Praca oryginalna *Original paper*

Immunohistochemical analysis of parvalbumin in the frontal cortex of Chinchilla lanigera

JADWIGA JAWORSKA-ADAMU, RADOSŁAW SZALAK, ALEKSANDRA KRAWCZYK, KAROL RYCERZ

Department of Animal Anatomy and Histology, Faculty of Veterinary Medicine, University of Life Sciences, Akademicka 12, 20-033 Lublin, Poland

Jaworska-Adamu J., Szalak R., Krawczyk A., Rycerz K.

Immunohistochemical analysis of parvalbumin in the frontal cortex of Chinchilla lanigera

Summary

Parvalbumin (PV), intracellular slow buffer regulates concentration of calcium ions protecting neurons from its excitotoxicity. This protein was described in inhibitory neurons of neocortex in many species of mammals, but its functions are not fully understood. In view of the absence of research on PV in the frontal cortex layers of chinchillas, we decided to trace the distribution of PV-immunoreactive neurons, demonstrate their intracellular locaition and describe their morphology. The aim of the study was also to compare the immunoreactivity of this protein in chinchllas and other species of mammals. Samples of the frontal cortex from 5 sexually mature chinchilla lanigera males was taken for the examination and after appropriate fixation, peroxidase-antiperoxidase immunohistochemical reaction was carried out with a monoclonal antibody against parvalbumin. In molecular layer (I) no nerve cells with PV expression of the examined protein were found but intensive PV immunoreactivity was observed in scattered neurons of the following layers: external granular (II), external pyramidal (III), internal granular (IV), internal pyramidal (V) and heteromorphic cells (VI). Within layer II and IV few, small, stellate, oval and fusiform cells were detected with intensive immunoreactivity in cell bodies and initial processes. Layers III and IV showed neurons of various sizes and shapes. Only layer V was characterized by the presence of the examined protein expression in pyramidal cells. Whereas, layer VI contained slightly more heteromorphic neurons with intensive PV immunoreactivity. In all examined layers of chinchilla's frontal cortex protein localized nuclearly and cytoplasmicly in cells. The obtained results of our own research concerning PV expression in different layers of frontal cortex show similarities and slight differences between species. Nerve cells containing PV should be considered as a specific subpopulation of GABAergic interneurons, which modulate the excitability of other neurons affecting the stability of neuronal nets in frontal cortex of chinchilla langera.

Keywords: chinchilla, frontal cortex, parvalbumin

Many brain areas contain parvalbumin (PV), a neurochemical marker for the subpopulation of GABAergic interneurons. This slow calcium buffer, belonging to the family of "EF-hand" proteins, regulates the concentration of calcium ions in the cell cytoplasm. The neocortex is made up of six layers: molecular (I), external granular (II), external pyramidal (III), internal granular (IV), internal pyramidal (V) and polymorphic cells (VI). Among the neurons of the neocortex layers there are two morphologically and functionally different basic types of cells. Principal excitatory neurons containing glutamate as a neurotransmitter do not show PV presence. They make up about 70% of all nerve cells of the cerebral cortex. Their long axons project to cortical and subcortical areas. The remaining 20--30% of cells are inhibitory interneurons, most of which contain γ-aminobutyric acid (GABA), and their short axons do not extend beyond the neocortex. They are local, morphologically and functionally heterogenous neurons modulating the functions of the neocortex. Interneurons have been classified into several subtypes because of the content of different calcium--binding proteins (parvalbumin-PV, calbindin D28k-CB, calretinin-CR), the presence of γ-aminobutyric acid (GABA), neuropeptides and morphological heterogeneity, as well as junctions with other nerve cells (6, 7, 19, 23). The distribution and morphology of interneurons containing PV immunoreactivity has been well

described in many areas of the neocortex in various mammalian species (4, 8, 9, 11, 18). However, calcium-binding proteins, including PV, are still a research topic because their functions are not fully understood. Interneurons are involved in changes in the excitability and activity of inhibitory and excitatory neurons, in the short-term plasticity of synapses, and they stabilize neuronal nets within the neocortex (3, 23).

