Chapter 8

Participation of Neurochemical Signaling in Adult Neurogenesis and Differentiation

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Additional information is available at the end of the chapter

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1. Introduction

The revealed peculiarities of structural and neurochemical organization and description of basic histogenetic processes (proliferation, migration and neuronal cell differentiation) during the brain forming in fish, which have signs of fetal organization, widen the existing knowledge about histogenesis of these structures in postembryonic development. It seems conceivable, that during postembryonic development in teleost fishes some neurotransmitters and gaseous mediators (NO and H_2S) act as factors, which initiate and regulate the cellular and the tissues processes of genetic program during the brain development. We suppose the presence of epigenetic control of adult neurogenesis in salmon brain via highly coordinated nonsynaptic cell–cell signaling. This communication engages the neurotransmitters GABA and dopamine whose extracellular concentrations depend on neuroblasts number and high affinity uptake systems in neural stem cells. Neuroblasts release GABA providing a negative feedback control of stem cell proliferation and instructing them on the size of the neuroblast pool. We suggest that in salmon brain exist strong control mechanisms of neuroblast production. The data provided by our study add to our general understanding, that peculiarities of distribution of classical neuromediators (GABA, catecholamines) and gasotransmitters (NO and H_2S) are directly connected with ability of the fishes brain to grow during the animal entire life. We suggest, that some classical neuromediators (GABA, catecholamines) and gasotransmitters (NO and H_2S) not only regulate functional activity of neurons and modulate synaptic transmission in mature neural networks, but also are regarded as inductors of the fishes brain development (morphogenetic factors) in postembryonic ontogenesis. We propose that dopamine and GABA act as homeostatic signals to regulate neuroblast production. This confirmation is proved by finding of the phenotypically immature elements, expressing the above mentioned molecules in proliferating brain areas, in the three-year-old salmon brain,

and of elements, which owe morphology of radial glia. The presence of enzymes, synthesizing gasotransmitters in the brain areas, which are expressing proliferative cell nuclear antigen PCNA, have proved their participation in regulation of postembryonic neurogenesis.

In the fishes, which preserve fetal state during long time (salmon and carp), such markers as NO and H_2S in periventricular proliferative areas may present in different ratios. This is consistent with the hypothesis that in functionally similar complexes in animals the different signal transduction systems may be involved. In contrast to widespread neurogenetic model *Danio rerio*, the development of the salmon and sturgeon nervous system occurs during long time. As it follows from our data, the development of different CNS structures in the *Oncorhynchus masou* brain is characterized by evident heterochrony, so the cells of caudal brain regions gain features of phenotypical specialization earlier than in the forebrain structures. We suggest that the brain of these animals during a long time preserves the signs of fetal organization and low differentiated cells presence confirms this hypothesis.

Last years, certain attention of neuroscientists of different profile was turned to participation in the work of the brain «gaseous intermediaries»: nitric oxide (NO) and hydrogen sulphide (H_2S). Their presence is found in the brains of representatives of different groups of vertebrates: from the Agnatha to human. The few data points to a high degree of variability in the distribution of NO-ergic neurons in the fish brain [1-3], and information about the involvement of nitric oxide and hydrogen sulphide in the functional activity of nervous system of fish is unordered and contradictory. This draws attention to the fact that the relative number of NO-synthesizing neurons and glial cells in the sensory, motor and integrative centers of the brain fish significantly exceeds that of terrestrial vertebrates, in particular, mammals [1, 4, 5]. This implies a wide and varied participation of NO in the metabolism of neurons and glial cells in the central nervous system of fish compared with mammals. However, information about the relationship of the NO-producing neurons of the brain of fish with the systems of classical neurotransmitters such as acetylcholine, catecholamines and GABA, are practically absent. Virtually nothing is known about the distribution of H_2S-producing systems in the CNS of bony fishes. These investigations are of particular importance in connection with the emerging data on morphogenetic the role of classical and gas intermediary in the formation of the central nervous system of vertebrates [6].

The brain of fish has a unique vertebrates feature - it grows with the organism during all life. In connection with this fish is a model object for the study of embryonic and postembryonic development of the CNS, to influence these processes of various factors. It is shown that in the brain of adult vertebrate a system of cambial elements remains, the activity of which allows to replenish the population of neurons and glial cells in the course of a long period after birth [7]. Currently the mechanisms of pre-and postnatal morphogenesis of the brain in the fish, which for a long time secures the larval state, virtually have not been studied [8-10].

Especially it concerns the role of the so-called «radial glial cells» in the processes of morphogenesis of the brain, the availability and distribution of proliferative areas in the brain of adult fish. The results of the research on *Danio rerio* showed that the newly formed cells moving from periventricular areas deep inside the brain, where they differentiate into neurons [11]. It was found that the centers of proliferation are localized along the rostro-caudal axis of the brain [7].

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The interest to the study of these processes in fish is caused by the fact that the «radial glia» may be connected with the processes of migration and differentiation of neurons and glial cells in the prenatal period, large quantities present in the brain of a fish and in the adult state (unlike other vertebrates). However, in spite of the available literature information, participation of the radial glia (RG) is in the process of neurogenesis adult animals and little studied. One of the reasons for the lack of such information is a small number of examined in the terms of species and groups of fish, the absence of reliable markers of the RG in lower vertebrates.

Sturgeon and salmon fish, which have become the main objects of our research, represent the most ancient group of vertebrates, which are the most primitive branches ray-finned fish [12-13]. The information about the development of the brain sturgeon and salmon, the relations of embryonic and a definitive parts in the structure of the pre-and postnatal neurogenesis, organization and formation of the neuromediating and modulating brain systems in the literature are extremely limited. This concerns especially the sturgeon fishes, the evolution of which was carried on the pedomorphosis way, which is characterized by the slowing of organs or of their systems and the preservation of the adult embryonic status of relevant features.

The purpose of this chapter is to explore the organization, projection features and relationships of signal-transduction systems, producing a classic neurotransmitters (catecholamines, acetylcholine, gamma-aminobutyric acid-GABA) and gazotransmitters (nitric oxide and hydrogen sulphide), in the brain of fish and evaluate their participation in the processes of the post-embryonic morphogenesis the CNS.

2. Methods

Molecular-biological approaches associated with identifying of histochemical and immunohistochemical activity of mediators or enzymes of their synthesis were used for characteristics of neurotransmitter systems structures of the brain and spinal cord fish. Specific antibodies are also used by us in identifying of proliferative cell nuclear antigen (PCNA), transcription factor Pax6 and calcium binding protein parvalbumin. To investigate the relationship of brain applied marking nerve fibers using carbocyanin dye DiI. To track ascending mediatorically specific projections of catecholaminergic cells was used the immunofluorescence method of marking tyrosine hydroxylase in combination with the marking of the DiI.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.

Fifty-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 [14]. 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.

For **histochemical staining of cholinergic neurons** in the brain of fish we used marking of choline acetyltransferase (ChAT; NF 2.3.1.6.). Method was performed on fishes whose brains were fixed at 4°C for 2 h in 1% solution of paraformaldehyde based on cacodylate buffer (0.1 M) with sucrose (0.32 M; pH 5.0). The material was washed out in cacodylate buffer (pH 5.2) with sucrose for 18 h with sevenfold change of this solution. Frontal and sagittal 50-μm-thick slices were prepared with a freezing microtome. To exclude nonspecific transferase activity, 20 mM diisopropyl fluorophosphate (DFP), 10 % sucrose, and 25 mM cacodylate buffer were added to the incubation medium (pH 6.0) cooled to 4°C ; this medium was placed on an ice bath (0-4°C) for 1 h. After preincubation, the slices were placed in the incubation medium (pH 6.0) with the following final concentrations (mM): cacodylate buffer, 25; DFP, 1.0; choline chloride, 4.0; lead nitrate, 1.0; acetyl-CoA, 0.3, and 5% sucrose. The sliced were thermostated at 37°C for 2 h, washed out in distilled water, and treated in 5% solution of ammonium sulfide. Then, the slices were post-fixed for 5 min in 5% solution of formaldehyde based on cacodylate buffer (0.1 M; pH 5.2) with sucrose (0.32 M), dehydrated, and embedded in balsam. To estimate the specificity of reactions to ChAT, we carried out a few control experiments. In the first control series, we excluded DFP from the incubation medium. In the second control series, cetyl-CoA or choline chloride were absent in the incubation medium. In the third control series, we added chloracetylcholine-perchlorate (10 mM) to the DFP-containing pre-incubation medium; the incubation period was increased to 1.5-2 h. In all control experiments, a positive reaction was absent.

Immunohistochemical methods. Fishes were kept in aquaria with aerated seawater at 15-17°C. Before experiments, fishes were anesthetized in the cuvette with 0.1% solution of tricaine methanesulfonate (MS-222; Sigma, USA) in seawater for 10-15 min. The brains of fishes were fixed for 2 h at 4°C in 4% solution of paraformaldehyde dissolved in phosphate buffer (0.1 M, pH 7.2). For morphological analysis, the obtained material was embedded in paraffin according to a standard technique and stained by Nissl. In the course of immunohistochemical studies, we identified the elements containing GABA, tyrosine hydroxylase (TH), parvalbumin (PA), neuronal nitric oxide synthase (nNOS), proliferative cells nuclear antigen (PCNA), transcription factor Pax6 and cystathionine β-synthase (CBS). For this purpose, we used indirect avidin-biotin-peroxidase (ABC technique) or streptavidin-biotin staining. The material was washed out for 24 h in 30% sucrose solution. Transverse 50-μm-thick slices of the fish brain were prepared using a freezing microtome. Free-floating slices were incubated at 4°C for 48 h in the presence of monoclonal mouse antibodies against GABA (ICN Biomedicals, USA; dilution 1:4000) and tyrosine hydroxylase, TH (Vector Laboratories, USA; dilution

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1:5000), PCNA (Dako, Denmark; 1:4000), monoclonal antibodies against human transcription factor Pax6 (Chemicon, USA; 1:3000), monoclonal antibodies frog against PA (ICN, Biomedicals, USA; 1:4000), rabbit polyclonal antibodies against nNOS (ICN, Biomedicals, USA; 1:5000), monoclonal antibodies mouse against CBS (Abcam ab54883, England 1:5000). Then, the slices were incubated with secondary biotin-conjugated horse antibodies against mouse immunoglobulins (Vector Laboratories, USA) for 2 h at room temperature and washed out three times in 0.1 M phosphate buffer. To reveal localization of NO-ergic neurons and fibers, we used a technique of indirect streptavidinbiotin immunohistochemical labeling of NOS. The slices were incubated with primary polyclonal rabbit antibodies against nNOS (ICN Biomedicals, USA; dilution 1:5000) at 4°C for 24 h. After three washings out in phosphate buffer, the slices were incubated with secondary biotin-conjugated goat antibodies against rabbit immunoglobulins (Biomedicals, Germany) at room temperature for 2 h. The material was washed out three times in phosphate buffer. Then, the slices were incubated in the presence of the streptavidin-peroxidase complex (Biomedicals, Germany) at room temperature for 2 h and again washed out three times in phosphate buffer. Immunohistochemical reactions were visualized using a standard avidinbiotin system (ABC; Vectastain Elite ABC Kit; Vector Laboratories, USA). To identify the reaction products, the slices were incubated in a substrate for detection of peroxidase (VIP Substrate Kit; Vector Laboratories, USA); the process of staining was controlled under a microscope. Then, the slices were washed out in three changes of phosphate buffer, mounted on slides, dehydrated using a standard technique, and embedded in balsam. To estimate the specificity of the immunohistochemical reaction, we used a technique of negative control. The masu brain slices were incubated in a medium containing 1% nonimmune horse serum (instead of primary antibodies) for 48 h, and then all procedures were performed as was described above. In all control experiments, the immunopositivity in the studied cells was absent.

