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РАСПРЕДЕЛЕНИЕ NADPH-ДИАФОРАЗА ПОЗИТИВНЫХ СТРУКТУР ОБОНЯТЕЛЬНОЙ ЛУКОВИЦЫ КРЫС В ОНТОГЕНЕЗЕ

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Цель. Установить особенности распределения NADPH-диафораза (NADPH-d) позитивных структур в обонятельных луковицах крыс разного возраста. ***Материалы и методы.*** Исследование проведено на 22 белых крысах самцах. Объект исследования – обонятельные луковицы новорождённых крыс – 1-3 суток, подсосного периода – 7, 14, 21 суток, инфантильного – 30 суток, ювенильного – 60, и зрелого – 180 суток. Исследование проведено на криостатных серийных срезах обонятельных луковиц (20 мкм). Для идентификации нитроксидэргических структур использовали гистохимическое маркирование NADPH-d (методом Хоупа). На стандартном срезе измеряли площадь NADPH-диафораза позитивных клеток по 100 в каждом случае, площадь гломерул, количество позитивных нейронов, окружающих гломерулу. ***Результаты.*** В результате исследования установлено, что в обонятельной луковице крыс изученных возрастных групп позитивность к NADPH-d проявляют только поверхностные и глубокие короткоаксонные нейроны, и перигломерулярные нейроны. Конечный продукт реакции распределяется в телах и отростках части клеток, плотность распределения зависит от слоя обонятельной луковицы и от возраста животных. Также позитивностью к ферменту обладают центральные части гломерул, причем распределение диафоразы зависит не от возраста, а от локализации гломерулы. ***Заключение.*** Возрастные преобразования позитивной субпопуляции нейронов обонятельной луковицы свидетельствуют об активном участии NO в процессах постнатальной дифференцировки, роста и развития обонятельного анализатора.

Ключевые слова: *нейрон, обонятельные луковицы, нейрогенез, NADPH-диафораза, онтогенез.*

DISTRIBUTION OF NADPH-DIAPHORASE POSITIVE STRUCTURES OF OLFACTORY BULB OF RATS IN ONTOGENESIS

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Aim: to identify peculiarities of distribution of NADPH-diaphorase (NADPH-d) positive structures in olfactory bulbs of rats of different age. ***Materials and Methods.*** The study was con-

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ducted on 22 white male rats. The object of research was olfactory bulbs of newborn rats – 1-3 days, suckling period – 7, 14, 21 days, infantile – 30 days, juvenile – 60, and mature – 180 days. The study was conducted on cryostat serial sections of olfactory bulbs (20 μm). Nitroxidergic structures were identified by histochemical labelling of NADPH-d (by Hope method). In a standard section, the area of NADPH-diaphorase-positive cells (100 in each case), surface area of glomeruli, the number of positive neurons surrounding a glomerulus, were measured. ***Results.*** In result of study it was found that in the olfactory bulb of rats of the studied age only groups of superficial and deep short-axon neurons and periglomerular neurons showed positivity to NADPH-d. The end product of reaction was distributed in somas and extensions of a part of cells with the density of distribution depending on the layer of the olfactory bulb and on the age of animals. Besides, positivity to the enzyme was also found in the central parts of glomeruli with distribution of diaphorase depending not on age, but rather on localization of glomeruli. ***Conclusion.*** Age-related transformations of positive subpopulation of neurons of olfactory bulb indicate active participation of NO in the processes of postnatal differentiation, growth and development of olfactory bulb.

Keywords: *neuron, olfactory bulbs, neurogenesis, NADPH-diaphorase, ontogenesis.*

Study of neurons containing nitroxide synthase has begun more than 40 years ago when cells with high activity of NADPH-diaphorase (NADPH-d) were found in cerebral tissues by histochemical methods. Activity of diaphorase in cells is determined by reduction of nitroblue tetrazolium into diformazan and serves as indication of the presence of NO-synthase [1]. But, however, due to existence of several isoforms of the given enzymes false positive results are possible [2,3]. The highest activity of the enzyme is found in neurons of the cerebellum and in astroglia. A lower level of activity is seen in the hypothalamus, mesencephalon, striatum, cortical representations, hippocampus and medulla oblongata [2,4]. Nitroxide synthase (NOS) are a family of enzymes that catalyze production of nitric oxide (NO) from L-arginine. Currently, three isoenzymatic forms of NOS are described: neuronal (n-NOS, or NOS-1), inducible by cytokines (i-NOS, or NOS-2) and endothelial (e-NOS, or NOS-3) [2,4].

