed in the nuclei as well as in cytoplasm. It concerned 68% of neurons located in layer II. In dorsal part, the reaction concerned less than 30% of neurons (Fig. 5). Other response relationship was observed in cytoplasmic reaction. In ventral part only a few percent of cells contained Er-β receptors. Nuclear reaction occurred in layer II in about 10% of neurons - similarly as in ventral and dorsal part of PPC. Within layer III, the differences in Er-β occurrence in neurons of vPPC and dPPC were not as clear as in the case of layer II (Fig. 6). The difference in cytoplasmic and nucleo-cytoplasmic reactions between these two areas was from a few to several percent. It was noted that more receptors of dPPC was localised in the cytoplasm and nucleus.

In the examined region, no glial cells were counted. They were differentiated on the basis of their size - a body of a glial cell is maximum about 20 µm, whereas a nerve cell is about 10 times bigger.

Discussion

The conducted studies have proven that PC has junctions with such brain structures as hippocampus, amygdale, hypothalamus, thalamus, enthorinal cortex, and perirhinal cortex (1, 3, 4, 12, 13). Luskin *et al*. (17) pointed out that olfactory structures have junctions and depend on brain structures governing emotions. The lack of research on the occurrence of α- and β-oestrogen receptors and the influence of oestrogen hormones on the piriform cortex inclined us to examine

this brain region using immunohistochemical staining. The demonstration of Er-α and Er-β receptors in PC in rabbit males indicates the influence of oestrogen on this brain area and at the same time the functions of these receptors associated with the smell. Just as in humans (10), in rabbit PC difference between ventral PC and dorsal PC was noted. The difference was primarily observed in layers II and III of nerve cells. However, it referred to the distribution of Er-β receptors while Er-α receptors were evenly distributed. For Er-β, the difference concerned the distribution of neurons in which cytoplasmic reaction occurred - in dorsal PC they were the most numerous in cell layer II, while nuclear and cytoplasmic reaction predominated in ventral PC. In both areas there was a different distribution of neurons with the reaction occurring in the nucleus - in dorsal part in layer III the number of neurons was twice as high as in ventral part. There was a different relationship between ventral and dorsal area of PC for Er-α, where no differences were observed in both areas. Er-α in comparison to Er-β localised primarily in neuron cytoplasm. This difference was observed not only in PC but in other brain areas such as amygdale and hippocampus (11, 15). The previous studies have shown that in PC, there are interneurons classified according to the expression of two types of cell markers (molecular markers) - calcium- binding proteins and neuropeptides (23).

In posterior and anterior PC, the presence of GAD-glutamate decarboxylase in layer II was observed. A small number of neurons with positive reaction occurred in layers III and I. The presence of calretinin was demonstrated in layers I, II, and III of posterior PC. Calbindin was also detected in neurons in the same layers of posterior PC. Parvalbumin was observed in layer II (29). According to some authors, GABA-ergic neuron density in posterior part of PC is much higher than in anterior part of PC (8, 9, 16). Haberly *et al.* (9) noted significant differences in distribution of cells showing GABA in anterior and posterior part of PC in the piriform cortex of opossum. In layer II, much more GABA-ergic cells occurred in posterior PC (30%) than in anterior part of PC (15%), while in cell layer III, mostly GABA labelled cells were demonstrated. In rats, GABA-ergic neurons occurred in higher density in layers II and III in posterior part of PC than in anterior one (16). Different results were presented by Mugnaini *et al.* (20), who found that in rats only a small percentage of neurons of PC were GABA-ergic.

Zhang *et al.* (28) examined the posterior part of PC in mice and found the presence of novel inhibitory network, which consisted of GAD65-EGFP positive neurons. They probably have a great significance for olfactory processes. Inhibitory neurons in posterior PC, which predominate in layer II, have a large influence on animal behaviour, their emotional states, and multisensory transformations (27). In the study, α-oestrogen receptors were detected in all layers of posterior PC, similarly but like in the papers of the mentioned authors, immunoreactivity was the greatest in layer II. Layer II contained most Er-β immunoreactive receptors, which were the most numerous in abdominal part of posterior PC. Taking into account the study on rabbits, layer II was shown to be particularly responsible for the reactions connected with oestrogen response.

Li *et al.* (14) have found that in kindled rat brain, synaptophysin immunoreactivity increased significantly in layers II and III of PC. They also found that in hippocampal CA1 and CA3 areas, and in the hilus of dentate gyrus, kindling increased synaptophysin immunoreactivity. In enthorinal cortex, there was no significant effect. The piriform cortex is susceptible to evoke epileptiform activity (21). Wójcik *et al.* (27) observed the influence of NO on PC functions in adult rabbits. They demonstrated the presence of nNOS and calretinine (CR) in PC. Results of their study pointed to low localisation of nNOS –ir and CR-ir in the neurons of the examined structure. The authors claimed the presence of PC in anterior and posterior part of PC. In both parts they demonstrated the presence of three cell layers. Immunoreactivity of nNOS in anterior and posterior part of PC was similar. The nNOS neurons were mainly localised in layers I and III of PC, in layer II there were the fewest of them.

According to Howard *et al.* (10), in humans, the posterior part of the piriform cortex is responsible for odour quality coding, as well as their categorisation, and storing of odours. By means of MRI, it was determined that in human brain posterior PC is responsible for determining the quality of human smell (odour quality). This was not observed in anterior PC.

Wagner *et al*. (25) proved that there is a difference in immunoreactivity of Er-α and progesterone receptors in neonatal mouse brain in relation to sex. Around the time of birth, mouse males demonstrated a higher level of progesterone receptors in medial preoptic nucleus (MPN) than females. This suggests that MPN in males is particularly sensitive to progesterone during this period. The expression of progesterone receptors depended on the expression of Er-α in this region.

Smell information from olfactory bulbs reach directly the piriform cortex. The presence of Ers α and β in neurons in both parts of piriform cortex may indicate the influence of estrogens on smell functions as well as on brain structures dependent on these functions.

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