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Chapter 5

PARTICIPATION OF CATECHOLAMINES, H_2S AND NO IN NEUROTRANSMISSION, NEUROMODULATION AND REGULATION OF ADULT NEUROGENESIS IN CARP BRAIN

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ABSTRACT

The chapter considers the overall organization of the main parts of brain in cyprinoid fish. It is described general cytoarchitectonical acpects of location, elements of neural structure and the system of relations in the most important centers of brainstem, and the forebrain - as the highest integrative center of the fish brain. It is presented a new data about adult neurogenesis and neurochemical (mediator) architectonics of the carp brain. It is described the some zones of neurogenesis in the adult carp brain, comparative data about immunolocalization of hydrogen sulphide producing enzyme the cystathionine β-synthase, NADPH-diaphorase and tyrosine hydroxylase in the different regions of carp brain. It is discussed the involvement of these neurotransmitters and gaseous intermediators in

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the regulation of neurotransmission in the different brain centers and processes of adult neurogenesis.

Catecholaminergic (CA-ergic) systems in the brainstem of carp were studied using immunohistochemical labeling of tyrosine hydroxylase. The peculiarities of localization of medullary neurons, morphology of the dendrites, and trajectories of the axon projections in the medulla of the carp allow us to differentiate three groups of neurons, namely interfascicular cells, units related to the *lobus vagus*, and cells localized within the *area postrema*. In the periventricular region of this cerebral zone, we found phenotypically immature forms of the TH-ip cells. We hypothesize that, in carp, dopamine functions as an inductor of development (morphogenetic factor) and is involved in adult neurogenesis in matrix zones of the brain.

H_2S-producing cells and fibers are located in all parts of the brain carp and may participate in the modulation other neurotransmitter systems of the brain. NADPH-d-producing cells have been identified in different parts of the brain: habenular nuclei, medial hypothalamus, isthmus, reticular formation and ventral column of spinal cord. Most of these nuclei in the brain cyprinoid fish are cholinergic centers. Thus, in carp brain nitric oxide can be considered as a modulator of cholinergic neurotransmission. In the carp brain have been found populations H_2S-producing cells in the areas of primary and secondary neurogenesis. H_2S-producing cells have been identified in the periventricular area of diencephalon, medulla oblongata and granular eminentia of cerebellum. We believe that H_2S may participate in the processes regulation of adult neurogenesis, cells migration and differentiation in these areas of the brain. Among all NO-producing cells in the adult carp brain was discovered population of NADPH-d positive cells, which had a high level of enzyme activity and located on the dorsal surfaces of telencephalon. This zone is a border of embryonic eversion, where activity of proliferative nuclear antigen was identified. Radial-oriented fibers and cells with a high activity of NADPH-d have been revealed in medial thalamus, marginal layer of optical tectum and external wall of brainstem. NADPH-d positive cells in these areas had a high level of enzyme activity, large nucleus located in central part of cells and high value of nuclear-cytoplasmic ratio. We believe that NO-producing cells are also involved in adult neurogenesis, because NADPH-d positive cells are located on the territory of secondary neurogenesis zones. However, H_2S-producing and NO-producing populations of cells in the carp brain have different spatial localizations. Thus, we believe that during the adult neurogenesis in carp brain may be involved various signaltransductor systems.

1. SHORT OVERVIEW OF THE STRUCTURE OF THE TELEOST FISH BRAIN

The Actinopterygii (ray-finned fish) are the largest group of the vertebrates, including around 27,000 species, and most of them comprise teleost fish (around 26,800 species). *Cyprinidae* is the largest teleost family in fresh waters fishes [1, 2].

Investigations of the morphological organization of the central nervous system in fish species have played an important role in our understanding of various theoretical and applied aspects in fisheries biology. Information from a detail analysis of the brain structure and function is a necessary for understand results physiological, biochemical and behavioral experiments.

1.1. Telencephalon

Telencephalon of the actinopterigian fishes develops in a fundamentally different way from that of all vertebrates. During the early studies of brain development, the lateral walls of the primordium forebrain evaginate, thereby leading to the formation of everted cerebral hemispheres, without lateral cerebral ventricles.

The cyprinidae (*Cyprinus carpio* L.) telencephalon consist of two olfactory bulbs and hemispheres. Olfactory bulbs are not everted and adjoin with hemispheres olfactory tractus. In the hemispheres are well defined dorsal (D, pallial) and ventral (V, subpallial) areas. Four zones can be distinguished in the pallium: dorso-medial (D.m.), dorso-dorsal (D.d.), dorso-central (D.c.) and dorso-lateral (D.d-l.) with subdivisions. (Figure 1). In caudal pole hemispheres dorso-posterior (D.p.) zone are distinguished. There is that at least a part of D.l., usually D-l.v., is homologous to the medial pallium in amphibians and other tetrapods. The least part, if not all, of D.m. in teleosts is homologous to the pallial amygdala in amphibians and other tetrapods. D.d. can be considered as a possible homologue of the dorsal pallium (isocortex) of tetrapods. Dorso-posterior telencephalic area (D.p.) is the major recipient of secondary olfactory input and is considered the lateral pallium (or olfactory cortex) homologue. The pallial lateral zone (D.l.) has been described as a visual area, the lateral, central (D.c.) and medial (D.m.) zones as lateral line mechanosensory, the lateral and medial zones as auditory, the medial and

central zones as somatosensory, and the medial zone as gustatory-recipient zones.

In the ventral areas four cytoarchitectonics zones are present: ventral (V.v.), dorsal (V.d.), intermedial (Vi.) and lateral (V.l.). It is separated from the dorsal areas by the *zona limitans*. Zones V.d. and V.i. can be considered as a possible homologue of the striato-pallidar complex tetrapod telencephalon. Ventral (V.v.) and lateral (V.l.) zones may be homologous to part of the septum and other medial subpallial regions of other vertebrates [3-5]. Neuronal organization of the telencephalon demonstrated that general level of neuronal differentiation is rather low. The pallium contains neurons three main types: fan-shaped (FN), radial (RN) and particular neurons medial zones (PN). Most neurons are represented by several varieties of poorly differentiated *isodendritic* cells, corresponding to Ramon-Moliner classification [6]. Morphological basis converge sensory information in the pallial zones is the presence of large *radial isodendritic* neurons (RN), those dendritic and axonal systems occupy large areas of hemispheres (Figure 1). The subpallial neurons is even less differentiated: majority of cells are the most primitive types of *isodendritic* neurons (*lepto- and lophodendritic* types) [7].

Thus telencephalon is the integrative center of the CNS in fish because its structure receive and process multimodal information from visual, olfactory, somato-sensory and octavo-lateral systems.

1.2. Diencephalon

The diencephalon is a large division forebrain, composed of several areas. The dorso-caudal area called the **pretectum,** ventral area called the **posterior tuberculum**. More rostrally, four areas are present: **epithalamus**, **dorsal thalamus**, **ventral thalamus**, and **hypothalamus** [5, 8].

Pretectum is a region that lies between the dorsal thalamus and the optic tectum. Its nuclei occupy **superficial**, **central**, and **periventricular zones.** The major inputs to the pretectum are from the retina and the optic tectum. The efferent pathways of the pretectum are involved in the regulation of eye and body movements in relation to prey and other features of the visual world.

Ventral to the area of the pretectum there is zone of migrated nuclei called the **posterior tuberculum.** Only in teleost fishes migrated nuclei of the posterior tuberculum are present. These preglomerular nuclei have a high degree of cytoarchitectonic differentiation. Its cells contain dopamine and may be homologous to *substantia nigra* and *ventral tegmental area* of amniote

vertebrates. The largest of these nuclei, the **preglomerular nuclear complex**, relays sensory information to the telencephalon from the auditory, the lateral line mechanosensory, the electrosensory, the gustatory, the somatosensory, and the visual systems. These pathways through the migrated nuclei of the posterior tuberculum are similar to the sensory pathways through the dorsal thalamic nuclei of amniotes [9, 10].

The **dorsal thalamus** includes nuclei that relay sensory information to the telencephalon. In anamniotes, a significant portion of the dorsal thalamic projections to the telencephalon are bilateral or ipsilateral. In most teleost fishes, only three dorsal thalamic nuclei are present. In contrast, amniotes have of many nuclei and/or nuclear groups [5].

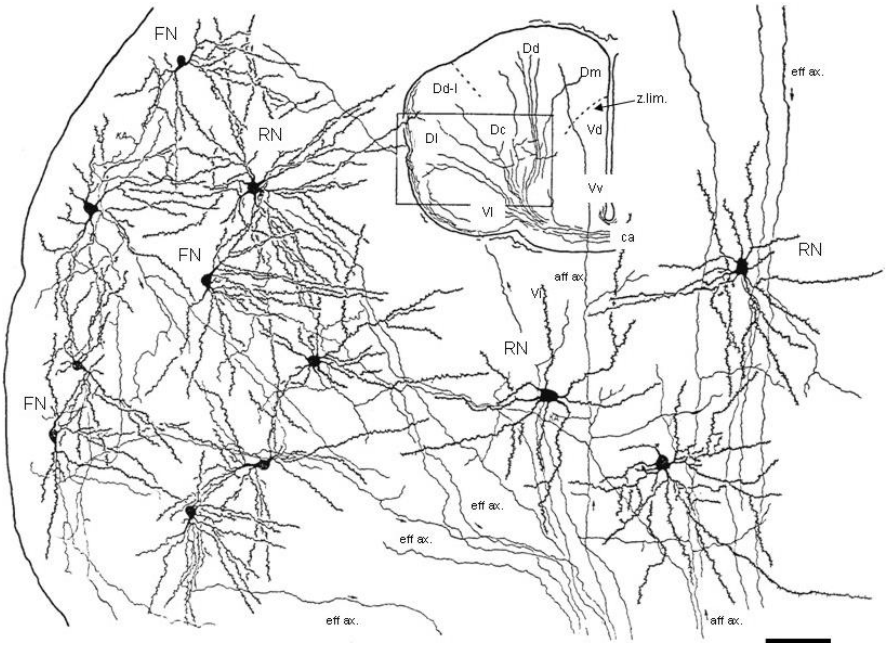

Figure 1. Fragment neuronal structure of the telencephalon of *Cyprinus carpio* L. Golgi-staning method. Drawings produced using a camera lucida. Abbreviations: ca – commissure anterior, D – area dorsalis telencephali (pallium), Dm, Dd, Dd-l, Dc, Dl – medial, dorsal,dorso-lateral, central and lateral zones of area dorsalis; V – area ventralis telencephali (subpallium), Vd, Vv, Vl, Vi – dorsal, ventral, lateral and intermedia zones ventral area; z.lim – zona limitans; FN, RN – fan-sheped, radial neuron isodendritic types; effax, affax – efferent and afferent axons. Scale bar 100 µm.

The **ventral thalamus** lies ventral to the dorsal thalamus and is involved with some sensory processing and with motor control systems. In most anamniotes, at least three nuclei are present in the ventral thalamus. In ray-finned fishes, these nuclei have been termed **nucleus intermedius**, **nucleus ventro-medialis**, and **nucleus ventro-lateralis**. These all nuclei receive retinal projections, but most of their other connections remain to be investigated.

The **epithalamus** is the most dorsal part of the diencephalon. In most vertebrates, include teleosts fishes, it is composed of two major parts: the **epiphysis** (**pineal gland** and related structures) and the **habenula,** which comprises the habenular nuclei. The habenula is asymmetrical in size (as in other vertebrates). In addition to pineal afferent connections, it receives inputs from a subpallial part of the telencephalon, called Vd (the dorsal nucleus of area ventralis). The epithalamus is involved with the maintenance of circadian rhythms in response to the light cycle and is part of a major pathway for the integration of limbic and striatal systems. The **hypothalamus** is a specialized region of the ventral diencephalon that is involved in regulation of the endocrine system, the autonomic nervous system and also is concerned with feeding and drinking, aggression, temperature regulation, and a number of other important biological functions. In cyprinid fishes, the hypothalamus consists of a dorsal, ventral divisions and region of the hypophysis. The inferior lobe ventral hypothalamus in cyprinid fishes appears to be specialized for gustatory input to the hypothalamus - it receives axons from the secondary gustatory nucleus.

1.3. Mesencephalon

The **mesencephalon** (midbrain) contains three major regions: **tectum**, **tegmentum** and **isthmus.**

In the most teleosts, the tectum is comprised of six tangential layers. The composition of these layers is differentiated by their fiber and cell constituents and the details of individual layers show some ecological correlations related to visual abilities and to eye structure. From the pia mater to the ventricle, the layers are typically named *stratum fibrosum marginale* (SM), *stratum opticum* (SO), *stratum fibrosum et griseum superficiale* (SFGS), *stratum griseum centrale* (SGC), *stratum album centrale* (SAC), and *stratum griseum periventriculare* (SPV). Functionally, distinction is between the superficial layers (SM, SO, SFGS), which receive mainly visual afferent fibers, and the deep layers (SGC, SAC, SPV) which receive multimodal sensory and motor

information from different sources (telencephalon; thalamic and pretectal areas; structures brainstem and other) [8].

As a result of the expansion of the tectal lobes, the **torus semicircularis** lies ventral to the tectal ventricle. The *torus semicircularis* is the site of termination of auditory and lateral line fibers. Its receives inputs from a variety of sources in addition to the octavolateral system, including the reticular formation, optic tectum, and diencephalon. The **torus longitudinalis** is a small structure that is unique to ray-finned fishes. It is part of a descending visual pathway to the *optic tectum* and *corpus cerebelli*.

The ventral portion of the mesencephalon is known as the **tegmentum**. The mesencephalic *tegmentum* contains a number of nuclei and fiber tracts, including the nuclei of two motor cranial nerves that innervate of the extraocular muscles for control of eye movements and transitional area called the **isthmus**, that contains cholinergic neurons, reciprocally and topographically connected with the optic tectum. Tegmentum consist of the rostral continuations of areas present in the hindbrain: the reticular formation, the oculomotor cranial nerve nuclei, and ascending and descending fiber systems. Additional nuclei, related to visuospatial functions, motor activities, and relay to the forebrain of ascending sensory information, are also present. The midbrain tegmentum is thus a gateway for incoming sensory information and outgoing motor responses to and from the forebrain.

1.4. Cerebellum

In teleosts the cerebellum can be divided into three major parts: the ***corpus cerebelli***, ***valvula cerebelli*** and ***vestibule-lateral lobe*** (***eminentia granuralis and lobus caudalis*****)**, based on morphology and their relationship to each other. The proportion of each region varies greatly among species. The *corpus cerebelli*, considered homologous to the vermis, lies in the central portion of the teleost cerebellum and expands dorsally. The *valvula* protrudes rostrally into the mesencephalic ventricle and is covered with the optic tectum, the major part of the primary visual center of the teleost brain. In some species including *Ciprinidae*, the *valvula* is subdivided into medial and lateral lobes. The valvula is unique to actinopterygian fish and is not obviously the homologue of any cerebellar components of other vertebrates. Corpus and valvula cerebelli, the latter as a rostral extension beneath the optic tectum, are intimately connected and appear to play roles in spatial orientation, proprioception, motor coordination, and eye movement.

