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IMMUNOCYTOCHEMICAL ANALYSIS OF CALRETININ IN THE FRONTAL CORTEX OF CHINCHILLA

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Abstract

The aim of the study was to define morphology and distribution of calretinin (CR) positive neurons in the frontal cortex of adult chinchilla males and intracellular localisation of the protein in this area. The brains of 5 adult chinchilla males were used in the study. CR immunoreactive neurons were shown with peroxidase-antiperoxidase immunohistochemical reaction using a specific monoclonal antibody. Intensive CR immunoreactivity was demonstrated mainly in few polymorphic neurons of II, III, and V layers. Cytoplasmic and nuclear reaction product in most CR positive neurons was diffuse and in some neurons of layer V in the form of granules localised peripherally. In a few cells more intensive staining was observed in the nuclei than in the cytoplasm. The results indicate the presence of heteromorphic CR positive neurons in specific layers of the frontal cortex. Nuclear localisation of CR in neurons suggests passive transport of this protein, which may affect the nuclear genes. This protein is a neuroprotector maintaining appropriate level of calcium, modulating neuronal activity, and synaptic conduction in the frontal cortex of chinchilla.

Key words: brain, chinchilla, calretinin.

Calretinin (CR) is a protein belonging to the "EF-hand" family of calcium binding proteins showing structural similarity to calbindin D28K. Proteins from "EF-hand" family build domains made up of two α-helixes and calcium binding loops, comprising 12 aminoacids. Calretinin contains six EF-hand domains, five of which have an affinity to bind calcium (29). Calcium ions constitute the basic integrating factor and they regulate many metabolic processes of the nervous system, but in excess they exert a toxic effect on neurons. Calretinin performs a neuroprotective function and it acts as a fast calcium buffer already in the earliest stage of brain maturation (25). Furthermore, like calbindin D28K, it can induce intracellular processes dependent on calcium ions as a sensory protein (6).

Cerebral cortex receives and analyses data from sensory organs, associates information, sends instructions determining motor reactions, and is also responsible for somatic sensation, vision, hearing, and higher mental activities, such as planning, thinking, and emotions. Approximately 95% of the whole human cerebral cortex is neocortex, while in animals only a quarter. Neocortex is made up of six layers: molecular layer (I), external granular layer (II), external pyramidal layer (III), internal granular layer (IV), internal pyramidal layer (V), and multiform layer (VI). Layer I is oligocellular and is mainly composed of dendrites. In layers I and II small granular cells dominate, while in layers III and V - triangular pyramidal cells. Layer VI contains small cells of irregular shape. Approximately 70%-80% of the total population of nerve cells make glutamatergic pyramidal neurons. The remaining are interneurons, most of which are inhibitory cells containing γ-aminobutyric acid (GABA) (11, 27).

Using specific antibodies and immunohistochemical analysis, the CR was shown in the neocortex in many species of mammals including rodents and primates (3, 9, 14, 15, 19-22, 28, 33). The aim of this paper was to define the morphology and distribution of CR positive neurons in the frontal cortex of adult chinchilla males. So far no one has determined intracellular localisation of this protein in the area in these rodents. The results obtained from our study were compared with similar results obtained in studies of other species of mammals.

Material and Methods

The brains of five 18-month-old chinchilla males, collected immediately after the slaughter, were used in the study. The material was fixed in 10% buffered formalin (pH 7.0) for 12 h at 4°C and then embedded in paraffin blocks using routine technique. Next, 6 µm-thick histological sections containing frontal cortex were placed on glass slides and held for 30 min at 56°C. Afterwards, the deparaffinised and hydrated sections were incubated in 0.4% H_2O_2 in phosphate

buffer at room temperature for 30 min in order to inhibit endogenous peroxidase activity. Then, they were washed in 0.5 M tris buffer (TBS-tris buffered saline), pH 7.6, and treated with normal goat serum (Sigma) at room temperature for 20 min in order to remove the staining of the background.

