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IMMUNOHISTOCHEMICAL MARKERS FOR NEUROBIOLOGY

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In neurobiological studies, crucial is the selection of most appropriate and informative experimental methods, one of which is immunohistochemistry. This review briefly summarizes the experience of adaptation of immunohistochemical methods to nervous system studies accumulated over years it the Laboratory of Functional Morphology of the Central and Peripheral Nervous System (Institute of Experimental Medicine). The aim of this work was to determine the most effective and reliable immunomarkers for neurobiological studies. The article contains theoretical basis and practical recommendations for use of key cytospecific and functional markers used in studies of structural and functional organization of brain and spinal cord of mammalian animals and human. In particular, the results of immunohistochemical reactions to neural markers (NeuN, neurofilament proteins, alpha-tubulin, alpha-synuclein), neurotransmitter synthesizing enzymes (tyrosine hydroxylase, glutamate decarboxylase, choline acetyltransferase, NO synthase) and glial markers (GFAP, glutamine synthetase, Iba-1, vimentin) are demonstrated. The presented methodology is useful for experimental neurobiology and clinical morphological diagnostics.

Keywords: neurobiology; immunohistochemistry; neural markers; glial markers; neurotransmitter systems.

ИММУНОГИСТОХИМИЧЕСКИЕ МАРКЕРЫ ДЛЯ НЕЙРОБИОЛОГИИ

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При проведении нейробиологических исследований важным представляется подбор наиболее адекватных и информативных методов, одним из которых является иммуногистохимическое исследование. В статье кратко обобщен многолетний опыт работы сотрудников лаборатории функциональной морфологии центральной и периферической нервной системы ФГБНУ ИЭМ, связанный с адаптацией современных методов иммуногистохимии для изучения нервной системы. Цель работы состояла в определении наиболее эффективных и надежных иммуномаркеров, которые целесообразно использовать при проведении нейробиологических исследований. Статья содержит теоретические сведения о важнейших цитоспецифичных и функциональных нейробиологических маркерах, применяемых в исследованиях структурно-функциональной организации головного и спинного мозга млекопитающих животных и человека, а также практические рекомендации по их использованию. В частности, представлены результаты постановки иммуногистохимических реакций на нейральные маркеры (NeuN, белки нейрофиламентов, альфа-тубулин, альфа-синуклеин), ферменты синтеза нейромедиаторов (тирозингидроксилаза, глутаматдекарбоксилаза, холинацетилтрансфераза, NO-синтаза), глиальные маркеры (GFAP, глутаминсинтетаза, Iba-1, виментин). Описанная методология перспективна для использования в экспериментальной нейробиологии и клинико-морфологической диагностике.

Ключевые слова: нейробиология; иммуногистохимия; нейральные маркеры; глиальные маркеры; нейромедиаторные системы.

List of abbreviations

GABA — gamma-aminobutyric acid; IF — intermediate filaments; TH — tyrosine hydroxylase; CNS — central nervous system; GAD — glutamate decarboxylase; GFAP — glial fibrillary acidic protein; GS — glutamine synthetase; nNOS — neuronal form of NO synthase; NOS — NO synthase.

In neurobiological studies, the identification of cells that respond to experimental influences and the determination of their functional status has always been an important task. In recent years, scientists have had access to a significant range of immunohistochemical, molecular, and genetic techniques to address these problems. Unfortunately, immunohistochemistry methods, which are the most accessible to neuroscientists, do not always produce high-quality results, as the antibodies used to identify specific structural and functional markers may not be suitable for certain tasks or cannot be combined with secondary reagents. Immunohistochemical labeling failures during experimental neurobiological studies also occur due to attempts to use antibodies created for use on human materials to detect similar markers in laboratory animals. Such extrapolations are not always possible, even when considering the recommendations of reagent manufacturers.

Due to the great interest in the field of neurobiology for immunohistochemistry methods capable of labeling various types of neurons and glial cells, in addition to the identification of progenitor, activated, and dying cells, this study aimed to determine the most effective and reliable immunomarkers that can produce high-quality results during neurobiological studies.

In this study, archival material was used, consisting of brain, spinal cord, and sciatic nerve sections from Wistar rats ($n = 50$). The materials were fixed in zinc–ethanol–formaldehyde (ZEF). The samples were dehydrated and embedded in paraffin, according to the generally accepted technique. Sections prepared on a microtome were glued onto adhesive-coated slide plates and dewaxed. Some of the preparations were subjected to thermal unmasking, using modified S1700 citrate buffer (Agilent, USA). Immunohistochemical studies were performed using primary antibodies, which are presented in the table.

Reveal Polyvalent horseradish peroxidase (HRP) 3,3′-diaminobenzidine (DAB) Detection System kits (SPD-015, Spring Bioscience, USA) and MACH2 Universal HRP Polymer Kit for mouse or rabbit (M2U522 G, H, L, Biocare Medical, USA) were used as the secondary reagents for light microscopy. An immunohistochemical reaction product was developed using DAB from the DAB+ kit (K3468, Agilent, USA). The reagents used for fluorescence microscopy included antibodies against goat immunoglobulins, labeled with biotin, from the R&D Systems kit (CTS008, R&D Systems, USA), donkey antibodies against murine immunoglobulins, labeled with biotin (#715-065-150, Jackson ImmunoResearch, USA); a monovalent Fab fragment from a donkey anti-rabbit immunoglobulin, conjugated to the Rhodamine Red-X fluorochrome (RRX) (#711-295-152, Jackson ImmunoResearch, USA), as well as a streptavidin-conjugated with the Cy2 fluorochrome (#016220-084, Jackson ImmunoResearch, USA). Cell nuclei were stained with aluminous hematoxylin, for light microscopy, or SYTOX Green nuclear fluorescent dye (S7020, Invitrogen, USA), for fluorescent and confocal laser microscopy. The resulting preparations were analyzed using a Leica DM750 light microscope (Germany) and photographed using an ICC50 camera (Leica, Germany). The images were processed using the LAS EZ program (Leica, Germany). The obtained fluorescent preparations were studied using an LSM 800 confocal laser microscope (Zeiss, Germany). A laser with a wavelength of 488 nm was used to excite Cy2 and SYTOX Green fluorescence, whereas a wavelength of 561 nm was used to excite Rhodamine Red-X. Obtained images were analyzed using the computer programs ZEN2012 and LSM Image Browser (Zeiss, Germany). More detailed information regarding the drug treatment protocols used during this study is presented in the works cited, which can be found in the last column of the table.