As the all vertebrates, the cerebellar cortex teleost consists of three layers: the molecular layer, the Purkinje cell layer, and the granule cell layer. The teleost Purkinje cell layer is referred to as the ganglionic layer because it includes not only the Purkinje cells but also the cells which are the projection efferent neurons of the teleost cerebellum. These efferent neurons project their axons to other brain regions using an excitatory neurotransmitter. The lack of the deep cerebellar nuclei is the one of the unique morphological features of the teleost cerebellum [5]. Teleost cerebellum receives inputs via both climbing and mossy fibers. The climbing fibers originate in the inferior olive; this is located in the ventromedial part of the caudal rhombencephalon and ventrolaterally adjacent to the raphe nucleus. Mossy fibers derive from many nuclei originating from the diencephalon, mesencephalon, rhombencephalon and spinal cord. The main functions of the teleost cerebellum are thought to involve control of movements, for the execution of swimming gait, controlling the vestibulo-ocular reflex, and emotional learning.

1.5. Myelencephalon (Rhombencephalon, Hindbrain)

The rhombencephalon is settle down between the spinal cord and the metencephalon. The boundary to the spinal cord is marked by the closure of the IVth ventricle. It is receives cranial nerve sensory information and provides information processing for reactions through the reticular formation as well as change of behaviour and vegetative functions [11]. As with the spinal cord, the myelencephalon consists of an alar plate, receiving and processing sensory information and a basal plate, include motoneurons centers cranial nerve and the reticular formation. The roof of plate is extended and attenuated to form the choroid plexus of the fourth (IVth) ventricle.

Within the central nervous system teleosts fishes, as the all vertebrates, the hindbrain contains all of the **mixed cranial nerves** (the trigeminal, facial, glossopharyngeus, and vagus nerves), the one cranial nerve with only branchial motoneurons (BM; the accessory nerve), and three of the four cranial nerves with only **somatomotor efferents** (SM; the hypoglossus, abducens and trochlearis nerves). The cranial nerve nuclei of the hindbrain receive general **somatosensory** information from the face (via the trigeminal, facial, and vagal nerves), **special visceral afferents** (taste information via the facial, glossopharyngeus, and vagus nerves) and some **special somatic afferents** (vestibular and auditory information via the vestibulocochlear nerve and lateral line mechanosensory and electroreceptive information via the lateral

line nerves. In the medulla, the mechanoreceptive lateral line fibers terminate primarily in two structures, the **eminentia granularis** of the cerebellum and part of the **lateralis column** [5, 8]. In bony fishes, the lateralis column consists of a large nucleus that forms most of the column's rostrocaudal extent, called **nucleus medialis** or **nucleus intermedius**. The more caudal nucleus in the column, **nucleus caudalis**, also receives mechanosensory input. **Visceromotor effernts** are associated with the facial, the glossopharyngeal and the vagus nerves [11].

2. HYDROGEN SULFIDE IN THE CARP BRAIN

The majority of studies on hydrogen sulfide have been focused on its toxic effects; however, recently it has been found that H_2S is a physiologically active mediator [12, 13]. This finding is based on the discovery of high concentrations of endogenous sulfides in the blood and brain tissues of mammals and some other vertebrates [14]. Endogenously H_2S is synthesized from L-cysteine by pyridoxal-5'-phosphate-dependent enzymes, cystathionine β-synthase (CBS), and cystathionine γ-lyase (CSE), which are expressed in many tissues [14, 15]. H_2S is involved in the relaxation of smooth muscles of mammals and humans [14]; physiological concentrations of this gas enhance the activity of NMDA receptors and facilitate induction of LTP in the hippocampus [15]. An analysis of the localization of CBS, which is an immunohistochemical marker of H_2S in the brain of bony fishes has been never made before. In this study, we study the localization of cystathionine β-synthase in the CNS and vessels of the brain of carp *Cyprinus carpio*.

2.1. Immunohistochemistry of CBS

We used 10 3-year-old *Cyprinus carpio*. The animals were anesthetized using a 0.1% solution of tricaine methanesulfonate (MS-222, Sigma, United States) for 10–15 min. The material was obtained from the Ryazan experimental-manufacturing fish-breeding plant in 2013. To identify H_2S-producing neurons, we used the method of indirect avidin-biotin-peroxidase (the ABC method) labeling of free-floating slices. The brain was fixed for 2 hours at 4°C in a 4% solution of paraformaldehyde made in 0.1 M phosphate buffer (pH 7.2). Material was washed for 1 day in 30% sucrose and 50 μm-thick cross-section slices were made in a cryostat. To block the activity of

endogenous peroxidases, the slices were incubated in a 1% solution of hydrogen peroxide in 0.1 M phosphate buffer for 30 min. Then slices were incubated for 1 day at 4°C with polyclonal mouse antibodies against cystathionine β-synthase (Abcam ab54883; UK) at a dilution of 1:5000. Next, the slices were incubated for 2 hours at room temperature with secondary biotinylated horse antibodies against mouse immunoglobulins (Vector Labs, Burlingame, United States); then washed three times with 0.1 M phosphate buffer for 5 min. The immunohistochemical reaction was developed using an ABC standard avidin–biotin system (Vectastain Elite ABC Kit, Vector Labs, Burlingame, United States). To develop the products of the reaction, we incubated slices with the peroxidase substrate (VIP Substrate Kit, Vector Labs, Burlingame, US) and controlled the development of blue staining under a microscope; the slices were washed, mounted on slides, dehydrated using a standard method, and placed in medium Bio-Optica (Italy). To evaluate the specificity of the immunohistochemical reaction we used a negative control. Brain slices were incubated with 1% nonimmune horse serum for 1 day instead of with primary antibodies and then were stained as described above. In all control experiments the immunopositive reaction did not occur. To compare the intensity of CBS marking in the fish brain, we measured the optical density of CBS immunostaining.

The measurements were performed using an Axiovert Apotome microscope and processed using Adobe Photoshop 7. The optical density (OD) in CBS-positive cells was evaluated using the following scale: high (130–160), moderate (100-130), and weak (50–80 units of OD) staining. The initial OD was measured in the control preparations. The data were analyzed using the parametric method (Student's *t* test). The data were processed using Statistica and Excel software.

2.2. Immunostaining of CBS

Immunostaining of CBS in *Cyprinus carpio* was found in neurons of the ventral column of the spinal cord (Figure 2A), medulla oblongata (Figure 3A, B), fibers and cells of the cerebellum (Figure 3C, D), optic tectum (Figure 3E), and telencephalon (Figure 3F). The morphometric characteristics of CBS-positive cells and data on the intensity of their labeling in carp and are shown in the Table 1. In all areas of the brain, the intensity of neuronal labeling varied between moderate and high. In medulla oblongata and spinal cord had intensely labeled vessels (Figure 2A, B). In the periventricular area of carp

was the presence of strongly stained round cells (Figure 2D, E). Figure 4 shows the histograms of optical density of CBS labeling in different structures of carp brain. CBS staining was observed in neuronal somas and proximal parts of their dendrites (Figures 2A, 3A, B), which helped to classify CBS-positive neurons in accordance with the neurochemical classification of Arevalo et al. [16].

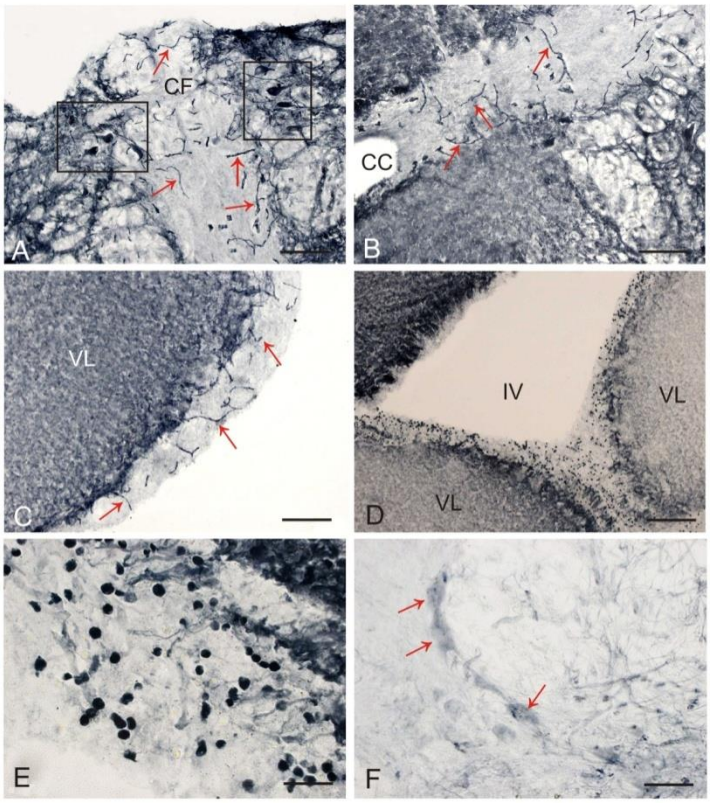

Figure 2. Immunolocalization of cystathionine β-synthase in the spinal cord, brainstem and periventricular area of carp *Cyrpinus carpio*. CBS-positive neurons in the ventral spinal column (square marks primary motoneurons) in ventral part (A) and middle part (B) red arrows mark vessels; C – CBS-staining in the vagal lobe of carp; D – CBS-positive cells in periventricular area of carp; E – cells of periventricular glia of carp at high magnification; F – negative control, CBS-immunonegative cells show by red arrows. Designations: CF – commissural fibers, CC – central canal, VL – vagal lobe, IV – fourth ventricle. Scale bar: A-D, F – 200 μm; E – 50 μm.

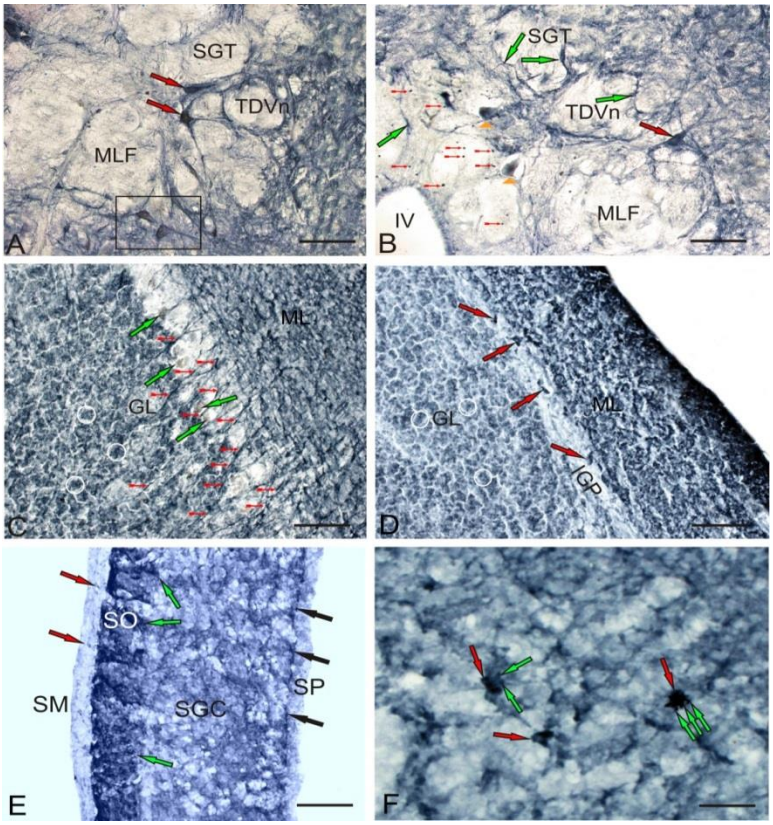

Figure 3. Immunolocalization of cystathionine β-synthase in the reticular formation, cerebellum, optic tectum and telencephalon of carp *Cyrpinus carpio*. A – interfascicle neurons of carp shown by red arrows, inlay shows immunonegative neurons of ventromedial reticular formation; B – reticulospinal cells (red arrow), interfascicle neurons of carp (yellow arrowhead), vessels are shown by green arrows, cells of periventricular glia are shown by small red arrows; C – CBS-staining in the cerebellum of carp, green arrows mark immunonegative Purkinje cells, small red arrows show immunopositive fibers, white ovals mark borders of glomerulus-like complexes; D – CBS-immunostaining in euridendroid cells (red arrows); E – CBS-staining in the tectum of carp, arrows show immunopositive cells of the tectum layers; F – CBS-staining in the telencephalon of carp, somas of oval cells of carp (red arrows) where converge immunopositive terminals (green arrows). Designations: SGT – secondary gustatory tract; TDVn – downstream path of trigeminal nerve; MLF – medial longitudinal fascicle; GL, granular layer; ML, molecular layer; IGP – infraganglionic plexus; SO – stratum opticum; SM – stratum marginale; SFGS – stratum griseum et fibrosum superficial; SGC – stratum griseum central; SAC – stratum album central; SP – stratum periventriculare. Scale bar: A-E – 200 μm; F – 50 μm.

According to the classification, there are five cell types: I, superlarge multipolar cells with 1–5 primary dendrites and soma size over 40 μm; II, cells with three or more dendrites with cell soma of about 25–40 μm; III, cells with 1–3 outgrowths without varicosities and soma size of 15–25 μm; IV, small cells with a round, oval, or bipolar shape of the soma and a size of 6–15 μm. Cells whose sizes were below 6 μm were considered as super small and related to the type V. The enzyme of hydrogen sulfide synthesis has a cytoplasmic localization in neuronal somas and proximal parts of their dendrites (Figure 2A, B). In addition to neurons, glial cells (Figure 2D, E, 3B) and fibers (Figure 3C, E) of the carp CNS were also CBS-positive. The labeled vessels and capillaries of the brain were widespread in carp (Figures 2A-C).

In the **spinal cord**, CBS was found in the ventral column of the spinal cord in neurons of the first and second types (Figure 2A). CBS-labeling was found in the commissural nucleus of Cajal (Figure 3B) and in the cells and fibers of dorso-medial funiculi. The intensity of staining of neurons was moderate or high. In hypertrophic vagal lobe (VL) had CBS immunoreactivity in both small neurons of fourth and fifth types and the neuropil (Figure 2C). Labeling was diffuse, OD was moderate.