To study projections of the preglomerular complex and glomerular nucleus, we used the carbocyanine dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, DiI (Aldrich, Sigma, USA). The brains of fishes were fixed for 24 h in 4% solution of paraformaldehyde; then, crystals of the above dye were introduced in the region of the anterior and medial preglomerular and mammillary bodies. The obtained preparations were incubated in 4% solution of paraformaldehyde with the addition of 0.01% ethylenediamine tetraacetic acid (EDTA) at room temperature. Frontal, sagittal, and horizontal slices (50 μm thick) were prepared using a vibratome (VIBRATOME 3000; Sectioning system, Germany) and embedded in glycerine. To visualize the marker, we used an optical system, AXIOPLAN-2, Imaging (Gerinang, Germany). Preparations with DiI-marked structures were photographed using a optical system AXIOPLAN-2, Imaging (Gerinang).

Immunofluorescent labeling of tyrosine hydroxylase (TH) combined with retrograde labeling of neurons with the carbocyanine stain DiI was used to study the brains of Amur bitterlings *Rhodeus sericeus*. Specimens were fixed in 4% paraformaldehyde for one day, after which crystals of stain were placed in the ventral part of the telencephalon. Specimens were incubated in 4% paraformaldehyde supplemented with 0.01% ethylenediaminetetraacetate (EDTA) at room temperature for one day. Frontal, sagittal, and horizontal vibratome sections of thickness

50 μm were cut and incubated with primary mouse monoclonal antibodies against TH (Vector Laboratories, Burlingame, USA) diluted 1:1000 at 4°C for two days. Sections were then incubated with secondary fluorescent antibodies conjugated with Alexa 546 (Invitrogen Molecular Probes, USA) diluted 1:300 overnight. TH localization was studied using a Leica DM 4500 fluorescent microscope (Germany). Labeled TH and the carbocyanine label were visualized using a Leica TSC SPE confocal laser system (Germany).

Immunoperoxidase labeling of fragmented DNA chains, (TUNEL-labeling). To reveal apoptotic cells, we used a technique for immunoperoxidase labeling of fragmented DNA chains. After 2-h-long fixation in 4% solution of paraformaldehyde based on 0.1 M phosphate buffer (pH 7.2), dissected parts of the brain were washed out for 24 h in 0.1. M phosphate buffer. Then, these samples were put in 30% solution of sucrose based on phosphate buffer (0.1 M) for cryoprotection and kept in this solution up to full immersion. Frontal and horizontal slices (20 μm thick) were prepared using a freezing microtome. To identify TUNEL-positive structures, we used a immuno-peroxidase identification system, ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon International Inc., USA). For blocking endogenous peroxidase, the slices were incubated in 1% solution of hydrogen peroxide for 3 min and then washed out two times for 5 min in phosphate buffer. The slices were covered with a smoothing buffer (75 μl) and kept for 10 sec at room temperature. Then, the slices were slightly dried, subjected to the action of TdT enzyme (55 μl/5 cm^2), incubated in a humid chamber for 1 h at 37°C, and immersed in a stop buffer for 10 min. The slices were washed out in phosphate buffer at room temperature (three times for 1 min with changing of the solution), again dried, covered with antidioxygenin conjugate (65 μl/5 cm^2), and incubated in a humid chamber for 30 min. To detect the reaction products, cerebral slices were incubated in the substrate for identification of peroxidase (VIP Substrate Kit; Vector Labs, USA) with control of the development of color under a microscope, washed out in three changes of phosphate buffer, and mounted on glass slides. The cell nuclei were subjected to final staining with methyl green according to the technique of Brasher [15]. The preparations obtained were dewatered using a conventional technique and embedded in balsam. Morphometric processing was performed using an inverted-stage microscope, Axiovert 200M, equipped with a module, ApoTome, and digital cameras, Axio Cam MRM and Axio Cam HRC (Carl Zeiss, Germany).

The measurements were performed at ×400 magnification in five randomly chosen fields of vision for each studied region. The proliferation index (PI) and apoptosis index (AI) were calculated per 1 mm^2 of the section using the following formulas:

PI = (n of the PCNA-positive nuclei × 100%) ÷ total n of the nuclei and

AI = (n of TUNEL-positive fragments × 100%) ÷ total n of nuclei

Parametric comparison (Student's t-test) was used for estimation of the intergroup differences. The data obtained were processed using Statistica and Excel software. Numerical data are presented below as Means ± s.e.m.

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3. Participation of classic neurotransmitters in the postembryonic neurogenesis in the fish brain

Studies suggest that in the salmon's brain at different stages of postembryonic development two forms of intercellular communications exist. The first form occurs in the early stages of postembryonic development and represents cells cooperation, carried out paracrinic in the period when cells have not developed processes and synaptic structure yet. However, such little differentiated cells are already able to express the specific synthetic machinery for some neurotransmitters and their synthesizing enzymes, gaseous intermediates, transcription factors and other substances (Fig. 1A-D). We suppose that most of the synthesized signals in this period are involved in the regulation of neuronal targets, differentiation and expression of their specific phenotype, acting as a morphogenetic factors. This is consistent with the Ugryumov concept [6] regarding the development of the mammalian brain in the embryonic period of ontogenesis. Already in the early stages of post embryonic morphogenesis of masou salmon, simultaneously two systems of neurochemical signaling exist, the dopaminergic and GABA-ergic systems, providing paracrinic and perhaps autocrinic influence on target cells until the formation of synaptic contacts and the beginning of neurotransmission with specific interneuronal connections. Study on the eal *Anguilla anguialla* showed that the maximum concentration of dopamine D_1 receptors is found in periventricular zones [16] which represent a matrix areas of the brain, where neurogenesis continues throughout the life of the animal. Consequently, the cells located in proliferating areas are targeted for the regulatory impact of dopamine. These cells are localized on the territory of the largest vascular plexus (forebrain and caudal medullar), and synthesize in these regions some substances, like a dopamine and GABA, which then may be excreted in the portal system blood flow and further into the general circulation system, providing regulatory endocrine effects on the peripheral organs [17]. Thus, there is considerable justification to suggest that in the hypophysotrophic areas of the diencephalon and medulla oblongata of the brain of juvenile salmons *O. masou*, dopamine and GABA in undifferentiated cells of periventricular and subventricular areas are inducers of development (morphogenetic factors).

Along with the specified form of intercellular signaling in salmon brain, in ontogenesis there is the development of specific system of forebrain activation and development of the system of remote intercellular signaling. The source of these directed connections are the nuclei of preglomerular complex [18]. Development of projective systems of salmon take place simultaneously with the formation of the structure of preglomerular complex [19]. In the brain of non mammalian vertebrates the volume of sensory projection zones increases during all their life and is provided due to the proliferation of neural stem cells located in the areas of special neurogenetic niches [10]. It is connected with the necessity of adaptation of the CNS of such animals to increase the size of the body and increased inflow of primary sensory information. We believe that a dopamine, GABA-and NO-ergic systems in the brain of salmon participate in regulation of some basis histogenetic processes, such as a cell migration and differentiation of neuro- and gliospecific lines, because the nuclei of preglomerular complexes contain morphologically and neurochemically heterogeneous cell populations (table. 1) represented

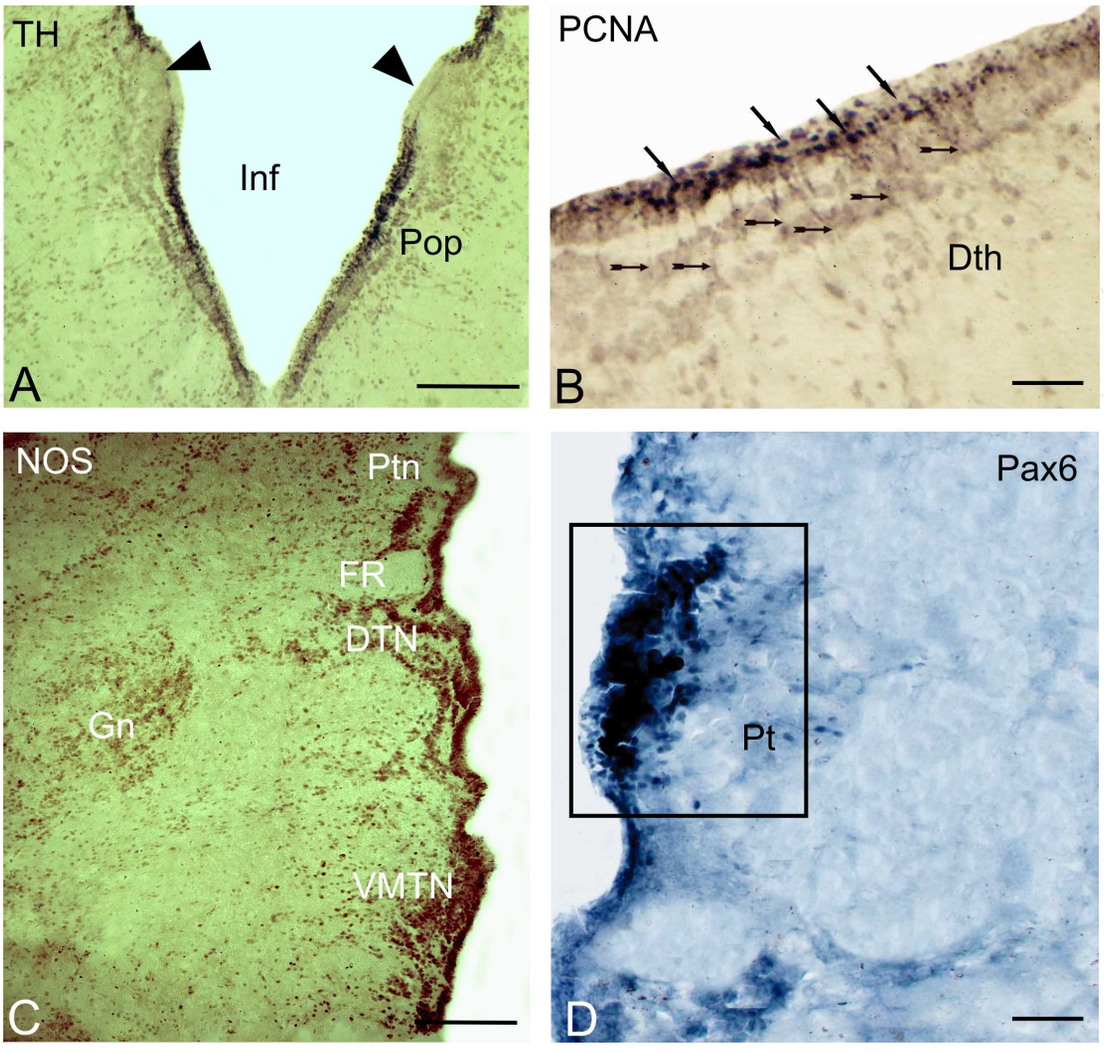

Figure 1. A - immunolocalisation of tyrosine hydroxilase (TH) in parvocellular preoptic nucleus (Pop), B - proliferative nuclear antigen (PCNA) in dorsal thalamus (DTh), C - neuronal nitric oxide synthase (NOS) in pretectal (Ptn), dorsal (DTN), ventro-medial (VMTN) thalamic nuclei, D - transcriptional factor Pax6 in periventricular diencephalon of 6-month-old *Oncorhynchus masou*. Immunonegative border of dorsal neuromers on A, delineated by a triangle, the cluster of immunopositive cells on D, delineated by rectangle. Inf – infundibulum, FR – fasciculus retroflexus, Pt – pretectum. Scale: A, C – 100 μm, B, D – 50 μm.