NO is proved to be an important cellular signaling molecule that is widely represented in structures of the autonomic nervous system and can function as a retrograde neurotransmitter. Functions of NO are very diverse: it controls oscillatory activity of neurons, is a neurotransmitter for nociception, thermal sensitivity, olfaction, it modulates vessel tone and cerebral circulation, participates in the angiogenesis and in development of the nervous system [3], plays the leading role in long-term potentiation processes and, consequently, in learning and memory [5]. The source of NO in the central and peripheral nervous system is non-adrenergic non-cholinergic nerves and glutamate neurons, and also endotheliocytes of vessels, microglial cells and astrocytes. This substance was found to participate in regulation of neurogenesis, in a mature organism as well, through triggering apoptosis of excessive progenitor cells (elimination of “unnecessary” set) [6]. A histochemical marker of nitroxidergic neurons is known to be NADPH-d which is metabolically related to neuronal NO-synthase. Identification of NO-synthase and NADPH-d

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in cells of proliferative zones of the brain confirms the role of NO as a modulator and regulator of proliferative processes in the central nervous system (CNS) [4].

Olfactory bulbs (OB) are multifunctional structures and the place of migration of neuroblasts from the subgranular zone of the hippocampus and the subventricular zone of the lateral ventricles through the rostral migration flow. Having reached the middle of the olfactory bulb, chains of neuroblasts disintegrate, cells begin radial migration and reach the outer cell layers where they undergo final differentiation. Migrating neuroblasts radially diverge into all layers of OB forming numerous synaptic contacts and integrating into the local neuronal network [5]. It is known that at different stages of the neurogenesis neurons can synthesize many specific proteins, signaling molecules, gas neurotransmitters identification of which shows the neuronal differentiation of "descendants" of the progenitor cells [7,8]. The currently existing data about the influence of nitroxidergic compounds on the course and activity of neurogenesis are controversial, there is no detailed information about distribution of NADPH-d-positive structures in different layers of olfactory bulbs of rats, which impedes evaluation of the direction of compensatory-adaptive reactions in experimental influences on the central nervous system.

The aim of study was to identify peculiarities of distribution of NADPH-d-positive structures in olfactory bulbs of rats of different age.

Materials and Methods

The work was conducted on 22 white male rats of *Wistar* line. The age of rats was selected according to the age-related periodization of a white rat ontogenesis proposed by I.P. Zapadnyuk, et al. (1974) on the basis of physiological peculiarities, growth intensity, behavioral reactions, change in the character of nutrition, body mass, functional maturity of animals. All stages of the work were conducted in compliance with European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Directive 2010/63/EU).

The object of research was olfactory bulbs of newborn rats of the age 1-3 days, suckling period – 7, 14, 21 days, infantile – 30 days, juvenile – 60, and mature – 180 days. The material was taken after preliminary transcardial perfusion with 10% buffered formalin with subsequent fixation within 24 hours at 4°C, washing and keeping for 24 hours in 30% saccharose solution. The study was conducted on cryostat parasagittal serial sections of the right and left olfactory bulbs of 20 μm thickness. For identification of nitroxidergic structures, histochemical labelling of NADPH-d was used. Activity of NADPH-diaphorase was determined by Hope method (Hope, Vincent; 1989) [1]. The sections were incubated in the medium containing 0.5 mM NADPH (Sigma, США), 0.5 mM nitro blue tetrazolium (Sigma, США), and 0.3% Triton X-100 in 0.15 M Tris-HCL buffer (pH-8.0) at 37°C within 60 min., after which the sections were washed in distilled water, dehydrated and placed into balm. For microscopy light microscope Optica DM-20 (Italy 2015) with built-in camera was used. In each standard section the number of positive neurons was determined. A standard section was considered a parasagittal section of the maximal surface area passing through the central zone of OB. The surface area of NADPH-diaphorase positive cells (100 cells in each case), surface area of glomeruli were measured, the number of positive neurons surrounding a glomerulus were determined. By the density of distribution of formazan in the

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cytoplasm of neurons of olfactory bulbs, three kinds of neurons were distinguished: neurons with high, medium and low degree of activity of NADPH-d. Analysis and processing of the obtained images were conducted using ImageJ program. Statistical processing of data was conducted by variation statistics method using Microsoft Excel 2010 and Statistica 10 programs. The mean values of the obtained parameters are presented in (M±m) form where M is arithmetic mean, and m is standard error of mean. Differences in parameters between the groups were analyzed using Student t-test. Reliable differences of compared parameters were considered those at $p<0.05$.

Results and Discussion

Use of parasagittal sections passing through the longitudinal axis of the bulb permitted to study distribution of positive cells in all six cytoarchitectonic layers arranged in the following order: a layer of the olfactory nerve, glomerular layer, external plexiform layer, mitral cell layer, internal plexiform layer, granular (granule cell) layer. In the center of the bulb the rostral migration flow terminates forming subependymal layer which smoothly continues to the granule cell layer (the central zone of the bulb).

According to the data of R. Spessert, S. Reuss, there are 5 morphological types of nerve cells in the olfactory bulb: superficial and deep short-axon cells, mitral cells, periglomerular cells and granule cells [9]. Superficial short-axon neurons (SSAN) are located in the periglomerular area and in the external plexiform layer. Deep short-axon neurons (DSAN) lie in the granular layer near its transition into subependymal layer. Mitral cells are arranged in a band forming a layer. Periglomerular cells reside in a glomerular layer and form associations with glomeruli; granular neurons make the most numerous subpopulation of the granular layer.