In the **medulla oblongata** of carp, we found CBS staining in interfascicular cells of the first type, which had moderate labeling (Figures 3A, B), and in fibers of the lateral longitudinal fascicle, commissural fibers, and medullary vessels (Figures 3A, B). Moderate CBS staining was found in neurons of types I and II in the secondary gustatory nucleus (Figure 3B). In addition to immunopositive cells, the medulla oblongata of carp also contained immunonegative reticulospinal cells (Figure 3F). In the periventricular area of the medulla oblongata (Figure 1C), dorsomedial, and medial subpial area (Figures 3B) of carp, we found strongly CBS-positive cells, which presumably correspond to cells of the periventricular glia. These cells were also labeled in proliferative areas of the cerebellum and medulla oblongata (Figure 2D, E).

In the **cerebellum**, CBS was found in fibers that penetrated the infraganglionic plexus, viz., cells of types IV and V in the molecular and granular layers (Figure 3C). Cells of the glomerular layer formed elongated glomerulus-like complexes surrounded by an immunonegative area (Figure 3C); the granular layer contained larger cells that presumably correspond to the Golgi cells of mammals and glomerulus-like structures that had an oval–elliptical shape. The molecular layer of the cerebellum also contained small CBS-positive cells of the fourth–fifth types, which had high optical density. In dorsolateral zone of infraganglionic plexus of the cerebellum had CBS-positive euridendroid neurons (Figure 3D).

Table 1. Morphometric characteristic and level of immunoreactivity of CBS-positive neurons (*M* ± *m*) of the brain and spinal cord of carp *Cyprinus carpio*

Brain area	Dimensions of CBS-immunopositive cells		Type of cells according to Arevalo et al., [16]	Intensity of immunoostaining (UOD)
	large diameter of cells (μm)	small diameter of cells (μm)		
Spinal cord				
Ventral column of motoneurons	59 ± 9.2	34.6 ± 1.8	I	79.1 ± 12
Medulla oblongata				
Reticulospinal cells	53.1 ± 19.7	36 ± 12.3	I	108.4 ± 6.1
Interfascicle cells	73.3 ± 12.3	30.9 ± 8.6	I	107 ± 10
Secondary gustatory nucleus	35.4 ± 3.4	20.4 ± 1.4	II	76 ± 15
Ventrolateral reticular formation	51.2 ± 6.2 29.4 ± 3.2	24.8 ± 0.8 21.2 ± 4.8	I II	74.9 ± 5.9 86.1 ± 8.9
Lobus vagus *Sensory layer* *Motor layer*	 6 ± 0.6 4.7 ± 0.7 9.5 ± 1.9	 3.8 ± 1 4.5 ± 0.2 5.5 ± 2.2	 IV V IV	 93 ± 9.1 103.3 ± 11.7 107.2 ± 13.2
Cerebellum				
Fibers of infraganglionic plexus		2.5 ± 0.6	-	121.1 ± 18.8
Glomerular cells	4.1 ± 0.4	3.8 ± 0.4	V	106.6 ± 6.6
Cells of molecular layer	9.3 ± 1.7	6.2 ± 0.8	IV	125.7 ± 13.8
Optic tectum				
Stratum griseum centrale	21 ± 4.1 9.6 ± 2.2	6.1 ± 1 5.4 ± 1.7	III IV	144.8 ± 13.8 129.8 ± 24.1
Stratum griseum et fibrosum superficiale	8.4 ± 2.8 4.4 ± 0.8	4.1 ± 0.9 3.4 ± 0.6	IV V	69.2 ± 5.5 71.5 ± 1.9

Brain area	Dimensions of CBS-immunopositive cells		Type of cells according to Arevalo et al., [16]	Intensity of immunoostaining (UOD)
	large diameter of cells (μm)	small diameter of cells (μm)		
Marginal and optical layer	8.7 ± 0.3	3.6 ± 0.4	IV	87 ± 16.2
Stratum periventriculare	8.9 ± 2.1	4.8 ± 1	IV	125.7 ± 5
Telencephalon				
Dorso-dorsal area	11.3 ± 2	6.4 ± 0.9	IV	143.5 ± 18
Dorso-central area	11.7 ± 1.8	8 ± 1.7	IV	125.3 ± 13.1
Dorso-lateral area	10.9 ± 2	7.4 ± 1.9	IV	129.4 ± 5.7
Ventro-dorsal area	9.1 ± 0.5	5.3 ± 0.7	V	81.3 ± 16.4
Ventro-lateral area	17 ± 2.5 12.9 ± 1.6	9.3 ± 2.3 9 ± 0.5	III IV	95.8 ± 8.1 97.8 ± 8.3
Ventro-ventral area	13 ± 1.7	8.8 ± 1.5	IV	76.9 ± 5

In the **optic tectum**, CBS was found in neurons of the fourth type in the periventricular layer, central cell layer, outer cell and fibrous layer, marginal and optic layers (Figure 3E). In the tectum of carp, CBS was found in neurons and fibers (Figure 3E). The most intense immunolabeling was detected in neurons of the third–fourth type in the central cell layer.

In the **telencephalon**, CBS staining was found in the cells of the dorsal and ventral areas. The OD of CBS-positive cells in the dorsal areas of the telencephalon of carp was higher than in ventral areas. In the telencephalon, CBS labeling was detected not only in somas of neurons of types III–IV and fibers but also in terminal ramifications of varicose outgrowths (Figure 3F).

In carp, CBS was found in the vessels of the caudal part of the medulla oblongata in the area adjacent to the *area postrema* (Figure 2A, B). The diameter of the immunopositive vessels was 4–7 μm or 13–18 μm. The largest CBS-positive vessels, with a diameter of 20–22 μm, were found in dorso- and ventro-medial areas of the spinal cord (Figure 2B). CBS was also present in the fibrous white layer surrounding the *lobus vagus* (Figure 2C). Studies of the mechanisms of the effects of hydrogen sulfide in the mammalian nervous system began only recently [14] and its role in the CNS of fishes has not been

studied. It seems that H_2S acts as a messenger that easily penetrates the membrane and regulates the enzymatic reactions of a cell. Analysis of the intracellular mechanisms of the effects of NO, CO, and H_2S during neuromuscular interactions helped to find the major targets for the gaseous mediators involved in the modulation of synaptic functions [12].

2.3. Morpho-Physiological Aspect of CBS Activity in Carp Brain

The CBS activity in carp brain was different in various brain areas (Figure 4). In the dorsal area of the telencephalon, we found bipolar and oval neurons of moderate and small size. High CBS activity was detected in the cytoplasm of bipolar cells of the fourth type in the dorsal nucleus of the ventral telencephalon; however, immunolocalization of CBS was also observed in fibers with varicose thickenings and their terminal apparatuses. The terminals of CBS-positive fibers ended on the somas of small and moderate oval CBS-positive cells. These data indicate that in the telencephalon of carp H_2S may act as a classical neuromediator and/or neuromodulator located in cell somas and axosomatic synapses. According to modern concepts, the dorsal and ventral areas of the telencephalon correspond to the pallial and subpallial areas of the mammalian brain [17]. It is known that the pallial area of cyprinoids, the highest representatives of euteleostean fishes, is a neurochemically heterogenous area of the telencephalon [3, 4]. In masu salmon, high neurochemical heterogeneity was found in the dorsal nucleus of the ventral telencephalon (which corresponds to the striatum) and in the ventral and lateral cell nuclei of the ventral telencephalon (which correspond to the septum area) [18]. GABA is the most widespread neuromediator in the dorsal and ventral areas of the telencephalon of carp [17]. It has been shown that H_2S regulates different subtypes of GABA-receptors located in the pre- and postsynapses [19].

The CBS activity in the fibers of the marginal and optic layers of the tectum was not high in carp. The majority of fibers that form the marginal layer are afferents of the cerebellum and the optic layer is formed by the afferents from the retina [1, 5, 11]. Thus, the differences in the immunolocalization of CBS in the fishes studied were found the midbrain structures that link an important sensory analyzer (the retina), a center of coordination of locomotor activity (the cerebellum), and the tectum.

The periventricular layer adjacent to the cavity of the midbrain contains a layer of cells that form neuroblasts migrating to the overlying layers of the

tectum [5, 8]. Carp had a lot of CBS-positive cells in the periventricular layer. The presence of H_2S-synthesizing elements in the periventricular layer of the tectum suggests that hydrogen sulfide may be involved in the morphogenesis of this brain region. In the cerebellum, we found CBS-positive fibers that penetrate the ganglionic layer. These fibers presumably are an analogue of the glutamatergic liana-like fibers of mammals [1, 5, 20]. It is known that H_2S selectively stimulates currents mediated by glutamate NMDA receptors [21].

The mechanisms underlying this phenomenon are unclear, although they may include redox modulation of the thiol groups [13]. The presence of a large number of fibers of varicose types and CBS-positive terminals in the infraganglionic plexus of the cerebellum presumably indicates that H_2S is released synaptically in this brain area of fishes. In the granular layer of the fish cerebellum, we found CBS-positive structures that resemble the glomerulus-like complexes in the cerebellum of mammals. In mammals, GABA and glutamate are the most important mediators in the glomeruli. H_2S in the granule layer may serve as a modulator of GABA-ergic synapses, as has been shown in mammals [14, 15]. H_2S in these neurons presumably acts as the main neuromodulator.

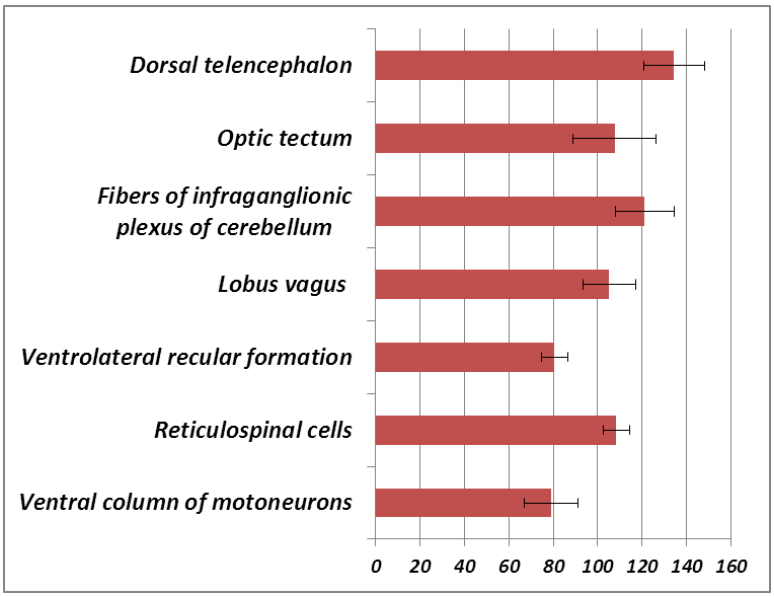

Figure 4. Densitometric analysis of the CBS activity in different brain areas of carp *Cyprinus carpio*. Abscissa axis, optical density (OD) of CBS-immunostaining; Ordinate axis, brain areas. Data are shown as $M \pm m$.

The CBS staining was found in carp in the vagal lobe, a laminar structure of the medulla oblongata that is somatotopically projected to the area of the oral cavity [5, 8, 23, 24]. GABA-ergic synapses in the vagal lobe of Cyprinoid fishes are located in the dense areas that coincide with layers of terminals of primary afferent fibers [25]. Our data indicate that the distribution of CBS in the vagal lobe of carp coincides with the previously described immunolocalizatoin of GABA. We believe that H_2S acts as a mediator that penetrates through the presynaptic membrane and modulates inhibitory neurotransmission at the level of postsynaptic receptors. NADPH-positive neurons in the nuclei of nerves V, VII, IX, X, and octavolateralis nerves were found in goldfish [26]. CBS was found only in the nucleus of nerve X of masu salmon and the vagal lobe of carp [20]. This distribution of nitric oxide and H_2S-synthesizing neurons in the nuclei of the medulla oblongata indicates that NO is a predominant neuromodulator of visceral sensory systems of the medulla oblongata and H_2S probably modulates only downstream locomotor systems. We found CBS in the interfascicle cells which function as local interneurons in the downstream locomotor networks of the medulla oblongata according to the classification of Ma [27] and in the spinal cord. We believe that interfascicle CBS-positive neurons compose a small proportion of the entire population of interfascicle cells of the medulla oblongata. In the medulla oblongata of carp, CBS activity was found in the commissural nucleus of Cajal and the secondary gustatory nucleus, which are visceral integrative centers of the medulla oblongata [5, 8, 28]. It is possible that the H_2S-producing systems in the fish brain modulates sensory functions related to intra- and extracranial evaluation of the spatial location of food and mechanisms of coordination of the mechanosensory, visual, and gustatory functions.

2.4. Immunolocalization of CBS in Vessels

Hydrogen sulfide acts on the blood vessels of mammals [29] and we were the first to find it in the brain vessels of fishes. High concentration of sulfites in the cerebrospinal fluid and blood of fishes suggests that H_2S plays an important role in the cardiovascular homeostasis of fishes [30, 31]. In trout, mRNA of CBS and CSE were found in the branchial, celiac, and mesenteric arteries and anterior cardinal vein [32]. H_2S regulates the vascular tone of fishes, constricts or dilates vessels, and produces different effects [30, 31, 33, 34]. H_2S induces monophasic contraction in the dorsal aorta of hagfish *Eptatretus cirhatus* and lamprey *Petromyzon marinus*. However, it does not

have this effect on the ventral aorta or afferent brachial arteries of these species, which indicates that H_2S has a selective vascular action. In sharks, H_2S suppresses spontaneous contractions in the dorsal aorta and induces relaxation at a concentration of 3×10^4 M in the dorsal aorta, ventral aorta, and afferent brachial arteries [29]. The very low spectrum of the dose-dependent effect is a unique characteristic of shark vessels. H_2S produces a multiphasic relaxation-contraction-relaxation response in trout efferent branchial and celiacomesenteric arteries, as well as in anterior cardinal veins, but only relaxes the *bulbus arteriosus* [29]. H_2S causes relaxation of mammalian vessels via hyperpolarization and opening of potassium ATP-dependent channels [14]. In some cases, H_2S interacts with NO and acts synergistically [30, 31]. Thus, interrelations between these two gaseous transmitters comprise an additional level of cardiovascular integration. The distribution of CBS in brain vessels of carp generally corresponds to the hight activity of NO-producing systems in the norm; however, NO presumably stimulates the metabolic activity of H_2S pathways. Studies in trout have shown that H_2S may have toxic effects on the cardiovascular systems of Salmonidae and the ability to regulate vascular tone may be determined by the ratio between exogenous and endogenous H_2S [31]. In the brain vessels of carp, CBS was found in the majority of brain areas, which means that H_2S may be considered as a prevailing vasoregulator in these species.

