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Calbindin Immunoreactivity in Rat Cerebral Cortex and Cerebellum Neurons

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Objective. To carry out an immunohistochemical assessment of the distribution of calbindin D28K in cerebral cortex (parietal and frontal) and cerebellum neurons in rats. **Materials and methods.** Experiments were performed on 18 mongrel white male rats weighing 200–250 g. Fragments of the frontal and parietal cortex and cerebellar cortex were collected. Paraffin sections were processed using primary polyclonal antibodies to detect calbindin D28K immunoreactivity. **Results.** In layer II of the frontal and parietal cortex, most neurons were moderately immunopositive, while layers III, V, and VI contained occasional neurons with high calbindin immunoreactivity. Individual nerve fibers, mainly the dendrites of pyramidal neurons, were clearly seen. Some of these showed a distinctive staining pattern: immunopositive parts alternated with immunonegative parts. In the cerebellar cortex, the bodies and dendritic branches of Purkinje cells were well stained. A significant proportion of neurons in the granular layer were moderately immunopositive, and included some afferent nerve fibers running from the white matter. **Conclusions.** Calbindin immunoreactivity in cerebral cortex structures in normal rats varied significantly and could be related both to the type of neuron and their functional state.

Keywords: calbindin, cerebral cortex, cerebellar cortex, rats.

Calbindin is needed for the activity of all cells, especially excitable cells, and is a universal intracellular regulator. Intra- and extracellular signals induce transient local increases in the calcium concentration, while various stresses or critical states significantly increase its concentration in the cytoplasm, which can lead to cell death. Calbindin protein is an important component of intracellular calcium homeostasis [9]. Its main function is to bind and store calcium. Endogenous calcium ion buffers such as parvalbumin and calbindin are modulators of neurotransmission [1]. Calbindin expression is used as a specific marker for identifying Purkinje cells in the cerebellum [6]. Overexpression of calbindin D28K protein in cerebellar cortex Purkinje cells may play a role in neuroprotection [7]. However, the distribution pattern of this protein in the cerebral cortex and cerebellum in rats in normal conditions has received insufficient study [3].

The aim of the present work was to carry out an immunohistochemical assessment of the distribution calbindin D28K in rat cerebral cortex (parietal and frontal lobes) and cerebellum neurons.

Materials and Methods. The study used materials from 18 mongrel white male rats weighing 200–250 g. Animals were kept in standard animal-house conditions. All experiments were conducted in compliance with the "Regulations for Studies Using Experimental Animals." The procedures were approved by the Ethics Committee of Grodno State Medical University (Protocol No. 1 of January 11, 2017).

In the morning hours (for time synchronization), after induction of anesthesia with ether vapor, animals were harvested and brains were extracted. Studies were run using fragments of the frontal (from –4.2 to 1.8 mm) and parietal (from –6.84 to –8.52 mm) lobes of the cerebral cortex and the cerebellar cortex (from –10.08 to –12.72 mm). The bregma was the null point for the stereotaxic coordinates in the anteropos-

