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Changes in the Immunohistochemical Characteristics of Neurons in a Number of Hypothalamic Nuclei on Aging

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Objectives. To identify changes in the immunohistochemical characteristics of neurons in the arcuate (AN), ventromedial (VMN), and dorsomedial (DMN) nuclei of the hypothalamus in rats on aging. **Materials and methods.** Studies were performed using male Wistar rats aged 3–4 months (five individuals) and 12–18 months (five individuals) using immunohistochemical methods. **Results.** Aging was associated with a decrease in the relative content of calbindin-immunopositive neurons in the DMN, while calretinin-immunopositive neurons in the DMN and VMN increased. The numbers of neurons immunopositive for neuronal nitric oxide synthase and the level of immunofluorescence for this enzyme were greater in all the nuclei studied in older animals. Older rats also showed a higher density of neuropeptide Y-immunopositive fibers in the VMN, with a smaller number in the DMN. **Conclusions.** Aging was associated with changes in the neurochemical composition of neurons, mainly in the ventromedial and dorsomedial nuclei of the hypothalamus.

Keywords: hypothalamus, ventromedial nucleus, dorsomedial nucleus, neurons, aging, immunohistology.

Introduction. The hypothalamus is a phylogenetically ancient part of the diencephalon and plays an important role in supporting the constancy of the internal milieu and integration of the functions of the autonomic, endocrine, and somatic, systems. It has been suggested that the hypothalamus is involved in the mechanisms of aging, with important roles being taken by the ventromedial (VMN) and dorsomedial (DMN) nuclei of the hypothalamus [12, 14]. The VMN and DMN are involved in regulating trophics and supporting energy homeostasis, and also in regulating peripheral circadian rhythms [11]. The most important nuclei in the diencephalic hypothalamic region with roles in maintaining homeostasis include the arcuate nucleus (AN) [12].

Age-related development is linked with changes in the cytochemical characteristics of neurons in the central and peripheral compartments of the nervous system. In particular, changes occur in the contents of calcium-binding proteins calbindin (CB) and calretinin (CR), neuropeptides, particularly neuropeptide Y (NPY), and nitric oxide synthase (nNOS) [1, 8]. Aging is associated with changes in the expression of various hypothalamic neurotransmitters and hormones [7]. There are relatively few data on the neurochemical characteristics of hypothalamic neurons in aging.

The aim of the present work was to identify changes in the immunohistochemical characteristics of neurons in the arcuate, ventromedial, and dorsomedial nuclei of the hypothalamus in rats on aging.

Materials and Methods. Studies were carried out on male Wistar rats aged 3–4 and 24–30 months (five animals in each group). Experiments were performed in compliance with the "Regulations for Studies Using Experimental

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TABLE 1. Intensity of Immunofluorescence of Neurons Immunopositive for Calbindin (CB), Calretinin (CR), and Neuronal Nitric Oxide Synthase (nNOS) in the Ventromedial, Dorsomedial (DMN), and Arcuate (AN) Nuclei of the Hypothalamus in Rats

Immunolabeling	Age, months	AN	DMN	VMN
CB	3–4	47 ± 2.8	43 ± 1.3	18 ± 1.2**
	24–30	49 ± 2.3	38 ± 1.4	16 ± 1.5**
CR	3–4	–	39 ± 2.4	40 ± 2.1
	24–30	–	37 ± 2.8	38 ± 1.6
nNOS	3–4	–	18 ± 1.4	18 ± 1.4
	24–30	27 ± 2.1	34 ± 2.4*	38 ± 2.5*

*Significant differences compared with young animals, $p < 0.001$; **significant differences compared with the AN and DMN, $p < 0.001$.