One of the primary tasks of contemporary neuromorphology is the determination of the type and chemical specificity of nervous system cells. The immunohistochemical detection of protein markers, neurotransmitters, and/or enzymes being synthesized in each cell represents a typical and effective method for determining cell types. The visualization of neurotransmitter synthesis enzymes is preferable to the identification of neurotransmitters themselves because monoamine molecules (serotonin, norepinephrine, and adrenaline) act not only as neurotransmitters but also as hormones (products of endocrine and mast cells) and, therefore, can be extracerebral in origin. In addition, amino acid neurotransmitters, such as glutamate and glycine, are involved in the metabolism of all

Primary antibodies used in neuroscience research
Первичные антитела, применяемые в нейробиологических исследованиях

Antigen under study	Primary antibody source	Clone	Catalog number, manufacturer	Works that describe the details of antibody use
Tyrosine hydroxylase	Rabbit	Polyclonal	ab112, Abcam, UK	[1]
GAD67	Mouse	Clone K-87	Ab26116, Abcam, UK	[2]
	Rabbit	Polyclonal	E10260, Spring Bioscience, USA	
Choline acetyltransferase	Goat	Polyclonal	AB144, Merck, USA	[3–5]
uNOS	Rabbit	Polyclonal	E393, Spring Bioscience, USA	[6]
Neurofilament protein Smi-32	Mouse	Clone Smi-32	SMI-32P, BioLegend, USA	[7]
α-Tubulin	Mouse	Clone DM-1A	MU121-5UC, BioGenex, USA	[8]
α-Synuclein	Rabbit	Polyclonal	E2684, Spring Bioscience, USA	[9]
NeuN	Mouse	Clone A-60	MAB377, Merck, USA	[10, 11]
GFAP	Rabbit	Polyclonal	Z033401-2, Agilent, USA	[12, 13]
	Mouse	Clone GA5	CM065 A, B, C, Biocare Medical, USA	
	Mouse	Clone spm507	E16510, Spring Bioscience, USA	
GS	Mouse	Clone GS-6	MAB302, Merck, USA	[7, 14]
Vimentin	Mouse	Clone V-9	M0725, Agilent, USA	[15, 16]
Iba-1	Goat	Polyclonal	ab5076, Abcam, UK	[17, 18]

cells, regardless of cell type or neurotransmitter specificity, and, therefore, can be found in cells of various natures. Therefore, to determine the transmitter specificity of neurons, the immunohistochemical identification of neurotransmitter synthesis enzymes, such as tyrosine hydroxylase (which synthesizes catecholamines), choline acetyltransferase (which synthesizes acetylcholine), and glutamate decarboxylase (which synthesizes gamma-aminobutyric acid [GABA]) is optimal.

Tyrosine hydroxylase (TH) is an enzyme that catalyzes the hydroxylation of the tyrosine amino acid in the presence of oxygen, tetrahydrobiopterin, and iron, which represents the rate-limiting step of catecholamine synthesis, including dopamine, noradrenaline, and adrenaline. These are well-known neurotransmitters that are involved in the regulation of various physiological and psychoemotional functions and reactions, such as stress, sleep and wakefulness, learning, attention, memory, and energy metabolism. Accordingly, disruptions in TH function represents a pathogenetic factor in the development of many neurological and psychiatric diseases, including Parkinson's disease, attention deficit and hyperactivity disorder, depression, schizophrenia, Alzheimer's disease, and drug addiction [19].

Catecholamines and TH are contained in nerve cells, which are distributed unevenly within the central nervous system (CNS). The majority of dopaminergic neurons and the conduction pathways formed by these neurons can be divided into three groups: nigrostriatal, mesocortical (or mesocorticolimbic), and tuberoinfundibular [20, 21]. Neurons in the zona compacta induce the nigrostriatal dopaminergic pathway, which ends in the caudate nucleus and the putamen, and plays a crucial role in the regulation of locomotor function in vertebrates [22]. Damage to nigrostriatal dopaminergic neurons in humans is associated with parkinsonism and Parkinson's disease [23].

Currently, a wide range of both monoclonal and polyclonal antibodies against TH are produced, which facilitate the detection of this enzyme immunohistochemically, in the CNS

and other parts of the human body and various animal species. Our previous studies revealed that one of the most successful antibody variants, which is capable of visualizing TH in catecholaminergic neurons and their processes in the brains of both laboratory animals (rats and mice) and humans is a polyclonal antibody against TH produced by Abcam (ab112), obtained against a purified protein from rat pheochromocytoma cells. The bodies and processes of TH-immunoreactive neurons are stained well in the substantia nigra and hypothalamus, and catecholaminergic nerve fibers are stained in the striatum (especially the putamen), the nucleus accumbens, the neocortex, septal nuclei, and olfactory bulbs (Fig. 1).

According to our experiments, the optimal detection of TH in the brains of humans and experimental animals was achieved using zinc-containing fixatives, especially ZEF, which resulted in high-quality immunohistochemical preparations for both light and confocal laser microscopy [24, 25].

Glutamic acid decarboxylase (GAD) is an enzyme that catalyzes the decarboxylation

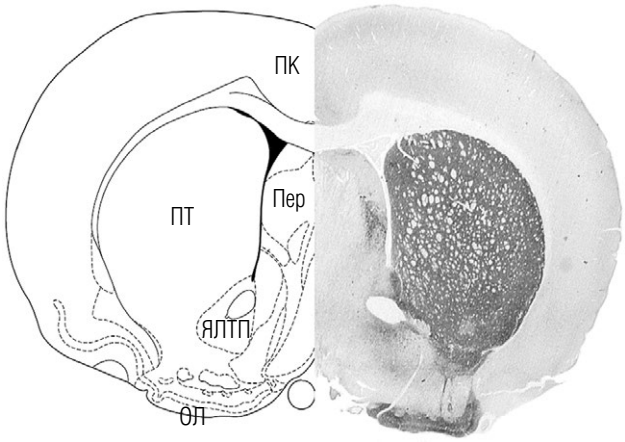

Fig. 1. The section of the rat brain through the corpus striatum. Left — figure of the rat brain areas, right — tyrosine hydroxylase immunohistochemistry (gray color). Catecholaminergic nerve fibers are predominantly distributed in the striatum (ПТ) and olfactory bulb (ОЛ). ПК — cingulate cortex, Пер — septum, ЯЛТП — bed nucleus of the stria terminalis

Рис. 1. Срез головного мозга крысы, проходящий через область полосатого тела (*corpus striatum*). Слева — карта областей головного мозга крысы, справа — иммуногистохимическое окрашивание среза головного мозга крысы на тирозингидроксилазу (серая окраска). Продемонстрировано преимущественное распределение катехоламинергических нервных волокон в полосатом теле (ПТ) и обонятельной луковице (ОЛ). ПК — поясная кора, Пер — перегородка, ЯЛТП — ядро ложа терминальной полоски

of glutamic acid to form GABA, which is the primary inhibitory transmitter in the CNS. Two primary GAD isoforms have been identified in the CNS, which differ in molecular weight (65 and 67 kDa). These isoforms are designated as GAD65 and GAD67 and are encoded by the genes *Gad1* and *Gad2*, respectively. GAD67 was detected in the bodies, processes, and synaptic terminals of neurons, whereas GAD65 was detected only in synaptic terminals [26]; therefore, antibodies against GAD67 are more often used for the immunohistochemical visualization of GABAergic brain structures.