by the different stages of ontogenesis of major cell types. Cells formed in the proliferative (PCNA-containing) diencephalic areas migrate to the region of preglomerular complex, where their subsequent differentiation and growth take place. These processes are regulated by dopamine and GABA, that indicates the presence of D_1 and D_2 dopamine receptors [16, 20] and $GABA_B$ benzodiazepine receptors [21] in these nucleus of fish. A critical step prevalence of paracrinic relations in the salmons brain can be considered the period before the formation of the blood brain barrier (BBB), which in salmon brain is formed during the first year of life (according to [22]). In the next period of ontogenesis, the formation of the specific connections and the development of cellular processes of neurons and synaptogenesis take place. Today much data exist about the participation of radial glia in the processes of postembryonal neurogenesis by asymmetric mitoses in which one daughter cell remains in the periventricular

area and has a rounded shape, while the other has a long process, which may later be eliminated through somal translocation [23]. It was shown that during embryogenesis of human, the predecessors of dopaminergic neurons in the basal part of the midbrain have the morphology of radial glia [24]. Immunolabeling of radial glia cells in salmon's brain in different ages (Fig. 2 (A-D), as well as evidence that the TH-and GABA-ip cells were located on the territory of PCNA-ip proliferative zones and together with PCNA marked the neuromeric structure of diencephalon and medullar part of the brain, certainly shows that dopamine and GABA-ergic signaling participates in the processes of postembryonic neurogenesis of the salmon's brain, as inductors of development. Our data are consistent with the labeling of some rhombomeres in the brain in an embryo of sharks *Scyliorhinus canicula* [25].

Nuclei	Neuronal nitric oxide synthase (nNOS)		Choline acetyltransferase (ChAT)		GABA		Tyrosine hydroxylase (TH)		Parvalbumine (PA)	
	Size of cells (μm)	Total number (%)	Size of cells (μm)	Total number (%)	Size of cells (μm)	Total number (%)	Size of cells (μm)	Total number (%)	Size of cells (μm)	Total number (%)
Glomerular	8-7 II 10-8 II 12-10 III	30±4	20-12 I 18-12 I 14-6 IV	18±2	9-6 II 7-7 II 12-6 IV 14-7 IV	50±6	9-7 II 10-6 II 15-6 III 17-8 IV	12±2	6-6 II 11-7 II 12-9 III 13-6 IV	48±4
Anterior Preglomer.	10-8 I 12-7 III 14-8 III 15-6 IV	24±3	12-9 III 13-8 III	9±1	20-13 I 12-12 III	47±5	8-6 II 10-7 II 12-9 III 14-6 III 12-6 IV	14±2	10-8 II 11-7 II 13-10 III	45±5
Medial Preglomer.	10-8 II 12-9 III 13-8 III	21±3	12-9 III 13-10 III 14-11 III	12±1	8-7 II 10-7 II 12-9 III	32±4	9-9 II 10-7 II 12-9 III 14-7 IV	8±1	9-7 II 10-8 II 13-10 III	30±3

Footnotes. Roman numerals (in brackets) indicate the cell type. Mean values of the large and small diameters of neurons (M ± m, μm) are separated by slashes.

Table 1. Morphometric characteristics and relative numbers of neurons belonging to different neurochemical types in the nuclei of the Preglomerular complex and also in the Glomerular nucleus of the *Oncorhynchus masou* brain.

Differentiation of cells in various parts of the salmon's brain presents a heterochronical process. In caudal part of brain some reticulospinal cells, cells of nucleus raphi, nuclei of V, VII, IX and X pairs of cranial nerves, much earlier acquire the features of phenotypic specialization than in the structures of forebrain. Measurements of fractal dimension and some morphometric parameters (total length of branches, number of terminal branches, number of branching

points, and cell area) were used for the quantification of morphological patterns of two spinal neuron groups in young *Oncorhynchus masou* at two ontogenetic stages [26]. During the 1st and 2nd years of life, the neurons of brainstem and spinal cord have enough developed dendrites and axons, which, however, have growth cones, indicating the continued postembryonic period of growth and development of these structures and their further differentiation. During the second year of life, the values of morphometric parameters and fractal dimension of neurons increased in both groups. Basic morphometric values correlated with fractal dimensions and conformed to morphological changes in the dendritic tree of the investigated neurons in ontogenesis. During the third year of life, in the nuclei of the brain and spinal cord large-differentiated cells expressing TH, GABA and parvalbumine in the motoneurons of ventral spinal column, nuclei of craniocerebral nerves, reticulospinal cells and some diencephalic nuclei were revealed [27].

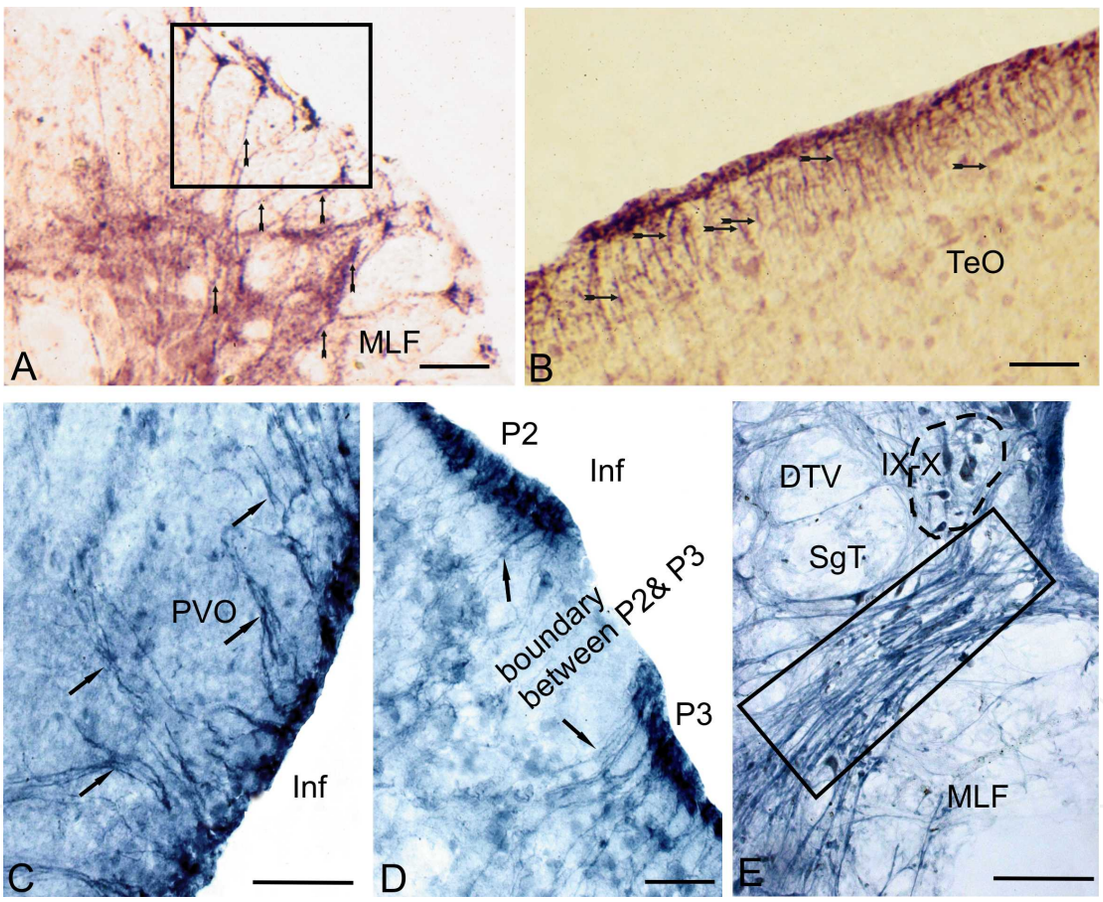

Figure 2. Immunohistochemistry of tyrosine hydroxylase in a spinal cord (A) and tectum (B) of a one-year old *O. masou*, in the periventricular diencephalic (C, D) and the medullar (D) departments of a 3-year-old fish. The arrows show the radial fiber; and: rectangle delineated areas of radial fibers, forming the «end feet»; D: on the border between dorsal neuromeres the immunolabeling of TH is absent; E: rectangle delineated by interfascicular area containing the radial fiber. Scale: A, B, D-50 μm; D-100 μm.

Along with systems synthesis of classical neurotransmitters, immunolocalisation of transcription factor Pax6 was investigated, the marking of which adequately reflects neuromeric

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structure of the salmon's brain in different ages (Fig 3 A, B). The early juveniles (3 and 6 months old) are the Pax6-ip cells do not have any processes and formed a small clusters corresponding to forebrain prosomeres (P1-P3), and in the medulla, such accumulations marked the rostral (R1-R2) rhombomeres (Fig. 3B). On the boundary of neuromeres labeling of PCNA and Pax6 were absent (Fig.3 A, D). In three-year old salmons the marking Pax6 was found in the cells and radial fibers, located in the periventricular and subventricular areas of diencephalon that corresponds to the data of labeling of Pax6 radial glia in the areas of postnatal neurogenesis of mammals [10]. On borders of the forebrain neuromeres the immunolabeling Pax6 in 3-years old individuals was absent. Expression of Pax6 was also found in glomerular nucleus and nuclei preglomerular complex that suggests about morphogenetic processes on the territory of the largest sensory center during postembryonal period. Immunolocalisation of Pax6 in specific cell clusters of glomerular nucleus, appropriated to some neuroanatomical zones, in which the differentiation of neurons, conducting various types of sensory signalization was revealed (Fig 3C. D). Studies suggest that factor Pax6 participates in the regionalization of the structure of the brain in postembryonal period, and its expression in different ages of salmon brain shows that the processes of neurodetermination and migration of cells, formed in proliferative areas of the brain in these age periods are regulated by means of this transcriptional factor. In the literature there are discrepancies regarding the organization and topography of dopamine, GABA-and NO-ergic complexes in the brain of different teleost fishes. Significant differences in the organization of the mediator systems in different fish species become more explainable, given the above mentioned scheme. We believe that neurotransmitter systems in the brain fish should be considered not only from the standard point of view of their definitive neuroanatomical structure, but must also take into account data on heterogeneous molecular phenotype of dopaminergic, GABA and, apparently, NO-ergic systems. Thus, for the establishment of homology, along with the systematic position, it is advisable to take into account the age, stage of development, physiological status and sex of the animal. In adult masou salmon and chum salmon the cells of Dc area of the telencephalon reach a high level of specialization and corresponding to the Ramon-Molener classification can be attributed to allodendric type.

