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Nucleolin and Nucleoli in Ependymocytes and Tanycytes of the Third Ventricle of the Rat Brain

D. A. Sufieva, O. V. Kirik, and D. E. Korzhevskii*

Institute of Experimental Medicine, St. Petersburg, 197376, Russia

**e-mail: dek2@yandex.ru*

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Abstract—The aim of the present study was to compare structure of the nucleoli of ependymocytes, tanycytes, and secretory cells of the subcommissural organ using immunohistochemical staining for nucleolin and confocal laser microscopy. The study was performed in samples from the diencephalon of adult male Wistar rats ($n = 6$). The samples were fixed in zinc–ethanol–formaldehyde, a fixative providing a high level of preservation of antigen determinants. In the present study, we estimated diameters of nucleoli and their number in various types of cells lining the third ventricle. We compared for the first time the nucleoli of different subpopulations of tanycytes and report data on the distribution of nucleolin protein in the cells lining the ventricles. The content and location of nucleolin reflect the functional state of the cell. Our data will promote understanding of the interrelationships between the indices of the nucleolar apparatus and the functional state of the cell under various conditions, including stress, neoplastic transformation, and other pathological conditions.

Keywords: nucleolus, nucleolin, ependymocytes, tanycytes, subcommissural organ, immunohistochemistry, confocal laser microscopy

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INTRODUCTION

It is presently known that various regions of lining of the third ventricle are heterogenous. For example, the lining of the circumventricular organs (CVOs), i.e., structures with a fenestrated capillary and without a blood–brain barrier, is formed by highly specified glial cells such as tanycytes of the median eminence, tanycytelike cells of the subfornical organ, the organum vasculosum laminae terminalis, and the area postrema (Langlet et al., 2013) or epithelial cells of the choroid plexus (Korzhevskii, 2003; Joly et al., 2007). The subcommissural organ (SCO) is an exception to this rule, because it does not contain fenestrated capillary and the lining of this area is formed by specific secretory ependymal cells (Guerra et al., 2015).

In the median region of the third ventricle, the structures of the CVO are located, such as the median eminence and the SCO. The ventricle lining this region is formed by tanycytes. In contrast to ciliated ependymocytes, tanycytes have a long basal process forming a contact with capillary of the adjacent nervous tissue. Tanycytes of this region form four subpopulations, α1–, α2–, β1–, and β2-tanycytes, which have different locations within the infundibular recess and some other structural, cytochemical, and functional features (Rodriguez et al., 2010).

The other CVO, located in the lining of median region of the third ventricle is the SCO. It is known that the SCO consist of two layers—ependymal and hypendymal. The cells forming these two layers release various substances, including SCO-spondin, transthyretin, and fibroblast growth factor, into blood or cerebrospinal fluid (CSF). Despite multiple studies that have been carried out on structural and cytochemical features of cells that form the CVO, this organ remains less examined (Kaur and Ling, 2017).

Due to their functional features, ependymocytes, tanycytes, and secretory cells of the SCO have a high level of protein synthesis. The level of protein synthesis correlates with organization of the nucleolar apparatus, which are mainly involved in biogenesis of ribosomes. It is known that the size of the nucleolus reflects its functional activity, whereas the number of nucleoli is related to the rate of cellular differentiation (Zharskaya and Zatsepina, 2007; Watanabe-Susaki et al., 2014). The issue of the organization of the nucleolar apparatus of these types of cells remains unstudied. Nucleolin is the main argyrophilous (Ag-NOR) proteins of the nucleolus and this allows to use this protein as a marker of nucleoli. This protein is mostly located in the dense fibrillary component and,

Abbreviations: SCO—subcommissural organ, CSF—cerebrospinal fluid, CVO—circumventricular organs.

to a lesser extent, in the granular component of the nucleolus; this protein may be also observed in the nucleoplasm, cytoplasm, and plasma membrane of various cells (Zenit-Zhuravleva, 2012). It is known that, in different cellular compartments, nucleolin plays various functions. Data in the literature show that nucleolar nucleolin is involved in biogenesis of ribosomes, cytoplasmic nucleolin participates in nuclear-cytoplasmic transport of various proteins and ribosomal subunits, and the nucleolin molecules of plasma membrane are capable to bind with various ligands and to function as mediators of extracellular signals (Tajrishi, 2011). It is considered that nucleolin is one of key factors regulating cell proliferation providing a high level of expression of this protein in actively proliferating cells, including the tumor cells (Chen and Xu, 2016). A comparative study of the nucleolar apparatus in tanycytes, secretory cells of the SCO, and classical ciliated ependymocytes can shed light on specific organization and functioning of these cells.