Animals" (USSR Ministry of Health Order No. 775 of August 12, 1977). The study was approved by the Ethics Committee of Yaroslavl State Medical University (No. 29, of February 21, 2019). Administration of a lethal dose of urethane (3 g/kg, i.p.) was followed by transcardiac perfusion with standard phosphate-buffered saline (PBS, 0.01 M, pH 7.4) (BioloT, Russia) and then 4% paraformaldehyde solution (Sigma, USA) in PBS. After perfusion, brains were extracted and parts of the hypothalamus containing the AN, VMN, and DMN were dissected out on the basis of coordinates from a rat brain atlas [10]; these were placed in the same fixer used for perfusion for 1–2 h. Serial sections of thickness 12 μm were cut on a cryostat. Neurons containing nNOS were detected using primary goat antibodies (Abcam, USA) diluted 1:300. CB, CR, and NPY were identified using rabbit antibodies (Abcam, USA) diluted 1:500, 1:100, and 1:500, respectively. Secondary donkey antigoat or antirabbit antibodies were conjugated with fluorochromes fluorescein isothiocyanate (FITC, Jackson Immunoresearch, USA) diluted 1:100, giving green fluorescence. Negative controls omitted primary or secondary antibodies. The proportion of immunopositive neurons was determined by labeling the whole neuron population with Neurological Trace Red (Molecular Probes, USA) diluted 1:500, this being a selective stain for Nissl substance with red fluorescence.

Preparations were analyzed using an Olympus BX43 fluorescent microscope (Tokyo, Japan) and the appropriate set of light filters with a Tucsen TCC 6.1 ICE cooled digital CCD camera with ISXCapture 3.6 software (China). The sizes and relative contents of immunopositive neurons on digital images of histological sections were determined using ImageJ (NIH, USA, http://rsb.info.nih.gov/ij/). The proportion of immunopositive neurons was determined as the ratio of neurons to the total number of neurons, taken as 100%. Analysis was applied to nerve cells whose nuclei were transected by sections. Neuron cross-sectional areas were determined by randomly selecting 100 neurons immunopositive for each of the study markers in each age group. Fluorescence intensity was analyzed quantitatively in relative brightness units from black (0) to white (255), also using ImageJ.

Data were processed mathematically in Sigma Plot (StatSoft, USA). All values are presented as arithmetic means ± errors of the mean ($M \pm m$). Significant differences were identified by ANOVA and the Wilcoxon and Mann–Whitney tests, $p < 0.05$.

Results. The results showed that CB, CR, nNOS, and NPY were detected in young and old animals, though immunoreactivity to these markers differed in young and old rats.

Calbindin-immunopositive (CB-IP) neurons displayed intense fluorescence in the AN and DMN (Fig. 1, Table 1).

CB immunofluorescence intensity in the VMN was lower ($p < 0.001$). The AN and VMN showed no differences in the percentage contents of CB-IP neurons between young and old animals (Fig. 2).

The percentage content of CB-IP neurons in the DMN was lower in old animals ($p < 0.001$). The immunofluorescence intensity of CB-IP neurons in all three nuclei remained unaltered on aging (see Table 1).

Calretinin (CR) was not detected in the AN of either group. intensely fluorescent CR-IP neurons were found in the DMN and VMN. On aging, the relative contents of CR-IP neurons in the DMN and VMN increased significantly (see Fig. 2). The level of immunofluorescence of CR-IP neurons in the DMN and VMN did not change on aging (see Table 1). nNOS was not found in the AN in young animals, while nNOS-IP neurons with weak immunofluorescence were present in the DMN and VMN. nNOS-IP contents in three-month-old rats were 41 ± 3.2% in the DMN and 58 ± ± 7.4% in the VMN. In old animals, the number of nNOS-IP neurons and the extent of immunofluorescence to nNOS increased in all the nuclei studied. Old rats showed more nNOS-IP neurons in the AN (86 ± 4.5%), while all neurons (100%) in the DMN and VMN became IP for nNOS.

Intensely fluorescent NPY-IP fibers were seen in the AN in young and old rats; the levels of immunofluorescence (77 ± 5.1 and 71 ± 3.9, respectively) of IP fibers were identical in the two age groups. Young rats showed a dense network of intensely fluorescent NPY-IP fibers in the DMN (47 ± 2.3), while the level of fluorescence of NPY-IP fibers in the VMN was significantly lower (15 ± 0.9). On aging,