Such cells have been found only in the most mature individuals (of 4-5 years old) going to spawn. One of the forms of specialization of these cells is that they have a network of basal spiny dendrites. This corresponds to the estimated specialization of such cells as associative spiny interneurons participated in communications with other parts of the dorsal area in telencephalon. Widespread TH and GABA in the telencephalon of adult chum salmon indicates that species to this period of development, along with paracrinic (volume) neurotransmission, there is a distant form of neurotransmission, which is becoming the predominant further ontogenetic development and ageing of the animal. We suggest that the acquisition of spiny apparatus by the neurons in the dorsal (Vd) and internal (Vi) areas ventral zone can be considered as one of the stages of ontogenetic development of neurons in the brain, indicating the age-related changes in the organization of the salmon's dopaminergic system. Formation of the system of neurochemical communication in the CNS of masou salmon in postembryonal period consists of two main stages. At the first stage the undifferentiated cells are located in matrix areas of the brain and expressed of specific syntheses (catecholamenes, GABA, NO,

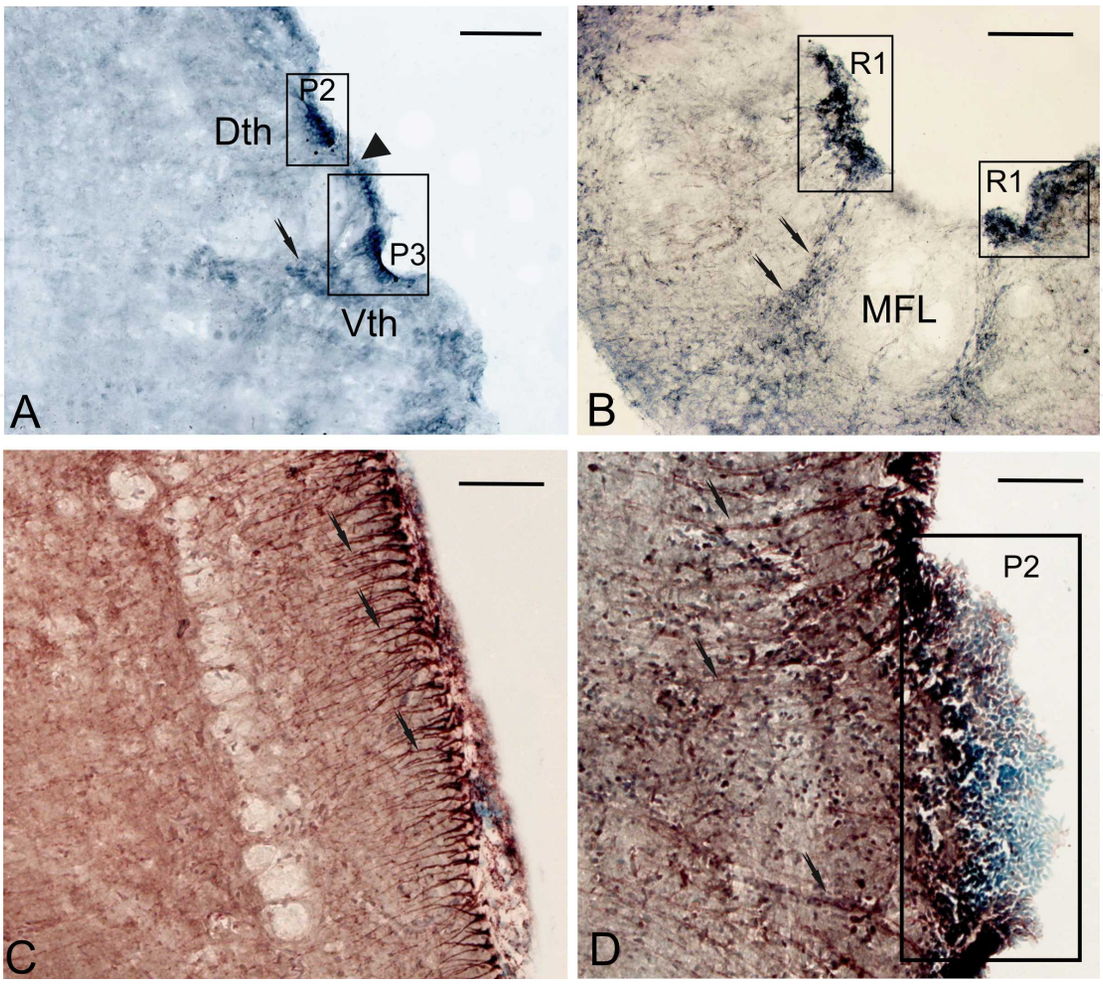

Figure 3. Expression of transcription factor Pax6 in the brain of 3-month-old salmon *O. masou* (A and B) and 3-year-old trout (C and D) (immunoperoxidase staining, light microscopy). Accumulations of immunopositive cells in the diencephalon (A) and medulla (B). Part of the brain (in rectangles) labels its neuromeric structure, the sites without immunolabeling constitute the borders of forebrain P2 and P3 prosomers (black edges of arrows), arrows with a cut show accumulations of migrating cells. Radial glia in the optical tectum (C) and around dorsal neuromer (P2) in the diencephalon (D). Scale: A-100 μm, B-200 μm, C and D – 50 μm.

some transcription factors). These substances are acting in paracrinic interaction and involved in regulating basis histogenetic processes: cell proliferation, cell migration, differentiation of target cells and expression of a specific phenotype. Under the influence of these factors on the second step is the formation of specific relations, development of processes of neurons and sinaptogenesis.

As a model to test an alternative hypothesis, we studied the CNS of amur bitterling *Rhodeus sericeus* (Cyprinidae), coming to sexual maturity in the first year of life. The literature of the late twentieth century actively discussed some issues relating to the organization and topography catecholaminergic system of vertebrate's brain detectable by methods formaldehyde-induced fluorescence (FIF) and IHC labeling of tyrosine hydroxylase. In this period, a hypothesis was formulated about the existence of dopamine deposited system in the brain of fish [28]. Data about neuromeric organization and molecular markers that define dopaminer-

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gic phenotype of neurons in *Danio rerio*, had recently published [29]. In the bitterling brain 3 main types of cells were verified. The first type consists of small round cells in the periventricular nucleus of the diencephalon and second one are formed by large pear-shaped or fusiform cells [30]. The cerebrospinalliquor-contacting cells (CSL) are the most common third type of catecholaminergic cells. According to the Meek classification [28], large cells and CSL-contacting cells at Amur bitterling can be attributed to the elements of dopamine deposited system. In the hypothalamus of bitterling were discovered a few CSL-contacting cells with a low level of activity TH, but cells were marked by gliocsalic acid. Some fish have similar features morphology of CSL-contacting cells (in particular, the presence of apical dendrite, turned into the lumen of the cerebral ventricle), and these cells are a FIF-positive, but do not contain enzymes synthesis of catecholamines (TH-negative). It was the reason for the assumption that such cells are not synthesizes catecholamines by themselves, but receives CE from external sources, in particular, liquor or from large dopaminergic neurons [31]. Data labeling catecholaminergic systems on other groups of vertebrates show that dopamine and norepinephrine dissolved in the cerebrospinal fluid are of greater importance for non mammalian vertebrates; but in mammals, the CSL-contacting cells at all have not been identified [32]. These confirm the observations obtained by us on the masou salmon.

Lack of Cyprinidae fish glomerular nucleus largely hinders establishing of homology between ascending sensory projections in the telencephalon with those of other fishes [33]. To identify sources of CA-ergic innervation of the ventral part of the telencephalon of bitterling investigated the projection of this area of the brain. Tracing part of dopaminergic fibers in the ventral telencephalon bitterling showed that along with intratelencephalic cell groups exists the extratelencephalic sources of innervation of the dorsal and ventral nuclei [30]. Sources of dopaminergic projections in the ventral part of the telencephalon are two populations of cells in posterior tuberculum of bitterling, namely large cells and small rounded cells. Such cells are projected on the dorsal and ventral areas of ventral telencephalic part respectively and are considered by us as the morpho-functional equivalents of meso-striatal and meso-limbic systems of mammals. Identification dopaminergic fibers in the dorsal region of telencephalon of *D. rerio* [31] suggests that teleostea have also equivalents of meso-pallial system.

The peculiarities of localization of medullary neurons, morphology of the dendrites, and trajectories of the axon projections in the medulla of the Amur bitterling allow us to differentiate three groups of TH-positive neurons, namely interfascicular cells, units related to the *lobus vagus*, and cells localized within the *area postrema* (Fig. 4 A-D). The 3-year-old masou salmon in all the above mentioned areas of the brain stem were also identified large TH-ip cells with clear features of phenotypic differentiation. However, along with differentiated TH-positive elements of masou salmon we revealed previously not described alternative TH-positive elements, namely small undifferentiated cells, located on the territory of proliferative periventricular and subventricular zones [27]; numerous radial fibers, having different localizations in medullar part of CNS (Fig. 2E). We believe that the presence of such elements with clear features of fetal organization, as well as radial fibers in the brain 3-year-old masou salmon connected with the processes of postembryonic (adult) morphogenesis of the brain. The differentiated TH-ip neurons in salmon brain are functionally active to this period of