In sexually mature animals activity of NADPH-d was identified in somas of neurons. Nuclei of neurons were not stained. In many cases NADPH-d-positive axons and dendrites were seen in a long distance reaching the adjacent neurons. On removal of NADPH from the incubation medium no staining was observed. Within the glomerular layer intensity of staining significantly varied. The brightest staining was seen in dorsally located glomeruli.

NADPH-d activity was identified in superficial and deep short-axon neurons, periglomerular and a part of granular neurons. Mitral cells were always negative. A similar distribution of the enzyme in sexually mature rats was described by B. Samama, C. Crespo [10,11]. No data were found in literature about age-related peculiarities of the distribution of the end product of reaction to diaphorase, but, evaluating the obtained data we found differences in the topography and density of distribution of the end product. Let us consider them by layers and by ages.

In granular and subependymal layers of the olfactory bulb in 1-day animals the only NADPH-d-positive neurons were deep short-axon Golgi neurons (DSAN) in the quantity of 8.7±0.85 in the standard section. These are large multipolar neurons with a high density of distribution of the product of reaction, with formazan densely filling the cytoplasm of cells and distinctly labelling extensions of neurons along a significant distance. The average surface area of these neurons was 126.2±13.64 μm^2. Their maximal accumulation was noted in the area of transition of the distal part of the rostral migration flow into subependymal layer of OB (Fig. 2B).

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In 7-day rats the number of DSAN in the granular and subependymal layers in the standard section of OB increased to 12.7±0.50 ($p<0.05$), with increase of the average surface area of their cross section to 170.4±18.30 μm^2 ($p<0.05$).

In 14-day animals neurons with low and medium activity of the enzyme were identified in the granular layer, they were having a large nucleus, a thin rim of cytoplasm and two long positive extensions coursing radially (Figs. 1A, 1B).

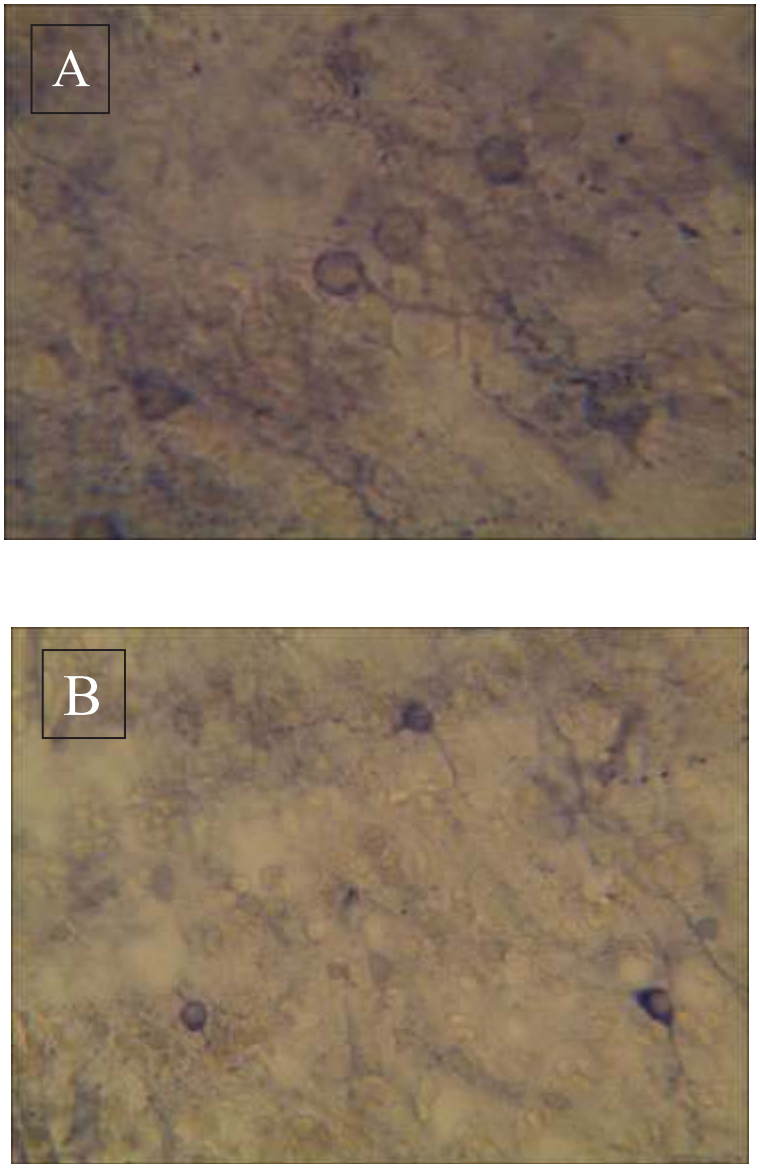

Fig.1. NADPH-d-positive cells of the granular layer of olfactory bulb of 14-day animals.
A – granule cells with low positivity. B – subpopulation of granule cells with medium positivity of the enzyme. Histochemical staining by Hope method (Hope, Vincent; 1989).
Objective magnification of 100 (A), 40 (B)