The aim of the present study is a comparative examination of the nucleolar apparatus of ependymocytes, tanycytes, and secretory cells of the SCO using immunohistochemical staining for nucleolin and confocal laser microscopy.

MATERIAL AND METHODS

The study was performed in samples from diencephalon of adult male Wistar rats ($n = 6$). The animals were maintained and sacrificed in accordance with the Declaration of Helsinki on humanity in experiments with animals and the Rules on Studies Using Experimental Animals of the Ministry of Healthcare of the Soviet Union, order no. 755, August 12, 1977. The brain was fixed in zinc–ethanol–formaldehyde fixative, dehydrated, and paraffinized. The frontal 5-μm sections were cut at the level from –3.24 and –4.44 mm relative to bregma (Paxinos and Watson, 2007). After deparaffination, the sections were subjected to thermal antigen retrieval. Then, double immunohistochemical staining was performed using antivimentin mouse monoclonal antibody (clone V-9, Dako, Denmark) diluted 1 : 100 and antinucleolin rabbit polyclonal antibody (Abcam, United Kingdom) diluted 1 : 200. Application of vimentin staining in this combination allowed clear identification of ependymal cells and tanycytes (Kirik and Korzhevskii, 2013; Sufieva et al., 2016). The secondary antibodies were donkey monovalent Fab-fragment of antirabbit immunoglobulin conjugated with the fluorochrome Rhodamine Red™-X (RRX) diluted 1 : 50 (Jackson ImmunoResearch, United States) and donkey monovalent Fab-fragment of antimouse immunoglobulin conjugated with the fluorochrome Cy2 diluted 1 : 50 (Jackson ImmunoReaserch, United States). The stained sections were coverslipped with water-soluble Fluorescence Mounting Medium (Dako, Denmark). The sections were examined using a Leica DM2500 fluorescent microscope and a confocal laser LSM 710 microscope (Zeiss, Germany). Analysis of aquired images and three-dimensional reconstruction of objects were performed using LSM Image Browser and Zen-2012 computer software (Zeiss, Germany). Quantitative analysis was performed in three animals. Measurement of the diameters of nucleoli was performed using ImageJ software in at least ten randomly chosen cells of each type. The nucleoli were counted in the nuclei of at least ten cells of each type if the nuclei completely appeared within the section.

RESULTS

Double immunohistochemical staining for vimentin and C23 protein revealed that the lining of the third ventricle is formed by a monolayer of cubical cells. The nuclei of ependymocytes were of oval or round form. The number of nucleoli was from one to four; in 80% of cells, there were two to three nucleoli, whereas in 20% of cells there was one nucleolus or four. The average diameter of nucleoli was 1.6 ± 0.1 μm (Fig. 1). In addition to nucleoli, positive immunohistochemical nucleolin staining was also observed in the nucleoplasm of ependymocytes. However, in contrast to the highly intense staining observed in the nucleoli, the protein was irregularly distributed in the nucleoplasm, where it appeared as small aggregates with unclear borders and located along the nuclear periphery.

The lining of the floor of the third ventricle in the region of the arcuate nucleus of the hypothalamus was formed by tanycytes, which could be divided into four subpopulations. α1-tanycytes were located in the most dorsal region. In the region where ependymocytes were replaced with α1-tanycytes, the structure of the lining became multilayered, after which it was again single-layered. The lateral walls of the floor of the third ventricle were covered by α2-tanycytes, which sent their processes to the arcuate nucleus of the hypothalamus. β1-tanycytes formed the lining of the infundibular recess, and β2-tanycytes formed the lining of the median eminence. The lining formed by β1-tanycytes also had a multilayered structure. The bodies of tanycytes were elongated and had the only process that started from the basal pole and could form branches. The processes formed contacts with blood capillaries of the surrounding nervous tissue. The nuclei of tanycytes were elongated in form and characterized by the presence of invaginations and protrusions of the nuclear membrane (Fig. 2). After immunohistochemical staining for nucleolin, we observed that the number of nucleoli and their sizes varied in different subpopulations of tanycytes. Thus, α1-tanycytes had from one to four nucleoli; one to three nucleoli were observed in 97% of cells, whereas four nucleoli were revealed in 3% of cells. The average diameter of this subcompartment was 1.7 ± 0.1 μm (see Fig. 1). In α2-tanycytes, we found one or two nucleoli in 95% of