2.5. Hydrogen Sulfide As a Regulator of Adult Neurogenesis in the Periventricular Area of Carp Brain

In carp, the periventricular area of the medulla oblongata and ventral and lateral areas of the cerebellum contained strongly CBS-stained cells without outgrowths. These CBS-positive cells were found in the periventricular zone corresponding to the area of primary proliferation [35]. It has been shown [36] that some neurotransmitters located in the cells-progenitors of the periventricular area of the brain may serve as regulators of adult neurogenesis. Hence, it is logical to hypothesize that H_2S may also work as a regulator of postnatal neurogenesis in the carp brain. It is known that a neuron begins to release typical signal molecules shortly after their formation from cells-progenitors and long before the formation of interneuronal connections [37]. A large proportion of all signaling molecules are involved in the autocrine and paracrine regulation of the differentiation of neurons-targets and function as morphogenetic and transcription factors [37]. In mammals, the duration of the

action of signal molecules is limited to certain periods of ontogeny when the differentiation of neurons-targets and the expression of specific phenotype are modulated by a long-term morphogenetic influence [38]. In adult fishes, postnatal neuro- and gliogenesis still occurs in the periventricular area [39, 40]. Previous studies have shown that the periventricular areas of masu salmon have NADPH-positive and NOS-positive cells [41]. In carp and other cyprinoids, the periventricular area is free of NADPH/NOS activity. It seems that H_2S may function as a signal molecule in the periventricular area of carp. It has been shown that hydrogen sulfide is involved in the regulation of cell proliferation and apoptosis [14, 42]. Another physiological function of H_2S in glial cells is presumably related to the induction of Ca^{2+} waves stimulating the activity of adjacent neurons [43 fin]. Administration of excitatory neurotransmitters, such as glutamate and ATP, as well as mechanical stimulation cause an increase in the level of intracellular Ca^{2+} in glial cells, which spreads to the adjacent cells as intracellular Ca^{2+} waves [44]. Calcium waves have been described in cultured astrocytes and acute hippocampal slices [45]. Some data suggest that there are reciprocal relationships between glial Ca^{2+} waves and neurons [46]. Glial cells are an important modulator element of synaptic neurotransmission [47].

2.6. Conclusion

Cystathionine β-synthase in the brain of carp *Cyprinus carpio* was found in neurons of the ventral spinal column and medulla oblongata, fibers and cells of the cerebellum, optic tectum, and telencephalon. In all brain areas, the intensity of CBS labeling in neurons varied between moderate and high. In carp, the medulla oblongata and spinal cord contained intensely marked vessels. In the brain of carp, H_2S presumably functions as a predominant vasoregulator. H_2S-producing systems in the brain of bony fishes have specific characteristics of organization and strong species related differences that correlate with the specificities of neuromediators, for example, NO-producing, systems.

In the periventricular area of the medulla oblongata and ventral and lateral zones of the cerebellum of carp, we found cells with strong CBS labeling that had no outgrowths. The sizes and location of cells in the brain and interrelation with H_2S-producing neurons indicate that the periventricular zones of the carp brain have H_2S-producing cells which participate in adult neurogenesis. We

believe that in carp H_2S serves as a regulator of postembryonic neurogenesis in the periventricular area.

The ventral area of the telencephalon and cerebellar infraganglionic plexus of carp have CBS-positive varicose fibers and highly immunogenic terminals which, presumably, point to synaptic release of H_2S in these brain areas of fishes. In the optic tectum, medulla oblongata, spinal cord, and granular layer of the cerebellum we did not find a varicose microstructure of afferents; there are only smooth fibers and diffuse CBS-immunolocalization, which may point to non-synaptic (paracrine) release of H_2S in these brain areas. We suggest that hydrogen sulfide may serve as a postsynaptic modulator of GABA receptors in the vagal lobe of carp.

3. NITRIC OXIDE IS REGULATOR OF NEUROTRANSMISSION, CELLS PROLIFERATION AND DIFFERENTIATION IN CARP BRAIN

Nitric oxide (NO) is a critical signaling molecule with multifaceted roles in the regulation of diverse systems, as a citotoxic signal in the immune system, as a neurotransmitter and neuromodulator in the nervous system and as a potent vasodilator with also haemostatic activity in the cardiovascular system [48]. This signaling molecule is synthesized from the substrates L-arginine, molecular oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) by three isoforms of the enzyme known as NO synthase (NOS): endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). Activity of NOS enzymes is finely regulated at transcriptional and posttranscriptional level [48, 49]. Many studies have revealed the free radical nitric oxide (NO) to be an important modulator of vascular and neuronal physiology. It also plays a developmental role in regulating synapse formation and patterning. Recent studies suggest that NO may also mediate the switch from proliferation to differentiation during neurogenesis [49-51]. Previously it was thought that NO is involved only in the growth of axons and dendrites of neurons [52]. The distribution of NOS-containing neuronal cell bodies, fibers, and putative nerve terminals were histochemically demonstrated in the diencephalons of the rainbow trout *O. mykiss* [53], in the brain of the goldfish *Carassius auratus* [26, 54] and tench *Tinca tinca* [16]. Immunohistochemical study of nNOS distribution in the brain of the Atlantic salmon *Salmo salar* [55] revealed main NO-producing centers. However, studies NO-producing

centers in the brain of adult fish in connection with data about adult neurogenesis and differentiation of the sensory centers in the brain of these animals were not previously considered. The aim of work was to study the distribution of NADPH-diaphorase, a marker for neuronal nitric oxide synthase in the brains of adult carp *Cyprinus carpio*, to determine the involvement of NO in the process of adult neurogenesis in the context of new data about zones of secondary neurogenesis in the brain of fish.

3.1. The Histochemical Reaction on NADPH-Diaphorase

The histochemical reaction on NADPH-diaphorase (NADPH-d, NF 1.6.99.1). Experimental procedures were conducted in accordance with European Community guidelines on animal care and experimentation. The animals were deeply anesthetized with 0.03% tricain methanesulfonate (MS-222, Sandoz) and perfused transcardially with 50 ml of 0,63% saline followed by 200 ml of a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brains were then removed from the skull, postfixed in the same fixative for 5 hours, washed in PB at 4°C overnight and then placed in a 30% sucrose solution for cryoprotection. Thirty-micron-thick transverse sections were cut on a cryostat and collected in cold PB and, after several washes in PB, processed for NADPH-diaphorase histochemistry. Free-floating sections were incubated in a medium made up of 1mM β-NADPH, 0.8 mM nitro blue tetrazolium, and 0.06% Triton X-100 in 0.1 M phosphate buffer (pH 7.6), at 37˚C for 2 hours [56]. All chemicals were purchased from Sigma. After incubation, the sections were rinsed in PB, mounted on gelatin-coated glass slides, and air-dried overnight. The following day they were dehydrated cleared in xylene, and coverslipped with Entellan (Merck, Darmstadt, Germany). In order to determine the specificity of the histochemical reaction, the following controls were carried out: incubation without the substrate β-NADPH, and incubation without the chromogen nitro blue tetrazolium in order to rule out possible nonspecific formation of reaction product. In all cases, no residual reaction was observed.

3.2. NADPH-Diaphorase Staining

In the adult brain of carp NADPH-d was found in all parts of the CNS. Many areas were characterized by diffuse labeling of NADPH-d, but a lot of

cells containing the labeled product of NADPH-d located in areas with neurogenic activity, mainly in secondary neurogenic zones stated in the external parts of thalamus, isthmus and hindbrain. The morphometric parameters of the NADPH-d-positive cells in the brain structures of carp and intensity of histochemical staining of these cells are shown in Table 2. In all areas of the brain, the intensity of neuronal labeling varied between moderate and high. NADPH-d staining was observed in neuronal somas and proximal parts of their dendrites. It made possible to classify the NADPH-d-positive cells in accordance with the neurochemical classification of Arevalo et al. [16]. NADPH-d has a cytoplasmic localization in the bodies of neurons and proximal parts of their dendrites (Figure 8, F-H). In addition to neuronal localization of NADPH-d in the CNS carp was found in astrocytes (Figure 7D), radial glia (Figure 5E, F, 7E, F, 8A, C-D), fibers (Figure 6E-H) and the capillaries of the brain (Figure 7D).

In **telencephalon** of carp the cells containing NADPH-d were identified in the medial (Dm) and dorsal (Dd) regions of Dorsal area. NADPH-d activity in Dd was revealed in cells of 4th and 5th types (Figure 5A). Morphometric parameters of NADPH-d positive cells in Dd present in the Table 2. Cells of 4th type were oval or spindle-shaped with homogeneously labeled cytoplasm; nucleus region remained free of NADPH-d-positive precipitate. In this area NADPH-d-positive cells either contacted directly with outer border of extra-telencephalic space or localized in small depth of the surface layer (Figure 5A). In Dd cells of 5th type were averaged 18.2% (Figure 5A, Table). This group of cells was represented by small rounded or oval elements, devoid of processes, with moderate or poorly labeled cytoplasm (see Table 2). The OD of NADPH-d-positive cells of 5th type was lower (107.7 ± 7.7 UOD) than in the cells of 4th type. In Dm NADPH-d activity was detected only in the cells of 4th type, located in the surface layer (Figure 5B). Morphometric parameters of NADPH-d-positive cells are presented in the Table 2. Density of distribution NADPH-d-positive cells in Dd to compare with Dm area was lower. The average intensity of NADPH-d activity in the cells of Dm is 126.7 ± 6.6 UOD.

In **midbrain** activity NADPH-d was found on the territory of medial and lateral parts of optic tract (Figure 5C, D), preoptic area, epithalamus (habenula and epiphyseal complex), thalamus, preglomerular area and hypothalamus.

Table 2. Morphometric characteristic and level of optical density of NADPH-d-positive cells and fibers (*M* ± *m*) of the brain and spinal cord of carp *Cyprinus carpio*

Brain subdivisions	Dimensions of NADPH-d-positive cells		Type of cells according to Arevalo et al. [16]	Total number of cells in profile field (%)	Optical density of NADPH-d-staining (UOD)
	large diameter of cells (μm)	Small diameter of cells (μm)			
Telencephalon					
Dorso-medial area	13,2 ± 1,6	8,4 ± 0,9	IV	100	126,7 ± 6,6
Dorso-dorsal area	12,5 ± 1,5 8,9 ± 1,1	8,8 ± 1,7 7,7 ± 1,5	IV V	81,8 18,2	128,9 ± 8,1 107,7 ± 7,7
Diencephalon					
Ventro-medial optic tract	16,4 ± 0,6 12,3 ± 1,1	8,2 ± 0,54 7,8 ± 1,47	III IV	37,5 62,5	114,6 ± 4,9 103,8 ± 9,9
Ventro-lateral optic tract	12,2 ± 1,6 8,5 ± 0,41	6,3 ± 1,25 6,7 ± 0,8	IV V	83,3 16,7	139,5 ± 6,9 116,6 ± 13
Habenula	13 ± 0,48 8,9 ± 0,45	8,48 ± 0,9 8 ± 0,54	IV V	33,3 66,7	138,3 ± 1,4 138,4 ± 1,1
Medial thalamus	6,34 ± 0,9	4,34 ± 0,9	V	100	114,9 ± 6,3
Preglomerular Complex					
Lateral preglomerular nucleus	11,82 ± 1 7,82 ± 1,2	7,84 ± 0,3 5,4 ± 0,97	IV V	10 90	143,6 ± 2,9 135,4 ± 2,6
Medial preglomerular nucleus	16,7 ± 2,7 11,2 ± 0,5 7,7 ± 1,3	8,38 ± 1,2 6,28 ± 1,8 4,59 ± 0,7	III IV V	11 33 55,9	137,8 ± 6,2 132,4 ± 7,7 130,5 ± 5,9
Subglomerular nucleus	7,8 ± 0,97	6 ± 0,83	V	100	150,7 ± 2,67
Cells migrated from lateral optic tract	11,5 ± 1 8,9 ± 1,1	6 ± 1 5,7 ± 1,1	IV V	60 40	133,7 ± 1,6 131,1 ± 7
Mesencephalon					
Optic tectum					
Radial glia	7,4 ± 1,35	4,16 ± 0,74	V	100	151,4 ± 0,9

Brain subdivisions	Dimensions of NADPH-d-positive cells		Type of cells according to Arevalo et al. [16]	Total number of cells in profile field (%)	Optical density of NADPH-d-staining (UOD)
	large diameter of cells (µm)	Small diameter of cells (µm)			
Caudal division of cells	24,1 ± 1,4 17,4 ±1,52	17,2 ± 1,3 12,5 ± 1,2	II III	45 55	100,2 ± 7,8 120,8 ± 3,5
Torus semicircularis	13,5 ± 1,3	9,6 ± 1,6	IV	100	137,4 ± 4,9
Periventricular area and secondary matrix zones of brain					
Ependyma	10,9 ± 0,5 8 ± 0,75	7,47 ± 0,2 6,37 ± 1	IV V	16,6 83,3	119,6 ± 8,4 115,8 ± 5,8
Thalamus					
Lateral area	17,9 ± 2,5 12,1 ± 1,1 8 ± 1,13	8,02 ± 1,2 7,8 ± 1,46 5,65 ± 1	III IV V	4,1 37,5 58,3	146,9 ± 6,3 153,2 ± 10,9 134,8 ± 8,37
Ventro-lateral area	16,3 ± 2,3 11,9 ± 1,8 7,96 ± 1,2	6,13 ± 1,4 5,8 ± 0,59 5,69 ± 1	III IV V	3,5 10,7 82,1	134,4 ± 2,1 141,6 ± 3,8 129,1 ± 4
Medulla oblongata					
Lateral area	25,1 ± 4,8 17,15 ± 1 13,9 ± 1,6	11,5 ± 1,7 10,2 ± 1,5 9,73 ± 1,4	II III IV	42 46 11,1	161,65 ± 4,9 161,4 ± 2,4 164,4 ± 3,1
Ventral area	22,2 ± 1,7 16,9 ± 1,5 12,1 ± 1,3 8,8 ± 0,47	7,17 ± 3,4 10,8 ± 2,1 6,6 ± 1,36 6,25 ± 1,1	II III IV V	4,3 30,4 39,1 26	148,2 ± 4,6 157 ± 7,9 140,2 ± 8 125,5 ± 5,2
Brainstem					
Ventro-lateral reticular formation	50,8 ± 0,5 29,3± 3,7	17,32 ± 5 17 ± 4,33	I II	23 76,9	168,8 ± 3,9 168,4 ± 2,8
Medial reticular formation	58,6 ±10,3 30,6 ± 5 18,3 ± 1,8	24,3 ± 2,3 20 ± 5,29 13,2 ± 1,6	I II III	44,4 33,1 22,3	171,2 ± 4,17 171,9 ± 2,87 158,2 ± 10,11
Spinal cord					
Ventral spinal column	51,4 ± 8,7 31,8 ± 5,5 14 ± 3,1	25,7 ± 8,3 18,7 ± 5,9 13,2 ± 2,1	I II III	47,1 51,9 0,96	159,3 ± 4 162,9 ± 5 145,3 ± 3,34

