

LOCALISATION OF PARVALBUMIN AND CALBINDIN D28K IN THE PERIAQUEDUCTAL GRAY MATTER (PAG) OF THE CHINCHILLA

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Abstract

The purpose of this study was to trace the immunoreactivity of the two calcium binding proteins, parvalbumin and calbindin D28k, in the periaqueductal gray matter of the chinchilla midbrain. The immunoreactivity of these proteins in this species has never been investigated. The localisation of the activity was examined by carrying out the peroxidase-antiperoxidase (PAP) reaction using specific antibodies against parvalbumin and calbindin D28k. Slightly different parvalbumin immunoreactivity was shown. In most neurons, with the exception of large neurons in the dorsal and dorso-lateral periaqueductal gray matter, a weak immunostaining for parvalbumin was observed. Extremely intense immunostaining for calbindin D28k occurred in all neurons in the examined area. The results obtained suggest a slightly different distribution of parvalbumin in the neurons of the periaqueductal gray matter of the midbrain of the chinchilla than in other animal species. This indicates that mainly calbindin D28k is involved in the regulation of intracellular calcium ion concentration in the periaqueductal gray matter of the chinchilla.

Key words: chinchilla, periaqueductal gray matter, parvalbumin, calbindin D28k.

Parvalbumin (PV) and calbindin D28k (CB) are neuroanatomic markers belonging to a large family of EF-hand calcium binding proteins regulating the intracellular concentration of calcium (1, 7). The common feature of these structural proteins is the presence of calcium binding domains. The EF-hand domain is formed by two α -helixes and a loop built from 12 amino acids. The uptake of calcium ions to the domain leads to its binding, and conformational change of the protein. Parvalbumin is classified as a calcium slow buffer, while calbindin D28k is a fast buffer calcium ion (20). Currently, there is an increasing interest in these proteins, since they are the most widespread, and specifically localised in some populations of neurons of the mammal central nervous system (CNS), and their functions have not yet been fully explained.

Previous studies have helped to determine the distribution of calcium binding proteins in neurons of many brain areas. In addition, it has been demonstrated that these proteins occur in conjunction with various neurotransmitters; hence it is believed that they are involved in the neuronal plasticity, synthesis, and release of neurotransmitters, as well as synaptic transduction (6). To date, examinations of calcium binding proteins in the periaqueductal gray matter (PAG) have been

carried out in rats and macaques (15, 17, 18). The morphology of PAG is well known in humans and many species of animals (5, 10, 11, 13). This area contains various neurotransmitters and is connected with many other centres of the CNS. Its functions are still the subject of research (2, 3).

PAG is known to be involved in changes in pain, emotional, and defensive reactions (including aggression), remembrance, micturation, sexual behaviour, and vocalisation (4, 12, 14, 19, 22).

Due to the lack of research on the distribution of the calcium binding proteins PV and CB in the brain of chinchilla, it was decided to investigate their immunoreactivity in the adult periaqueductal gray matter.

Material and Methods

The brains of 10 sexually mature (about 1.5 years old) male chinchillas originating from the "Raba" farm in Myślenice were used. The brains were prepared immediately after slaughter. The material was fixed in a fresh 10% buffered formalin for 12 h at 4° C. After fixation, the 6 μ m-thick frontal slices of midbrains were prepared using the routine paraffin technique. Slices

containing the periaqueductal gray matter were placed on basic slides, and kept for 30 min at 56° C. After deparaffinisation and hydration, the slices were incubated in 0.4% H₂O₂ in the phosphate buffer at room temperature for 30 min in order to inhibit endogenous peroxidase activity, and then washed in fresh 0.5 M Tris buffer (TBS-Tris Buffered Saline) (pH of 7.6). In order to remove the staining of the background, the slices were treated with normal goat serum at room temperature for 20 min.