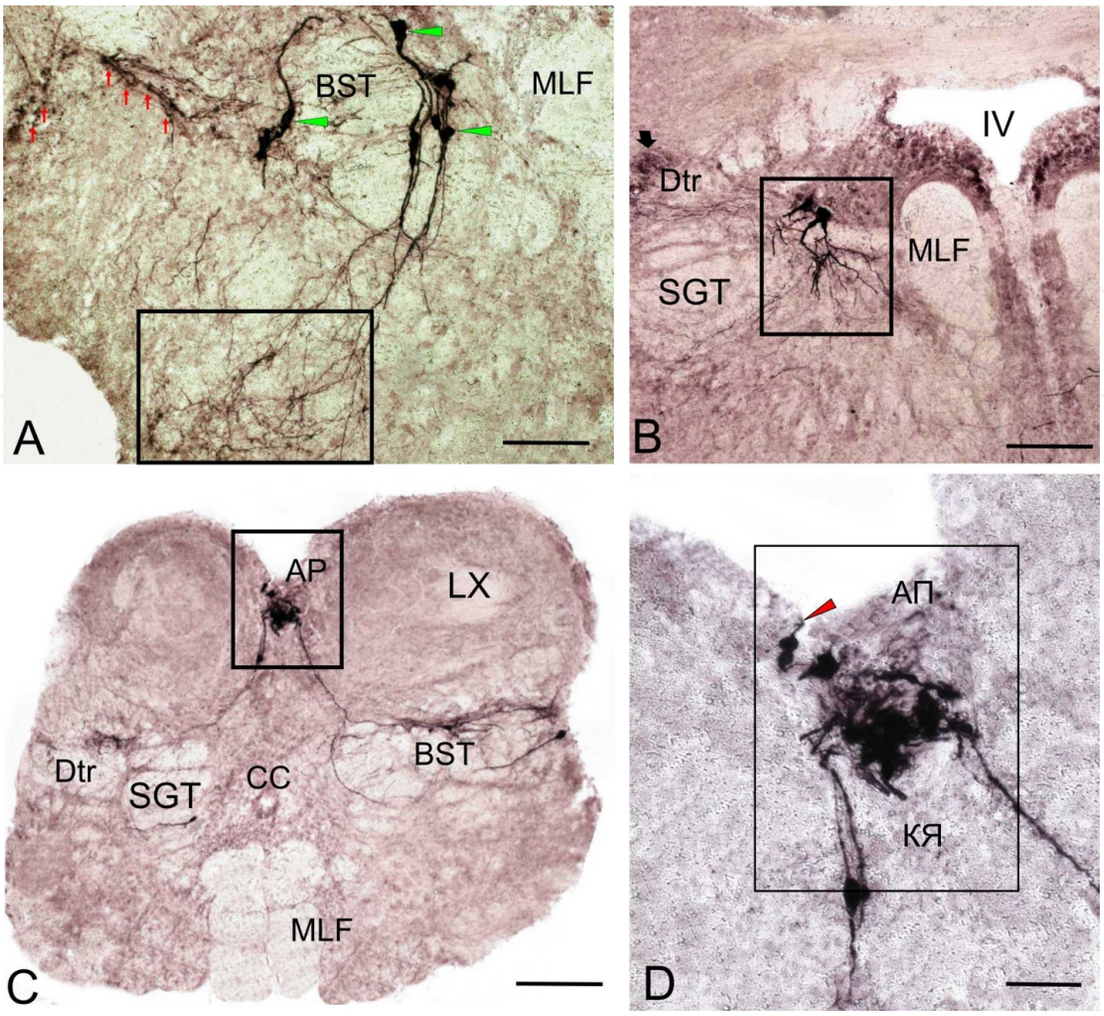

Figure 4. Tyrosine hydroxylase in the neurons of the medulla oblongata Amur bitterling *Rhodeus sericeus*. A and B-neurons of interfascicular group, C, D-neurons of area postrema. Scale: A-C-100 μm; D-50 μm.

ontogenesis elements of CA-ergic system. Study of the CA-ergic system in the medullary part of bitterling found pronounced features of specialization associated with the organization of medullary CA-ergic complexes. Analysis of these characteristics showed that of bitterling 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 Amur bitterling brain allows one to regard their functional specialization as local interneurons, since they form intensely branched dendritic networks (Fig. 4A, B). All three groups of medullary TH-ip neurons of bitterling project their terminals to the longitudinal catecholaminergic tract. 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 which are relay centres, between the primary sensory nuclei of medulla oblongata and sensory centers of the ventral thalamus. 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 (Fig. 4C, D). On the

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other hand, these two neuronal groups in the Amur bitterling 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 Amur bitterling 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. In the masou salmon brain phenotypically mature types of TH-ip cells localized in similar areas of the medulla oblongata, can have a similar functional specialization (Fig. 2E). However, part of the identified by us TH-ip elements is located in the proliferative (PCNA-marked) areas of medulla oblongata [27] at the earlier stages of ontogenesis mark neuromeric structure of medulla oblongata. At later stages localization TH found in the fibres of radial glia in interfascicular region, on the territory of fossa romboidea, as well as in populations of small cells in periventricular and subventricular areas (Fig. 2E).

In the Amur bitterling the density of the distribution of such phenotypically not mature cell forms in the periventricular area of the brain is significantly lower than the masou salmon. We believe that the features allocation in medullar part of masou salmon and Amur bitterling confirm the assumption about the participation of dopamine as a morphogenic factor regulating brain development of fish in postembryonal period.

4. Participation gaseous intermediators in the modulation of classical neurotransmitters in fishes brain

Study of the modulating influence gaseous intermediators to the classical system of neurotransmitters in the brain of fish previously had not been carried out. In our studies showed that the total nitroxidergic products in the nuclei of the brain stem in different fish species significantly exceeded the measure set for other groups of vertebrates and, particularly mammals. So, it is normal for different fish species NO-producing neurons were verified somato- and viscerosensor and visceromotor nuclei of medulla oblongata (V, VII, IX, X nuclei of craniocerebral nerves, Fig. 5), 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 brain stem involved in the innervation of brachiomotor muscles and some sensory inputs from the somatosensory, gustatory extra- and intraoral system, mechanosensory, octavolateral receptors. In fish due to low level of cephalization brain the most of the sensory inputs from the somatosensory (nucleus V), octavolateral, 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 (see the diagram on Fig. 5). Despite significant interspecific morpho-adaptative differences, in Perciformes and Cyprinoid fish were identified similarities in the organization of medullar and spinal NO-producing centres. Participating NO in modulation of sensor systems in forebrain of mammals it was proved today [34]. We assume that in the medulla fish NO performs modulation of primary sensory centers, located in the nuclei of craniocerebral nerves. In the masou salmon brain all of the above mentioned nuclei,

located in the stem and isthmus region are cholinergic and express nNOS (see the diagram on Fig. 5). Primary sensory nuclei (V, VII, VIII, IX and X), and secondary relay nuclei (secondary gustatory nucleus, the nucleus of the isthmus) in tha masou salmon brain, processing heteromodal sensory the information in the nuclei of preglomerular complex modulated by NO (Fig. 5). We assume that in the masou salmon brain NO is modulator of sensory and motor cholinergic centers.

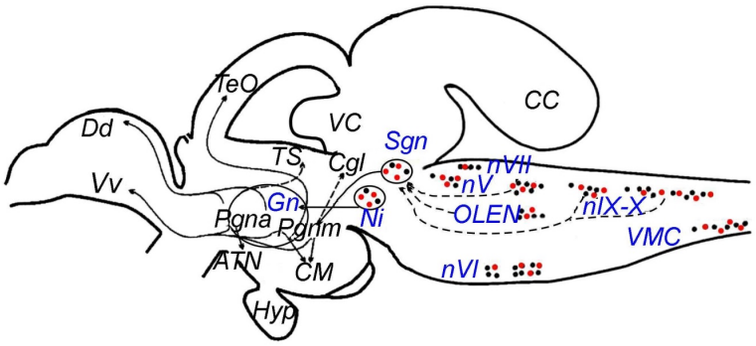

Figure 5. Schematic diagram of sensory signals ascending from the nuclei V, VII, IX, X and octavo-lateral nerves of medulla oblongata to the telencephalon. In the left part are demonstrated the efferent ascending and descending projection, anterior, medial preglomerular nuclei and mammilar body O. masou labeling by the DiI [19]. NO-ergic nuclei of brainstem are shown by black, cholinergic-red circles. The other explanation see text.

The most important sensory center, conducting nociceptive information in fish's brain is a nucleus raphi. We found that in different species of teleost fish the most of the neurons of the nucleus raphi superior and nucleus raphi inferior are expressed NADPH-d. This confirms the data installed on mammalian and human brain, that NO is a mediator of nociception [35]. The presence of nNOS-producing neurons and high level of activity NADPH-d in the nuclei of raphi, dorsal spinal cord fibers and sensory part of the nucleus of trigeminal nerve indicates participation of nitric oxide in the modulation signals of nociceptive and somatosensor centers of the medulla oblongata in fishes brain. Study of the localization of nNOS in some periventricular hypophysotropic nuclei in diencephalon of adult specimens of Amur bitterling showed that TH-ip and NO-producing system in periventricular and subventricular nuclei in general have similar localization and area of colocalisation these transmitters is periventricular nucleus of posterior tuberculum, where nNOS and TH were localized in small cells, forming ascending projections on the ventral telencephalon. In these cells NO can modulate synaptic plasticity of dopaminergic neurons and regulate the excretion of dopamine.

Study of physiological activity of hydrogen sulfide in the nervous system of mammals began recently [36], and identifying its role in the central nervous system of fish previously had not been carried out. The results of researches conducted on fish suggest that hydrogen sulfide acts as an intermediary, regulating a number of enzymatic reactions cells. Distribution of the enzyme synthesis of H_2S in the CNS of fish is expressed species-specific features, perhaps reflecting their adaptation character and functional status of the animal. Cystathionine β-synthase in the brain of masu salmon *Oncorhynchus masou* and carp *Cyprinus carpio* was found

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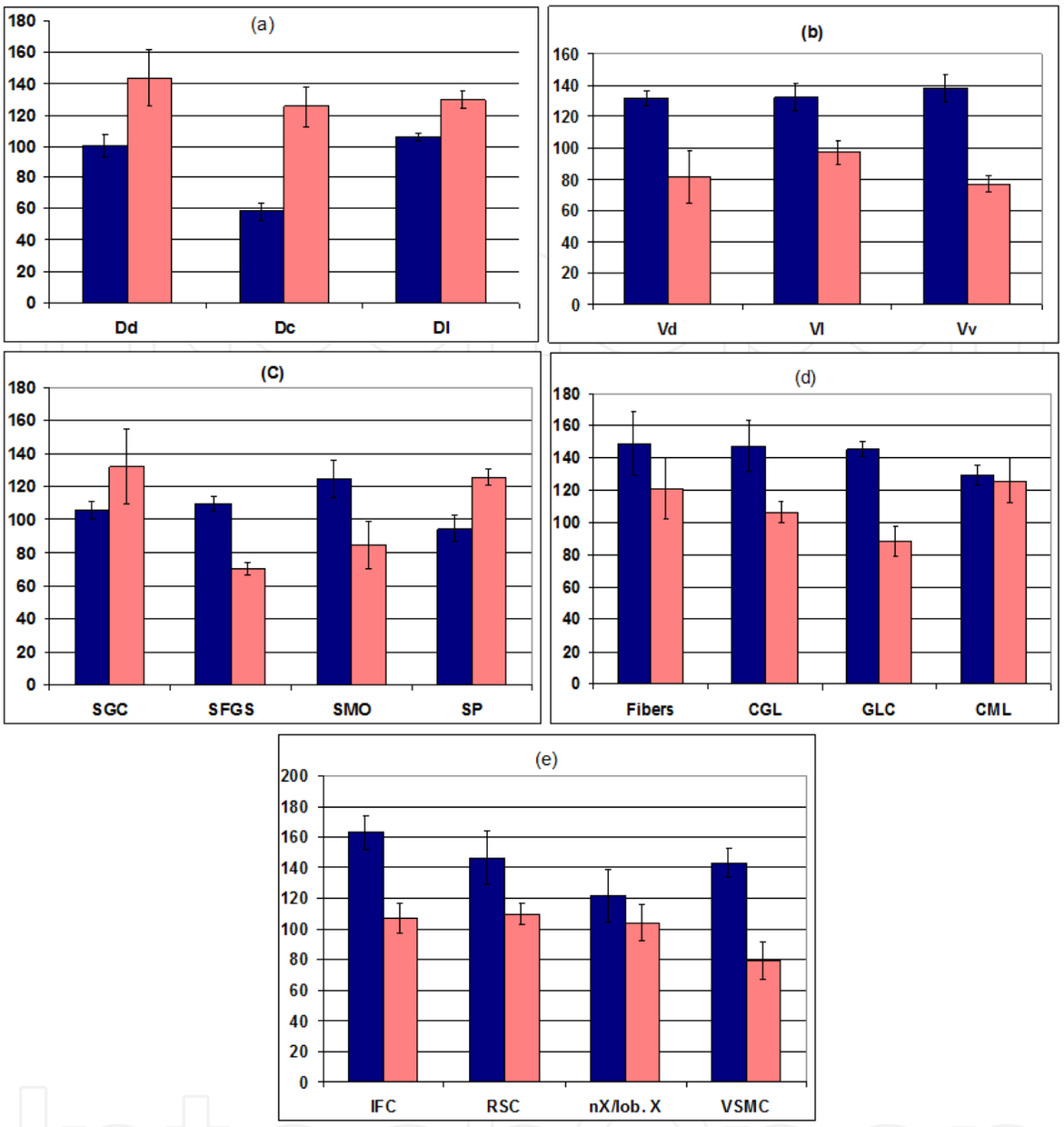

Figure 6. Densitometric analysis of the CBS activity in different brain areas of masu salmon Oncorhynchus masou and carp Cyprinus carpio. Abscissa axis, brain areas; Ordinate axis, optical density (OD). Data are shown as M ± m. (a) CBS activity in neurons of dorsal telencephalon; (b) CBS activity in neurons of ventral telencephalon; (c) CBS activity in the optic tectum; (d) CBS activity in the cerebellum; (e) CBS activity in the spinal cord and medulla oblongata. Designations: Vv, Vd, Vl, ventral, dorsal, and lateral cell nuclei of the ventral telencephalon; Dd, Dc, Dl, dorsal, central, and lateral cell nuclei of dorsal telencephalon. blue columns-masu salmon; pink columns-carp.