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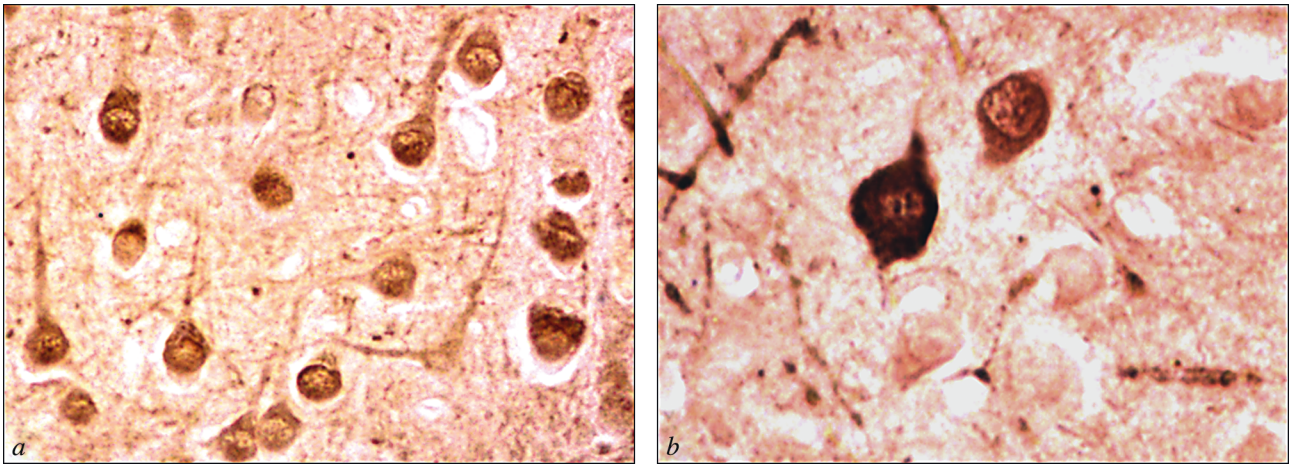

Fig. 1. Calbindin immunoreactivity in the rat frontal cortex. *a*) Outer granular layer; *b*) inner pyramidal layer. Immunohistochemical reaction for calbindin. Digital photomicrograph. Magnification ×400.