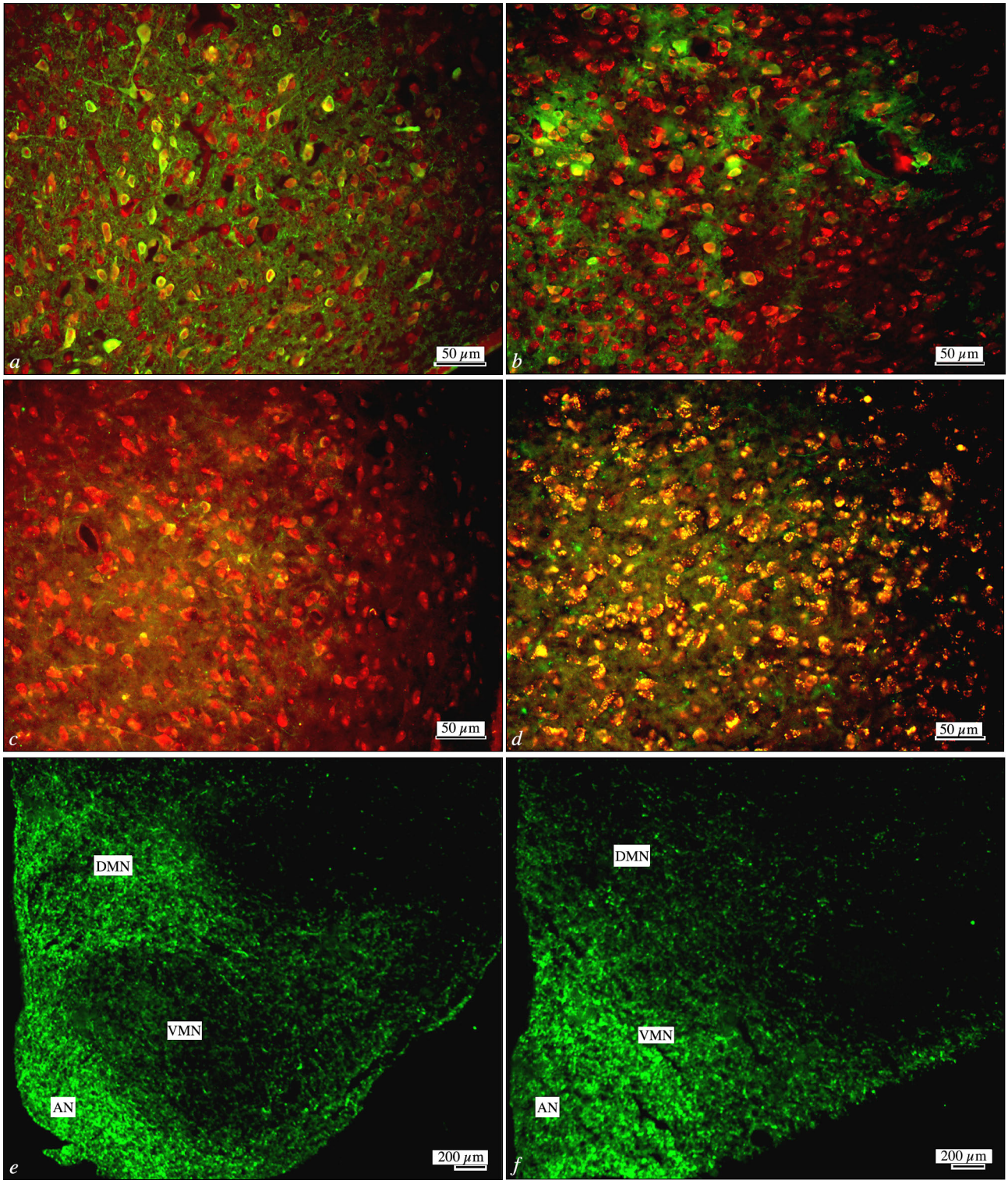

Fig. 1. Immunohistochemical reactions for calbindin (*a*, *b*), and neuronal nitric oxide synthase (*c*, *d*) neuropeptide Y in the dorsomedial nucleus (*a–d*) and a group of nuclei in the hypothalamus (*e*, *f*) in young (*a*, *c*, *e*) and old (*b*, *d*, *f*) rats. Double immunofluorescent labeling: calbindin, neuronal nitric oxide synthase, and neuropeptide Y (green, FITC) and Neurological Trace Red (red). VMN – ventromedial nucleus; DMN – dorsomedial nucleus; AN – arcuate nucleus.

inverse changes in the distribution of NPY-IP fibers were seen in the DMN and VMN. In old rats, immunofluorescence to NPY was significantly greater in the VMN (61 ± 3.7) and significantly lower in the DMN (23 ± 1.8).