To determine the expression of the two selected calcium binding proteins, slices from five animals for each protein were used. The peroxidase-antiperoxidase (PAP) reaction was carried out using specific antibodies against parvalbumin and calbindin D-28k (21). For immunostaining, a set of antibodies and reagents dissolved in 0.5M TBS was used. Likewise, the same buffer solution was used for washing the preparations after the application of each antibody.

The slices were treated with relevant monoclonal immunoglobulins (IgG), and incubated in the monoclonal PAP complex developed for the appropriate species of animals. DAB (3,3'-diaminobenzidine tetrahydrochloride, Aldrich) was used as a chromogen for all proteins. The incubation with DAB was conducted at room temperature for 30 min. The insoluble brown reaction product of different intensity was received. The slices were then washed in distilled water, dehydrated, overexposed, and closed in DPX (Fluka).

For the immunocytochemical techniques, specificity control was performed, where the first antibody against antigens was omitted or replaced by a normal serum.

The parvalbumin and calbindin D-28k immunopositive, dorsal, dorso-lateral, and ventro-lateral PAG neurons were observed and photographed under an Axiolab (Zeiss) light microscope.

Results

The positive reaction to parvalbumin was observed in the dorsal, dorso-lateral, and ventro-lateral PAG, in the surrounding aqueduct of the midbrain, and in small and medium-sized round, oval, pyramidal neurons. Almost all the cells in the examined area were characterised by a relatively weak immunoreactivity for parvalbumin. In the dorsal and dorso-lateral PAG, large oval or star-shaped neurons showed intensive cytoplasmatic and nuclear PV immunostaining in the form of brown granulation. Some large stellar immunostained neurons in the primary processes departed from the body cell (Figs 1, 2, 3).

Positive immunocytochemical reaction for calbindin D28k was observed in parts of the dorsal, dorso-lateral and ventro-lateral PAG, and in oval, round, and pyramidal neurons. All these cells showed very intensive immunoreactivity for the protein in the form of dark brown granules spaced evenly throughout the neuroplasm surrounding the cellular nucleus.

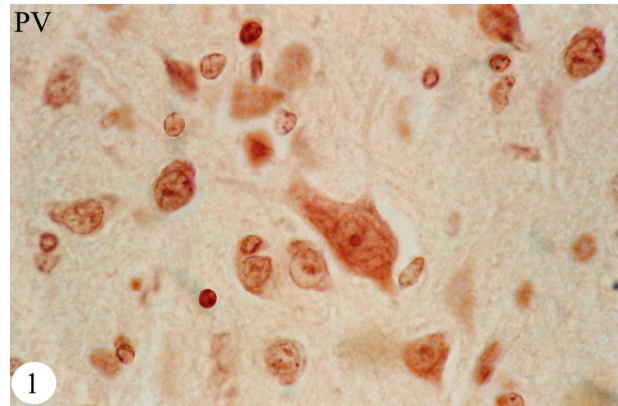


Fig. 1. Dorsal PAG, immunoreactivity of PV.
1,000x.

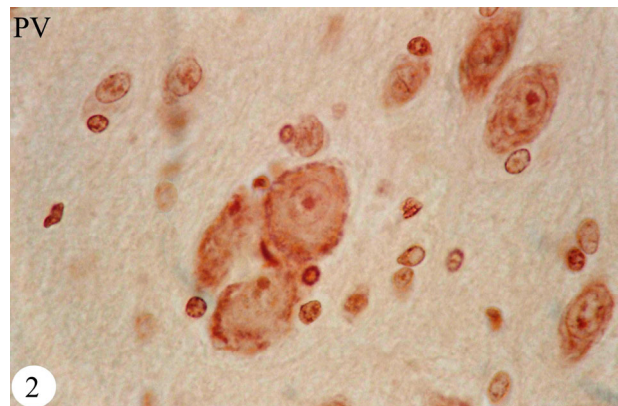


Fig. 2. Dorso-lateral PAG, immunoreactivity of PV.
1,000x.