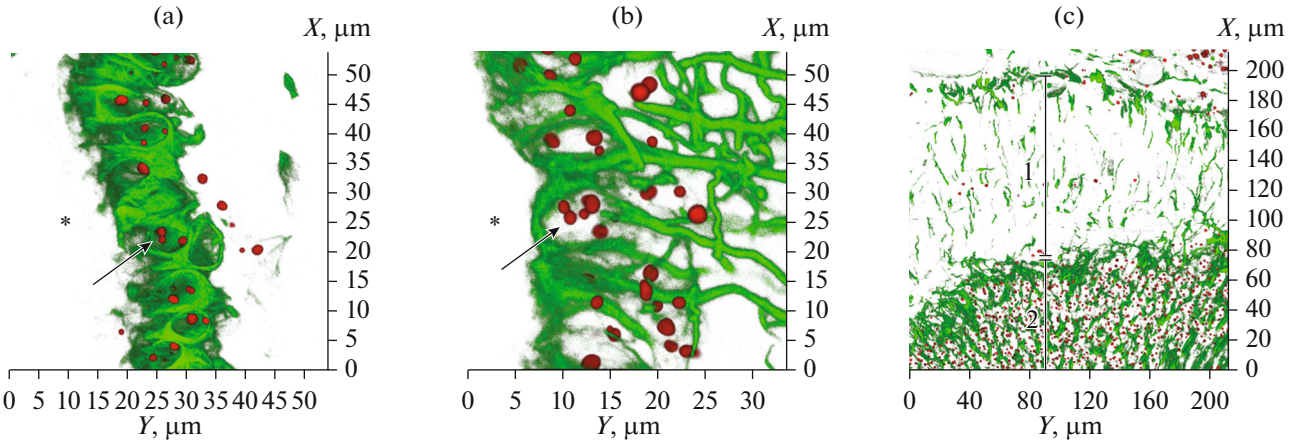

Fig. 1. Lining of the third ventricle of the rat brain. 3D reconstruction of cellular elements. Double immunohistochemical staining for vimentin and nucleolin (visualization using fluorochromes Cy2, green color, and RRX, red color, respectively). Confocal laser microscopy. (a) Ependymocytes of the third ventricle, (b) α1-tanycytes of the third ventricle floor, and (c) (1) hypendymal and (2) ependymal layers of the SCO. Step size of Z-stacks: (a) 10 and (b, c) 5 μm. Number of optical sections: (a) 34, (b) 16, and (c) 14. Lenses: (a, b) C-Apochromat 63×/1.20 W Korr M27 lens; (c) LD C-Apochromat 40×/1.1 W Korr M27 lens. Reconstruction was performed using a ZEN 2012 software module (Zeiss, Germany). Asterisk, cavity of the third ventricle; arrows, nucleoli.

cells, and only in 5% of cells did we observe three nucleoli. The mean diameter of the nucleoli in this subpopulation of tanycytes was 2.2 ± 0.1 μm (Fig. 3). As a rule, β1-tanycytes had one or two relatively large nucleoli in 60 and 40% of cells, respectively, with an average diameter of 2.4 ± 0.1 μm (see Fig. 3). Similarly, most of the β2-tanycytes, 63%, had one relatively large nucleolus, while more rarely, in 37% of cells, two nucleoli were observed. The mean diameter of the nucleoli in this subpopulation of tanycytes was 2.2 ± 0.1 μm (see Fig. 3). As in ependymocytes, staining was observed in nucleoplasm of tanycytes; the protein was irregularly distributed and formed aggregates and was also located in periphery of the nucleus (Fig. 2).

The ependymal layer of the SCO was a mass of cells that formed a multilayered structure. At the same time, the cell bodies of the ependymal layer were not located in the cavity of the ventricle, but rather in the deep structure, and their apical parts were in contact with the CSF (Fig. 3). The hypependymal layer, which was located more deeply inside, consisted of a small number of cells that were distributed in the rat SCO irregularly. In the hypependymal layer, we also observed separate parallel processes of the secretory bipolar cells of the ependymal layer that formed contacts with the surrounding capillaries of the nervous tissue and blood vessels of the meninges (Fig. 1). The nuclei of secretory cells of both ependymal and hypependymal layers were of oval shape. Immunohistochemical nucleolin staining of the ependymal layer cells revealed two or three nucleoli in 75% of cells and, rarely, one or four nucleoli in 25% of cells; the average diameter was 1.4 ± 0.1 μm. The nuclei of 80% of cells of the hypependymal layer contained one nucleolus; in a few cases, i.e., 20% of cells, two nucleoli were observed. The mean diameter of the nucleoli was 1.6 ± 0.1 μm. The nucleoplasm of cells was weakly stained for nucleolin. In cells of the ependymal layer, the protein was distributed relatively regularly, while nucleo-