Normally, the expression of GAD67 is noted in the neurons of the cortex, cerebellum, striatum, olfactory bulb, paleostriatum, reticular zone of substantia nigra, hippocampus, and inferior colliculus [2, 27–29]. Enzyme expression can be impaired during various neurological and psychiatric diseases (brain ischemia, schizophrenia, and Parkinson's disease).

The techniques used in this study are intended for the application to both the light-optical and immunofluorescence detection of GABAergic structures, in the brains of mice, rats, and humans. To detect GAD67 in rats, a murine monoclonal (clone K-87) antibody (Abcam, UK) or a rabbit polyclonal antibody (Spring Bioscience, USA) can be recommended. Staining using the murine monoclonal (clone K-87) anti-GAD67 antibody on rat cerebellar cortex slices are presented in Fig. 2, *a*. GAD67-immunopositive structures are present in all layers of the cerebellar cortex. Purkinje cell bodies are intensely stained, as are the fibers of basket cells, which form "baskets" around Purkinje cells. In the granular layer, GAD67 can be found in the axons of Golgi cells, found in the peripheral region of the cerebellar glomeruli. In the molecular, ganglionic, and granular layers, many GAD67 immunopositive fibers can be identified (see Fig. 2, *a*).

Thus, the use of antibodies against GAD67 enables the distribution of GABAergic structures to be identified.

Choline acetyltransferase (CAT) is a widely used marker for cholinergic neurons. CAT is a cytosolic protein that serves as the key enzyme for the synthesis of the neurotransmitter acetylcholine, from acetyl coenzyme A and choline [30]. Choline acetyltransferase is synthesized in the rough endoplasmic reticulum within the neuron body before being transported by the

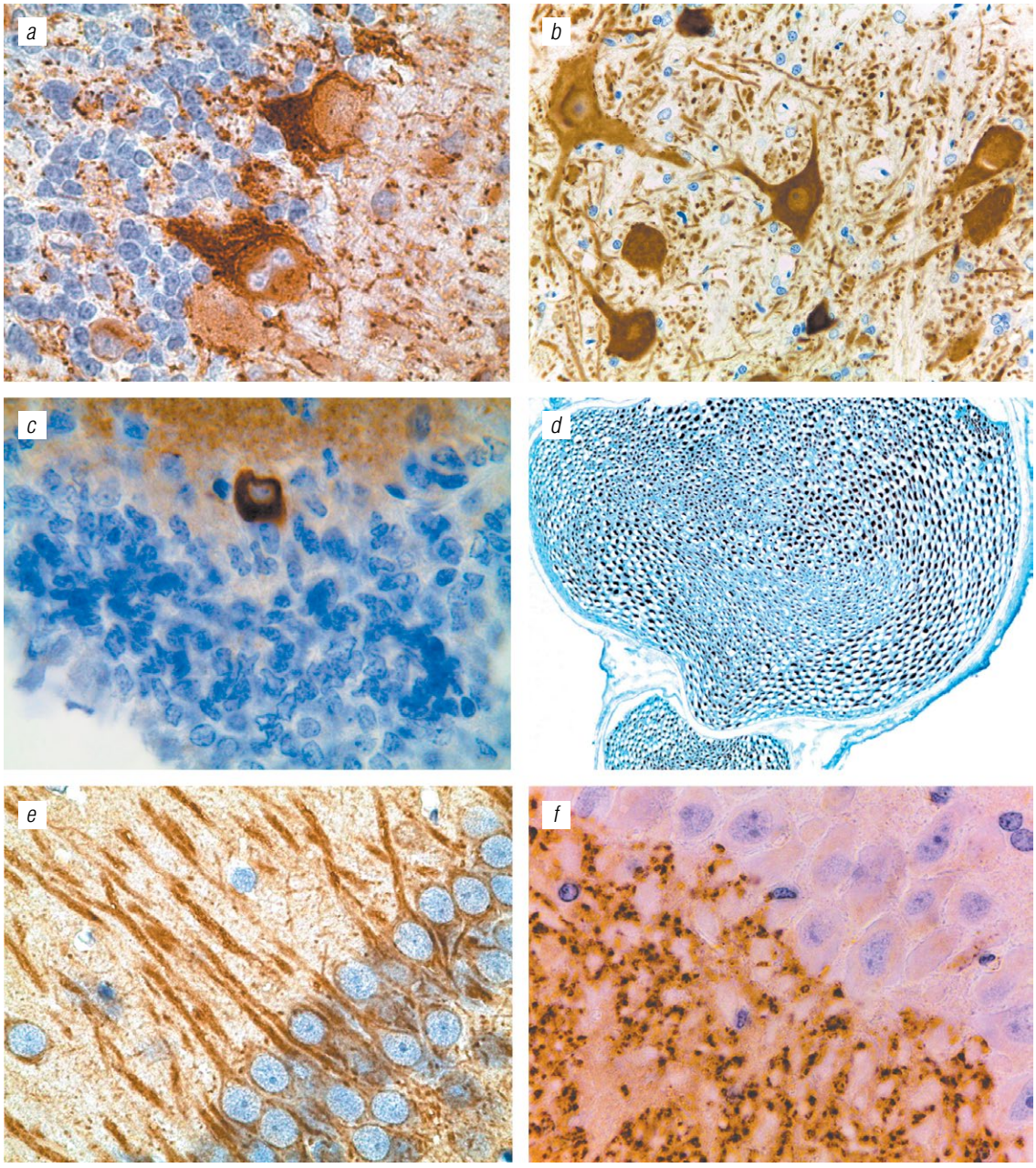

Fig. 2. Immunohistochemical visualization of various neural markers in the rat brain using light microscopy. *a* — GABA-ergic structures in the cerebellum, GAD67 immunohistochemistry, ob. ×100; *b* — cholinergic motor neurons of the spinal cord, choline acetyltransferase immunohistochemistry, ob. ×100; *c* — NOS-immunopositive neuron in the subventricular zone of the lateral ventricle, NOS immunohistochemistry, ob. ×100; *d* — neurofilaments in the axial cylinder of the nerve fibers of the rat sciatic nerve (cross section), SMI-32 immunohistochemistry, ob. ×10; *e* — alpha-tubulin in the processes of pyramidal neurons in the CA1 zone of the hippocampus, alpha-tubulin immunohistochemistry, ob. ×100; *f* — alpha-synuclein in giant synapses of mossy fibers of the CA3 zone of the hippocampus, alpha-synuclein immunohistochemistry, ob. ×100; *a–c*, *e*, *f* — nuclear counterstaining with alum hematoxylin; *d* — counterstaining of tissue structures with astra blue