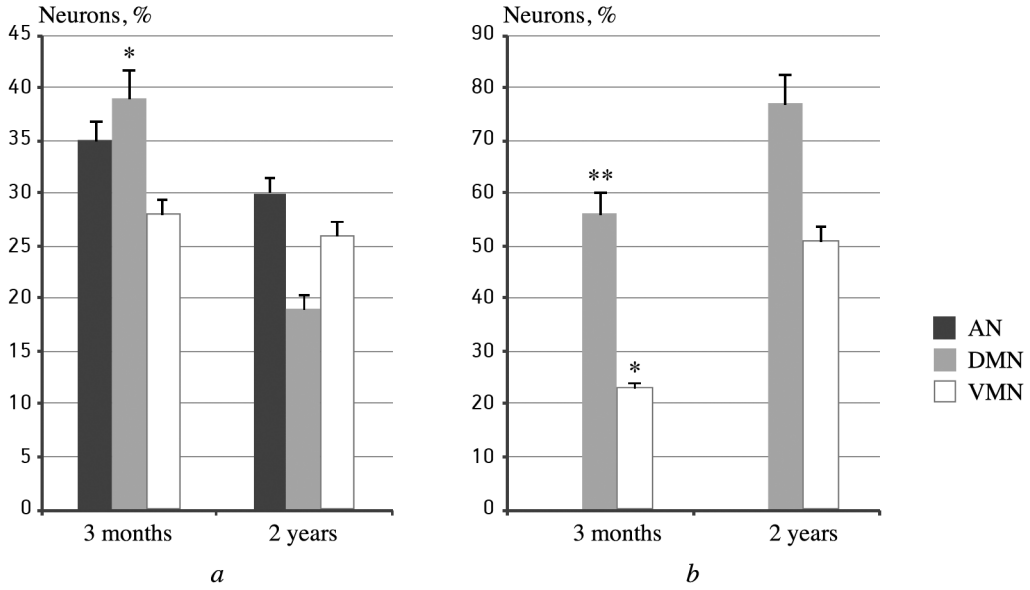

Fig. 2. Percentage compositions of neurons immunopositive for calbindin (*a*) and calretinin (*b*) in the ventromedial (VMN), dorsomedial (DMN), and arcuate (AN) nuclei. *Significant differences, $p < 0.01$, compared with old rats.

The mean cross-sectional area of IP neurons in the AN, DMN, and VMN varied from 119 ± 9.6 to 137 ± 8.2 μm^2 in rats of both groups. Neurons in different nuclei IP for different markers showed no differences in this parameter.

Discussion. One of the best known and most developed concepts in gerontology is the elevation theory of aging, which assigns key value to age-related increases in the threshold of sensitivity of the hypothalamus to homeostatic signals [6]. The VMN and DMN of the hypothalamus, which are responsible for regulating metabolism and energy in the body, are presumed to play an important role in aging processes [4].

Among the factors controlling the development of signals and their intensity, the most significant are the maintenance of a specific concentration of calcium ions. CB and CR operate as calcium sensors and take part in maintaining calcium levels in cells [13]. The literature contains data on age-related decreases in CB and CR expression in the cerebral cortex in rodents, which makes cells more sensitive to changes in calcium ion concentrations [2]. Excessive inward calcium currents in neurons and a characteristic overloading of neurons with calcium are currently regarded as important mechanisms of brain aging, particularly neuron degeneration and the development of age-related pathology [9].

Expression of the NO synthesis enzyme neuronal nitric oxide synthase (nNOS) is significantly restricted to areas of the hypothalamus involved in controlling such body functions as energy balance and multiplication [3]. The data obtained here provide evidence that nNOS immunofluorescence increases in the AN, DMN, and VMN on aging, which is consistent with previous data on increases in nNOS expression in the preoptic area and the supraoptic and paraventricular nuclei, as well as in the AN in older rats [6].