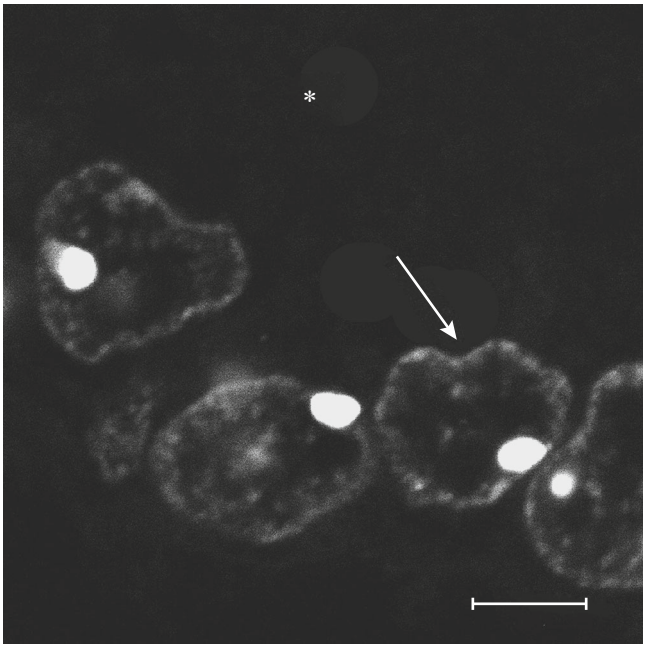

Fig. 2. Distribution of nucleolin in tanycytes. Immunohistochemical nucleolin staining visualized using the RRX fluorochrome. Confocal laser microscopy. Single optical section. Plan-Apochromat 100×/1.40 Oil DIC M27 oil-immersion lens. Scale bar, 5 μm. Asterisk, cavity of the third ventricle; arrow, invagination of tanycyte nuclear membrane.