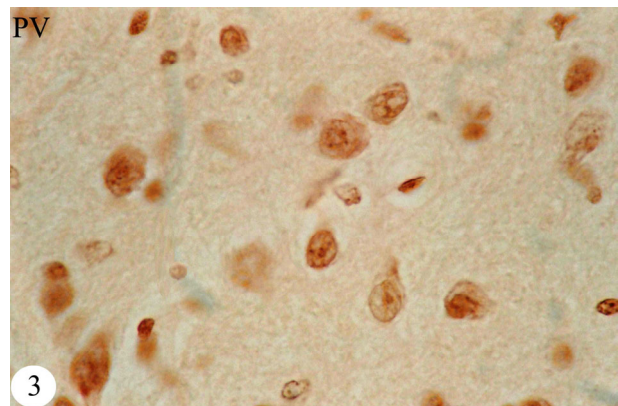


Fig. 3. Ventro-lateral PAG, immunoreactivity of PV.
1,000x.

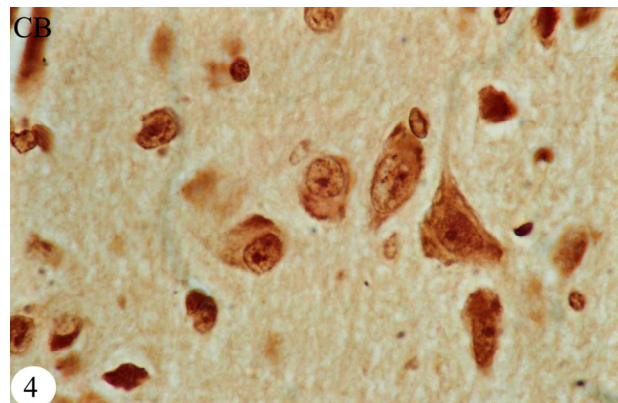


Fig. 4. Dorsal PAG, immunoreactivity of CB.
1,000x.

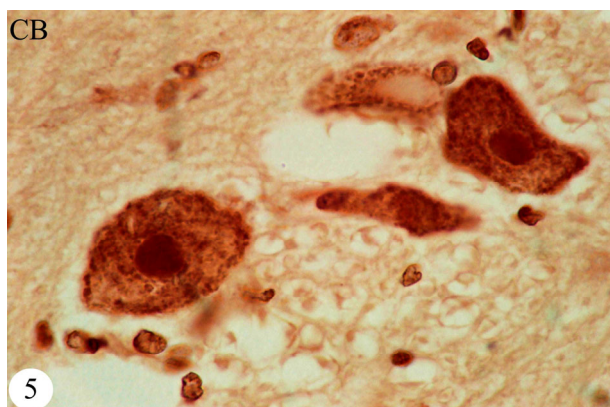


Fig. 5. Dorso-lateral PAG, immunoreactivity of CB.
1,000x

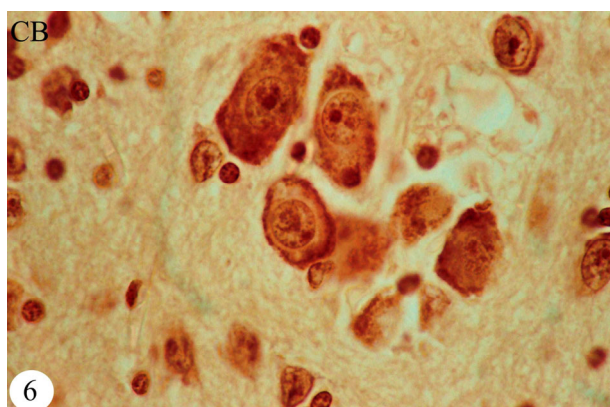


Fig. 6. Ventro-lateral PAG, immunoreactivity of CB.
1,000x.