In view of the absence of research on PV in the frontal cortex layers of *Chinchilla lanigera*, we decided to trace the distribution of neurons immunoreactive for this protein, describe their morphology, demonstrate their intracellular location and compare the results of our examinations with similar studies on primates and other mammals.

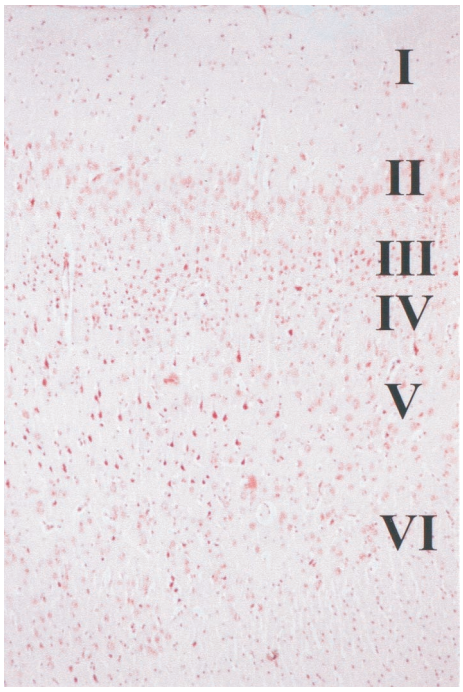

Fig. 1. PV immunoreactivity, layers I-VI of frontal cortex (magn. approx. 100 ×)

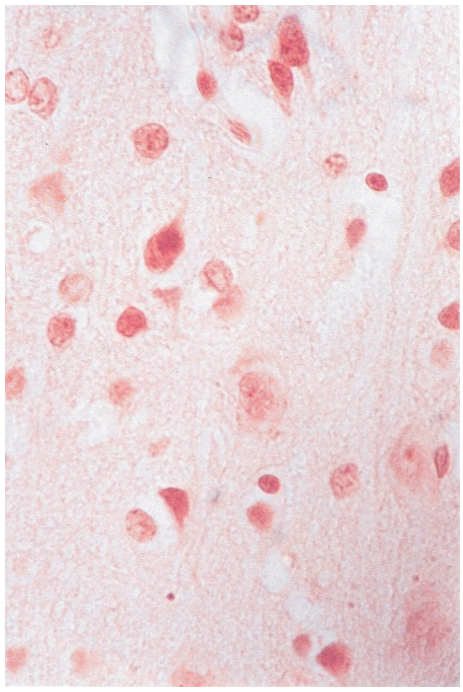

Fig. 2. PV immunoreactivity, layer III of frontal cortex (magn. approx. 1000 ×)

Material and methods

Our examinations were carried out on 5 sexually mature (about 1.5 year old) chinchilla males from the RABA farm in Myślenice. Immediately after the slaughter, their skulls were opened, the brains were removed, and the frontal cortex was excised. The frontal cortex was fixated, embedded in paraffin blocks by the routine histological technique, and cut into 6 µm-thick slices. The slices were exposed to 0.4% H_2O_2 for 30 min. in order to inhibit endogenous peroxidase activity. To remove the background staining, the slices were treated with 10% goat serum for 20 min. Peroxidase--antiperoxidase immunohistochemical reaction (PAP) was carried with a mouse-specific monoclonal antibody against PV (24). Next, a secondary monoclonal antibody against mouse immunoglobulins and a monoclonal peroxidase--antiperoxidase complex were used. Diaminobenzidine (DAB) was used as a chromogen. Reaction specificity control was carried out by omitting the primary antibody or replacing it with normal goat serum. Brown PV immunopositive neurons were analyzed and photographed under a light microscope Axiolab (Zeiss).