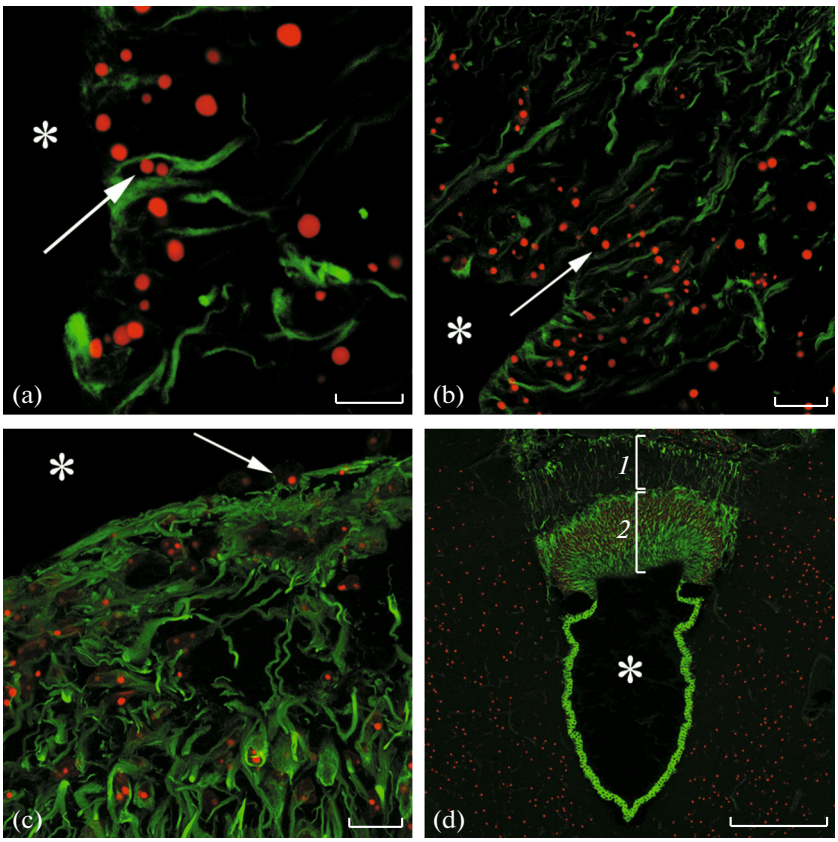

Fig. 3. Tanycytes and secretory cells of the subcommissural organ. Double immunohistochemical staining for vimentin and nucleolin (green and red colors, respectively). Confocal laser microscopy. (a) α2-tanycytes, (b) β1-tanycytes, and (c) β2-tanycytes. Images show a superposition of ten optical sections aquired with an interval of 0.3 μm. C-Apochromat 63×/1.20 W Korr M27 lens with zoom (a) 2.5 and (b, c) 1.0. (d) Subcommissural organ. Single optical section. EC Plan-Neofluar 10×/0.30 M27 lens. Scale bars: (a) 10, (b, c) 20, and (d) 200 μm. Asterisk, cavity of the third ventricle; arrows, nucleoli; (1) hypendymal and (2) ependymal layers of the subcommissural organ.

lin was predominantly located at the periphery of the nucleus in cells of the hypependymal layer. Quantitative indices of nucleoli in the studied cell types are summarized in the Table 1.

DISCUSSION

The nucleolus is an evolutionary conservative domain of the cellular nucleus, which is present in most eukaryotic cells. This structure is involved in formation of ribosomal subunits and in the regulation of cell cycle, apoptosis, and aging (Németh and Längst, 2013). It is known at present that the sizes of nucleoli and their number vary in different cells of the same body depending on the synthetic activity of the cell and the level of its differentiation (Treré et al., 2004). It has been shown that, during cell differentiation, these subcompartments of the nucleus tend to merge into larger nucleoli. A positive correlation has been observed between the size of nucleolus and the synthetic activity of the cell (Wallace, 1963; Pena et al., 2001). These studies have usually been performed to trace the relationship between these indices and cancer diseases (Derenzini et al., 2009). Therefore, several questions concerning the mechanism of formation of different numbers of nucleoli and the relationship of the functional activity of cells with synthetic processes still remain open. In this regard, literature data often demonstrate the necessity of comparative analysis of these parameters for different types of cells in normal and pathological states (Farley et al., 2015). It is important that the nucleolar apparatus of the nervous tissue has become a subject of study only in the last decade due to accumulation of data on the role of nucleolar stress in the pathogenesis of neurodegenerative diseases (Hetman and Pietrzak, 2012; Parlato and Kreiner, 2013). However, those studies were focused on studying the role of the nucleolus in neurons, whereas glial cells remain practically undescribed. There are only a few studies of the nucleolar apparatus of glial cells, specifically astrocytes, related

Table 1. Quantitative indices of the nucleoli of ependymocytes, tanycytes, and secretory cells of the SCO

Cell type	Number of nucleoli per nucleus	Mean diameter of the largest nucleus, μm
Ependymocytes	1–4	1.6 ± 0.1
α1-tanycytes	1–4	1.7 ± 0.1
α2-tanycytes	1–3	2.2 ± 0.1
β1-tanycytes	1–2	2.4 ± 0.1
β2-tanycytes	1–2	2.2 ± 0.1
Ependymal layer of the SCO	1–4	1.4 ± 0.1
Hypendymal layer of the SCO	1–2	1.6 ± 0.1

to their involvement in the development of cancer and prion diseases, such as glioblastomas and Creutzfeldt–Jacob's disease (Lafarga et al., 1993; Holmberg Olausson et al., 2015). Thus, the study of morphological features of nucleolus in the nervous tissue and, in particular, in glia, is an important task of modern neuroscience. In the present study, we have studied the nucleolar apparatus in three different types of glial cells (ependymocytes, tanycytes, and secretory cells), which are involved in the formation of the lining of the middle part of the third cerebral ventricle of the rat under the normal conditions.