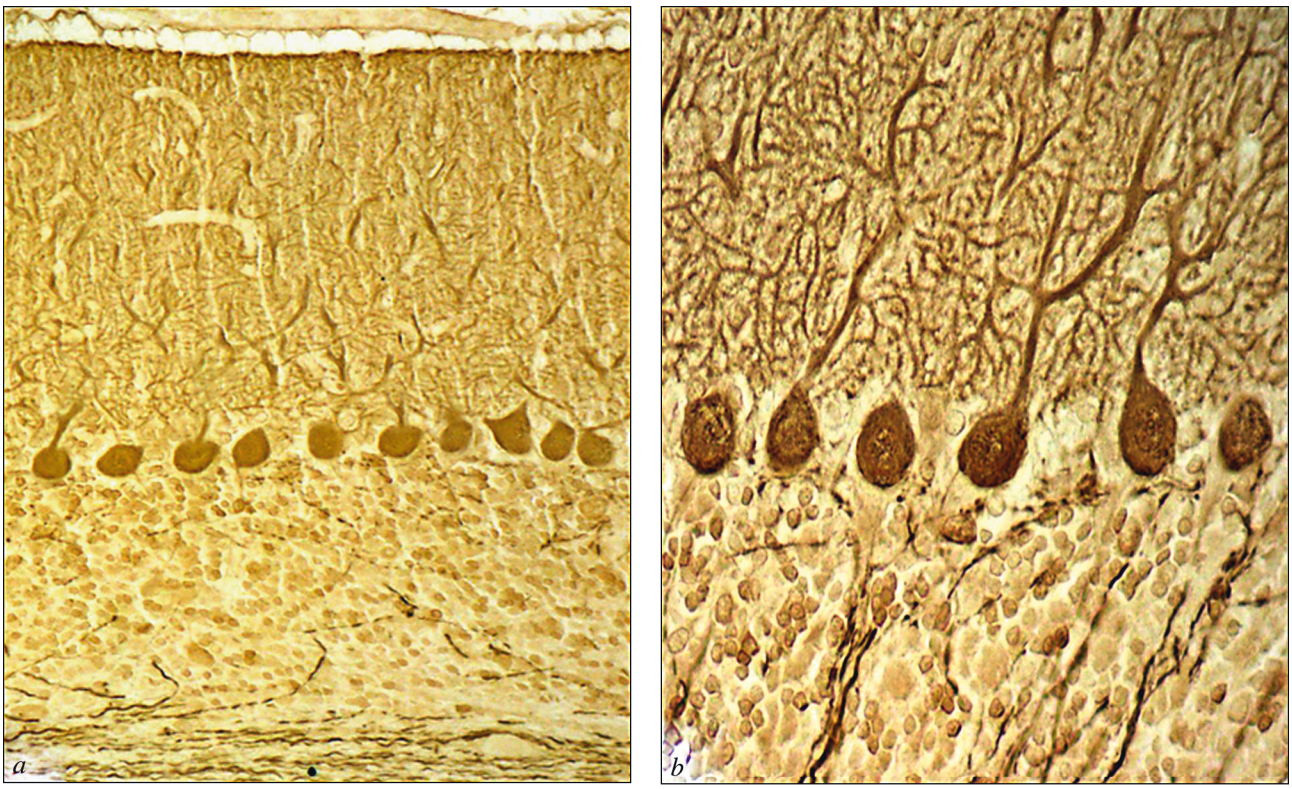

Fig. 2. Calbindin express in the rat cerebellar cortex. Immunohistochemical reaction for calbindin. Digital photomicrograph. Magnification *a*) ×100, *b*) ×200.

terior direction, frontal planes behind the bregma being designated with negative numbers [8]. Samples of cortex from all animals were processed in parallel in identical conditions. Fixation was in zinc-ethanol-formaldehyde at +4°C overnight and was followed by embedding in paraffin. Standard paraffin sections of thickness 7 μm were cut on a microtome (Leica RM 2125 RTS, Germany) and mounted on slides.

Post-fixing antigen demasking was not used. Immunohistochemical detection of calbindin D28K was with primary polyclonal rabbit antibodies (Abcam, UK, ab. 11426) diluted 1:1200 at +4°C, exposure for 20 h in a humid chamber. Bound primary antibodies were detected using an EXPOSE Mouse and rabbit specific HRP/DAB detection IHC kit (Abcam, UK, ab. 80437 for rabbit antibodies).

The negative control consisted of samples processed using normal rabbit serum instead of primary antibodies (no immunopositive staining produced). The internal negative control consisted of meninges (immunopositive staining should not occur with this tissue) and the positive control consisted of cerebellar Purkinje cells, known for their high calbindin immunoreactivity.

Studies of immunohistochemical preparations, microphotography, and cytophotometry were performed at different magnifications using an Axioskop 2 Plus microscope (Zeiss, Germany), with a Leica DFC 320 digital video camera (Leica Microsystems, Germany) and Image Warp image analysis software (Bit Flow, USA).

Results were processed using nonparametric statistical tests in Statistica 8.0 for Windows. Differences between the experimental and control groups were regarded as significant at $p < 0.05$ (Mann–Whitney U test).

Results. *Frontal and parietal cortex*. In layer II of the cerebral cortex, most neurons were moderately immunopositive for calbindin. Immunohistochemical reaction products in the form of dark brown small and minute granules were distributed throughout the whole of the perikaryon and visible parts of processes, mainly dendrites (Fig. 1).

Layers III, V, and VI of the cortex showed occasional neurons with high immunoreactivity, along with neurons with moderate and low immunoreactivity, though most of the neurons in these layers were immunonegative. It is interesting to note that neurons with high calbindin immunoreactivity in these layers were, in terms of quantity (0.3–0.5% of all neurons), shape, and distribution, very reminiscent of hyperchromic neurons detected by Nissl staining in normal animals. In some neurons, staining of the perinuclear area was accompanied by staining of the nuclei themselves, the pattern being reminiscent of the distribution of nuclear chromatin (see Fig. 1). Along with neuron bodies, vertically distributed individual nerve fibers were also detected, which were mainly the dendrites of pyramidal neurons. Some of these showed distinctive staining: areas with intense staining alternated with immunonegative areas.