Results and disscusion

No nerve cells with parvalbumin expression (PV) were detected in the molecular layer (I). Intensive parvalbumin immunoreactivity was observed, however, in scattered neurons of the other layers of the male chinchilla's frontal cortex: external granular (II), external pyramidal (III), internal granular (IV), internal pyramidal (V) and heteromorphic cells (VI) (Fig. 1). Nerve cells showed heterogeneity in terms of the size and shape of the cell bodies. In external granular (II) and internal granular (IV) layers few small fusiform, stellate and oval cells characterized by intensive PV expression were observed. The external pyramidal layer (III) contained quite a number of stellate, oval, round and fusiform neurons of various sizes and shapes with intensive brown PV reaction product in cell bodies and initial nerve processes (Fig. 2). The internal pyramidal layer (V), just like the external pyramidal layer (III), was characterized by intensive PV immunoreactivity in numerous heteromorphic nerve cells. Fusiform, oval and mostly stellate neurons of large sizes were scattered within the internal pyramidal layer (V). Intensive PV immunoreactivity in this layer was also shown by pyramidal neurons (Fig. 3). The polymorphic cell layer (VI) contained slightly more neurons with intensive PV expression in comparison with layers II and IV (Fig. 4). In all analyzed layers of the male chinchilla's frontal cortex, the protein was located in oval or spherical cell nuclei, as well as cytoplasmically in perikaryons and initial processes of neurons. Brown, intensive nuclei and cytoplasm immunostaining was similar in most cells. Some large heteromorphic and pyramidal cells were characterized by more intensive nuclei staining in comparison with the cytoplasm. Especially in pyramidal neurons brown granules located near neurolemma were observed (Fig. 3).

The layered arrangement of PV-immunopositive neurons in the frontal cortex of *C. lanigera* shows similarities and differences between species. In our studies, neurons with the expression of the calcium--binding PV was not observed in layer I of the frontal cortex, just as in other mammalian species (2, 5, 11, 12, 18, 20). Scattered PV-immunopositive nerve cells were revealed in all other layers of the chinchilla's frontal cortex. Numerous neurons with parvalbumin expression were found mainly in layers III and V, less

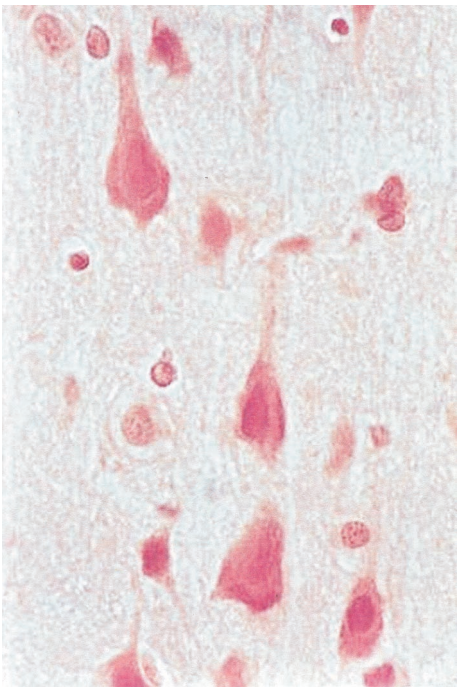

Fig. 3. PV immunoreactivity, layer V of frontal cortex (magn. approx. 1000 ×)