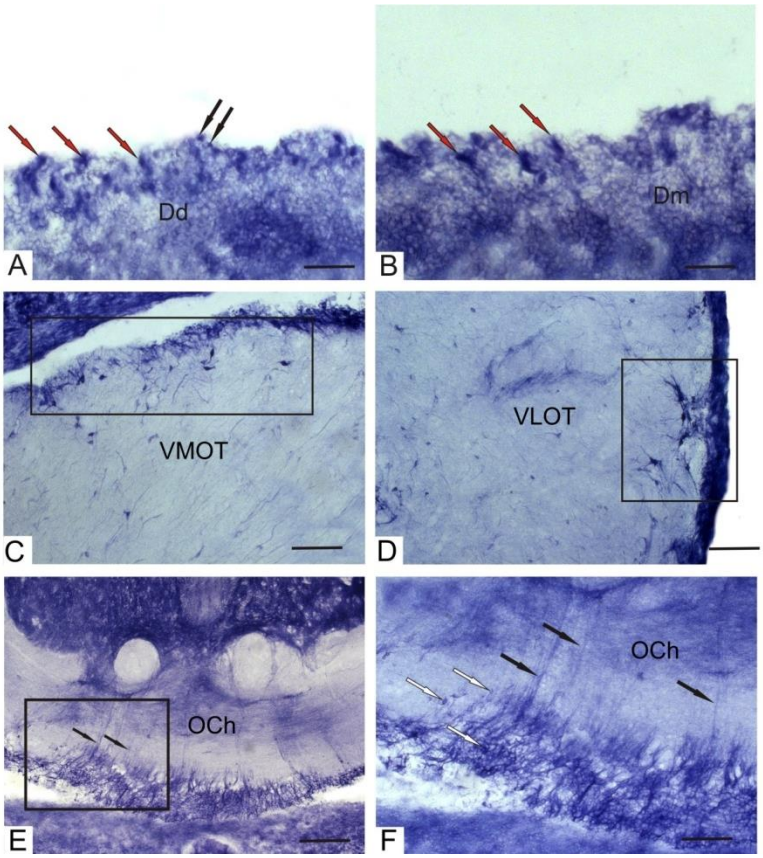

Figure 5. Distribution of NADPH-d in the telencephalon and diencephalon of adult carp *Cyprinus carpio*. A and B ─ NADPH-d-positive cells in dorsal telencephalon on A - cells of 4th type (red arrows) and 5th type (black arrows) in dorsal area (Dd); B – cells of 4th type in medial area (Dm); C and D ─ NADPH-d-positive cells (contoured by rectangle) in ventro-medial (VMOT; on C) and ventro-lateral (VLOT; on D) optical tract; D and E ─ radial glia (black arrows) and migrating cells along radial fibers (white arrows) in the basal diencephalon of carp, fragment of chiasm (OCh) contoured by rectangle in E, shown in F at high magnification. Scale bar: A, B ─ 50 μm, C, D, F - 100 μm, E ─ 200 μm.

High activity of NADPH-d was found in optic tract, and in distal part of optic nerve (Figure 5C, D). These areas contained NADPH-d-positive cells and elements of radial glia (Figure 5D, E). In anterior commissure were found NADPH-d-positive cells of 4th and 5th types. Morphometric parameters of

NADPH-d-positive cells in ventro-lateral and ventro-medial parts of optic tract are shown in the Table 2. Such cells had 1-2 outgrowths; drop-shaped or oval somas, nucleus in cells was clearly visualized; level of NADPH-d activity usually was moderate or weak. In the surface layers of the ventro-medial and ventro-lateral parts of the optical tract were revealed larger and more intensely stained cells (Figure 5C, D). Such cells in the lateral area formed clusters of closely spaced and highly active NADPH-d-positive cells of 4th and 5th types (Fig 5D; Table 2). The intensity of NADPH-d staining in the cells of 4th type was 139.5 ± 6.9 UOD, in 5th type was 116.6 ± 13 UOD. In the medial region of optic tract were found larger cells of third type with cytoplasm intensely labeled by NADPH-d (Figure 5B). These cells usually had a bipolar morphology; the nucleus in such cells does not delineate or poorly contoured.

Cells of third types were singly located or formed the small groups with cells of 4th type. The number of cells of third type to profile field was 37.5%; the intensity of NADPH-d staining was 114.6 ± 4.9 UOD. Numerous radial glia (RG) fibers were revealed in anterior commissure (in basal diencephalon, Figure 5D, E). RG fibers were directed radially inward from outer boundary of brain at various distances. Among individual fibers of RG were detected NADPH-d-positive cells of 4th and 5th types with high or medium levels of enzyme activity.

Epithalamus. Cells mainly concentrated in the surface layers of the pineal complex with high levels of NADPH-d activity were identified in epithalamus of carp (Figure 6A). Network of NADPH-d-positive fibers with an average level of enzyme activity was identified in epithalamo-epiphyseal complex. **Ependyma**. In periventricular areas of di- and mesencephalon always are identified ependymal cells with moderate activity of NADPH-d (Figure 6B, Table 2). The mean optical density in ependymal cells was 117.7 ± 2.6 UOD. Among ependymal cells were identified elements of 4th and 5th types (see table). Small ependymal cells (5th type) were average 83.3% and larger cells (4th type) - 16.6%. NADPH-d staining in such cells has been detected in the peripheral layers of cytoplasm; the central part of cells, comprising nucleus usually has NADPH-d-negative. Subventricular region does not contain an expression activity of NADPH-d.

Habenulo-interpeduncular system. High activity of NADPH-d was found in asymmetric habenular nuclei of carp (Figure 6B, D). NADPH-d staining in habenula was uneven; along radial rows of NADPH-d-negative cells were revealed areas of dense and intense NADPH-d-staining. Mean optical density of NADPH-d-positive cells of 4th and 5th types was quite high (138 UOD; see Table 2).

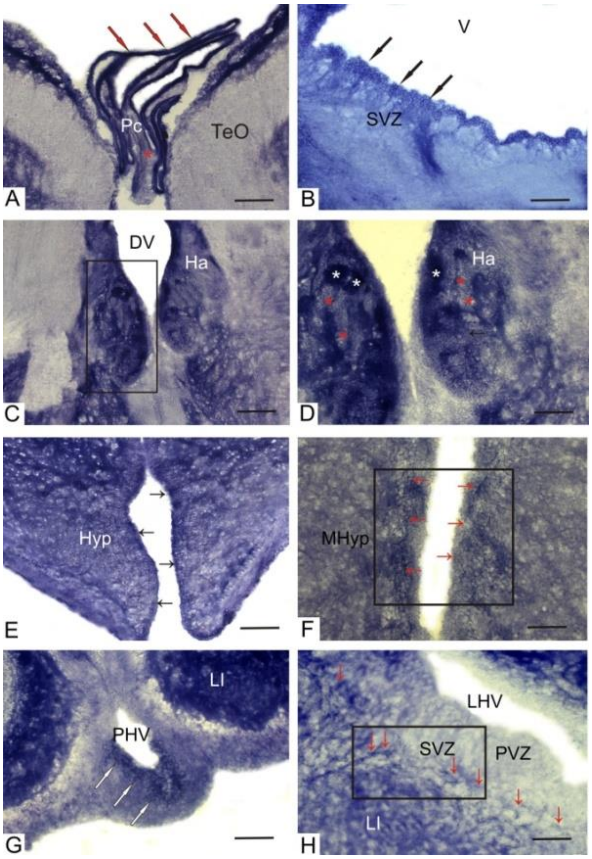

Figure 6. Distribution of NADPH-d in diencephalon of adult carp *Cyprinus carpio*. A – NADPH-d-positive cells (red arrows) in pineal complex (Pc); red asterisk denotes zone Pc containing NADPH-d-positive fibers, TeO – tectum opticum; B – ependymal cells (black arrows) with low level activity of NADPH-d, SVZ – subventricular zone, V – ventricle; C and D – NADPH-d in habenular nuclei (Ha); rectangle bounded by fragment in C, which is shown in D at higher magnification, white asterisks show loci with high activity of NADPH-d, red asterisk - with low activity, DV – diencephalic ventricle; E – NADPH-d in medial hypothalamus (Hyp), black arrows show periventricular NADPH-d-positive cells; F – NADPH-d-positive innervation (red arrows) in subventricular zone (bounded in square) of medial hypothalamus (MHyp); G - NADPH-d-positive cells in the posterior hypothalamic bay (white arrows), PHV - posterior hypothalamic ventricle, LI – *lobus inferior*; H – NADPH-d-positive innervation in subventricular zone (bounded in rectangle) of lateral hypothalamus, LHV - lateral hypothalamic ventricle, PVZ – periventricular zone. Scale bar: A, C, E - 200 µm, B, D, F, G – 100 µm, H – 50 µm.

Hypothalamus. NADPH-d-positive innervation, density of which varied in the dorsal-ventral direction was revealed in hypothalamus of carp (Figure 6E-H). NADPH-d-positive fibers located in the subventricular area of medial hypothalamus had some varicose thickness intensely labeled by NADPH-d (Figure 6E). Density of distribution and intensity of NADPH-d-labeling in such fibers were the highest in subventricular area and decreased in periventricular zone (Figure 6F). Small NADPH-d-positive cells with intense or moderate intensity of enzyme activity were revealed in posterior hypothalamic bay (Figure 6G). Subventricular NADPH-d-positive fibers with varicose thickness, similar to those in the medial hypothalamus were found in lateral ventricles (Figure 2B). Activity NADPH-d was not revealed in the periventricular region of the lateral hypothalamus.

Preglomerular area. Preglomerular complex (PGC) is projection´s area of mechanosensory, gustatory, octavo-lateral and other sensory modalities in carp brain. PGC includes medial, lateral and subglomerular nuclei. In the PGC of adult carp was identified high level of NADPH-d activity, both in cells and fibers of glomerular tract (Figure 7A). Mean optical density of NADPH-d in nuclei of PGC was in the medial nucleus 133.6 UOP, in lateral nucleus 139.5 UOD in subglomerular nucleus 150 UOD. In medial nucleus were identified cells of 3-5 types, in the lateral nucleus - 4th and 5th types and in subglomerular nucleus – cells of 5th type. Ratio of morphological parameters and level of NADPH-d activity are presented in the Table 2. Along of NADPH-d-positive fibers we observed the presence of NADPH-d-positive cells clusters. Such conglomerates NADPH-d-positive cells and fibers were found close to all PGC nuclei. Thus, a large cluster of labeled neuropil and cells was identified in the lateral preglomerular nucleus (LPGN) (Figure 7B). At least a major conglomerate was found in the medial preglomerular nucleus (MPGN). The average diameter of neuropil components was 12.0 ± 0.7 μm in long and 8.2 ± 1.9 μm in width. Mean optical density of NADPH-d staining in the neuropil was 140.1 ± 2.0 UOD. We believe that the labeled fibers and NADPH-d-positive cells represent a single cells migrating from the zones of secondary neurogenesis and periventricular region of the nucleus in PGC. Investigation of cells in nuclei of PGC on serial sections of the brain allows us to conclude that labeled fibers and cells in LPGN and SGN of carp are likely to migrate from the secondary neurogenic zones of thalamus and lateral optic tract.

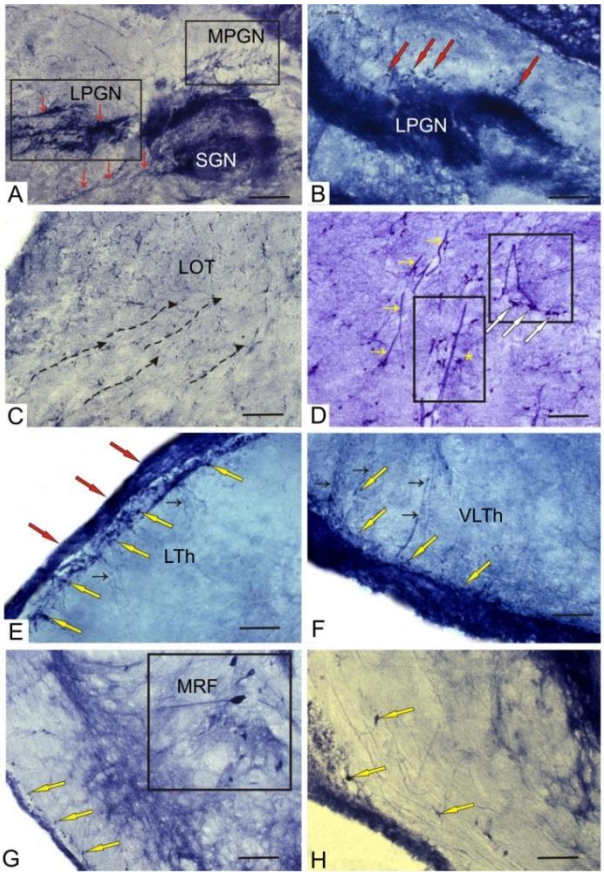

Figure 7. NADPH-d in preglomerular complex and SMZs of thalamus and medulla oblongata of adult carp *Cyprinus carpio*. A – NADPH-d-positive nuclei of preglomerular nuclear complex, migrating cell´s clusters contoured by rectangles in lateral (LPGN) and medial (MPGN) nuclei, red arrows show positive fibers in glomerular tract, SGN - subglomerular nucleus; B – NADPH-d-positive cells (red arrows) migrate to LPGN; C – NADPH-d-positive cells migrating from the lateral optical tract (LOT), dashed lines with arrows indicate the direction of cell´s migration; D – NADPH-d-labeled vessels (yellow arrows) and conglomerates of NADPH-d-positive cells and vessels (delineated by rectangles) in optical tract, the yellow asterisk marks an astrocyte, white arrows show conglomerates of astrocyte-like cells; E and F – SMZs in thalamus, on E – lateral area (LTh), red arrows show NADPH-d-positive cells in external wall, the yellow arrows show NADPH-d-positive cells in SMZ, black arrows show radial fibers; F – ventro-lateral area of the thalamus (VLTh); G and H – zone of secondary neurogenesis in medulla oblongata, in the square NADPH-d-positive cells shown in medial reticular formation (MRF); H – NADPH-d-positive cells in SMZ at high magnification. Scale bar: A, C, G – 200 μm, B, D-F – 100 μm, H – 50 μm.

This is confirmed by the large number of NADPH-d-positive cells of 4th and 5th types in LOT forming spatially organized rows of cells along radial glial fibers (Figure 7C, Table 2). In this zone were identified individual NADPH-d-stained vessels, which were located close to the clusters of astrocyte-like cells (Figure 7D).