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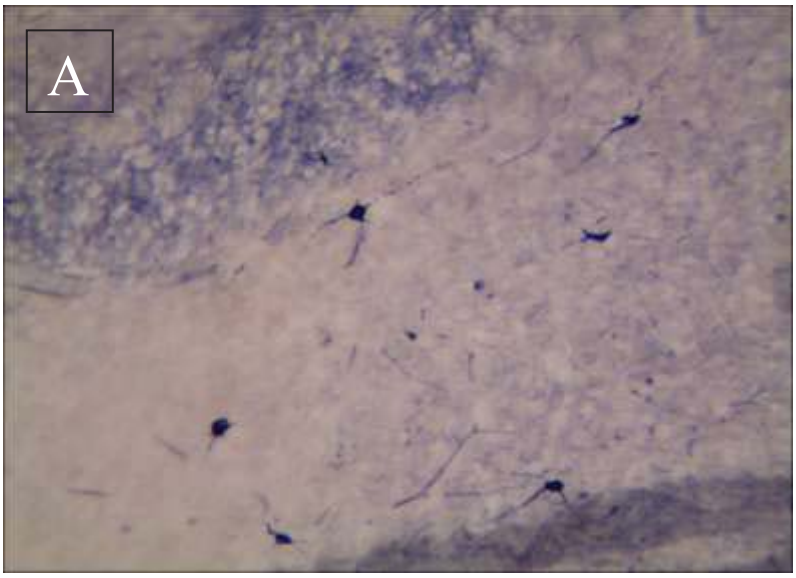
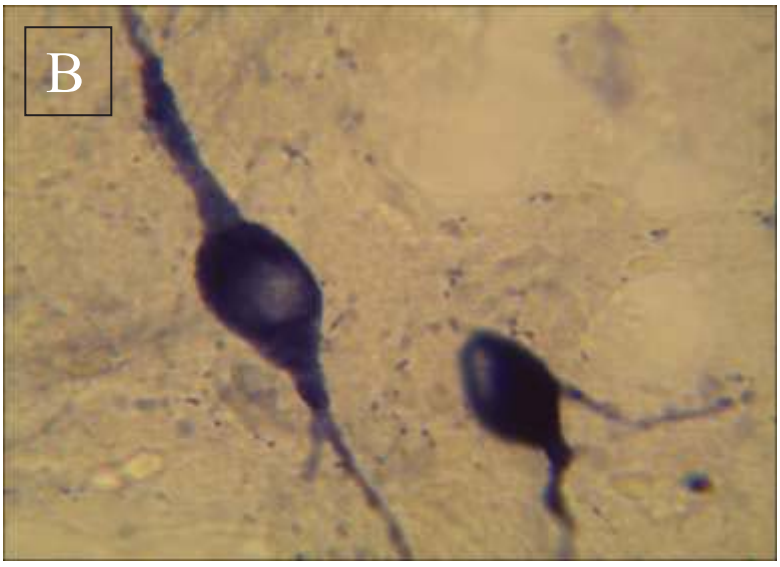
All the rest of neurons of the granular layer were enzyme-negative. DSAN of subependymal layer revealed the highest activity of the enzyme, their quantity increased two-fold in comparison with newborn animals and reached 16.7±0.68 in the standard section. The average surface area of this subpopulation of neurons was 325.7±16.40 μm^2 (Figs. 2A, 2B). The extensions, as a rule, branch off and in some places enlace a vessel forming an evident neuropile (Fig. 2B).

In the period from 14 to 21 days the number of DSAN decreased to 12.1±0.60 ($p<0,05$).

In age groups from 21 to 180 days the quantity of positive DSAN in the standard section did not show reliable changes, activity of the enzyme in the cytoplasm remained high. No cells with medium and low activity of the enzyme were identified.

Mitral cells in all the studied age groups of animals did not show nitroxidergic positivity. Activity of NADPH-d in the layer of olfactory nerve was high and did not depend on the age of animals.