Рис. 2. Иммуногистохимическое выявление различных нейральных маркеров с использованием световой микроскопии: *a* — ГАМК-ергические структуры в мозжечке крысы. Иммуногистохимическая реакция на GAD67, об. ×100; *b* — холинергические мотонейроны спинного мозга крысы. Иммуногистохимическая реакция на холин-ацетилтрансферазу, об. ×100; *c* — NOS-иммунопозитивный нейрон в субвентрикулярной зоне бокового желудочка головного мозга крысы. Иммуногистохимическая реакция на NO-синтазу (NOS), об. ×100; *d* — нейрофиламенты в осевых цилиндрах нервных волокон седалищного нерва крысы (поперечный срез). Иммуногистохимическая реакция с применением антител против белков нейрофиламентов (SMI-32), об. ×10; *e* — альфа-тубулин в отростках пирамидных нейронов в зоне CA1 гиппокампа крысы. Иммуногистохимическая реакция на альфа-тубулин, об. ×100; *f* — альфа-синуклеин в гигантских синапсах мшистых волокон зоны CA3 гиппокампа. Иммуногистохимическая реакция на альфа-синуклеин, об. ×100; *a–c*, *e*, *f* — подкраска клеточных ядер гематоксилином Джилла, *d* — подкраска тканевых структур астровым синим

axoplasmic flow to the nerve terminal. CAT is concentrated at the nerve terminals, where it synthesizes neurotransmitters [31]. Cholinergic neurons play important roles in the regulation of learning, memory, and sleep. They also provide motor functions and are involved in the regulation of gastrointestinal motility [32–36].

In modern morphological studies, cholinergic neurons are identified, as a rule, by the presence of CAT [4, 37]. Antibodies against CAT can be used to detect cholinergic structures in immunohistochemical studies of both the CNS and the peripheral nervous system, in normal and pathological conditions. CAT is particularly relevant to Alzheimer's disease, the pathogenesis of which has been associated with the significant loss of cholinergic neurons in the basal forebrain nuclei [38, 39]. In patients with cognitive impairments, the densities of CAT-containing fibers and neurons in the frontal cortex and amygdala were reduced [39].

In modern studies, both monoclonal and polyclonal antibodies against CAT have been used. The high-quality staining of paraffin tissue sections can be achieved using a staining protocol that we previously proposed [4]. Various types of fixation materials are acceptable for histological materials; however, the optimal staining results were obtained using ZEF [24, 25]. Using this method, the distribution of rat cholinergic nerve cells in the spinal cord (SC) was studied at different stages of embryonic development, during the early postnatal period, and in adult animals [3, 40, 41]. Primary goat polyclonal antibodies (AB144, Merck Millipore, Chemicon, USA) were used to detect the CAT [3, 41].

In the substantia grisea centralis (Rexed lamina X), CAT expression has been observed in the cytoplasm of small interneurons and their processes, some of which reach the Rexed lamina IX. In the intermediate zone of the SC gray matter, spindle-shaped, mediolaterally oriented neurons are expressed on the border between the Rexed lamina VI and VII of the SC. In the gray matter of the SC anterior horns, in the region of the Rexed lamina IX, large CAT-containing motor neurons, with a large number of immunopositive processes that form the SC ventral roots, were identified (Fig. 2, *b*). The study shows that CAT was localized not only in the neuronal cytoplasm but also in the nuclei of individual motor neurons. When examining the immunohistochemical reaction against CAT in dendrites and the bodies of large and medium neurons in the anterior horns, immunopositive synaptic buds were detected (see Fig. 2, *b*). Similar synapses are present on immunonegative nerve cells, located in the region of the Rexed lamina VIII–IX.

When studying the expression of CAT in the cervical SC of an adult rat, by light microscopy, several areas with intense immunohistochemical reactions were revealed. In the region of the SC posterior horns, the network-forming processes of neurons and single cells of the Rexed lamina II–IV were immunopositive. In the substantia grisea centralis (Rexed lamina X), the expression of CAT was noted in the cytoplasm of small interneurons and their processes, some of which reach the Rexed lamina IX. In the intermediate zone of the SC gray matter, spindle-shaped, mediolaterally oriented neurons were expressed on the border between the Rexed lamina VI and VII of the SC. In the gray matter of the anterior SC horns, in the region of the Rexed lamina IX, large CAT-containing motor neurons, with a large number of immunopositive processes that form the SC ventral roots, were identified (see Fig. 2, *b*). The study shows that in individual motor neurons, CAT was found to localize not only in the cell cytoplasm but also in the nucleus. Immunohistochemical reactions against CAT revealed its expression in dendrites and the bodies of large and medium neurons of the anterior horns, and immunopositive synaptic buttons can be detected (see Fig. 2, *b*). Similar synapses are present on immunonegative nerve cells, which are located in the region of the Rexed lamina VIII–IX. Thus, the method used to visualize cholinergic neurons was highly effective.

NO synthase

In the late 1980s, nitrogen monoxide (NO) molecules were discovered to have a vasodilative effect and to be involved in interneuronal connections, as a type of neurotransmitter. Nerve cells that synthesize and use NO as a neurotransmitter are scattered throughout the brain and SC and form the nitroxidergic (nitrergic) system of the brain, which is important for the general regulation of nervous system function. The regu-

latory role played by NO in the processes of memory, neurogenesis, sleep, participation in the stress-limiting system, and the perception of auditory signals has been demonstrated.

NO is a key participant in free radical processes and glutamate excitotoxicity and is, therefore, involved in the pathogenesis of many neurodegenerative and psychiatric diseases. NO has been demonstrated to both contribute to and counteract cellular degeneration [42–44]. Therefore, all aspects of NO in the nervous system are of importance for neurology and psychiatry.

In cells, NO is produced by the oxidation of the amino acid arginine (with the simultaneous formation of citrulline), by the enzyme NO synthase (NOS). Three isoforms of this enzyme have been identified: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). eNOS and nNOS localize primarily in endothelial and neuronal cells, respectively, and iNOS is expressed in different types of cells, regulated by cytokines and other signaling molecules [45].

We studied the localization of various NO-ergic cells, using a universal rabbit polyclonal antibody from Spring Bioscience (USA), which predominantly detected nNOS [6]. In the subventricular zone of the forebrain, individual, rounded, nNOS-immunoreactive cells were detected. Fig. 2, *c*, presents a part of the subventricular zone, in the dorsal angle of the rat brain lateral ventricle. Among the numerous immunonegative cells, a large nNOS-immunopositive neuron can be observed. The reaction product is evenly distributed throughout the cytoplasm and is absent in the nucleus (Fig. 2, *c*). Other NOS-immunopositive structures in the subventricular zone were not detected using this antibody. Deeper in the striatum neuropil, numerous small immunoreactive fibers form a dense plexus.

The participation of NO in the regulation of neurogenesis, in both the growing and adult brain, is widely accepted [46]. The NO molecule is short-living, does not have time to diffuse far from the location of synthesis and release; therefore, the neurogenesis regulatory properties in the subventricular zone are most likely manifested by NO molecules released by cells located directly in this region, indicating the functional significance of the subventricular zone of the adult brain and the few NOS-immunopositive cells that control this process. The conducted reaction fully meets the requirements of neurobiological studies.