For the immunohistochemical analysis, based on peroxidase-antiperoxidase reaction (PAP), the tissue sections were incubated with specific monoclonal mouse antibody directed against CR (1:2,000, Sigma) for 48 h at 4°C (30). Next, the samples were rinsed with 0.5 M TBS and incubated for an hour with a monoclonal antibody against mouse immunoglobulins (1:50, Sigma), and later with a monoclonal complex of PAP (Sigma). The sections were rinsed with the same buffer (0.5 M TBS) after each antibody. In the next step, the samples were sequentially treated with 3,3- diaminobenzidine tetra-hydrochloride (Fluka) at room temperature for 30 min. Then, the sections were washed in distilled H_2O, dehydrated, and closed in DPX (Fluka). In the performed immunocytochemical technique, the specificity control comprised a sample, in which the primary antibody was omitted, or replaced with normal serum.

Neurons of the frontal cortex immunopositive for CR were observed and photographed under a light microscope Axiolab (Zeiss).

Results

The examination results revealed the presence of few heteromorphic neurons with an immunoreactivity of CR scattered in the frontal cortex. In the molecular (I), internal granular (IV), and multiform (VI) layers of the frontal cortex, most neurons were characterised by a very weak immunoreactivity for CR (Fig. 1).

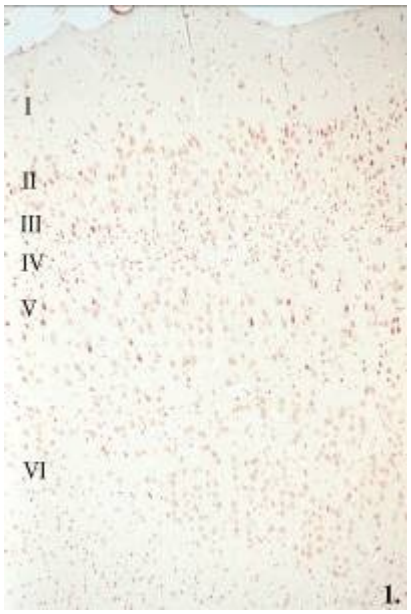


Fig. 1. Differentiated calretinin immunoreactivity in neurons of layers I-VI in chinchilla's frontal cortex. About 100x.

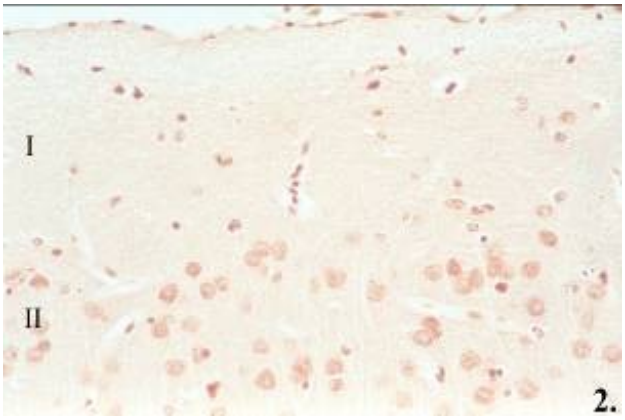


Fig. 2. Fusiform and stellate neurons with intensive calretinin immunoreactivity in molecular layer (I) of chinchilla's frontal cortex. About 400x.

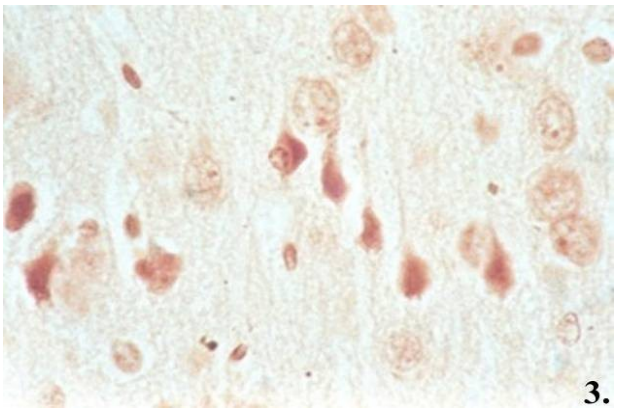


Fig. 3. Intensive calretinin immunoreactivity in fusiform, oval, and stellate neurons of external granular layer (II) in chinchilla's frontal cortex. About 1,000x.