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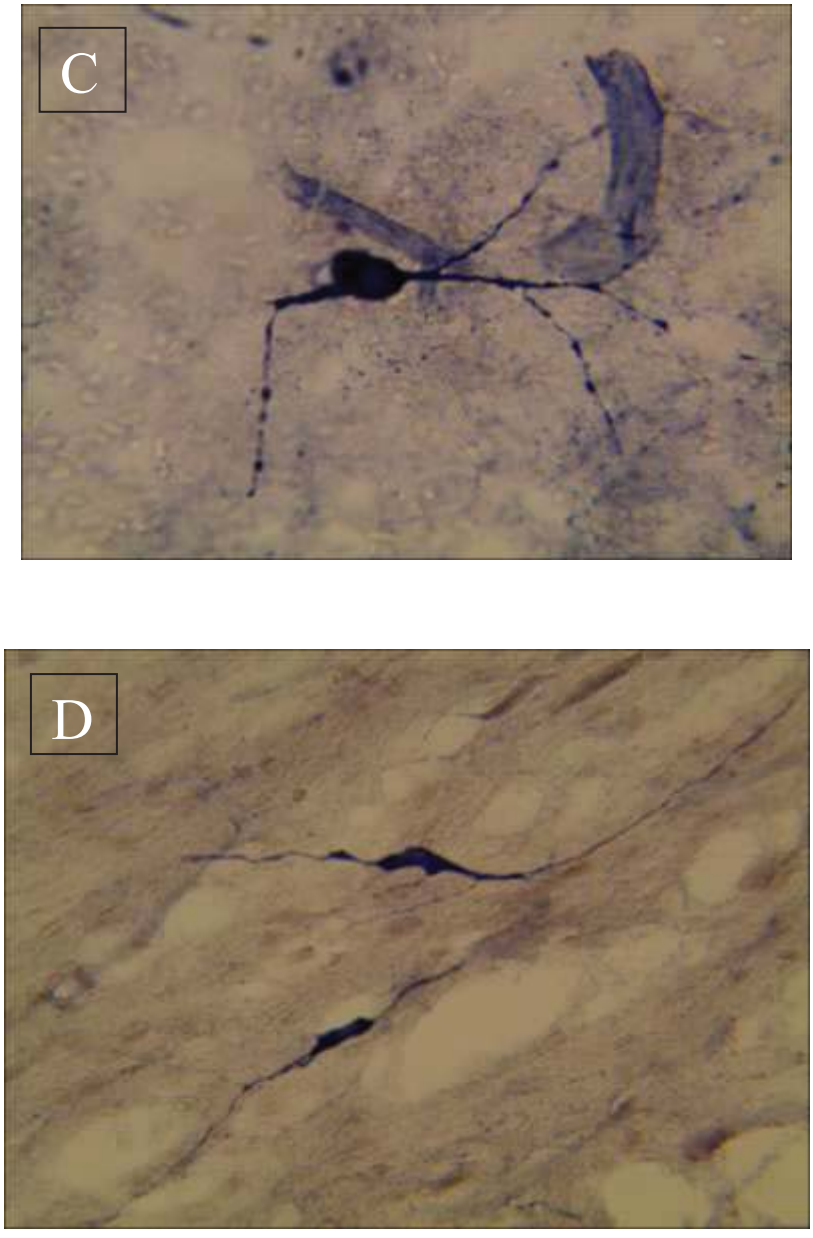

Fig. 2. NADPH-d-positive cells of subependymal layer of OB of 14-day animals.
A B, C – subpopulation of Golgi DSAN with high positivity of enzyme and evident neuropile.
D – single diaphorase-positive DSAN. Histochemical staining by Hope method
(Hope, Vincent; 1989). Objective magnification 10 (A), 100 (B), 40 (C, D)

In the region of transition of the glomerular layer into the external plexiform layer large single bipolar neurons (superficial short-axon neurons, SSAN) with high activity of NADPH-d were identified oriented parallel to the row of mitral cells. The number of these neurons in standard section did not exceed 1-2, the surface area ranged from 160 to 210 μm^2 irrespective of the age (Fig. 2D).

In the glomerular layer of OB of a rat of 6 months age evident age-related peculiarities of distribution of the enzyme were also noted.

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The glomerular layer in newborn rats was poorly developed and consisted of superpositive oval glomeruli arranged in one row mainly on the dorsal surface and apex of OB (Fig. 3A). The surface area of glomeruli was 1107.9±117.96 μm^2. Periglomerular neurons did not exhibit positivity to NADPH-d.

In 7-day animals glomeruli were arranged in a row along the perimeter of the bulb around which positive periglomerular neurons were located. They were small rounded cells that non-uniformly surrounded glomeruli (4-8 cells around each glomerulus), with the majority possessing the medium level of NADPH-d-positivity. The average surface area of periglomerular neurons was 42.9±5.33 μm^2. The neuropile of these neurons was negative (Fig. 3B).

In 14-day animals the area of glomeruli increased 3-fold (2998.1±513.13 μm ^{2}). Glomeruli were positioned close to each other and arranged in groups of 3-4 cells. Maximally positive to enzymes were glomeruli located dorsally and on the apex of OB. Glomeruli on the ventral surface exhibited medium and low activity. Each single glomerulus was surrounded by 5-16 cells having a positive perikaryon with a single extension directed toward the glomerulus Figs. 3C, 3D). The average area of their cross section increased to 51.9±2.86 μm ^{2}.