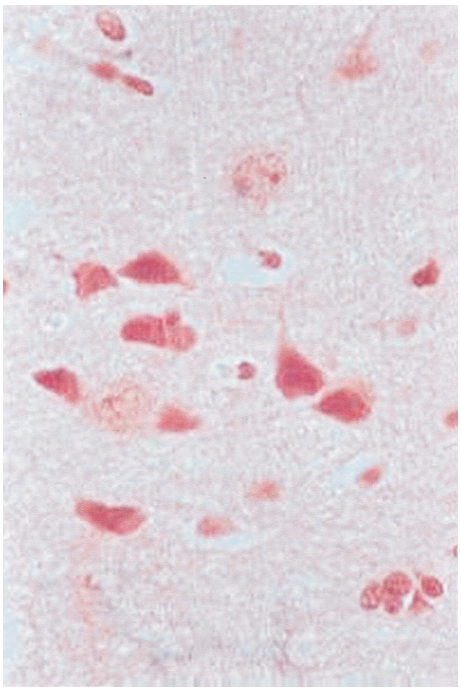

Fig. 4. PV immunoreactivity, layer VI of frontal cortex (magn. approx. 1000 ×)

numerous in layer VI, and few in layers II and IV. Similarly, most neurons with intensive PV immunoreactivity were demonstrated in neurons of layers III and V, and in layers IV and VI of the rabbit's cerebral cortex. The most numerous neurons with parvalbumin expression were found in layers III and V but also numerous in the remaining layers of the dog's neocortex (12). In the chinchilla's layer II few cells with parvalbumin expression were observed, just as in the rat's, mouse's and rabbit's visual cortex (10, 11, 15, 18). In comparison with some mammalian species, layers IV and VI of the chinchilla's cerebral cortex contain less PV-immunoreactive neurons (1, 12, 16, 18). However, as in the chinchilla, less numerous neurons with PV expression in these layers were also demonstrated in cats, mice and hamsters (5, 17). In the rat's visual cortex most PV-positive neurons were observed in layer IV-72.8% and in layer V-69.9%, less in layer VI-52.8%, whereas layers II and III contained 37.2% of them (10, 11). PV-immunoreactive neurons of the frontal cortex in the examined species of rodents expressed heterogeneity in terms of the morphology and size of cell bodies. Different sizes of round, oval, stellate and sometimes fusiform nerve cells were observed. These types of neurons were similar to those identified in the rat's frontal cortex and in the visual cortex in humans, monkeys (1), cats (5), dogs (12), mice (11), hamsters (17), rats (14, 15) and rabbits (18). In all mammalian species PV-positive neurons were defined as nonpyramidal nerve cells. Our studies reveal that some large pyramidal neurons are characterized by intensive PV expression. These neurons are distributed only in layer V of the chinchilla's frontal cortex, as in the neocortex of dogs and in the motor and somatomotor area of monkeys and humans (12, 20). Pyramidal PV-immunoreactive neurons were also observed in the neocortex of gerbils and in the CA1 area of the dog's hippocampus (2, 13).

Our examination results reveal a similar intracellular location of parvalbumin in all neurons of layers II-VI of the chinchilla's frontal cortex. PV expression was observed in the neuroplasm of cell bodies in the initial segments of nerve processes and in nuclei. Some neurons of layer V showed brown granules located near neurolemma. Many cells were characterized by more intensive immunoreactivity in cell nuclei in comparison with the cytoplasm. Parvalbumin was considered primarily as a cytoplasmic buffer of calcium ions present in the perikaryons and axons of nerve cells. Furthermore, on the basis of examinations of Purkinje's neurons of the cerebellar cortex, PV was also labeled in cell nuclei, which suggests that this mobile protein of low molecular weight can be transported passively through nuclear pores and affect gene expression (21, 22).