NO-producing zones of secondary neurogenesis in the adult brain of carp. NADPH-d-positive cells located at the outer layers of brain were revealed throughout the carp brain (Figure 7E-H). Such cells in different brain regions generally were represented by morphologically heterogeneous elements of 2-5 types (see Table 2). Cells of 4th and 5th types were revealed in carp thalamus. Cells were arranged in small groups under the *pia mater* and generally had a high level of NADPH-d activity (Table 2). Cells of third type represented a small part of general cell´s population. The thickness of the *pia mater* was varied in different regions, but throughout all brain´s parts remained high NADPH-d activity (Figure 7E, F). On the territory of thalamus, similar regions were found in the lateral and ventro-lateral regions (Table 2). The density of cell´s distribution in these areas was very high; the ratio of different cell types is shown in the table 2. Cells with high levels of NADPH-d activity often were labeled adjacent to the radial glial fibers (Figure 7F, G). Cell´s migration pattern could be observed along of RG fibers (Figure 7F). Activity of NADPH-d in migrating cells was lower than NADPH-d activity of cells near the *pia mater*. Ventral and lateral areas containing NADPH-d-positive cells were identified in the medulla oblongata (Figure 7G, H). Cells of 2-4 types with a high level of NADPH-d activity were revealed in lateral area (Table 2). Ventral area contained cells of 2-5 types; total level of NADPH-d activity in this area was lower (Table 2). Similar with thalamic patterns of NADPH-d distribution were found in medulla oblongata (Figure 7G). Cells with high enzyme activity were located in the adjacent areas with *pia mater* (Figure 7H).

Optic tectum and torus semicircularis. In the *optic tectum* NADPH-d activity was detected in the fibers of *stratum opticum* (SO), *stratum fibrosum at griseum superfaciale* (SFGS), as well as radial glia, located in the *stratum marginale* (SM; Figure 8A). NADPH-d-positive cells of 5th type had high level of enzyme activity (151.4 ± 0.9 UOD). Another group of NADPH-d-positive cells was represented in surface layer of caudal tectum by large elements of the 2nd and 3rd type with a moderate level of enzyme activity (Figure 8B).

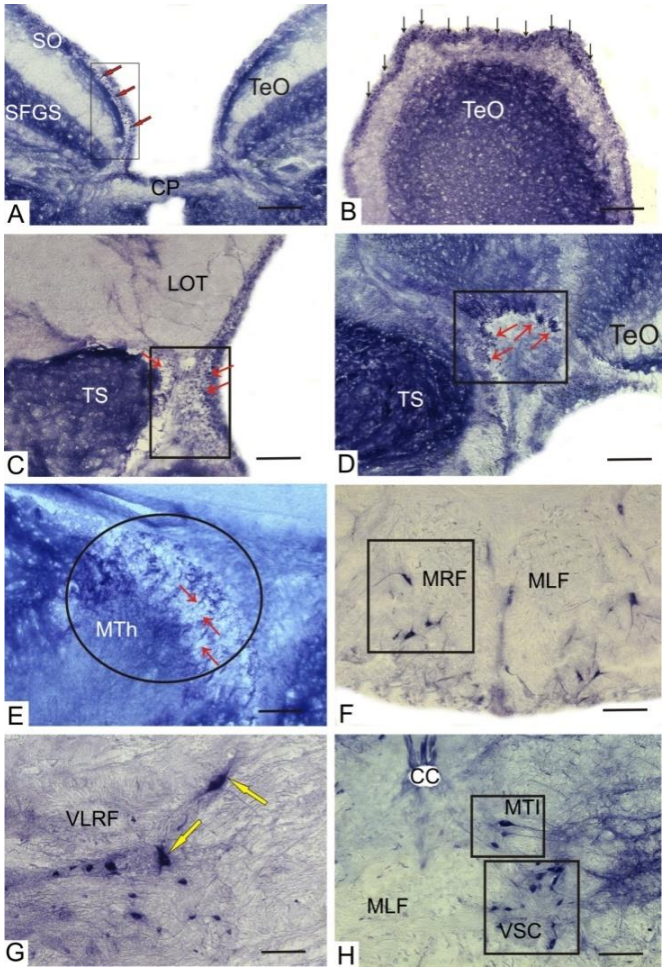

Figure 8. NADPH-d activity in tectum opticum, medial thalamus, medulla oblongata and spinal cord of adult carp *Cyprinus carpio*. A – NADPH-d-positive fibers in *stratum opticum* (SO) and in *stratum fibrosum at griseum superfaciale* (SFGS), a fragment containing NADPH-d-positive RG (red arrows) delineated by rectangle, CP - commissure posterior; B – NADPH-d-positive cells (black arrows) in caudal tectum; C and D – SMZs (in rectangles) in rostral (C) and caudal (D) levels of *torus semicircularis* (TS), red arrows show RG; E – fragment of medial thalamus (MTh, in oval) containing RG (red arrows); F and G – NADPH-d-positive cells of reticular formation, on E – medial (MRF, in square) on F – ventro-lateral (VLRF, shown by yellow arrows); H – NADPH-d-positive neurons (in rectangles) in ventral spinal column, primary neurons (MTI) and secondary neurons (MTII), CC – central canal. Scale bar: A-D, F, H – 200 μm, E – 50 μm, G – 100 μm.

Such cells were morphologically similar with RG found in mechanosensory and auditory center of midbrain – *torus semicircularis* (TS; Figure 8C and D, Table 2). NO-producing cells in TS were morphologically resembled with radial glia from *optic tectum*, but cells from *torus semicircularis* were larger (4th type) and had a more compact localization. In medial thalamus were found cluster of cells 5th type, same morphological structure (Figure 8D) with a moderate level of NADPH-d activity (see Table 2).

Brainstem. In brainstem, NADPH-d activity was detected in cells of medial reticular formation (MRF; Figure 8E) and ventro-lateral reticular formation (VLRF; Figure 8F). Parameters of NADPH-d-positive cells and the level of optical density are presented in the Table 2. In reticular formation the large cells 1st and 2nd type had the highest level of enzyme activity. Thus, in VLRF average level of activity NADPH-d was 168 UOD. In neurons of MRF (1st and 2nd type) activity of NADPH-d was 171 UOD, and in cells of third type - 158 UOD. All cells had 1-3 dendrites, intensively labeled cytoplasm and unlabeled centrally located nucleus (Figure 8F, G).

Spinal cord. In spinal cord NADPH-d activity was found in the ventral spinal column of neurons (VSC; Figure 8H). The enzyme activity was detected in large cells of 1st type and in secondary neurons located in ventro-lateral part. NADPH-d staining in VSC was same to that in cells of the reticular formation. Morphologic parameters and the level of NADPH-d activity are shown in the Table 2.

3.3. Role of NO in the Fish´s Central Nervous System

The distribution of NOS-containing neuronal cell bodies, fibers, and putative nerve terminals were demonstrated in the different parts of brain and spinal cord of fishes [26, 53-55]. Development of NADPH-diaphorase activity from 20 h after fertilization to 5.5 days was demonstrated in the CNS of the cichlid fish, *Tilapia mariae* [57]. In the sunfish brain, NADPH-diaphorase histochemistry stained tanycytes almost exclusively [58]. Qyan et al., [59] found that cloning and sequencing of NOS isoforms in the cerebellum and optic tectum of the Atlantic salmon *Salmo salar* revealed a partial gene sequence of 560 bp corresponding to mammalian nNOS from cerebellum complementary DNA (cDNA), and the predicted protein sequence of identified salmon nNOS possessed 85% identity to that of mammalian nNOS, suggesting that the arising of different vertebrate NOS isoforms is an evolutionarily old event. NOS immunoreactivity and NADPH-diaphorase

activity partly coincided with the nNOS mRNA expression in the brain of the adult zebrafish but was present also in additional neuronal and non-neuronal cell types, indicating the occurrence of different NOS isoforms in the fish brain [60]. The nNOS may participate in neurotransmission or neuromodulation and in mechanisms related to the growth and neuronal plasticity. Cioni et al. [61] noted that an NADPH/Ca^{2+}-dependent NOS activity is present in the soluble and in the particulate fractions of teleost caudal spinal cord homogenates, both activities being inhibited by calmodulin inhibitors and by L-NAME. Western blot analysis using either anti-nNOS or anti-eNOS antibodies showed that the soluble enzyme corresponded to nNOS of mammals, whereas the particulate one was likely attributable to nNOS or eNOS (or both). In comparison with mRNAs encoding α- and β-subunits of soluble guanylylcyclase, nNOS-immunoreactive and NADPH-diaphorase-positive neurons were more widely distributed in many cerebral neurons in the rainbow trout; however, wide overlaps of guanylyl cyclase subunit mRNAs and nNOS distribution were observed, suggesting a role for soluble guanylyl cyclase in various neuronal functions possibly via NO/cyclic GMP signaling in the fish brain [62].

Cyclic GMP immunoreactivity was determined in pineal photoreceptor cells, whereas NADPH-diaphorase-positive structures were located adjacent to these photoreceptor cells, suggesting a role for NO in pineal function in, for example, cyclic GMP-related events in the phototransduction process and the light-dark control of melatonin synthesis [63]. Cioni et al. [61] provided evidences that caudal neurosecretory cells of teleosts express nNOS and that NO acts as a putative modulator of the release of urotensins from the neurosecretory axon terminals. The nNOS was colocalized with arginine-vasopressin in a subpopulation of neurosecretory neurons of the teleost *Oreochromis niloticus*, suggesting that NO is implicated in the modulation of hormone release [64].

3.4. Involvement of Nitric Oxide in Modulation of Cholinergic Brain Centers

Some brain structures contained NADPH-d-positive cells (habenular nuclei, centers of the reticular formation at the isthmic level, ventro-lateral and ventro-medial reticular formation and ventral spinal column) were revealed in the brain of adult carp. All of the above mentioned structures of brain and spinal cord are conservative cholinergic centers, which have been shown in

studies on different species of Cyprinoid fish [65, 66]. Despite the high interspecies variability inherent for mediator-specific centers of fish brain, these areas, according to various authors, labeled antibodies against acetylcholine [65]. Identification of centers with NADPH-d activity in the brain of adult carp confirms previously established data about the presence of NADPH-d in cholinergic sensory and motor centers of spinal cord and medulla oblongata in Perciformes, Cyprinoid and Salmonidae [41, 67, 68]. This confirms our previous hypothesis put forward that nitric oxide can be considered as a modulator of sensory and motor cholinergic centers of brainstem and participates in the modulation of ascending cholinergic projections of reticular formation, comprising so-called non-specific system of telencephalon´s activation [69]. These cholinergic centers of the brain are highly conserved among all vertebrates [65, 66]. It is possible that similar neurochemical mediator-modulatory relations among different vertebrate groups could occur in the early stages of evolutionary development. Study of the modulating influence of gaseous intermediators to the classical system of neurotransmitters in the brain of fish previously had not been carried out. It was shown that total NO-ergic production in the nuclei of the brainstem in different fish species significantly exceeded [55, 60, 67] the measure set for other groups of vertebrates and, particularly mammals. So, it is normal for different fish species NO-producing neurons were verified in somato- and viscerosensor and visceromotor nuclei of medulla oblongata (V, VII, IX, X nuclei of craniocerebral nerves, efferent octavo-lateral neurons, the nuclei of the isthmus, secondary gustatory nuclei, the nuclei of oculomotor complex (III, IV and VI nuclei of cranial nerves). Most of these nuclei in fish brain are cholinergic centers of brainstem involved in the innervation of brachiomotor muscles and some sensory inputs from the somatosensory, gustatory extra- and intraoral system, mechano-sensory, octavo-lateral receptors [70]. In fish due to low level of cephalization brain the most of the sensory inputs from the somatosensory (nucleus V), octavo-lateral, gustatory extraoral (nucleus VII), intraoral (nucleus IX) are concentrated on the territory of medullary part; therefore this sector is perceived by a large volume of incoming sensory information [69]. Despite significant interspecific morpho-adaptative differences, in Perciformes and Cyprinoid fish were identified similarities in the organization of medullar and spinal NO-producing centres [67, 68]. Participating NO in modulation of sensor systems in forebrain of mammals it was proved today [71]. We assume that in the medulla oblongata of fish NO performs modulation of primary sensory centers, located in the nuclei of cranio-cerebral nerves. In the masou salmon brain all of the above mentioned

nuclei, located in the brainstem and isthmus region are cholinergic and express nNOS [69]. Primary sensory nuclei (V, VII, VIII, IX and X), and secondary relay nuclei (secondary gustatory nucleus, the nucleus isthmus) in fish brain, processing heteromodal sensory information in the nuclei of preglomerular complex modulated by NO. We assume that in the fish brain NO is modulator of sensory and motor cholinergic centers.

3.5. Nitric Oxide in Secondary Matrix Zones of Carp Brain

Results of morphological studies and experimental investigations in teleost fish have shown that NO-produced neurons in CNS involved in different functional mechanisms associated with regeneration [72] differentiation [73, 74], a communication between neurons and glial cells, synaptic transmission and neuromorphogenesis [57, 72, 75]. NO plays special roles in proliferative processes in the matrix areas of fish´s brain which are not limited by embryonic stage of development and continue throughout life [76]. Regulation of neurogenesis in the adult brain is a topic of great interest to researchers and clinicians seeking new therapeutic avenues for treating age and injury-related impairment of neural functioning. Progenitor cells that can give rise to both neurons and glia have been described in adult brain regions of several species, including humans [48, 49]. In addition to the regulation of neuronal proliferation in the developing brain, NO may play a role in controlling the generation of new neurons from these stem cells in the adult. In the adult brain *Danio rerio* mRNA of nNOS expressed by cells associated with proliferative zone of brain located in subependymal layer [60, 77]. In our studies, NADPH-d distribution in the brain of adult carp has been found in centers where NO works as a neurotransmitter or modulator for another neurotransmitter systems, for example cholinergic. From other side, we found NADPH-d-positive cells in zones of brain contained proliferating cells. These zones, according to [60] produce neurons in fish´s brain throughout life and are the centers so-called secondary neurogenesis. In the brain of adult carp were found same areas in the external walls of thalamus, brainstem (isthmic region, medulla oblongata), as well as in areas contained RG cells which morphologically resembling that in sensory areas of midbrain (*optic tectum* and *torus semisircularis)*. Amount of NO-producing areas where nitric oxide acts as a regulator of neurogenesis in adult carp´s brain sufficiently large to compare with zones in which the nitric oxide performs the role of the neurotransmitter / neuromodulator. From other hand, in zones of secondary

neurogenesis the level of NADPH-d activity is sufficiently high and comparable with activity of neurons in neurotransmitter/neuromodulatory centers of carp´s brain (see table 2; Figure 9).