SMI-32 antibodies against neurofilament proteins

Antibodies against cytoskeletal proteins can be used to label nerve cells and nerve fibers. Thus, the visualization of neurofilaments detected using SMI-32 antibodies is widely used in neurobiological and clinical studies to label neurons and their processes. Using SMI-32 antibodies, the non-phosphorylated epitope of the heavy subunit of neurofilaments can be visualized. Neurofilaments are one of the primary elements of the cytoskeleton and play important structural roles in nerve cells. In addition, they participate in slow axonal transport, regulate the state of other cytoskeleton proteins, and link the cytoskeleton and cytoplasmic structures.

The SMI-32 marker is contained in the perikaryon of large nerve cells, which facilitates the study of their shape and size, as well as the distribution of these neurons in different parts of the nervous system. Therefore, it is widely used as a marker of ganglionic [47] and amacrine cells [48] in the retina, pyramidal neurons in the neocortex [49], neurons in the substantia nigra in norm [50] and Parkinson's disease [51], SC motor neurons [52], and axons in white matter [53]. Using SMI-32 antibodies, the brains of humans and laboratory animals have been examined [50, 54]. SMI-32 is a convenient marker for studying emerging neurons during postnatal ontogenesis [55, 56].

In our studies, a monoclonal murine antibody against the SMI-32 protein (Bio Legend, USA) was used to study the nerve fibers in rat normal sciatic nerves before and after trauma. The reaction was conducted on paraffin sections, after fixing the material in ZEF [24, 25].

The study of histological preparations allowed the visualization of the axial cylinders of nerve fibers with different diameters (1.5–14.0 microns) on transverse sections through the intact rat sciatic nerve, in the region of the upper third of the thigh. In this case, the other structural elements of the nerve (Schwann cells, myelin sheaths, perineurium) were not stained, and the reaction proceeded without background (Fig. 2, *d*).

According to our own and others' published data, the high selectivity of this marker, the

reproducibility of the reaction on paraffin and frozen sections, and the ability to evaluate the material using light, fluorescence, and laser confocal microscopy facilitate the morphological assessments of nerve guide restoration. We intend to use our developed method in further studies, to assess nerve regeneration after trauma and experimental cell therapy.

Alpha-tubulin

Alpha-tubulin is another important marker of the nerve cell cytoskeleton. The tubulin molecule, the primary microtubule protein, is a heterodimer, consisting of two subunits, alpha- and beta-tubulin. Tubulin is localized in the nervous system, in the bodies of mature neurons, their dendrites, and axons, as well as in the synaptic membrane and the postsynaptic density. Tubulin is a component of microtubules during the formation of the neuron cytoskeleton and their processes and is involved in the growth of axons, the formation of synapses, and axoplasmic transport. Genetic disorders of tubulin synthesis (tubulopathies) result in severe brain damage [57], and many diseases of the nervous system are associated with quantitative and qualitative disorders of neuronal tubulin [58]. The widespread prevalence of tubulin (including alpha-tubulin) in the nervous system and the universality of its functions indicate the importance of studying this protein in the CNS.

In the rat brain, uneven staining was noted when establishing an immunohistochemical reaction to alpha-tubulin. The most intense reaction was registered in the superficial (first) layer of the neocortex, especially in the cingulate cortex, and intense staining was revealed in the pyriform cortex, olfactory tubercles, optical chiasm, and anterior commissure. The neocortex also has a band of increased immunoreactivity against alpha-tubulin in the middle (III–IV) and lower (VI) layers of the neocortex. A significant immune response is typical for the hippocampus and the medial part of the septum. In the area of the lateral ventricles, a fimbriate band of ependyma from the ventricle, which marks the cilia, is highlighted with a bright color, whereas the cytoplasm of the ependymocytes and the vascular plexus cells are immunonegative for alpha-tubulin.

Alpha-tubulin is present in cells that correspond to neurons and astrocytes, according to morphological characteristics. Alpha-tubulin was not detected in microglia. In all cases, alpha-tubulin-immunopositive cells, regardless of size, demonstrated a characteristic staining pattern, showing intense staining of the peripheral zone of the cytoplasm and the visible portions of axons and dendrites, with the complete absence of color in the nucleus and the perinuclear zone (Fig. 2, *e*); therefore, the immunohistochemical reaction to alpha-tubulin somewhat outlines the contours of nerve cells, providing an image of a neuron, similar to that observed following silver impregnation the Golgi method.

Thus, immunohistochemical staining for tubulin enables the efficient detection of nerve cells and processes, in all areas of the brain, and is promising for studying the cyto- and myeloarchitectonics of various brain structures.

Alpha-synuclein

Alpha-synuclein is a small molecule, weighing 19 kDa, which belongs to the synuclein protein family, along with β- and γ-synuclein. The protein is encoded by the *SNCA* gene, located on the long arm of the fourth human chromosome (4q21.3–q22). Due to alternative splicing, α-synuclein isoforms of 140, 126, 119, and 98 amino acids in length can be formed, with the 140 aa isoform representing the primary isoform [59]. The protein structure includes the N-terminal domain (1–60 amino acids), the central hydrophobic domain (61–95 amino acids), also known as the non-amyloid component, and the acidic C-terminal domain (96–140 amino acids). The N-terminal region is characterized by the presence of 7 highly conserved repeating sequences, consisting of 11 amino acids. This region forms an amphipathic alpha-helix and mediates the protein binding to membrane lipids. The central domain is amyloidogenic and can form protein aggregates. The C-terminus consists of charged amino acid residues, undergoes post-translational modifications, and mediates the binding of α-synuclein to other proteins, ligands, and metal ions, and its chaperone activity [60, 61]. Alpha-synuclein can either be identified in the native, soluble, unfolded protein form, or as a membrane-bound protein, which is accompanied by the conformational transition into an alpha-helix [62].

To date, the function of α-synuclein has not been established. α-Synuclein may be involved

in a variety of physiological processes, including vesicular neuronal transport, calcium regulation, mitochondrial homeostasis, gene expression, protein phosphorylation, and fatty acid-binding [59]. In a number of studies, α-synuclein has been shown to be able to affect intracellular dopamine contents by directly acting on the proteins involved in dopamine synthesis [63].

Today, most studies of α-synuclein aim to establish its role in the development of Parkinson's disease, although the fibrillar form of this protein has also been associated with other neurodegenerative diseases, which are collectively referred to as synucleinopathies, including Lewy body dementia, multiple system atrophy, and Bradbury-Eggleston syndrome [59, 64].

In our laboratory, a polyclonal rabbit antibody (Spring Bioscience, USA) was used to study α-synuclein in various brain structures. Figure 2, *f* shows the distribution of α-synuclein in the CA3 region of the rat brain hippocampus. Immunopositive alpha-synuclein granules were diffusely distributed in the *stratum lucidum* and *stratum radiatum*. In the pyramid layer, no reaction to α-synuclein was observed. Immunopositive granules have a predominantly rounded shape, whereas oval- and rod-shaped structures were less common (Fig. 2, *f*). In the in the *stratum lucidum* and *stratum radiatum*, numerous rounded structures of various sizes were well-contoured, where the reaction to α-synuclein is completely absent. These structures, apparently, represent the sites of pyramidal cell dendrites that have laterally entered the section. Around these areas, immunopositive α-synuclein granules were unevenly distributed inside the layer, which appear to represent conglomerates of α-synuclein-immunopositive axodendritic and axospiny synapses of giant mossy fiber terminals (Fig. 2, *f*).