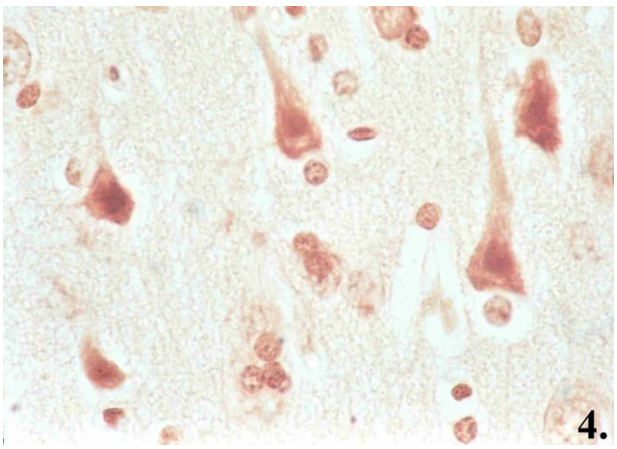


Fig.4. Intracellular cytoplasmic and nuclear calretinin immunoreactivity in neurons of internal pyramidal layer (V) in chinchilla's frontal cortex. About 1,000x.

In molecular layer few small-sized cells of fusiform shape horizontally arranged and stellate cells showed intensive immunoreactivity of the examined protein in both: the cytoplasm and the cell nuclei (Fig. 2).

Intensive immunoractivity of CR was detected mainly in unevenly scattered neurons of external granular

l (II), external pyramidal (III), and internal pyramidal (V) layers of the frontal cortex.

Intensive immunoreactivity in external granular layer (II) was observed in small vertically oriented neurons of fusiform, oval, and stellate shape (Fig. 3).

Cell bodies and initial processes contained brown, diffuse reaction product, which was also visible in round or oval cell nuclei. Similar intensive immunoreactivity of CR was disclosed in neurons of external pyramidal layer (III).

Internal pyramidal layer (V) was characterised by the presence of few heteromorphic neurons intensively stained for CR. These cells were located among weakly immunoreactive neurons. Large perikaryons of nerve cells with immnonoreactivity of the examined protein took fusiform, stellate, and pyramidal shapes. Intracellular, cytoplasmic, and nuclear immunoreactivity was observed. Cytoplasmic brown reaction product appeared in the diffused form or granules localising peripherally near the cell membrane. In some neurons more intensive staining was observed in oval or round nuclei rather than in the cytoplasm (Fig. 4).

Discussion

Intensive immunoreactivity for CR was observed in heteromorphic neurons of neocortex in many animal species. Most of the neurons containing CR are vertically oriented bipolar cells with fusiform or oval perikaryon (10, 17, 21, 33). Multipolar neurons are also observed, including double bouquet cells and horizontally arranged Cajal-Retzius cells localised exclusively in the first cortical layer. The immunoreactivity of the examined protein was also demonstrated in few pyramidal neurons in some species of mammals (3, 9, 21).

Distribution of neurons with CR immunoreactivity is differentiated in respect to species as well as between areas and layers of the cortex. In the monkey, numerous CR immunoreactive neurons were observed in visual cortex, less numerous in prefrontal, motor, and premotor cortex, and the least numerous in primary sensory cortex (12). In the man, the number of CR immunoreactive neurons was significantly lower in the frontal cortex in comparison with temporal, occipital, and parietal cortex (4). In addition, it was shown that the neocortex in primates is characterised by significantly higher number of CR positive neurons than in rodents. In rat's medial prefrontal cortex neurons containing the examined protein made about 4% of the total population of nerve cells, while in humans 8% and in monkey 11% (16, 32).

Our results correspond with the results obtained in primates and rodents, in which neurons showing CR immunoreactivity are located mainly in the layers II and III of the cerebral cortex (3). In rabbit's visual cortex, 35% of CR positive neurons were observed in layer II, about 28% in layer III, and less than 15% in other layers (28). In dolphins, almost all neurons containing CR were localised in layer I of the visual cortex (15). In felids, many neurons positive for CR were found in contrast to humans, monkey, rabbit, rat, as well as the examined chinchilla, in which only few cells with the expression of this protein were observed (22, 28). In canids and felids, there were very large CR immunoreactive neurons in layer III in the motor cortex. They are morphologically similar to the neurons found in layers III, V, VI of the whole cerebral cortex in large artiodactyls such as giraffe, llama, and camel. In smaller ruminants and pigs these cells were less numerous (21). In artiodactyls just as in chinchillas, CR was expressed by fusiform, bipolar, and multipolar neurons of layers I and II as well as superficial layer III, which corresponded to bipolar and bittufted neurons of layer II in other mammals such as rats, predators, and primates (2). Moreover, large pyramidal neurons, weakly immunoreactive for the examined protein, were observed in dolphins' layer III (21). On the other hand, in layer III of the dog and macaque and in human cortex pyramidal neurons with an intensive immunoreactivity of CR were described (19, 20). In addition, the neocortex of primates and rodents, including chinchillas showed few CR positive neurons also in the layers V and VI (20).