Moreover, dark-brown immunostaining for the protein was observed also in the cellular nuclei (Figs 4, 5, 6).

No immunoreactivity for PV and CB was observed in the control slices.

Discussion

PV and CB belong to a large family of EF-hand proteins covering more than 200 members, which are located specifically in certain subpopulations of neurons in many mammal brain areas. Calcium-binding proteins (PV and CB) are involved in maintaining the homeostasis of calcium ions inside their cells, preventing excessive concentration and thus protecting neurons against their toxic effects. They play a regulatory function affecting the changes in the activity of neurons by controlling the concentration of calcium in the cell. Moreover they participate in the release of neurotransmitters and synaptic conductivity (20).

Our study has shown weak PV immunostaining in most small and medium-sized PAG neurons in the male chinchilla. In the rat, this protein was not observed in this area of the brain (8, 15). Intensive PV immunoreactivity in large neurons in dorsal and dorso-lateral PAG in the chinchilla is similar to that described in the macaque (18). Slightly different results obtained in our study compared to those carried out in rats and

macaques may suggest intraspecies differences. Intensive PV immunoreactivity in large PAG neurons may be a witness to their neurotransmitting nature, because the protein locates itself very specifically in neurons containing γ -aminobutyric acid (GABA). The PAG in the cat revealed about 36% of GABA-ergic neurons, but no studies were carried out for the presence of PV. According to other authors, this protein is involved in intracellular calcium buffering by modulating the excitability and activity of GABA-ergic neurons (1, 3, 7).

Our findings revealed very intense immunoreactivity of CB neurons in all parts of the PAG in the male chinchilla, similar to that described by other authors in rats and macaques. The protein is mainly located in the non-GABA-ergic neurons and may affect the synaptic activity, as it is involved in the release of neurotransmitters from synaptic terminals. Calbindin D28k, as a buffer protein found in many areas of the brain, was assigned to modulate the transitional cytoplasmic concentration of calcium ions (8, 15, 18). PV and CB may colocalise in neurons in certain CNS areas. Neurons containing both proteins are GABA-ergic, and also contain other neurotransmitters and neuromodulators (16). It remains to be determined whether the coexistence of PV and CB with neuropeptides is a general feature of the subpopulation of neurons. Our study has shown that the intracellular location of both PV and CB is in the neuroplasm, as well as in cellular nuclei, which is consistent with previous studies, demonstrating that CB can enter the nucleus through passive diffusion. It is known that calcium signals are transmitted to the nucleus where they regulate gene expression and affect the proper functioning of neurons (9).

PAG is particularly noteworthy, despite the fact that its structure is well known in many species of mammals (5, 10, 11, 13). PAG neurons contain various neurotransmitters, *e.g.* noradrenaline, glutamate, acetylcholine, histamine, serotonin, GABA, and neuropeptides, *e.g.* substance P, neurotensin, enkephalins, dynorphins (2, 3). This area connects the ascending and descending pathways, among others, with the cerebral cortex, thalamus, hypothalamus, basal forebrain, brain stem nuclei, and spinal cord, and affects their functions. The presence of calcium binding proteins in PAG neurons indicates that they are involved in the modulation activity of neurons, which affect the functions of PAG, *e.g.* the maternal behaviour and lordosis, vocalisation, changes in pain (analgnesia), emotional and defensive reactions, remembrance, and sexual behaviour (4, 12, 14, 19, 22).

The difference in the distribution of PV and CB in PAG neurons indicates the differences in their properties to carry out functional capabilities (1). PV is detected in "quick-firing" neurons, which suggests that this protein may regulate the calcium activated potassium channel involved in communication between neurons. CB reduces the concentration of free calcium in the cytoplasm, and changes the voltage-dependent calcium channel (8). The physiological functions of calcium binding proteins have not yet been fully

explained. Further studies are necessary to determine the nature of neurotransmitters and their common occurrence with PV and CB in the PAG neurons in the chinchilla.

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