In 21-day animals the average surface of glomeruli was maximal and reached 4225.5±314.80 μm^2. The amount and morphology of these neurons did not change (Figs. 3E, 3F). The average area of these neurons was 65.1±4.43 μm^2.

In one-month and six-month animals the morphology of glomeruli practically did not change in comparison with 21-day animals, the glomeruli reached the maximum size on the 30th day (3724.4±316.15 μm^2), and by the 180th day gradually decreased to 2504.2±133.23 μm^2. The area and quantity of periglomerular positive cells in this period of observation kept on the reached values.

In all age groups the central parts of glomeruli were positive to NADPH-d, glomeruli of the dorsal surface and the apex of OB were superpositive, and those of the ventral surface possessed medium and low positivity, here, distribution of diaphorase depended not on age, but rather of location of a glomerulus: glomeruli of the dorsal surface of OB possessed high activity, and glomeruli of the ventral surface possessed low and medium activity. This may serve as an additional evidence of functional differences between glomeruli of the olfactory bulb that was noticed in the works of R. Spessert and E. Layes [9]. Positivity of glomeruli may be possibly related with the activity of diaphorase of the primary olfactory sensory axons incorporated into the glomeruli [12].

Thus, NADPH-d was identified in three heterogenous subpopulations of neurons of OB, and age-related changes in the quantity and activity of this enzyme analogous to the activity of NO-synthase [4,13] have common and specific features.

DSAN were identified since birth, they possessed high activity, their quantity in a standard section increased 2-fold within 21 days and after that did not change. During the time of observation the area of neurons of this population increased almost two-fold. Location of these cells in the orifice of rostral migration flow, that is, in the region of maximal accumulation of neuronal precursors, indicated their role in regulation of cell composition of OB, and participation of NO in regulation of apoptosis of neurons was described in works of H.G. Kuhn and C. Crespo [6,11]. The similar age-related dynamics of NADPH-

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d activity in some hypothalamic nuclei was described in works of D.K. Obukhova, V.I. Dunaj, L. Villani conducted on fish, guinea-pigs and rats [4,14,15].

SSAN located in the region of transition of the glomerular layer to the external plexiform layer were few in number, possessed high activity and did not increase in size within 180 days of observation. Taking into account the direction of extensions of these cells parallel to the surface, it is possible to consider them a subpopulation of association inhibitory neurons, since many authors describe GABA-positive neurons in this zone [8,11]. Thus, it may be suggested that short-axon neurons are single active cells that participate in regulation of the blood flow in microcirculatory bed through gas-neurotrans-mitter NO-system.

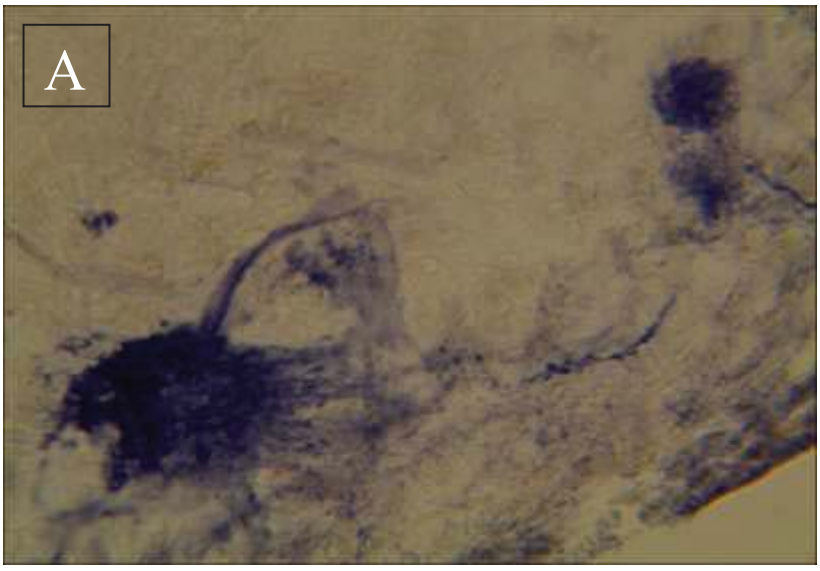
Periglomerular neurons were characterized by low or medium level of activity of the enzyme. Here, during observation the activity in them gradually increased from the zero level in newborns to the medium level in 14-day rats. The size and number of periglomerular neurons reached the definite level as early as by the 21st day and after that showed no reliable changes. Such early maturation of subpopulation occurred in parallel with reduction in the activity of neurogenesis identified in the previous works by the activity of its markers [13].

Conclusions

1. In the result of study it was found that in the olfactory bulb of rats of examined age groups from newborn period to 180 days, positivity to NADPH-d was observed only in superficial and deep short-axon neurons located in the subependymal, external plexiform layers, and in periglomerular neurons.