Glutamatergic pyramidal neurons are main stimulating neurons, that occur in all layers of the neocortex, containing stimulating glutamate as a neurotransmitter, never inhibitory γ-aminibutyric acid (GABA). Interneurons are, on the other hand, represented by two types of cells: spiny stellate cells and smooth non-pyramidal cells. Stellate cells are localised in medial layers of the cortex, especially in layer IV, where they play a role of stimulating neurons. Nonpyramidal cells are present in all layers mainly as GABA-ergic inhibitory neurons. They are morphologically and structurally diverse (9).

Most of CR immunopositive neurons show immunoreactivity for GABA and therefore belong to interneurons. In the visual cortex of the rat and monkey, more than 90% of CR positive neurons were GABA-ergic (9, 28). However, the neocortex of magachiropterans is characterised by a complete lack of CR in interneurons (21). In many mammal species, co-occurrence of CR and different neuropeptides in neurons of the neocortex are also described. In the monkey, in medial prefrontal cortex, 86% of CR immunoreactive neurons showed expression of vasoactive intestinal peptide (VIP) (9). In layers V and VI of rat's neocortex, 94% of CR positive neurons contained VIP, whereas in layers II and III about 71% (26). Colocalisation of CR and VIP with choline acetylotransferase (CHAT) was also observed (7, 14). Only few CR immunopositive neurons showed expression of nitric oxide synthase (NOS). On the other hand, there was no co-existence of CR with neuropeptide Y (NPY) and somatostatin (SOM) (9).

Interneurons affect the activity of pyramidal neurons forming two functional networks: slowly and fast responding to stimulation. In the brain of rodents, neurons with immunoreactivity of CR are slowly,

regularly communicating with nonpyramidal cells (RSNP) and very slowly and rapidly communicating with nonpyramidal cells (BSNP) (23, 24, 31). In cortical layers II and III in mice, CR positive bipolar neurons have shown abrupt discharge, whereas multipolar neurons communicated regularly. It is suggested that multipolar cells with CR immunoreactivity are one of the elements of interneuronal network, which in a synchronised way of controlling inhibition of pyramidal neurons. Bipolar neurons immunoreactive for CR abolish this action as so-called disinhibitors (3).

CR as a neuroprotective protein protects neurons from calcium cytotoxicity, reducing its intracellular concentrations. Neurons showing CR immunoreactivity seem to be resistant to various neuropathological factors. CR immunoractivity in neurons does not change in disease states, such as schizophrenia, depression, Alzheimer's disease, and multiple sclerosis (5, 8, 13, 18). Similarly in states of epilepsy, a number of CR positive neurons of the neocortex remains stable or declines less evidently in comparison with immunoreactivity of other calcium binding proteins, such as PV and CB (1, 3). A slight decrease or no change in CR immunoreactivity may indicate its neuroprotective role, which is not sufficiently understood (13).

Our research presented above provided new data on the morphology and distribution of CR immunopositive neurons in the layers of the neocortex in chinchilla. In addition, a differentiated intracellular localisation of this protein was demonstrated. In some publications, the nuclear CR locations shown in neurons of the frontal cortex of chinchillas are described. CR as a protein of low molecular weight is transported passively through the nuclear pores, which can affect the expression of genes in the nucleus (29). The presence of CR in the chinchilla's neocortex, as well as in the neocortex of other animal species, points to its involvement in neuroprotective maintenance of proper level of calcium ions, modulation of neuronal activity, and synaptic conduction. Distribution of neurons in the frontal cortex of chinchilla in comparison with other species of mammals is differentiated. Further examination is necessary to determine colocalisation of CR with neurotransmitters and neuropeptides in neurons of the neocortex in chinchilla.

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