Using the immunohistochemical double labeling of neurons for parvalbumin and inhibitory neurotransmitter of γ-aminobutyric acid (GABA), it was found that they represent about 39% of all GABAergic cells in the adult mouse's visual cortex, and about 50.8% in rats. In the rat's frontal cortex they were demonstrated in about 40% of all GABAergic cells. In addition, few GABAergic neurons containing PV simultaneously showed immunoreactivity for calbindin D-28k and constituted 6% in the rat's frontal cortex located in deeper layers V and VI and 4% in the mouse's visual cortex (10, 11, 25). Parvalbumin buffering intracellular calcium ion concentration performs a neuroprotective function and affects the regulation of synaptic plasticity. This chemical marker is contained in two subpopulations of inhibitory GABAergic interneurons. One of them is made up mostly of large vertically oriented multipolar basket interneurons (basket cells-Bcs) located mainly in layers III-VI and in layer II of the cerebral cortex. Their axons do not extend beyond the neocortex and appear in the form of baskets on the bodies and proximal dendrites of target neurons, i.e. other interneurons and excitatory nerve cells. Axonal nerve endings forming electrical and chemical synapses harmonize cortical activity and are involved in the plasticity of neuronal transmission. In this way they affect the synchronization and oscillatory activity of a much larger population of excitatory pyramidal

neurons. Other, less numerous interneurons are heteromorphic: oval, stellate and fusiform chandelier cells innervating the initial segments of the pyramidal axons of excitatory neurons and forming inhibitory, symmetrical synapses with them. They are scattered, like basket cells in layers II-VI, and they are non-spinous interneurons which do not innervate other interneurons. Some of them contain not only PV, but also CB. Both subpopulations of GABAergic interneurons were included in the category of fast-spiking cells, modulating not only the activity of GABAergic cells, but also excitatory pyramidal neurons, which stabilize neuronal nets in the neocortex (3, 6, 22, 23).

The present study provides new information on the morphology and distribution of neurons immunopositive for parvalbumin in the layers of the chinchilla's frontal cortex. The distribution of neurons in the frontal cortex in chinchillas is slightly different than but similar to that in other mammalian species. The data obtained suggest that there can be two subpopulations of basket and chandelier GABAergic interneurons. PV expression in these cells appears to be involved in the regulation of calcium levels and thus participate in the stabilisation of neuronal nets.