Published data indicate that the AN age is associated with a decrease in the content of proopiomelanocortin and NPY [7]. We found that aging is associated with an increase in the density of NPY-IP fibers in the VMN and a decrease in the DMN.

Conclusions. Thus, aging alters the neurochemical composition of the hypothalamic nuclei studied here. These changes consist of a decrease in the proportion of CB-IP and increases in the proportions of CR-IP and nNOS-IP neurons, with changes in the density of NPY-containing fibers in the VMN and DMN.

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REFERENCES

1. P. M. Masliukov, A. D. Nozdrachev, and A. I. Emanuilov, "Developmental characteristics of the expression of calcium-binding proteins in neurons in the ganglia of the autonomic nervous system," *Usp. Gerontol*., 29, No. 2, 247–253 (2016).
2. J. H. Ahn, S. Hong, J. H. Park, et al., "Immunoreactivities of calbindin D28k, calretinin and parvalbumin in the somatosensory cortex of rodents during normal aging," *Mol. Med. Rep*., **16**, No. 5, 7191–7198 (2017), doi: 10.3892/mmr.2017.7573.
3. K. Chachlaki, J. Garthwaite, and V. Prevot, "The gentle art of saying NO: how nitric oxide gets things done in the hypothalamus," *Nat. Rev. Endocrinology*, **13**, No. 9, 521–535 (2017), doi: 10.1038/nrendo.2017.69.
4. P. A. Dacks, C. L. Moreno, E. S. Kim, et al., "Role of the hypothalamus in mediating protective effects of dietary restriction during aging," *Front. Neuroendocrinol*., **34**, No. 2, 95–106 (2013), doi: 10.1016/j.yfrne.2012.12.001.

5. V. M. Dilman and V. N. Anisimov, "Hypothalamic mechanisms of ageing and of specific age pathology – I. Sensitivity threshold of hypothalamo-pituitary complex to homeostatic stimuli in the reproductive system," *Exp. Gerontol.*, **14**, 161–174 (1979).
6. M. Ferrini, C. Wang, R. S. Swerdloff, et al., "Aging-related increased expression of inducible nitric oxide synthase and cytotoxicity markers in rat hypothalamic regions associated with male reproductive function," *Neuroendocrinology*, **74**, No. 1, 1–11 (2001).
7. K. Kim and H. K. Choe, "Role of hypothalamus in aging and its underlying cellular mechanisms," *Mech. Aging Dev.*, **177**, 74–79 (2019), doi: 10.1016/j.mad.2018.04.008.
8. P. M. Masliukov, A. I. Emanuilov, K. Moiseev, et al., "Development of noncatecholaminergic sympathetic neurons in para- and prevertebral ganglia of cats," *Int. J. Dev. Neurosci.*, **40**, 76–84 (2015).
9. M. P. Mattson, "Calcium and neurodegeneration," *Aging Cell*, **6**, No. 3, 337–350 (2007).
10. G. Paxinos and C. Watson, *The Rat Brain in Stereotaxic Coordinates*, Academic Press, Elsevier (2013), 7th ed.
11. A. Satoh, S. I. Imai, and L. Guarente, "The brain, sirtuins, and ageing," *Nat. Rev. Neurosci.*, **18**, No. 6, 362–374 (2017), doi: 10.1038/nrn.2017.42.
12. P. Seoane-Collazo, J. Fernø, F. Gonzalez, et al., "Hypothalamic-autonomic control of energy homeostasis," *Endocrine*, **50**, No. 2, 276–291 (2015), doi: 10.1007/s12020-015-0658-y.
13. B. Schwaller, "The regulation of a cell's Ca(2+) signaling toolkit: the Ca (2+) homeostasome," *Adv. Exp. Med. Biol.*, **740**, 1–25 (2012).
14. Y. Zhang, M. S. Kim, B. Jia, et al., "Hypothalamic stem cells control ageing speed partly through exosomal miRNAs," *Nature*, **548**, No. 7665, 52–57 (2017), doi: 10.1038/nature23282.