Thus, the reaction to α-synuclein can be successfully used both for the analysis of neurodegeneration and for the study of the hippocampal synaptic apparatus during experiments.

NeuN

In neurobiological experiments, of importance is determination of not only neurotransmitter specificity of neurons, but also the typical specificity of the cells. Currently, a large number of immunohistochemical markers can be used to determine the types of cells under study, including whether they represent neurons, glia, or other types of cells. One of the most common neuronal markers is the NeuN nuclear protein. This protein is localized in the nucleus and perinuclear cytoplasm of most mammalian CNS neurons and is absent in astrocytes. The protein highly conserved and can be detected using the same antibodies in mammals, including humans [65], birds [66], amphibians [67], and fish [68]. This neuronal marker was discovered in 1992 when a group of researchers managed to obtain monoclonal antibodies (clone A60) against a previously unknown nuclear protein [65]. However, the nucleotide sequence of the gene encoding this protein was not deciphered until 2009, when NeuN was discovered to be the product of the *Fox-3* gene, one of the *Fox* genes that regulate splicing [69], and is synthesized during the late stages of differentiation among postmitotic neuroblasts [70]. However, NeuN, which is a typical neuronal marker, cannot be identified in a number of nerve cells, in particular, neurons in the cerebellum (basket cells, stellate cells, unipolar brush neurons, Purkinje cells, Golgi cells, Lugaro cells, dentate nucleus neurons), neocortex (in Cajal–Retzius cells [65, 71], inferior olive neurons, mitral cells of the olfactory bulbs [65], spinal gamma-motor neurons [72, 73], and sympathetic ganglia neurons [74]). The substantia nigra neurons in the brain of experimental animals and humans revealed only weak immunohistochemical staining of NeuN or were not stained at all [10].

The reaction to NeuN was widespread during our experimental studies. With the development of pathological conditions, NeuN detection in cells can be altered, including the complete disappearance of the reaction, as observed following ischemic damage to striatal neurons [11]. In pathomorphological studies, the reaction to NeuN can be used as a sensitive test for the detection of early autolytic changes in biological objects, which has been associated with the fairly rapid catabolism of NeuN protein [75]. These data indicated that NeuN protein may not be detected in some neurons; however, if NeuN is visualized in cells, this interaction reliably demonstrated their neuronal nature.

In our laboratory, a murine monoclonal (clone A60) antibody against the NeuN protein,

made by Merck Millipore (formerly Chemicon), USA, was used. To demonstrate the results of the immunohistochemical reaction to NeuN, a section of the dentate fascia of the rat hippocampus is presented (Fig. 3, *a*). The nucleus of neurons were clearly visible, due to the high-intensity reaction against NeuN (Fig. 3, *a*, green). Within the hippocampal granular zone, neurons were localized in dense rows, whereas in the subgranular zone and the chyle zone, they were located at considerable distances from each other. In the nucleus of neurons, the NeuN protein is distributed in the form of small discrete clusters, throughout the entire volume of the nucleus. NeuN is also present in the cytoplasm of the perinuclear region. In some neurons of the hilus hippocampi, the reaction product was also identified in the initial segments of the processes (Fig. 3, *a*, green).

In addition to neurons, micrographs containing astrocytes can be detected by an immunofluorescence reaction against the glial fibrillary acidic protein (GFAP) (Fig. 3, *a*, red). In the CNS, the neuronal and astrocytic functions are closely interrelated; therefore, the simultaneous detection of nerve cells and astroglia represents an urgent task for most neurobiological studies. Conducting the NeuN/GFAP double reaction enables the simultaneous detection of neurons and astrocytes, facilitating the assessment of structural aspects and the functional status of each of these cell types, in addition to studying their mutual arrangement, relative to each other.

Glial fibrillary acidic protein-a marker of astrocytes

Astrocytes are multifunctional glia cells that serve a number of functions in the CNS, such as providing neurons with an energy substrate (lactate), participating in synaptogenesis, synaptic plasticity, and the modulation of synaptic transmission, removing neurotoxic glutamate from the synaptic cleft after signal transmission between neurons, participating in the formation of the blood-brain barrier, the regulation of microcirculation, and maintaining the water ion balance [76]. In addition to numerous functions in the CNS, astrocytes respond to brain damage through a process called reactive astrogliosis [77]. Therefore, the study of astrocytes represents the aim of a huge number of neurobiological studies, indicating the need for a reliable marker that can be used to study the structurally functional characteristics of this cell population. Currently, more than 20 proteins are known to serve as astrocyte markers, among which GFAP is used the most widely [78, 79]. GFAP is a class III intermediate filaments (IF), which is the primary protein that composes the astrocyte cytoskeleton, ensuring the stability of neuronal body and process morphologies, and is involved in the regulation of astrocyte volume and the modulation of their movement. GFAP localization in astrocytic bodies and processes enables the identification of this cell population and the creation of high-quality 3D astrocyte reconstructions, which can be used to study the features of their structural organization and spatial mutual arrangement [12]. In addition, GFAP can act as an important functional marker for astroglia. The increased expression of this protein has been observed in many CNS pathologies, including ischemia, neurodegenerative diseases, tumor development, and traumatic brain damage.

The catalogs of many manufacturers present various poly- and monoclonal antibodies against GFAP. Three types of these antibodies have been used in our laboratory, including a rabbit polyclonal antibody made by Agilent (formerly Dako), USA, and two clones of murine (monoclonal) antibodies (clone GA-5, made by Monosan, Netherlands, and clone SPM507, made by Spring Bioscience, USA). These antibodies allow the specific detection of astrocytes in paraffin-embedded brain sections from laboratory animals (mouse, rat, rabbit, and cat) and humans. Fig. 3, *a* (red), shows the results of using the rabbit polyclonal antibody against GFAP (Agilent, USA), to identify astrocytes in the rat hippocampus. Astrocytes have the appearance of arborizing cells, and are mainly stellate. In the subgranular zone and hilus, the bodies and processes of astrocytes were clearly visible, whereas in the granular zone, only the astrocytic processes that penetrate the dense rows of neurons can be identified (Fig. 3, *a*, red).