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. We found interspecies differences in the immunolocalizatoin and optical density of CBS in different brain structures of masu salmon and carp. In carp, the medulla oblongata and spinal cord contained intensely marked vessels that were absent in masu salmon. 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.

Comparative studies of localization CBS and densitometric data in various structures of the masu salmon and carp showed interspecific differences, having obviously adaptive value (Fig. 10). In different areas of the masu salmon brain revealed varicose or smooth microcytosculpture of afferents and their endings, which may indicate to synaptic or paracrinic (volume) methods of H2S release in different areas of the fishes brain.Currently shows the involvement of GABA in the regulation of the endocrine activity of hormones preoptico-pituitary complex at the early juveniles *Salmo trutta fario* [37]. On larval and early juvenile of this salmon species showed the participation of GABA-ergic innervation in the regulation of synthesis of peptide hormones of the pituitary, namely metencephalin and galanin [37]. In our research on different age groups of masu salmon, it was found that GABA-ip neurons are present in various parts of the brain: in the medulla oblongata, periventricular nuclei of diencephalon, mesencephalic tegmentum, the brain stem, the cerebellum and spinal cord (Fig. 11). In addition to the neural localization of GABA, it was identified small undifferentiated cells and radial fibers, localized in areas where the proliferative activity of cells persists in adults animals (Fig. 11A, D). These zones have been identified in the diencephalon on the territory of preoptical area, posterior tuberal, thalamic and hypothalamic areas; in the region of the central gray matter of mesencephalic tegmentum; in the interfascicular area of brain stem and in the periventricular zones in nuclei IX-X pairs of cranial nerves and the spinal cord. Patterns of distribution GABA-ergic elements in the masu salmon brain is similar with the distribution of TH-ip structures. This similarity manifests itself in the presence of both phenotypically mature cell forms and undifferentiated elements with periventricular and subventricular localization and marking of neuromeric structure of the brain. This immunomorphology of GABA-ergic structures, discovered in the different age groups of masu salmon, may indicate that, like dopamine, GABA should be also considered as morphogenetic factor affecting of postembryonic brain development.GABA-ergic neurotransmission characterized by a high variability of synaptic responses. In mammals, hydrogen sulfide regulates the condition of GABA-receptor of different subtypes, localized pre-and postsinapticaly [38]. In adult masu salmon in different areas of the brain and spinal cord, containing large projection cells, namely the dorsal tegmental nuclei, medial reticular formation, reticulospinal cells, neurons in the ventral spinal column were installed joint localization of GABA, PA and CBS (Fig. 7). These large-cells structures in the fish brain participate in the organization of fast motor responses [39]. In medullary regions of the medial RF and VSC of the masu salmon, the level of colocalization of CBS, GABA, and PA is rather high. It is believed that the presence of PA promotes the formation of buffer calcium systems that provide generation of repeated action potentials in neurons with high-frequency discharges (Fig. 7). The high level of colocalization of PA, cytochrome oxidase, and 2-deoxyglucose also indicates that the PA content is typical of neuronal systems characterized by a high level of oxidative metabolism [40]. It was demonstrated that the concentration of intracellular calcium in neurons and glial cells upon the action of H_2S reversibly increases (due to the release of calcium from intracellular stores and its entry into the cell through the plasma membrane) [41, 42]. Such adenylate cyclase-dependent

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mechanisms of activation can also be realized in the magnocellular CBS-and PA-ip populations of myelencephalic cells of the masu salmon brain identified in our experiments. As was found, inhibition of H_2S synthesis results in a significant decrease in the level of intracellular calcium. This confirms the conclusion on the appreciable effect of H_2S-dependent pathways on the time characteristics of processes related to calcium homeostasis in the neurons [41].

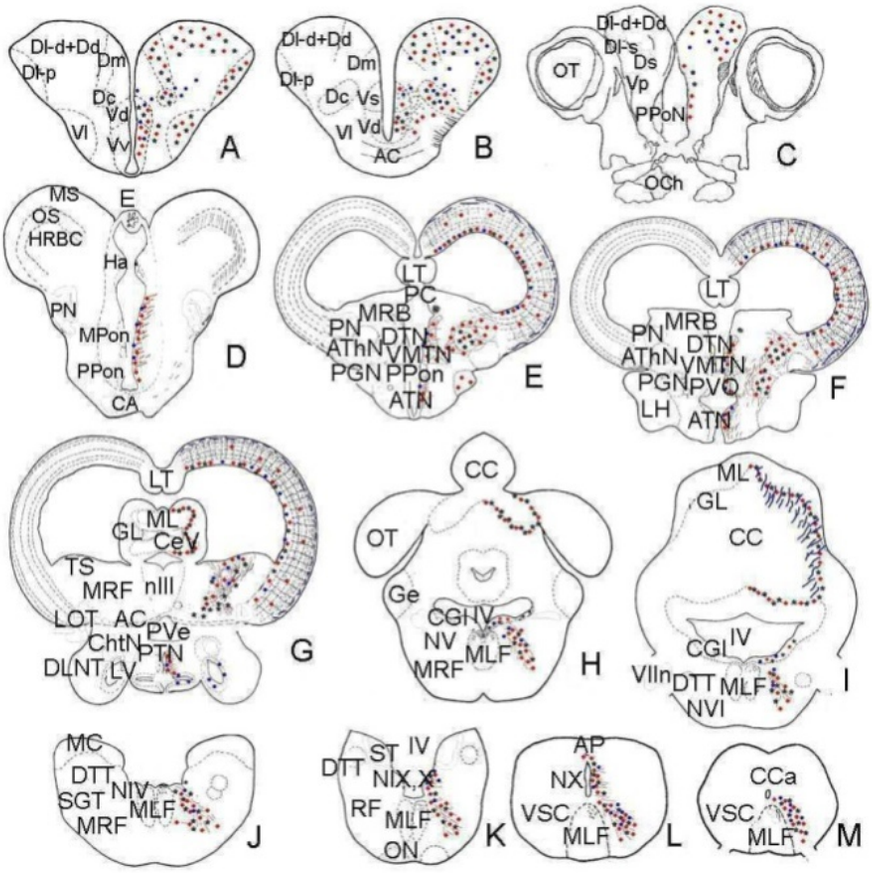

Figure 7. Schematic diagrams of distribution of cystathionine-β-synthase (CBS)-, GABA-, and parvalbumin (PA)-immunopositive loci in frontal CNS slices of the masu salmon, Oncorhynchus masou (A-M). Zones of immunopositivity with respect to CBS, GABA, and PA are indicated by blue circles, red diamonds, and black asterisks, respectively. AC) Ansular commissure, AP) area postrema, Vv, Vd, Vl, and Vs) ventral, dorsal, lateral, and supracommissure zones of the ventral region, respectively, SGT) secondary gustatory tract, VMTN) ventromedial thalamic nucleus, VSC) ventral spinal column, Ha) habenula, GE) granular eminence, Gl) granular layer, Dd, Dl, Dm, and Dc) dorsal, lateral, medial, and central zones of the dorsal region, respectively, DLNT) dorsolateral nucleus of the tegmentum, DTN) dorsal thalamic nucleus, PVe) posterior ventricle, PC) posterior commissure, PTN) posterior tuberal nucleus, rV) root of the trigeminal (V cranial) nerve, MPoN) magnocellular preoptic nucleus; LH) lateral hypothalamus, LVe) lateral ventricle, LOT) lateral optic tract, CC) corpus cerebelli, MeRF) mesencephalic reticular formation, CeV) cerebellar valve, CeCh) cerebellar chiasm, PPoN) parvicellular preoptic nucleus, MLF) medial longitudinal fascicle, MRF) medial reticular formation, CeMl) cerebellar molecular layer, DTT) descending tract of the trigeminal nerve, OLen) octavolateral efferent neurons, MRB) Meynert's retroflex bundle, OT) optic tectum, OCh) optic chiasm, ON) olivary nucleus, SlT) semilunar torus, PVO) paraventricular organ, PGN) preglomerular nucleus, AC) anterior commissure, LT) longitudinal torus, ATN), anterior thalamic nucleus, ATbN) anterior tuberal nucleus, PN) pretectal nucleus, RF) reticular formation, ST) solitary tract, CHtN) central hypothalamic nuclei, CC) central canal, CGl) central gray layer, NIII) oculomotor nucleus, NIV) nucleus of the trochlear nerve, NIX-X), nuclei of the glossopharyngeal and vagus nerves, respectively, NV) nucleus of the trigeminal nerve, NVII) nucleus of the facial nerve, IIIn) oculomotor nerve, IV) fourth ventricle, and VIIn) facial nerve.

Significant heterogeneity of CBS-ip, GABA-ip,and PA-containing subpopulations of neurons in all regions of the masu brain is indicative of the fact that such units belong to different neurochemical and electrophysiological systems. The density of CBS-, PA-, and GABA-ip cells in the masu salmon is maximum and constant in the magnocellular caudal cerebral regions, namely in the regions of localization of the reticulo-spinal neurons, "high-frequency" Mauthner cells, and ventral spinal cord (VSC) neurons. Cells of these types in fishes are involved in the organization and control of rapid motor reactions [43]. H_2S-dependent regulation of calcium release with participation of PA can influence the parameters of impulse activity due to shortening of the refractory period in the corresponding neurons after generation of action potentials and, therefore, can provide the animal with certain behavioral evolutionary preference. Thus, in the population of large inhibitory neurons containing enhanced concentration of intracellular Ca^{2+}, the excretion of GABA in our opinion can be arranged with the help of hydrogen sulphide.