References

1. *Blümcke I., Hof P. R., Morrison J. H.*: Distribution of parvalbumin immunoreactivity in the visual cortex of old world monkeys and humans. J. Comp. Neurol. 1990, 301, 417-432.
2. *Brückner G., Seeger G., Brauer K., Härtig W., Kacza J., Bigl V.*: Cortical areas are revealed by distribution patterns of proteoglycan components and parvalbumin in the Mongolian gerbil and rat. Brain Res. 1994, 658, 67-86.
3. *Caillard O., Moreno H., Schwaller B., Llano I, Celio M. R., Marty A.*: Role of the calcium-binding protein parvalbumin in short-term synaptic plasticity. PNAS 2000, 97, 13372-13377.
4. *DeFilipe J., Hendry S. H. C., Jones E. G.*: Visualization of chandelier cell axons by parvalbumin in monkey cerebral cortex. Proc. Natl. Acad. Sci. 1989, 86, 2093-2097.
5. *Demeulerneester H., Arckens L., Vandesande F., Orban G. A., Heizmann G. A., Pochet R.*: Calcium binding proteins and neuropeptides as molecular markers of GABAergic interneurons in the cat visual cortex. Exp. Brain Res. 1991, 84, 538-544.
6. *Druga R.*: Neocortical inhibitory system. Folia Biologica (Praha) 2009, 55, 201-217.
7. *Elston G. N.*: Cortex, cognition, and the cell: new insights into the pyramidal neuron and prefrontal function. Cereb. Cortex 2003, 13, 1124-1138.
8. *Ferrer I., Zujar M. J., Admella C., Alcantara S.*: Parvalbumin and calbindin immunoreactivity in the cerebral cortex of the hedgehog (Erinaceus europaeus). J. Anat. 1992, 180, 165-174.
9. *Galarreta M., Hestrin S.*: Electrical and chemical synapses among parvalbumin fast-spiking GABAergic interneurons in adult mouse neocortex. Proc. Natl. Acad. Sci. 2002, 99, 12438-12443.
10. *Gonchar Y., Burkhalter A.*: Three distinct families of GABAergic neurons in rat visual cortex. Cereb. Cortex 1997, 7, 347-358.
11. *Gonchar Y., Wang Q., Burkhatler A.*: Multiple distinct subtypes of GABAergic neurons in mouse visual cortex identified by triple immunostaining. Frontiers Neuroanat. 2008, 1, 1-11.
12. *Hof P. R., Bogaert Y. E., Rosenthal R. E., Fiskum G.*: Distribution of neuronal populations containing neurofilament protein and calcium-binding proteins in the canine neocortex: regional analysis and cell typology. J. Chem. Neuroanat. 1996, 11, 81-98.
13. *Hof P. R., Rosenthal R. E., Fuskum G.*: Distribution of neurofilament protein and calcium-binding proteins parvalbumin, calbindin, and calretinin in the canine hippocampus. J. Chem. Neuroanat. 1996, 11, 1-12.
14. *Kawaguchi Y.*: Physiological subgroups of nonpiramidal cells with specific morphological characteristics in layer II/III of rat frontal cortex. J. Neurosci. 1995, 15, 2638-2655.
15. *Kawaguchi Y., Kubota Y.*: GABAergic cell subtypes and their synaptic connections in rat frontal cortex. Cereb. Cortex 1997, 7, 476-486.
16. *Lim H.-R., Kong J.-I., Jeon Ch.-J.*: Calcium-binding proteins calbindin D28k, calretinin, and parvalbumin immunoreactivity in the rabbit visual cortex. Mol. Cells 2000, 10, 206-212.
17. *Park H.-J., Hong S.-K., Kong J.-H., Jeon C.-J.*: Localization of calcium--binding protein parvalbumin-immunoreactive neurons in mouse and hamster visual cortex. Mol. Cells 1999, 9, 542-547.
18. *Park H.-J., Lee S.-N., Lim H.-R., Kong J.-I., Jeon Ch.-J.*: Calcium-binding proteins calbindin D28k, calretinin, and parvalbumin immunoreactivity in the rabbit visual cortex. Mol. Cells 2000, 10, 206-212.
19. *Povysheva N. V., Zaitsev A. V., Rotaru D. C., Gonzales-Burgos G., Lewis D. A., Krimer L. S.*: Parvalbumin-positive basket interneurons in monkey and rat prefrontal cortex. J. Neurophysiol. 2008, 100, 2348-2360.
20. *Preuss T. M., Kaas J. H.*: Parvalbumin-like immunoreactivity of V pyramidal cells in the motor and somatosensory cortex of adult primates. Brain Res. 1996, 712, 353-357.
21. *Schmidt H., Arendt O., Brown E. B., Schwaller B., Eilers J.*: Parvalbumin is freely mobile in axons, somata and nuclei of cerebellar purkinje neurons. J. Neurochem. 2007, 100, 727-735.
22. *Schwaller B., Meyer M., Schiffmann S.*: „New" functions for „old" proteins: the role of the calcium-binding proteins: calbindin D-28k, calretinin and parvalbumin, in cerebellar physiology. Studies with knockout mice. Cerebellum 2002, 1, 241-258.
23. *Siucińska E.*: Neuroprzekaźnik hamujący w plastyczności kory mózgu. Kosmos 2005, 54, 195-212.
24. *Sternberger L. A.*: Immunohistochemistry. John Wiley and Sons, New York 1986.
25. *Uematsu M., Hirai Y., Karube F., Ebihara S., Kato M., Abe K., Obata K., Yoshida S., Hisabayashi M., Yanakawa Y., Kawaguchi Y.*: Quantitative chemical composition of cortical GABAergic neurons revealed in transgenic venus-expressing rats. Cereb. Cortex 2008, 18, 135-330.

Author's address: dr hab. Jadwiga Jaworska-Adamu, prof. nadzw. UP, ul. Akademicka 12, 20-033 Lublin; e-mail: jadwiga.jaworska@up.lublin.pl