We believe that in adult carp´s brain exist several populations NO-ergic cells involved in morphogenetic processes. The first population includes NADPH-d-positive RG detected in the sensory areas: *optic tectum* and *torus semicircularis*. Similar observations have been described in sunfish [58], masu salmon [41] and in Muller cells of retina in goldfish, catfish and salamanders [78]. RG-like cells were identified in the medial thalamus of carp. The second population presents NADPH-d-positive cells detected in external ventro-medial and lateral walls of thalamus and the medulla oblongata. Such cell (III, IV and V types) were arranged near the secondary matrix zones (SMZ). However, these cells only surrounded SMZ and cell´s morphology indicates these cells were not proliferating elements. Cells expressing nNOS were identified among the progenitor cells of *dentate gyrus* in hippocampus of guinea pig [50]. Carp as another teleostean fish has telencephalon of everted type and Dm and Dd telencephalic areas of telencephalon containing external zones with proliferative activity. NADPH-d-positive cells of 4th and 5th types were identified in Dm and Dd of adult carp´s telencephalon. In adult rats and mice, neuronal progenitors and stem cells have been identified in the subventricular zone (SVZ), which lies inside the external wall of the lateral ventricles [50, 79]. These progenitors migrate tangentially to the olfactory bulb, where they divide and differentiate into granular and periglomerular neurons [49, 50]. This region of migration is referred to as the rostral migratory stream (RMS). NADPH-d and NOS-immunostaining have revealed a putative source of NO in the SVZ and RMS, intermingled with regions of proliferating and undifferentiated progenitor cells [49]. NO may then serve to prevent the division and differentiation of neural progenitors as they migrate along the RMS, confining these events to their destination in the olfactory bulb. We believe that in telencephalon and areas of secondary neurogenesis in adult fish brain nitric oxide acts as a regulator of proliferative activity of neural progenitors. It is possible that nitric oxide is a negative regulator of cell proliferation in central nervous system of carp.

The third population presents by NADPH-d-positive migrating cells. They were found along the fibers of RG in SMZ of thalamus and medulla oblongata. Another area contained same cells have revealed on territory PGC surrounded by fibers of optical tract. Previously detection of same patterns in distribution NADPH-d-positive structures in adult fish´s brain has not been specifically studied and interpreted. Our findings in adult brain of carp are fully consistent

with those in mammals brain, where NO has been shown to participate in the regulation of cell proliferation and neuronal differentiation [48, 49, 52, 80].

3.6. Nitric Oxide in the Sensory Centers of the Brain - The Factor of Migration and Differentiation of Cells

Investigation of distribution NADPH-d in PGC of adult carp have revealed a high level of NADPH-d activity in LPGN. It allows us to conclude that nitric oxide can act as a regulator of cells migration and differentiation in this polysensory thalamic center. It was particularly evidenced by patterns of distribution NADPH-d-positive fibers and cells directed to the MPGN, LPGN and SGN observed by us. NADPH-d-positive cells migrating along the RG fibers in optical tract confirmed facts of cell´s migration in histological slices. Optical tract may be a source of new cells in the LPGN and SGN that can be traced by examining of serial sections of carp´s brain. We believe that cells in the MPGN migrate from the periventricular region of diencephalon.

Thus, in carp´s brain nitric oxide in SMZ may act as a regulator adult neurogenesis limiting NSC proliferation and possibly controlling the migration and differentiation of cells originated in SMZ in reticular formation, thalamic nuclei (especially PGC) or other sensory centers, such as *optic tectum* or *torus semicircularis*.

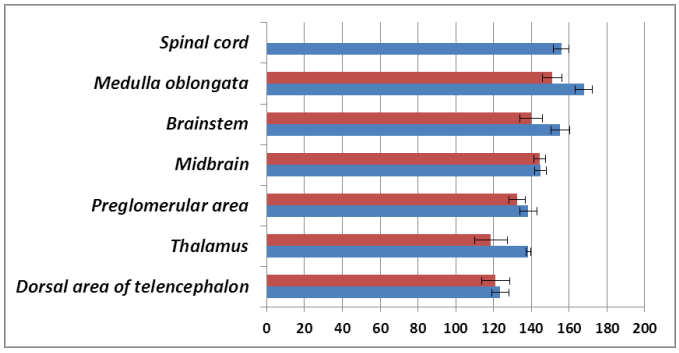

Figure 9. Densitometric analysis of NADPH-d activity in some neurotransmitter-contained centers of brain (red bars) and secondary matrix zones (blue bars) in brain of adult carp *Cyprinus carpio*. Abscissa axis, optical density (OD) of NADPH-d staining; Ordinate axis, brain areas. Data are shown as $M \pm m$.

3.7. Nitric Oxide and Hydrogen Sulfide Are Gaseous Mediators Involved in Adult Neurogenesis of Carp

In the brain of adult carp high enzyme activity of NADPH-d has been found in SMZ of thalamus and brainstem, but in ependymal cells of periventricular area we revealed low activity NADPH-d (Table 2). Thus, in primary zone of neurogenesis in adult carp was reported low activity of NO-producing enzyme. However, in periventricular region we previously have detected cells containing cystathionine-β-synthase (CBS), an enzyme synthesizing hydrogen sulphide [20]. Periventricular region of the brain, as well as zones of secondary neurogenesis surrounding the *lobus vagus* and *lobus impar* (special part of facial nerve) contained small cells, without outgrowths intensively labeled by CBS [20]. According to established data, sensory areas containing NADPH-d in adult carp´s brain are presented in *optic tectum*, and *torus semicurcularis*, but not detected in *lobus impar* and *lobus vagus*. Thus, in the adult brain of carp NO- and H_2S-producing cell populations located in territory of the primary (periventricular) and secondary matrix zones are separate, non-overlapping populations of cells having properties of spatial specificity. Thus, in primary matrix zone have demonstrated high activity of CBS, but not have found the activity of NADPH-d. In SMZs located on territory of thalamus and medulla oblongata have showed a high activity of NADPH-d and not found expression of CBS. SMZs associated with *lobus vagus* and *lobus impar* have high immunohistochemical activity of CBS detected in cells morphologically similar with cells of periventricular area and revealed no activity of NADPH-d. Such observations allow us to suggest that, in brain of carp during adult in neurogenesis occur same zones with proliferative activity, which are modulated by various gazotransmiters systems.

4. CATECHOLAMINES IN THE CARP BRAIN

Dopaminergic neurotransmission functions in the CNS of all classes of vertebrates; it is obvious that it developed before the appearance of this taxonomic group. Many molecular components of the dopaminergic neuronal systems of vertebrates (enzymes of biosynthesis of this transmitter, its transporters, and receptors) are also typical of other monoaminergic systems, e.g., serotonergic one. This is indicative of a possible common origin of the above systems [81]. In the mammalian CNS, dopaminergic neuronal systems

are rather numerous and diverse; they participate in perception of visual and olfactory information, sensory/motor programming, in the formation of motivations, memory, and emotions, as well as in endocrine regulation [81, 82]. Some these functions are realized in a few groups of vertebrates, while other are typical only of some animals, which is reflected in certain anatomical and functional peculiarities of organization of the medullary part of the brain typical of different taxonomic groups.

Catecholaminergic (CA-ergic) systems of the fish brain are characterized by high morphological heterogeneity [27, 83, 84]. Recently, it was found that an isoform of the main enzyme of synthesis of catecholamines, CAs (tyrosine hydroxylase, TH_2), which is absent in other vertebrates (in particular in mammals), is expressed in a representative of the family Cyprinidae, *Danio rerio* [85]. In the Actinopterygia characterized by broad evolutionary divergence, significant specificities in the organization of CA-ergic systems of the forebrain division related to the peculiarities of histogenesis of the corresponding structures were found [9]. In the medulla of bony fishes, the number of CA-ergic cellular groups is very limited; they include dopaminergic and noradrenergic neurons [27]. Taking into account the morphological heterogeneity and divergent nature of interspecies phenotypical differences in *Teleostea*, we believe that comparative studies of the organization of CA-ergic structures of the *medulla oblongata* of these animals are rather expedient. In our study, we tried to elucidate morphological characteristics of CA-ergic cellular groups in the *medulla oblongata* of the carp *Ciprinus carpio* and examined peculiarities of projection of the corresponding medullary neurons.

4.1. Immunohistochemistry of Tyrosine Hydroxylase

To identify CA-ergic neurons, we used immunohistochemical labeling of these cells containing the main enzyme of CA synthesis (tyrosine hydroxylase) using an indirect avidin-biotin peroxidase ABC technique for staining. The brains from five fishes were fixed for 2 h at 4C in 4% solution of paraformaldehyde based on 0.1 phosphate buffer (pH 7.2). The material was washed out five times for 24 h in 30% sucrose solution at the same temperature and frozen in a cryostat. Serial frontal and horizontal 50-μm-thick slices prepared using a crystate (Cryo-star HM 560 MV, Germany) were washed out three times in phosphate buffer for 5 min. Slices were incubated with monoclonal murine antibodies against TH (Vector Laboratories, USA) in dilution of 1: 5000 at 4°C for 24 h and then washed out in three changes of 0.1

M phosphate buffer (pH 7.2) for 5 min. Incubation of the slices with secondary biotin-conjugated horse antibodies against murine immunoglobulins (Vector Laboratories, USA) was performed during 2 h at room temperature and accompanied by washing out in three changes of 0.1 M phosphate buffer (for 5 min). Then the slices were incubated for 2 h with a standard avidin-biotin visualization system (ABC Vectastain Elite ABC Kit, Vector Laboratories, USA) at room temperature in in the dark and again washed out three times in phosphate buffer. To estimate the products of reaction, the slices were incubated in the presence of a substrate for detection of peroxidase (VIP Substrate Kit, Vector Laboratories, USA); the process of development of staining was controlled under a microscope.

4.2. Distribution of TH in Carp Brain

Peculiarities of localization of the neurons, morphology of their dendrites, and trajectory of axon projections in the medulla of carp allowed us to differentiate the following three groups of TH-positive neurons, namely, interfascicular cells, units related to the *lobus vagus*, and cells connected with the neurosecretory region of the *area postrema*; a population of phenotypically immature cells in the periventricular region should also be mentioned. The first three cellular groups can be considered components of the dorsal reticular formation (RF) at the level of the nuclei of the X nerve and *area postrema.* Accumulation of TH-positive cells is arranged between the medial longitudinal fascicle (MLF), fibers of descending fascicles of the trigeminal (V cranial) nerve (DTr), and secondary gustatory tract (SGT).

Interfascicular group of TH-positive neurons. In the longitudinal axis of the medulla of the carp, there are two main systems of conduction tracts. In parallel to the midline on both sides, the largest motor cerebral tract (MLF) is localized. Larger and less structured tract systems, the lateral longitudinal fascicle (LLF), descending fascicle of the *n. trigeminus* (DTr), and descending secondary gustatory tract (SGT) are located more laterally. Between these tract systems, mid-sized neurons forming longitudinal columns within each half of the brain are present. In the caudal part of the medulla of the carp, such cells form an interfascicular group of TH-containing neurons (Figure 10A, B). The column of interfascicular neurons in the brain of the carp begins beyond the anterior border of the *lobus vagus* and spreads to the caudal border of the facial nerve. Interfascicular neurons are localized near the bulbo-spinal tract;

their dendrites frequently go around separate fascicles of this tract. The cells are multipolar and characterized by a rostro-caudal gradient in the sizes of their somata. Rostrally localized cells are relatively large diameter of somata and have most widely ramifying dendrites (Figure 10A, B). In neurons of the interfascicular group, there are up to five typical dendrites in the slice plane. Caudally localized interfascicular TH-positive cells are smaller than the rostral units and have similar but more spatially limited zones of the dendrite branchings (Figure 10B). Interfascicular cells frequently form clusters consisting of four to six cells. The total number of such neurons is 30-50 units per each brain side. Morphology of their dendrites and trajectories of axons are similar to those in larger neurons localized more rostrally.

The most caudal group of interfascicular neurons is localized around the *lobus vagus* at the level of motor nuclei of the X nerve. On each side, we found three to four cells with drop-shaped somata, which were shifted dorsally and laterally with respect to other interfascicular neurons. Near the ventricular clearance, we observed large bipolar cells with thin long somata.

TH-Positive cells of the *Area postrema*. In the carp, we observed a compact group of intensely marked neurons within the *area postrema*. On the cross-sections, such cells formed a densely packed accumulation that, in turn, formed a "septum" along the midline of the brain (Figure 10C, D). The TH-positive cells of the *area postrema* in the carp were, as a rule, bipolar or drop-like and were characterized by relatively small sizes. Their dendrites spread in the rostro-caudal and caudo-dorsal directions. Caudo-dorsal (or apical) branches of the dendrites are relatively short; they form small fascicles of thickenings under the dorsal surface of the *area postrema* (Figure 10D). The rostro-ventral (or basal) branch projected to the Cajal nucleus and formed a dense TH-positive neuropil.

Periventricular TH-positive cells and localization TH in areas secondary neurogenesis. Along with large-sized differentiated cells possessing well-developed dendrite arborization, we found in the medulla of the carp a population of phenotypically immature cells characterized by periventricular localization, and location in peripheral areas of *lobus vagus*, cerebellum and *lobus impar* (Figure 11A-F). The sizes of the somata of such cell were about 6-8 μm; the relative level of TH staining in these cells was very high (Figure 11C, E). At different levels of the medulla of the carp, we found groups of periventricularly localized cells between radial processes of different lengths (Figure 11B, F). The somata of such cells were comparatively hight marked with respect to TH; they were localized periventricularly, while radial fibers reached deep cerebral layers (Figure 11E). In the subventricular

region, in the zone along radial fibers, we found the somata of undifferentiated cells also containing TH (Figure 11F). Sizes of the somata of undifferentiated TH-positive cells in the periventricular zone varied within a wide range, from 11.5/7.3 μm in the smallest cells to 19/9.8 μm in the largest units.

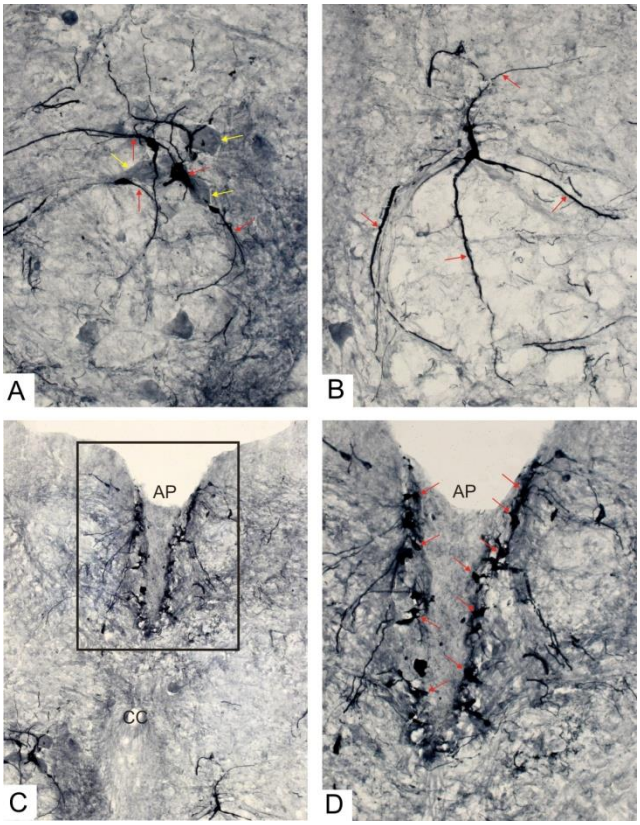

Figure 10. Tyrosine hydroxylase-immunopositive neurons in the interfascicular zone and *area postrema* of the carp brain. A – group of TH-positive interfascicular cells in the rostral zone (red arrows) near with TH-immunonegative cells (yellow arrows); B – stained interfascicular neuron (limited by a square) localized in the rostral part of the brainstem; red arrows indicate the typical dendrites of cell; C – distribution of TH-positive cells at the level of the *area postrema* (in rectangle); D – fragment of the dorsal part of the slice shown in fragment C (at a higher magnification); red arrows show a immunopositive cells of *area postrema* (AP). Scale bar: A, B, D – 50 μm; C – 200 μm.