Study of the relationship between NO and H_2S-producing systems in the masu salmon brain revealed that they were separate, non-overlapping system of intra-and intercellular signaling. The study of the distribution of NADPH-d positive, nNOS-and CBS-ip elements in different areas of the masu salmon brain, and some features immunolabeling of cells and fibers indicate that NO and H_2S-producing systems are independent neural complexes that perform specialized functions in the work of local neural networks.

In the dorsal region of the telencephalon in masu salmon NO is predominant gasotransmitter, the effects of which release by paracrinical way. In the ventral region of the telencephalon prevails system of hydrogen sulfide synthesis. In the ventral region of the telencephalon high activity CBS was revealed. Perhaps this system has synaptic localization, significant morphological heterogeneity of cells in the dorsal nucleus (Vd) and varicose cytosculpture of the afferents. Apparently, in the telencephalon of masu salmon way to release the gasotransmitters affect the nature of their neuromodulatory effects.

In the periventricular area of diencephalon and optic tectum masu salmon were populated by both CBS and nNOS and NADPH-d-producing cells. The presence of NO and H_2S-producing elements in these areas indicates possible participation of hydrogen sulfide and nitric oxide in morphogenesis these compartments of a brain. In masu salmon brain has been identified CBS-ip fibers of varicose type that penetrate the layer of Purkinje cells. The presence of such fibers and CBS-ip endings in interganglionic plexus of corpus cerebelli, possibly reflecting the sinaptical method of release of H_2S in this area of the masu salmon brain. The presence of NO-ergic cells and fibers was shown in the cerebellum on different species of fish by histochemical marking of NADPH-d [4, 44, 45]. Detection of nNOS in eurydendroid cells of masu salmon cerebellum confirms received our earlier data on histological labelling of NADPH-d in the neurons of this type of fish [46]. According to Ikenaga with co-authors [47], most of the eurydendroid cells in fish are aspartat-ergic and receive GABA-ergic impulses from the Purkinje cells. According to our data, the population of eurydendroid cells of masu salmon in cerebellum contains GABA-ergic and PA-ergic cells. Identified in the of masu salmon cerebellum thin nNOS-ip fibers, in our opinion, are the axons of eurydendroid neurons. Thus, nitric oxide, and being located in the projection eurydendroid cells, can acts as a modulator of

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aspartat-ergic signals in structure of efferent fibres to various parts of the masu salmon brain. Localization of nNOS, NADPH-d and CBS in interfascicular cells of masu salmon, by the classification [48], identified for the first time. We believe that interfascicular CBS-and nNOS-ip neurons of masu salmon are separate subpopulations of cells of the reticular formation, which modulating GABA-and cholinergic system in the medulla oblongata, respectively.

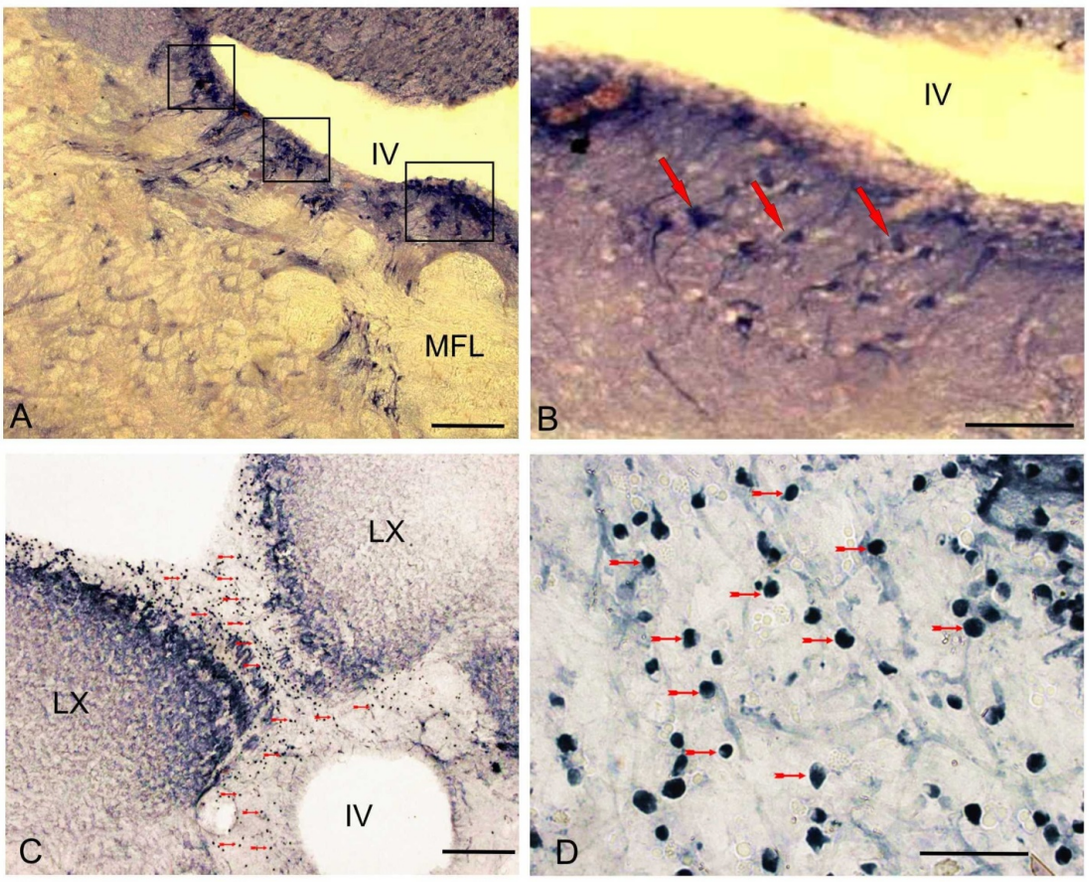

Figure 8. A – clusters of NADPH-d-producing cells (delineated by rectangles) in periventricular area of medulla oblongata of Oncorhynchus masou; on B in a large magnification. C - cystathionine β-synthase (CBS) producing cells (red arrows) in periventricular area of Cyprinus carpio brain, on D in a large magnification. LX – lobus of vagal nerve, IV – forth ventricle, MLF – medial longitudinal fascicle. Scale: A, C – 200 μm, B, D – 50 μm.

Secondary gustatory nucleus is seen as a visceral integrative centre in medulla oblongata in fishes brain [46]. In Carp in this nucleus was found the CBS immunolocalization, and in the masu salmon the secondary gustatory nucleus is CBS-immunonegative, but contains NADPH-d and nNOS. We believe that with the participation of H_2S and NO-producing systems in the brain fish is carried out sensory modulation functions related to the evaluation of food in space and coordination of mechanosensor, visual and gustatory features. In Carp brain the main neurotransmitter of the gustatory system is hydrogen sulfide, and in the masu salmon brain is nitric oxide, which confirms the assumption about the use of fish of various signal transductor systems to transfer the neurochemical information in functionally similar complexes.

We have revealed the existence of NO and H_2S-producing neurons in brainstem and isthmus regions of masu salmon brain. nNOS-ip and NADPH-d positive neurons were discovered in the composition of somato- and viscerosensoric (V, VII, IX-X) and visceromotoric (III, IV and VI) of craniocerebral nerves, octavo-lateral efferent complex, medial reticular formation. CBS in the medulla of masu salmon was detected in neurons of the nucleus X nerve, reticulospinal cells and ventro-lateral reticular formation. Distribution of NO and H_2S-producing neurons in the nuclei of medulla oblongata of masu salmon indicates that NO is the predominant neuromodulator of somato-and viscerosensoric and visceromotoric systems of medulla oblongata, and H_2S probably modulates viscerosensoric systems associated with the nucleus X nerve, and descending motor systems. NO and H_2S-producing systems in the fishes brain: 1) are independent neural complexes which are carrying out specialized functions in the work of local neural networks; 2) represent separate, non-overlapping systems of intra-and inter-cellular signaling, modulating the activity of choline-, GABA-and catecholaminergic systems, respectively; 3) regulate the processes of adult neurogenesis in the matrix areas of the brain.

5. Gaseous mediators as a regulators of adult neurogenesis

Unlike mammals, the fish brain has a high neuronal plasticity and can produce new cells throughout life [49]. The results of our investigations indicate the existence of nNOS and NADPH-d in neurons and glial cells in the masu salmon brain. It is shown that NO plays the role of signaling agent, regulating the processes directed growth of axons and dendrites and migration of differentiating neurons [50]. It is established that in the subventricular zone of mammalian forebrain is surrounded by NO-producing neurons [51, 52]. Cells expressing nNOS were identified among progenitor cells of dentate girus in the hippocampus of Guinea pig [53]. These areas of the brain are considered zones adult neurogenesis in which the proliferation of the cells is maintained throughout the life of animals and human. The results of our investigation (Fig. 8A, B) suggest that in the periventricular area of the medulla oblongata in masu salmon containing PCNA-ip proliferating cells in different age periods, NO can act as a regulator of adult neurogenesis, which confirms the data obtained on mammals.

In the periventricular area of the medulla oblongata, ventral and lateral areas of the cerebellum of carp are considered matrix areas of the brain of this species [54], identified highly CBS-immunogenic cells, without any processes (Fig. 8C, D). The sizes of cells, their location in the brain and correlations with H_2S-producing neurons indicate the presence of H_2S-producing of glia in the matrix zone of carp brain. In similar areas of the masu salmon brain such cells were not found. As currently participation of gaseous mediators in the regulation of post-embryonal neurogenesis of mammals was shown [55], we believe that in carp brain H_2S can act as such an agent, as the presence of CBS in proliferative areas of brain we consider as one of the proofs of this. One of the mechanisms regulating the in fish producing the large number of cells, educated including postembryonal period is apoptosis [7]. Study of a 60-day old sturgeon fry showed the presence of intensively proliferating zones containing PCNA-ip cells in forebrain. The active proliferation of cells in this period of the sturgeon's development is complemented by the formation of secondary neurogenetic zones.