In this study, it has been shown that different types of investigated cells differ in size of the nucleoli and their number. In addition, there is variability in these parameters within the same cell type. Thus, in different subpopulations of tanycytes, the mean diameter of nucleoli varies from 1.7 to 2.4 μm and the number of nucleoli varies from one to two to one to four per cell. These differences probably reflect specificity of functioning of the cells studied. Despite the fact that tanycytes are a monomorphic group, their division into four subpopulations is due to the fact that different subpopulations of tanycytes interact with different structures of the mediobasal hypothalamus and, thus, perform different functions that is primarily reflected in the synthetic activity of these cells. Here, we show that there is a decrease in the number of nucleoli and an increase of their sizes from α1 cells located dorsally to the most ventrally located β2-tanycytes. This distribution may have several causes. First, according to the literature data, a subset of α1-tanycytes have gap junctions, in contrast to other types of tanycytes, and no types of tanycytes have cilia. The functional significance of gap junctions in a subset of α1-tanycytes remains unknown. It has been suggested that, unlike the other types of tanycytes, α1-tanycytes do not perform transport and secretory functions (Rodriguez et al., 2010). Therefore, the synthetic activity of these cells should be lower in comparison with other types of tanycytes. We have shown that this cell subpopulation is characterized by the smallest diameter of the nucleoli, which may be evidence in favor of the hypothesis of decreased activity of their biosynthetic machinery. Secondly, since there are data on the correlation between the degree of differentiation of cells and the number of nucleoli (Wallace, 1963), there is a possibility that the differences in the number and size of the nucleoli may be associated with the different proliferative capacities of specific subpopulations of tanycytes. It has been suggested that α2-tanycytes in the adult brain are neural stem cells that can give rise to both neurons and glia (Goodman and Hajihosseini, 2015). This may explain the presence of more numerous and smaller nucleoli in the nuclei of α-tanycytes with more extensive proliferative potential than β-tanycytes.

The SCO cells and ependymal and hypendymal layers also differ in the number and size of nucleoli. According to literature data, a SCO is a CVO of secretory type, cells of which release different substances into the CSF (cells of the ependymal layer) or into the blood and subarachnoid space (cells of the ependymal and hypendymal layers) (Grondona et al., 2012; Kaur and Ling, 2017). However, we have found that SCO cells have relatively small nucleoli, which indicates a low synthetic activity of these cells. There is evidence that the SCO, in addition to its secretory functions, also performs a receptor function. This organ has many receptors for various neuropeptides and neurotransmitters (Nurnberger and Schoniger, 2001). It is believed that such enriched and diverse innervation is required for regulation of secretory activity of SCO cells during development and in response to brain activity such as the sleep–wake cycle and hibernation. In human beings and bats, the SCO operates only in embryogenesis and undergoes regression after birth. However, in rats, this CVO functions throughout life (Miranda et al., 2001). The estimated low synthetic activity of cells in the CVO of adult rats, which is based on the diameters of the nucleoli, may be related to the fact that either the secretory function is not a priority for these cells or the secretory activity of the SCO is absent in the adult brain.

Ependymal cells have relatively small nuclei as compared to tanycytes, with their high synthetic activity. It is known that the main function of these cells is formation of the CSF-brain barrier, transport of various substances, for example, glucose, from the CSF to the nervous tissue, and detoxing of heavy metals (Del Bigio, 2010). Despite the fact that these cells can synthesize different substances, such as growth factors and protein S100, our data on the diameters of the nucleoli may indirectly indicate a low rate of synthetic processes in them.

In addition to in the nucleoli, nucleolin staining was observed in the nucleoplasm of all the studied types of cells, but not in the cytoplasm or in the region of the plasma membrane. This staining may be due to the fact that the content of nucleolin in the cytoplasm and in the plasma membrane is below the threshold level necessary for the methods of immunocytochemistry and confocal laser microscopy. However, these data may be related to the fact that, in the studied cells, nucleolin is mainly involved in nucleolar and nuclear processes and is not involved in nuclear-cytoplasmic transport and transmission of intercellular signals. In the nucleoplasm, the protein C23 was irregularly distributed in the form of aggregates with indistinct borders or was located in the periphery of the nucleus. It is known that nucleolin can function as a chaperone of histones, participating in remodeling of the chromatin structure (Tajrishi et al., 2011). From this point of view, the heterogeneity of the distribution of nucleolin may be explained by a local modification of transcriptional activity in some areas of chromatin of these cells.

The nucleolar apparatus is today one of the most intensely studied structures of the cell nucleus; however, in the literature, there are few studies devoted to the examination of the nucleolus in the cells lining brain ventricles, which were studied in detail only by electron microscopy. The present study consists in a comparative analysis of nucleoli with the use of modern immunohistochemical techniques and confocal laser microscopy, which permits high-accuracy determination of such parameters as the number of nucleoli and their diameters. In this study, we have shown that there is heterogeneity in the organization of the nucleolar apparatus of different types of cells forming the lining of the third ventricle. We compared for the first time the size and number of the nucleoli in different subpopulations of tanycytes, in addition to providing data on the distribution of the multifunctional protein nucleolin in the cells lining the ventricles. The location and content of nucleolin reflect the functional state of the cell. These data will promote the establishment of the interrelationship between the parameters of the nucleolar apparatus and functional state of the cell under different conditions, including stress, malignization, and other pathological conditions.

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