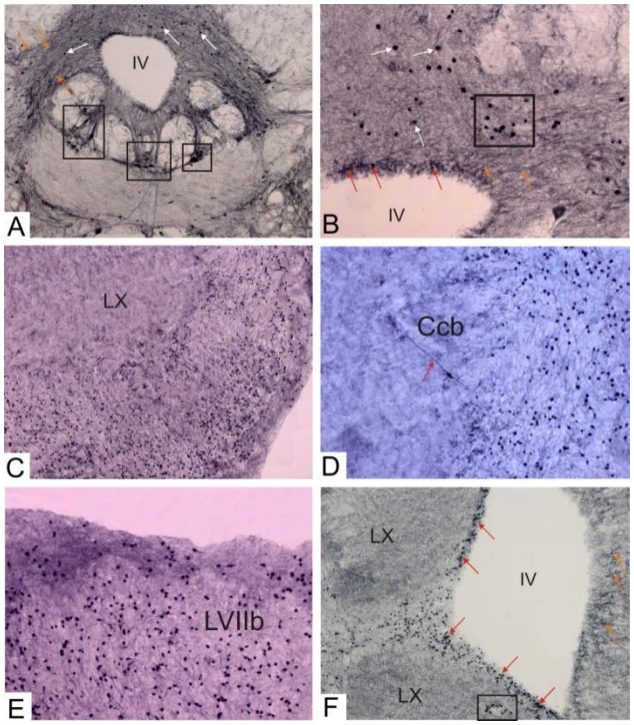

Figure 11. Tyrosine hydroxylase-ip cells in primary and secondary of neurogenic zones of carp brain. A – TH-ip cells (white arrows) and radial fibers (orange arrows) in periventricular zone of *medulla oblongata*; cells migrated from periventricular zone in the rectangle; B – morphological heterogeneity of TH-ip elements of periventricular zone, red arrows show immunopositive fiber endings and cells, another designations as in A; C – TH-ip cells in the latero-basal part of *lobus vagus* (LX), D – immunopositive cells in the basal part of cerebellum, red arrow shows the ectopic TH-ip cell; E – TH-ip cells in basal part of *lobus inferior* (LVIIb); F – TH-ip cells in periventricular area (red arrows), fragment migrated immunopositive cells bounded by a rectangle. Scale bar: A, C, F – 200 μm; B – 50 μm; D, E – 100 μm.

4.3. Morpho-Functional Acpects of CA-Ergic System in Carp Brain

According to the published data [1, 5, 11, 27, 86, 87], CA-ergic cells in neuronal networks of the medulla can fulfill the functions of local interneurons, projection long-axon neurons, neurosecretory units, or sensory units. The morphology of interfascicular TH-positive cells in the carp brain

allows one to regard their functional specialization as local interneurons, since they form intensely branched dendritic networks [88]. The most medullary TH-positive neurons project their terminals to the LCT. Therefore, it is appropriate to hypothesize that all these cells are relatively long-axon neurons projecting to the rostral part of reticular formation, isthmus, and secondary gustatory nucleus. The TH-positive cells of the vagus region and *area postrema* (supposedly dopaminergic) have access to the fourth ventricle; likely, these neurons are chemosensory units responsible for the relations between the cerebrospinal fluid and neuronal medullary systems. On the other hand, these two neuronal groups in the carp brain differ from others in an extremely high level of TH activity; it cannot be ruled out that they can serve as a source of dopamine coming to the cerebrospinal fluid. The morphology of these neurons allows one to hypothesize that each of the three groups of medullary CA-ergic neurons in the carp is involved in realization of at least two functions of the above-listed ones, while the cells associated with the *lobus vagus* can combine all three functions. We believe that a crucial question in understanding the principle of functioning of CA-ergic neurons in the carp CNS is the following: "How do single neurons identify or coordinate these functions?" The relationship of all three groups of medullary CA-ergic neurons with the sensory systems attracts special interest. Neurons of the vagus region and *area postrema* provide abundant innervation of the commissural Cajal nucleus, the main visceral center in fishes [89], and also of the *lobus vagus*. All three CA-producing cellular groups in the carp project to the LCT, whose fibers are directed toward cholinergic cells of the secondary gustatory nucleus (which is the major integrative center [28]) and of the isthmus nuclei (which is the secondary visceral structure). The medial octavo-lateral nuclei receiving primary mechanosensory projections from receptors of the lateral line [90] are the most intensely innervated structures of the medulla. In the motor region, interfascicular CA-ergic neurons project to motoneurons of the nuclei of *glossopharyngeal* (IX) and *vagus* (X) nerves. We believe that these cells provide neurohumoral relations between the cerebrospinal fluid and different centers of reticular formation. Therefore, all three groups of CA-ergic cells of carp medulla can realize neurosecretory and sensory functions related to estimation of the location of food in space and provide local or "distant" neuron-to-neuron connections using coordination of mechanosensory, visual, and gustatory functions, respectively.

In the carp medulla, there is no system of coherent conduction pathways. Axons of medullary CA-ergic neurons usually do not pass along the external cerebral surface. Throughout the carp medulla, the LCT is closely related to

the SGT. Medullary CA-ergic neurons of all three types project their axons to the LCT. Axons of such cells belonging to the *vagus group* and *area postrema* compose, probably, a greater part of the tract. In addition, the LCT is the largest fiber structure at the level of the *lobus vagus*. In the caudal direction, this tract possesses a cone-shaped narrowing, and it, little by little, disappears at the level of the spino-medullary bunch. This allows us to believe that medullary TH-positive neurons cannot be considered a source of the main descending projections. The decrease in the LCT volume rostrally to the *lobus vagus* is explained, perhaps, by the fact that the axons leave the bunch and, at the same time, innervate adjacent structures of reticular formation.

Interfascicular cells in the isthmus region and the rostral part of the carp medulla are morphologically heterogeneous. According to the existing classifications [1, 27, 83], we believe that specialized neurons and neurons belonging to the region, which is localized along the rostro-caudal column of interfascicular cells, are units of the tegmental motor nuclei. In this column, Mauthner neurons, groups of reticulo-spinal neurons, and neurons of the *locus coeruleus* are most typical. The fact that interfascicular cells are localized near large conduction bundles of the brainstem and that these units belong to the medial RF allow us to suppose that such neurons are involved in spatial integration of neuronal activity, in particular in coordination of the reflex activity. It was found that interfascicular neurons in fishes are heterogeneous from the neurochemical aspect and, at the same time, can be addressees of several stimuli of different modalities [1, 5, 20, 27].

In the carp, large multipolar interfascicular neurons possess typical peculiarities of morphological organization, which can provide realization of such functions. We believe that interfascicular TH-positive neurons in the carp brainstem form only a small subpopulation of interfascicular cells. In interfascicular TH-positive neurons and some reticulo-spinal neurons of the carp, certain similarity in the morphology of dendrites is observed. For example, the main ventrally and ventrolaterally projecting dendrites of interfascicular CA-ergic neurons resemble those of Mauthner neurons and reticulo-spinal cells [87]. In such cells, dendrites have long and thick primary branchings. Branches of the subsequent orders form dense trees within the ventral reticular formation.

Projection regions of the terminals cover the ventral and lateral segments of the white matter along the periphery of the medulla [5, 8]. It seems possible that such regions receive input signals from the transbulbar systems. The coefficient of volumetric expansion of terminals in interfascicular cells of carp medulla indicates that such neurons are involved in integration and/or

coordination typical of certain types of reticular activity. For example, their relation to the motor zone of the *lobus vagus* and secondary gustatory nucleus is, probably, indicative of the fact that they simultaneously influence the orobranchial and oropharyngeal motor systems, whose activities provide the main components of alimentary behavior.

Catecholaminergic cells of the Lobus vagus. Tyrosine hydroxylase-positive cells related to the nucleus of the *lobus vagus* are less numerous than interfascicular units. The presence of apical terminals in vagus CA-ergic cells in the carp allows us to consider such units a neuronal group that maintains contacts with the surface of the cavity of the cerebral ventricle in the definitive state; this is indicative of possible chemosensory specialization of such units [91]. Therefore, TH-positive cells related to the *lobus vagus* can participate in the control of functions of the circumventricular organ and/or in regulation of the chemical composition of the liquor. It is hypothesized that paraventricular vagus CA-ergic neurons in the carp excrete dopamine into the cerebrospinal liquor or participate in dopaminergic modulation of functions of the ependyma. The morphological structure and location of medullary CA-ergic neurons of this group in the carp and liquor-contacting cells distributed along the ventral field of the central canal throughout the entire length of the spinal cord in the eel [92] and trout [93] and Amur butterling [88] are similar in many aspects. In different fish species, apical terminals of such cells provided with cilia project to the clearance of the spinal tract, while thickened basal terminals form propriospinal reticular branchings in the lateral and ventral regions of the spinal cord [83, 92, 93]. Results of the studies of ultrastructural peculiarities of such CA-containing thickenings in the brain of the eel and trout showed that they participate in the formation of pre- and postsynaptic structures in the spinal cord [92, 93]. The structural and neurochemical similarity indicates that the above-mentioned two cellular groups can promote similar functions within the medulla and spinal cord.

CA-ergic neurons of the Area postrema. Recently, it was demonstrated that the dense wedge-shaped population of CA-ergic cells in the zone of the *area postrema* is a unique feature of this region of the fish medulla [27, 83, 88, 92]. In such wedge-shaped accumulation in the *Danio rerio*, TH-immunonegative cells are also present [27]. Apical terminals of neuronal processes of the cells of this group occupy a strategically important position from the aspects of both control of the chemical composition of the plasma and release of neurogenic substances into the circumventricular system of animals. Basal processes of TH-positive neurons of the *area postrema* in the carp are branched and distributed at significant distances and form powerful

projections in the medial part of the Cajal nucleus. A part of the basal dendrites in neurons of the *area postrema* is combined with lateral processed of vagus CA-containing cells at the site of their entry into the LCT; they participate in innervation of the nuclei of the isthmus region. It was demonstrated that injections of a retrograde marker into the secondary gustatory nucleus of the golden carp result in staining of neurons in the *area postrema* [89]. The neurochemical status of stained neurons in this fish species remains unknown, but a high density of distribution of TH-positive neurons around the *area postrema* does not allow one to rule out the possibility that some such units are CA-ergic. Collaterals of the basal processes project (along the ventrolateral border of the *lobus vagus*) to the superficial region and terminate in this region as a ribbon with final broadenings. The *lobus vagus* in the carp is weakly stratified; this is why it does not correspond completely to the structure described in the golden carp [89] and *Danio* [27]. In the former species, primary processes of visceral afferents are found in the superficial layer of the *lobus vagus* [94], among which there are also CA-containing fibers [28]. If the organization of the *lobus vagus* in the carp is similar to that in the *Danio* and gold carp, TH-positive neurons of the *area postrema* in the carp play, apparently, the role of a gating mechanism for the activity incoming along visceral afferents.

4.4. Involvement of Dopamine in Adult Neurogenesis of the Carp Brain

The fish brain possesses a unique peculiarity, which is absent in other vertebrates. Namely, it continues to grow as the organism grows during the entire period of life of the fish. It was demonstrated that, in the brain of fishes, the system of cambial elements, whose differentiation allows one to replenish populations of neurons and glia during the entire period of life of the animal, is preserved [69, 95]. We believe that the existence of undifferentiated CA-ergic cells demonstrating a high positivity with respect to TH in their terminals, as well as of elements of radial glia, at different levels of the periventricular zone of the medulla of the carp should be considered to be related to the involvement of these cellular cerebral systems in the processes of adult neurogenesis in carp. Analysis of the morphological parameters of the somata of undifferentiated cells in the periventricular cerebral region of the carp showed the presence of several size groups of the cells similar in their morphology. Their relative number in the periventricular region and areas of

secondary neurogenesis is varies. We believe that small rounded cells are at an early stage of cellular differentiation after going out of such units from the proliferative cycle in the matrix cerebral zone. Larger cell units, which comprise about 20 and 40%, are, apparently, subsequent stages of growth and differentiation within the period preceding the formation of terminals of these cells. Expression of TH in different cell types within development stages where morphological differentiation of the cells is weak or practically absent indicate that dopamine synthetized by these cells plays, probably, the role of a morphogenetic factor controlling proliferation, migration, and differentiation of nerve cells [69]. The density of distribution of phenotypically immature cells in the periventricular cerebral region and peripheral areas of sensor centers (*lobus vagus* and *lobus impar*) of the carp is very high, which shows that they can be a target for the paracrine way of neurotransmission in this cerebral region.

The presence of radial glia in the periventricular region and localisation of the somata of undifferentiated TH-positive cells distributed along the radial fibers, indicate, apparently, that these cells in the periventricular cerebral zone of the carp actively migrate. According to the data obtained in experiments on the eel, the maximum concentration of dopamine D1 receptors was found in the periventricular regions [96], i.e., in the matrix cerebral zones where neurogenesis lasts during the entire period of life of the animal. It is obvious that the cells localized in the proliferative regions are targets for a regulatory influence of dopamine. Therefore we believe that the peculiarities of distribution of TH in the medullary cerebral region of the carp are directly related to the ability of fishes to grow and to continue neurogenesis during the entire period of life. Such interpretation of the data obtained in our experiments allows us to conclude that dopamine is not only a regulator of the functional activity of neurons and a modulator of synaptic neurotransmission in mature neuronal networks. In primary and secondary matrix cerebral zones, this cathecholamine serves as an inductor of development (a morphogenetic factor) that functions in the course of adult neurogenesis of fishes. As evidence, we can consider the presence of phenotypically immature elements with expression of TH in the medullary proliferative zone and also of elements whose morphology in the carp brain corresponds to that of radial glia.

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