Glutamine synthetase

When conducting neurobiological studies, the type of cell must be identified and its functional state must be determined. One marker that can be used to studying the function of astroglia is glutamine synthetase (GS). GS is a ligase class enzyme that catalyzes the reaction of

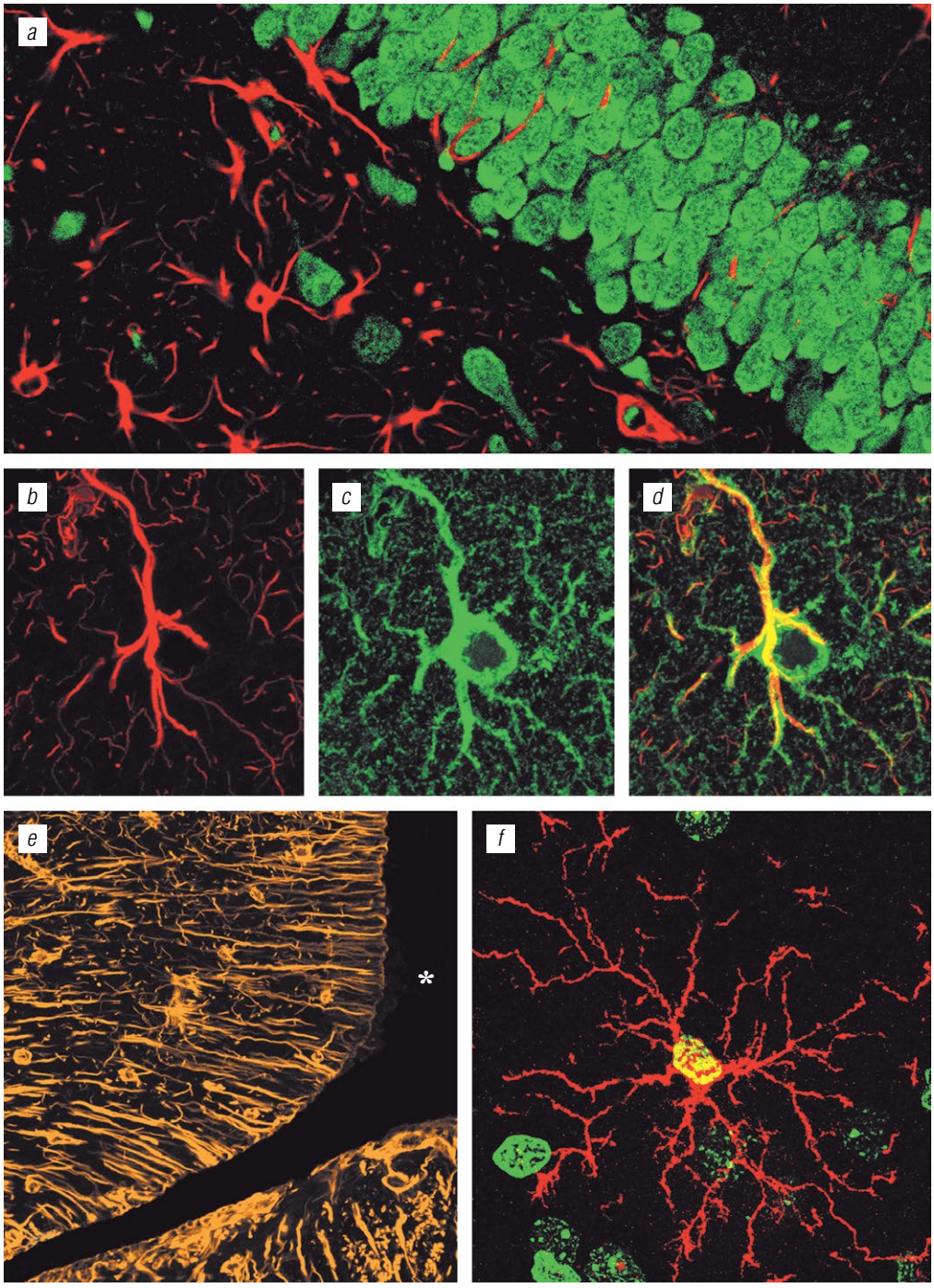

Fig. 3. Fluorescence imaging of neural and glial markers (confocal microscopy): *a* — fascia dentata of the rat hippocampus. Double immunofluorescence staining for NeuN (green) and GFAP (red), ob. ×63; *b–d* — astrocyte in the striatum of the rabbit brain. Double immunofluorescence staining for GFAP (red) and glutamine synthetase (green). Confocal images obtained in single channels are shown in (*b*) and (*c*) while merged images are shown in (*d*), ob. ×63; *e* — tanycytes of the third ventricle of the rat brain. Vimentin immunohistochemistry, ob. ×63, the asterisk marks the ventricular cavity; *f* — microgliocyte in the striatum of the rat brain. Iba-1 immunohistochemistry (red color) with nuclear counterstaining with SYTOX Green (green color), ob. ×63

Рис. 3. Флуоресцентная визуализация нейральных и глиальных маркеров (конфокальная микроскопия): *a* — зубчатая фасция гиппокампа крысы. Двойная иммуногистохимическая реакция на NeuN (зеленый цвет) и GFAP (красный цвет), об. ×63; *b–d* — астроцит в стриатуме головного мозга кролика. Двойная иммуногистохимическая реакция на GFAP (красный цвет) и глутаминсинтетазу (зеленый цвет), раздельное (*b*, *c*) и совмещенное (*d*) представление красного и зеленого каналов, об. ×63; *e* — таниците третьего желудочка головного мозга крысы. Иммуногистохимическая реакция на виментин, об. ×63; звездочка — полость желудочка; *f* — микроглиоцит в стриатуме головного мозга крысы. Иммуногистохимическая реакция на Iba-1 (красный цвет) с подкраской ядер клеток красителем SYTOX Green (зеленый цвет), об. ×63

ATP-dependent binding of glutamate with ammonia to produce glutamine. In the brain, GS is synthesized primarily in astrocytes. GS represents a key enzyme in the glutamate-glutamine cycle, during which astrocytes absorb extracellular glutamate, which has neurotoxic properties and converts it to non-toxic glutamine, using GS [80–82]. As a result of the same reaction, an excess amount of neurotoxic ammonia becomes inactivated, which accumulates during liver pathologies and acts as a pathogenic factor in hepatic encephalopathy [83].

Currently, GS is widely used as a functional marker of astrocytes, in both experimental and clinical neurobiological studies. Unlike other widely used astroglial markers, such as GFAP, GS is present in all astrocyte subtypes, which facilitates the identification of this cell population most fully [84].

Antibodies against GS that are suitable for immunohistochemical studies are currently supplied by many manufacturers (for example, Thermo Fisher Scientific, BioLegend, Merc, and others). In our laboratory, a murine monoclonal (clone GS-6) antibody against GS, generated by Merck (formerly Chemicon), USA, was used. According to the manufacturer's instructions, these antibodies can be used to work with the tissues of sheep, rats, mice, and humans. According to our studies, they are also applicable to the study of the rabbit brain. Fig. 3, *b–d*, presents the results of the double-immunofluorescence GFAP/GS reaction, in rabbit brain preparations. GFAP (red) is primarily localized in the body and large processes of astrocytes. However, regions characterized by the absence of GFAP immunoreactivity were visible in the perinuclear region, and the fluorescence intensity in thin processes is low (Fig. 3, *b*). Unlike GFAP, GS (green) can be detected in all parts of the perinuclear cytoplasm of astrocytes, as well as in large and small cell processes, and the amount and intensity of fluorescence were visually increased compared with GFAP immunostaining (Fig. 3, *c*). Fig. 3, *d*, with the combined representation of the green and red channels, demonstrates that GFAP and GS did not completely colocalize within astrocytes in the rabbit striatum. GFAP appears to be distributed in the bodies and processes of astrocytes, in the form of fibrous structures (Fig. 3, *b*), whereas GS staining is characterized by a discrete distribution and is identified in the form of numerous small clusters (Fig. 3, *c*).