2. Each of the subpopulations was characterized by its own dynamics of the activity of the studied enzyme.

3. Age-related transformations of positive subpopulations of neurons indicated active participation of NO in the processes of postnatal differentiation, growth and development of the olfactory bulb.



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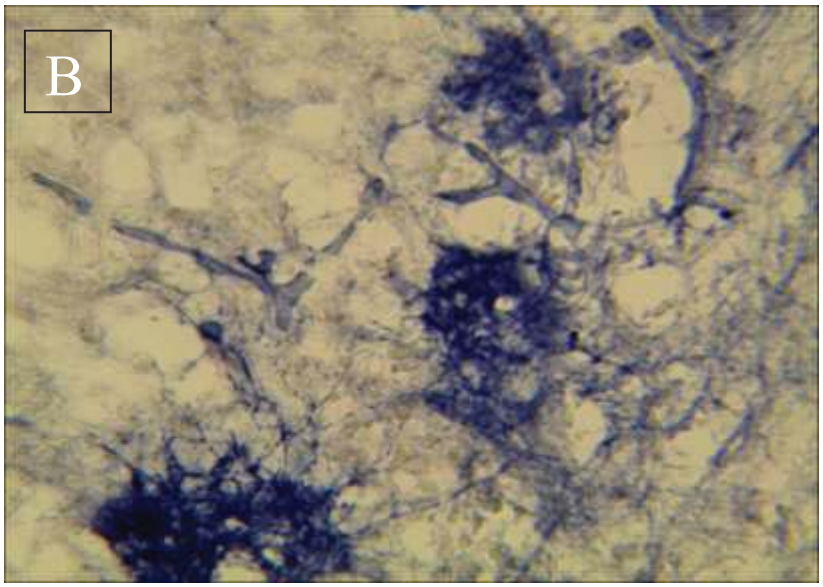

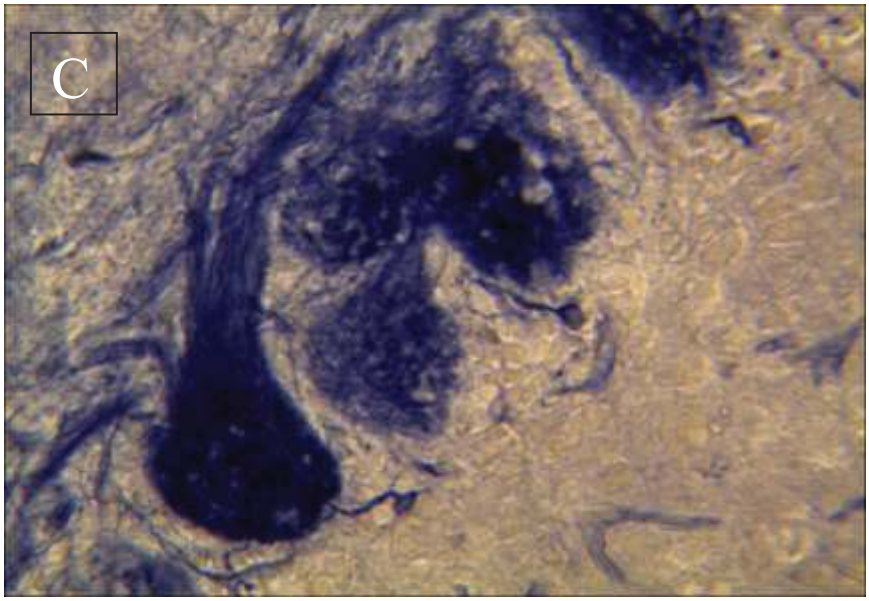

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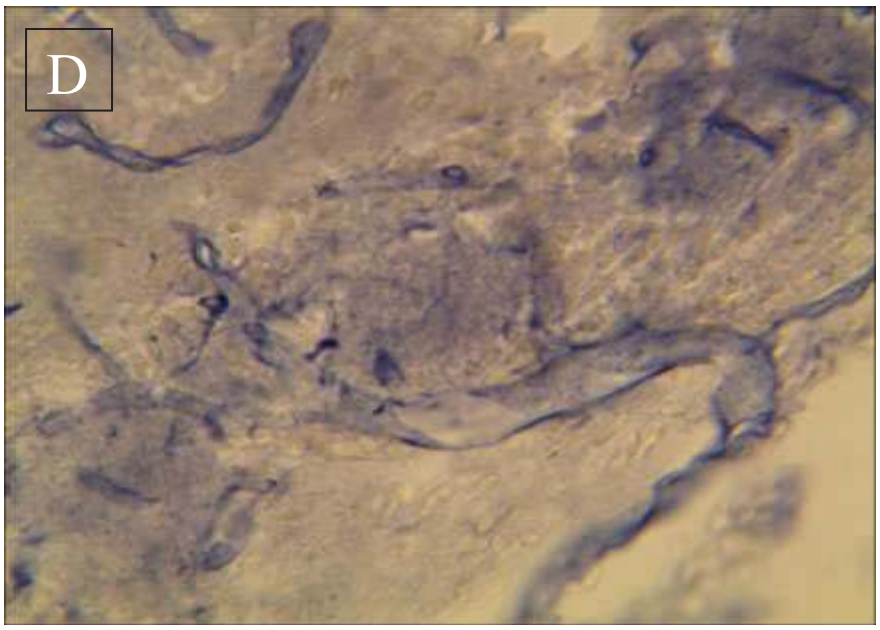