Cerebellum. Immunohistochemical staining for calbindin expression in the cerebellar cortex demonstrated the absence of staining of the meninges and intense immunopositive staining of the bodies and dendrites of Purkinje cells. The molecular layer showed not only large, but also multiple small branches of Purkinje cell dendrites, and their axons remained unstained (Fig. 2). It is interesting to note that all neuron bodies in the molecular layer were immunonegative, while a significant proportion of neurons in the granular layer were moderately immunopositive. In addition, the granular layer of the cerebellum showed very clear staining of several afferent nerve fibers running from the white matter. As these did not run in the Purkinje cell layer or the molecular layer, they could be identified as mossy fibers (see Fig. 2).

Nonidentical immunoreactivity of calbindin in Purkinje cells on the surface and in the depths of the cerebellar gyri should be noted: staining of these cells closer to the surface was less marked than in the intermediate parts, while the strongest staining was seen in the deep parts of the gyri and the flexure itself. Thus, while the mean optical density in the perikarya of Purkinje cells was 0.298 ± 0.006 U, cells closer to the meninges stained 10.8% more weakly, while those in the deep parts of the gyrus stained 9.4% more strongly.

Discussion. These experiments demonstrated the absence of calbindin immunoreactivity in the meninges and glial cells. In the superficial layers of the cerebral cortex and cerebellar gyri, immunoreactivity was greater or the number of stained neurons was larger. This feature has also been noted by other authors, who have demonstrated that calbindin expression in neurons in the deep layers of the cerebral cortex in guinea pigs was greater than in the superficial layers [5]. This may be linked with the rate of penetration of fixative into brain fragments and, thus, the duration of exposure, such that it is an artifact. However, it is also possible that there are different quantities of calbindin in different types of neurons in the superficial and deep layers. The staining of nuclear structures in a manner reminiscent of chromatin and the area of the nuclear envelope also raises questions. However, this is not an artefact, as the presence of calbindin D28K in the nuclei of different types of brain neurons, including cerebellar Purkinje cells, has been demonstrated in a specific study [4]. This is due to the ability of calcium to penetrate into nuclei, where it can regulate gene expression, and the presence of calbindin in the nucleus is important for cell functioning.

Some nerve fibers in the cerebral cortex, more rarely in the granular layer of the cerebellar cortex, stained in a distinctive way: parts with intense staining alternated with immunonegative parts. Judging from their locations, these were neuron axons running into the cortex. The uneven distribution of calbindin within these axons may be associated with the characteristics of nerve impulse conduction along them. The dendrites of cerebellar Purkinje cells were always stained uniformly.

Overall, it can be suggested that calbindin immunoreactivity is linked with the characteristics of calcium metabolism, excitability, or some other functional feature of neurons. This is supported by the fact that fewer than 1% of neurons in the deep layers of the cerebral cortex were immunopositive. Some types of neurons were not seen at all. Identical neighboring neurons had significantly different calbindin contents: from sharply immunopositive to immunonegative. The sizes, shapes, and spatial organization of neurons with high calbindin immunoreactivity in the deep layers of the cerebral cortex coincided with the sizes of hyperchromic neurons seen using the Nissl method. Only a small proportion of nerve fibers was immunopositive; these often showed distinctive immunostaining (regions with intense staining alternating with immunonegative regions). However, the link between calbindin immunoreactivity and the functional state of neurons requires further investigation.

We can expect calbindin immunoreactivity in cerebral neurons to change significantly in response to experimental treatments and pathological states. Thus, the action of propofol (an anesthetic) during the development of the brain in mice leads to a decrease in the number of calbindin-immunopositive Purkinje cells and a decrease in the length of their dendrites in the molecular layer [10]. In degenerating Purkinje cells with signs of apoptosis, caspase-3 expression is comparable with calbindin expression [2].

Thus, calbindin immunoreactivity in cerebral and cerebellar cortex neurons in normal rats varies significantly and may be linked both the cell type and the functional state of cells.

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