Thus, GS represents a convenient astrocyte marker, which in combination with GFAP, enables the full evaluation of astroglial structural aspects, during both normal and pathological conditions.

Vimentin

In some studies, the selective labeling of the cells that line the brain ventricles (ependymocytes and tanycytes) is necessary, for which vimentin can be used as a marker [15]. Vimentin belongs to class III intermediate filaments and forms homo- and heterodimers with other IF proteins, including nestin and desmin. Vimentin is a highly conserved protein among vertebrates, has a molecular weight of 57 kDa, and consists of 466 amino acids.

The primary cellular function of vimentin is the maintenance of cellular integrity, ensuring rigidity, mechanical stability, and the maintenance of cell shape. Vimentin filaments are involved in the intracellular distribution of organelles and proteins in the cytoplasm, as well as organelle transport, cell adhesion, migration, and intracellular signal transmission. A number of studies have demonstrated the role played by vimentin in cell proliferation, differentiation, and apoptosis.

Normally, vimentin is a component of brain barrier structures. Vimentin IFs are expressed in large amounts in ependymocytes, tanycytes, astrocytes, meningocytes, and endotheliocytes [16]. In addition, along with nestin, vimentin is a component of the cytoskeleton in neural stem and progenitor cells. During embryogenesis, vimentin is expressed in radial glial cells [15]. Under the influence of various types of damaging factors, astrocytes become reactive and were characterized, in addition to GFAP overexpression, by the expression of vimentin (which is normally non-characteristic for astrocytes in the adult mammalian brain). The expression of IFs is believed to be necessary for the neuroprotective functions of astrocytes [85].

In our laboratory, vimentin expression studies were performed, using a monoclonal antibody (clone V9) made by Agilent (formerly Dako), USA. Fig. 3, *e* presents the floor portion of the rat brain third ventricle. Vimentin-immunopositive tanycytes form the lining of this area of the brain, which appear as bipolar cells, with a long basal process. Vimentin fila-

ments were evenly distributed throughout the cell cytoplasm and were present in both the bodies and the processes of tanycytes. One basal process branch from the base of the tanycytes body, which then branches into smaller branches. The processes were long, thin, and differ in a wavy course. They end on the vessels of the hypothalamus, containing endotheliocytes, which were also vimentin-immunopositive (endotheliocytes, smooth myocytes, and adventitia cells were stained).

Thus, the immunohistochemical reaction to vimentin enables the characterization of the structural and spatial organization of the hypothalamus circumventricular zone.

Iba-1

The Iba-1 protein is a reliable marker for microglial cells. Currently, the nervous and immune systems are known to function in close cooperation and mutually affect each other [86]. Various aspects of neuroimmune interactions have been the subject of a large number of current scientific studies. The CNS is characterized by the presence of its own immune system, formed by microglial cells, which are tissue macrophages of mesenchymal origins. The great interest of researchers in the study of this cell population is likely due to the fact that microglia represent a key factor during the neuroinflammation process, which is associated with neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and Huntington's chorea. Therefore, researchers require selective microglial markers, which facilitate the evaluation of quantitative changes and the functional status of this cell population.

Iba-1 calcium-binding protein (ionized calcium-binding adaptor molecule-1), also known as AIF-1 (allograft inflammatory factor-1), has been widely used as a selective marker for microglia [17, 18, 87, 88]. Using an antibody against the Iba-1 protein, both activated amoeboid microglia and resting ramified microglia could be detected, as well as all intermediate states. The uniform distribution of Iba-1 in the cytoplasm of microglia enables the full characterization of the structural features of these cells [89, 90].

Currently, a variety of commercial antibodies against Iba-1/AIF-1 (for example, made by Wako Chemicals, Abcam, and others) are available to researchers. An Abcam goat polyclonal antibody against Iba-1 (ab5076) is currently being used for research in our laboratory, and is suitable for electron microscopic immunohistochemistry, immunohistochemical studies on paraffin and free-floating sections, western blotting, and immunofluorescence. The immunogen for these antibodies is a synthetic peptide that corresponds to the C-terminal portion of the human Iba-1 molecule. These antibodies are specific for microglia and macrophages and do not cross-react with neurons, astrocytes, or oligodendrocytes. They are suitable for the immunostaining of brain tissue in rats, rabbits, guinea pigs, cows, dogs, primates, and humans. These antibodies enable high-quality preparations to be obtained for light, fluorescence, and confocal laser microscopy. The use of antibodies against the Iba-1 protein and SYTOX Green fluorescent nuclear dye provides good results. The tinting of cellular nuclei greatly facilitates the orientation of structures in the preparation and enables the assessment of functional cell states (based on the sizes of the nucleus and nucleolus and the state of chromatin). Figure 3, *f* presents the results of the immunofluorescence response to the Iba-1 protein, on sections of the brain from a mature rat, with nuclei stained with SytoxGreen (Invitrogen, USA). Iba-1 protein is evenly distributed in the cytoplasm of the microglial cell, allowing both the body of the cell and its many thin, complex branching processes to be detected (Fig. 3, *f*, red). Using the fluorescent nuclear stain SYTOX Green enables the identification of microglia nucleus and clearly demonstrates the intranuclear localization of the Iba-1 protein, as well as the identification (based on the size and structure of nucleus) of neighboring cells, and the evaluation of their functional status (Fig. 3, *f*, green).

Thus, the immunohistochemical reaction to the Iba-1 protein represents a robust tool for assessing the microglial cell population, both in normal and experimental studies.

Conclusion

This article summarizes our experiences with the use of the 12 significant markers for neurobiological studies, for which the optimal combinations of primary and secondary antibodies are presented. The combination of these immunocytochemical approaches with the fixation of samples in ZEF enables the high selectivity

of cell labeling to be achieved while maintaining the structure and tinctorial properties of the nervous tissue. This, in turn, enables high-quality preparations to be obtained, using both immunohistochemical markers and classical histological staining. This methodology is promising for use in experimental neurobiology and clinical and morphological diagnostics.

Additional information

The study was conducted within the state task of the Institute of Experimental Medicine.

The study was approved by protocols No. 1/14 of 04/21/2014, No. 3/17 of 11/30/2017, and No. 3/19 of 04/25/2019 of the local ethics committee of the Institute of Experimental Medicine.

Conflict of interests. The authors declare no conflict of interest.

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