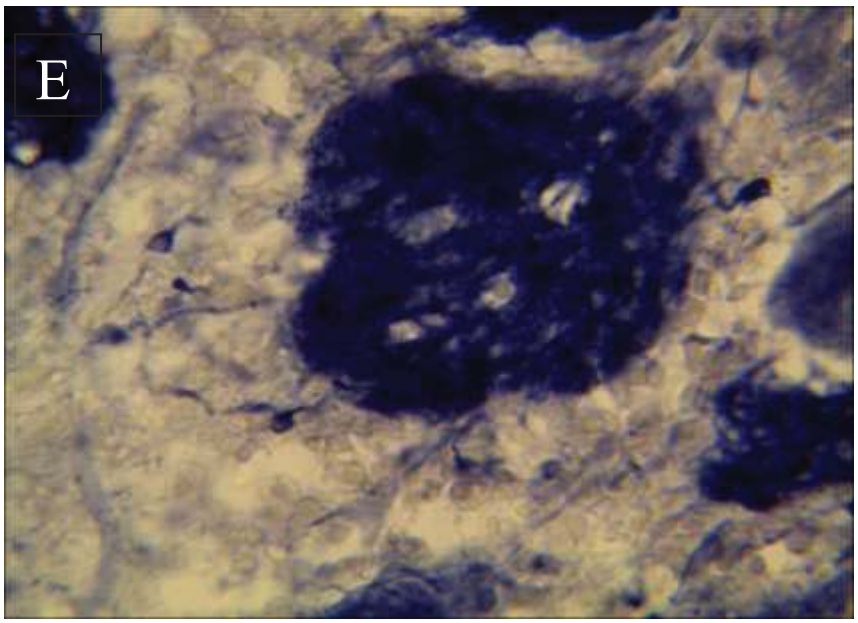

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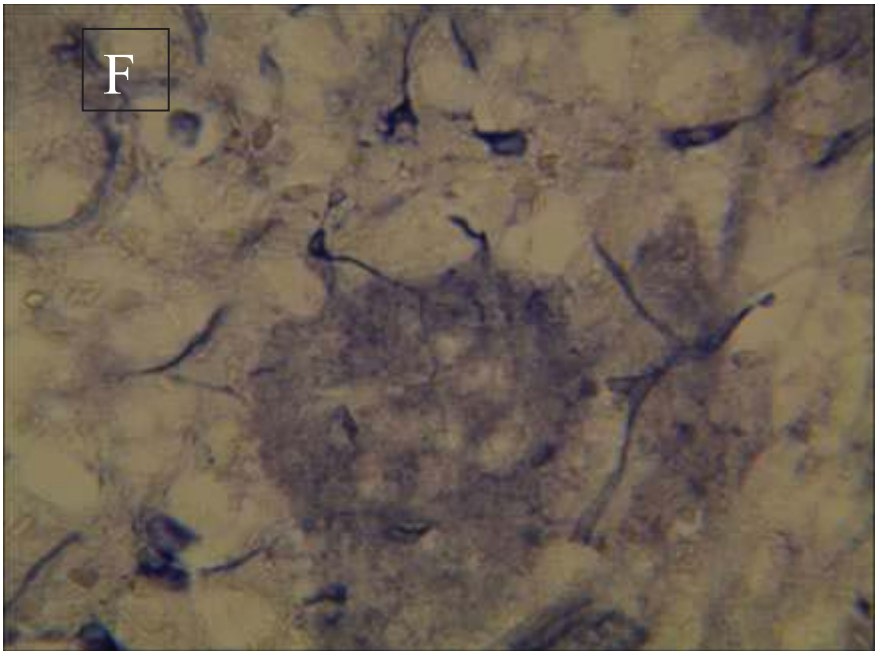

Fig. 3. Distribution of NADPH-d-positive structures in glomerular layer of OB of animals of different age. A – glomeruli with medium positivity of enzyme (1 day). B – glomeruli with medium positivity of enzyme with single NADPH-d-positive periglomerular cells and vessels (7 days). C – glomeruli with high positivity of enzyme on the dorsal surface of OB (14 days). D – glomeruli with low positivity of the enzyme with association of periglomerular cells on the ventral surface of OB (14 days). E – glomeruli with high positivity of enzyme on the dorsal surface of OB (21 days). F – glomeruli with low positivity of the enzyme with association of periglomerular cells on the ventral surface of OB (21 days). Histochemical staining by Hope method (Hope, Vincent: 1989). Objective magnification 10

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