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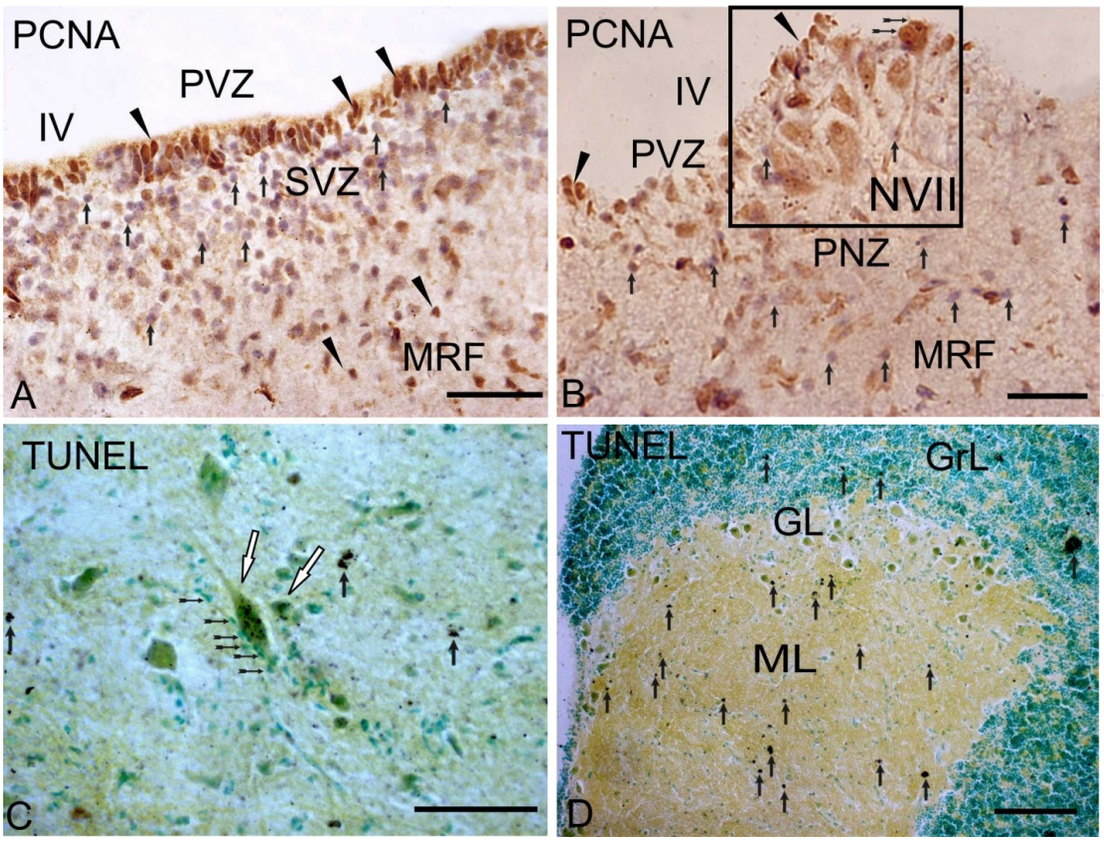

Figure 9. Proliferative activity (A and B) and apoptosis (C and D) in the brain of a three-year sturgeon *A. schrenckii*. PCNA-ip cells are shown triangular arrow, TUNEL labeled elements – are shown black arrows. Scale: A, B-100 μm; C-50 μm; D-200 μm.

The 3-year olds sturgeons' zone proliferation and apoptosis in various parts of the intact CNS saved (Fig. 9A, B). The highest proliferative activity was detected in periventricular zone of medulla oblongata, that allows considering this area as a major area of adult neurogenesis (Fig. 14A, 15B). In the medial reticular formation, dorsal nuclei of the thalamus, the inner fibrous layer of tectum opticum and lateral hypothalamus were discovered maximum number of apoptotic elements. This circumstance allows us to suppose that these regions in the sturgeon brain correspond with the areas of postmitotic neuroblasts localization. In the sensory centers (tectum opticum and nuclei V, VII and X nerves were revealed variable ratio processes of proliferation and apoptosis (Fig. 10A), which indicates different rates of growth and differentiation of visual and chemosensory centers of the sturgeon brain. In contrast to mammals in which central divisions of sensory systems are completely formed and correspond strictly to the number of sensory receptors at the moment of birth and/or immediately after this event, sensory projections in the fish brain continue their growth and development during the entire life. Such a peculiarity of the fishes is related to the fact that the CNS organization must adapt to a significant permanent increase in the size of the body and, correspondingly, to a rise in the volume of incoming sensory information. Our studies of projections of the somato- and viscerosensory nuclei of the myelencephalon and tectum opticum of the sturgeon confirmed in general the hypothesis that adult neurogenesis and apoptosis exert significant effects on the

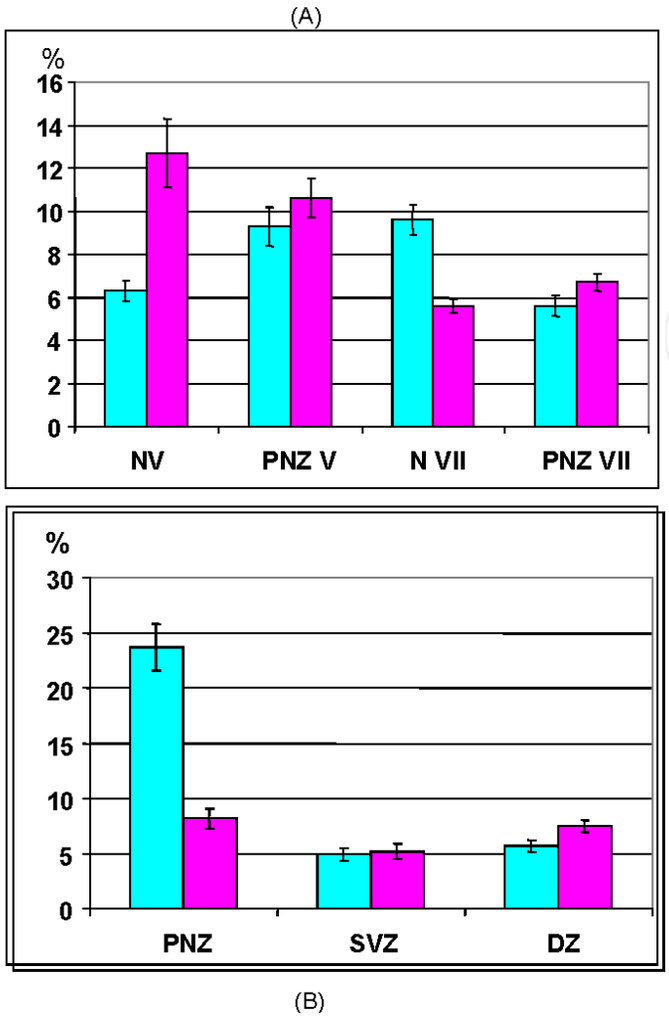

Figure 10. Intensity of the processes of proliferation and apoptosis in different parts of the myelencephalon of the Amur sturgeon Acipenser schrenckii. Data are shown as M ± m. A) In the nuclei of trigeminal and facial nerves (NV and NVII, respectively) and perinuclear zones adjacent to these nuclei (PNZ V and PNZ VII, respectively). B) In the lobe of the vagus nerve. PVZ, SVZ, and DZ-periventricular, subventricular, and deep zones, respectively. Ordinate axis-proliferation index, PI (blue columns) and apoptosis index, AI (pink columns), %.

peculiarities of postnatal development of the sensory systems. Our findings agree with the published data on intensification of differential growth in primary sensory regions in the lobe of the nucl. vagus of the carp, as well as in the Danio retina and tectum, compared with other cerebral regions [56].

Up to now, it remains unknown whether all types of neurons develop and are integrated into the corresponding networks of the growing brain of fishes. It seems probable that some initial level of organization of neuronal networks in fishes is already preformed at the moment of their hatching, and only some types of neurons continue their formation and integration into existing networks during the later period of life. It is believed that the weak ability for substitution or development of new neurons in the mammalian brain is related to the limited ability of such cells in animals of this class to be integrated into mature neuronal networks [58]. It is hypothesized that neurons formed de novo in adult animals are distinguished by a higher

plasticity compared with that of preexisting cells [59, 60]. This viewpoint agrees well with our findings on the sturgeon and allows us to suppose that postembryonic (adult) neurogenesis correlates with coordinated growth of the sensory systems and sensory structures of the brain. Therefore, this phenomenon can open possibilities for the processing of new ontogenetic experience. Incorporation of new cells into the neuronal networks existing earlier in the sensory regions is directly related, first of all, to an increase in the size of the brain in the course of growth of the fish. However, it should be taken into account that fishes, immediately after hatching, possess relatively well preformed sensory and motor systems making possible rather rapid training for complex behavioral habits, e.g., active catching of food and avoidance of predators. This indicates that some parts of the CNS of fishes, which are responsible for information processing and realization of functional needs of the organism necessary within a certain life period, begin to function before hatching. The later postembryonic growth can be considered a process of delayed development related to the maintenance of the functions necessary in future, e.g., for the formation of zoosocial communication or sexual behavior. Therefore, our conclusion that some parts of the sturgeon brain remain, in fact, in the neotenic state over a rather long postembryonic period seems to be quite logical. This hypothesis explains the high indices of proliferative activity in some brain regions in cartilaginous ganoid fishes.

The particular relevance of the results obtained acquire the communications regulatory functions of nitric oxide and hydrogen sulfide, regarded as regulators of adult neurogenesis in the fish brain. We have highlighted nNOS-ip fiber varicose type in subventricular area of the spinal cord, as well as the presence of PCNA-and nNOS-ip cells in the composition of the periventricular area of diencephalon and medulla oblongata in sturgeon and salmon indicates the presence of NO-producing elements in zones containing proliferating cells. On the other hand, detection of NO-ergic activity in TUNEL positive areas of the brain sturgeon indicates the involvement of nitric oxide in the regulation of apoptosis. Thus, it is possible that in the brain sturgeon NO is as proapoptogenic and regulatory proliferation factor exercising to maintain a balance between the two processes. Cytotoxic and neuroprotective effects NO can be viewed as interrelated elements of a single action: if the excess output of NO potentiates the mechanisms of apoptosis in the zones of localization of postmitotic neuroblasts, the factors reducing NO production can be considered as compensatory. In the basis of post embryonic morphogenesis of sturgeon's A. schrenckii brain, and particular, development of sensor systems are founded on certain ratio of the processes of proliferation and apoptosis, having NO-dependent mechanism of regulation.

6. Conclusion

Thus, we believe that the peculiarities of the distribution of the investigated systems synthesis of classic neurotransmitters (GABA, catecholamines), as well as gaseous mediators (NO and H_2S) is directly linked to the ability of the brain fish grow throughout life. We interpret the obtained results in this context. This led us to the conclusion that some of the classic neurotransmitters (dopamine, GABA), as well as gaseous intermediaries (NO and H_2S) are not only

regulators of the functional activity of neurons and modulators of synaptic transmission in mature neural networks, but also are considered as inductors of development (morphogenetic factors) in the brain during postembryonic fish ontogenesis. Proof of this is a detection of the phenotypic immature elements in the masou salmon brain adult age, expressing the above mentioned molecules in proliferative areas of the brain, as well as elements that have the morphology of the radial glia. Presence of markers of gaseous intermediaries in the areas of expressing proliferative nuclear antigen (PCNA), proves the involvement of gaseous intermediaries in the regulation of post-embryonal neurogenesis. The fish with the prolonged cycle of development (salmon, and carp) such markers (NO and H_2S) in periventricular proliferative areas of the brain may differ, which is consistent with the notion that in functionally similar complexes in animals can be used different signal transduction systems. Development of the nervous system salmon and sturgeon, in contrast to the widespread neurogenetic model D. rerio occurs over a long period of time. According to our data, the different structures of the CNS of masou salmon characterized by severe heterochrony, i.e. the cells of caudal parts of the brain in much earlier than neurons of forebrain departments, acquire the features of phenotypic specialization. We are convinced that the brains of these animals for a long time keeps features of fetal organization, and the presence of first and second years of life low differentiated phenotypically immature cell forms, confirms this hypothesis. The data presented in this study open a new trend in investigation of cellular mechanisms of shaping in structural organization in the postembryonic fishes brain and in examination of morpho-functional manifestations concerning histogenetic processes in different periods of postembrionic ontogenesis. The new priority data received are connected with development of nervous tissue in the pacific salmon brain and with dynamic of the brain shaping and distribution of classical neurotransmitters and gaseous mediators in a context of incessant postembryonic neurogenesis.

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