ISSN 1062-3604, Russian Journal of Developmental Biology, 2019, Vol. 50, No. 2, pp. 39–58. © Pleiades Publishing, Inc., 2019.
Russian Text © E.V. Pushchina, A.A. Varaksin, D.K. Obukhov, 2019, published in Ontogenez, 2019, Vol. 50, No. 2, pp. 84–105.

MECHANISMS OF CELL PROLIFERATION AND DIFFERENTIATION

Cystathionine β-Synthase in the Brain of the Trout *Oncorhynchus mykiss* after Unilateral Eye Damage and in Conditions of in vitro Cultivation

E. V. Pushchina[a, b, *], A. A. Varaksin[a], and D. K. Obukhov[c]

[a] *National Scientific Center of Marine Biology, Far East Branch, Russian Academy of Sciences, Vladivostok, 690041 Russia*
[b] *Bogomoletz Institute of Physiology, National Academy of Sciences of Ukraine, Kyiv, 01024 Ukraine*
[c] *St. Petersburg University, St. Petersburg, 199034 Russia*
**e-mail: puschina@mail.ru*

Received September 28, 2018; revised November 14, 2018; accepted November 20, 2018

Abstract—Expression of cystathionine β-synthase (CBS) in the brain of adult trout under normal conditions and 1 week after an eye injury was assessed using Western blot analysis. The study of CBS distribution in the brain of intact trout and after a mechanical eye injury in the telencephalon, tectum, cerebellum, and brainstem was carried out by the method of immunoperoxidase labeling on free-floating sections. The results of the study showed an increase in CBS expression in different brain divisions after an eye injury. In the visual projection center of the brain (tectum), radial glia cells expressing CBS were revealed after the injury. The emergence of CBS+ heterogeneous radial glia in the trout tectum after eye injury indicates the ability of the reactive neural stem cells (NSC) to synthesize hydrogen sulfide. CBS+ fibers and cells were found in the proliferative zones of cerebellum (*valvula*) and telencephalon (ventral region). In the intact trout tegmentum, CBS expression was observed in large neurons of the dorsal region and in the proliferative zones. After the eye injury, reactive neurogenic niches appeared in the brainstem, and no CBS-immunopositivity was detected in the periventricular zone. The increased expression of H_2S-producing CBS enzyme after a traumatic impact can probably be explained by the neuroprotective functions of hydrogen sulfide, which are implemented in the matrix zones of the brain associated with reparative neurogenesis. A cultivation of trout brain cells showed the formation of neurosphere-like complexes, a part of which exhibited CBS-immunopositivity in the monolayer.

Keywords: hydrogen sulfide, cystathionine β-synthase, optic nerve, reparative neurogenesis, radial glia, neurosphere-like complex, primary cell culture

DOI: 10.1134/S1062360419020048

INTRODUCTION

Over the past two decades, there has appeared increasing evidence of the involvement of hydrogen sulfide (H_2S) in various biological processes. H_2S is expressed in the brain, performing a number of functions, both in normal conditions and in various pathological states (Nagpure and Bian, 2015). The potential of the use of slowly releasing H_2S donors for therapeutic correction in various central nervous system (CNS) disorders, including Alzheimer's and Parkinson's diseases, ischemic stroke, and traumatic brain injury, is currently studied (Zhang and Bian, 2014). The neuroprotective properties of H_2S, particularly its antioxidant, antiinflamatory, and antiapoptotic effects in pathological conditions, have been determined (Wang et al., 2014).

H_2S modulates neurotransmission by exerting an effect on glutamate NMDA-receptors and secondary mediators, increasing the intracellular Ca^{2+} concentration, and modulating the intracellular cAMP content (Zhang and Bian, 2014). In different divisions of the brain and spinal cord of juvenile masu salmon, *Oncorhynchus masou*, CBS-producing neurons and fibers can be spatially conjugated with parvalbumin-

Abbreviations: CBS–, cystathionine β-synthase-negative; CBS+, cystathionine β-synthase-positive; $_K$ATP, ATP-sensitive potassium channels; Vd, dorsal zone of the telencephalon ventral region; GABA+, gamma-aminobutyric acid-immunopositive cells; DTN, dorsal tegmentum nuclei; Dc, central zone of the telencephalon dorsal region; ODU, optical density units; IHC, immunohistochemistry; MRF, mesencephalic reticular formation; SM, *stratum marginale* (marginal layer); NSC, neural stem cells; ON, optic nerve; OD, optical density; OL, layer of optic fibers; OT, optic tectum; PA, parvalbumin; PA+, parvalbumin-immunopositive cells; PVZ, periventricular zone; PVL, periventricular layer; RG, radial glia; RF, reticular formation; SVZ, subventricular zone; SGAC, *stratum griseum et album centrale* (central gray and white layer); SGC, *stratum griseum centrale* (central gray layer).

and GABA-producing systems (Pushchina and Varaksin, 2011; Pushchina et al., 2011).

Along with other gas transmitters, such as nitric oxide (NO) and carbon monoxide (CO), H_2S is considered a gaseous mediator and endogenous neuromodulator playing a substantial role in some pathophysiological conditions of CNS, particularly in secondary inflammation (Wang et al., 2014). In case of acute disorders of neuronal activity, including stroke and traumatic damage to the brain and spinal cord, the processes of secondary neuronal damage play a key role. The endogenous H_2S level in the brain is much higher than in peripheral tissues; it is mainly formed due to the enzyme cystathionine β-synthase (CBS) in astrocytes and is released in response to neuronal excitation. The mechanism of secondary neuronal damage, amplifying the effect that emerges as a result of traumatic impact, includes a disturbance of cerebral microcirculation mediated by glutamate excitotoxicity, oxidative stress, inflammatory reactions, and apoptosis of neurons against the background of increased calcium synthesis. H_2S dilates brain vessels by activating ATP-sensitive potassium channels ($_K$ATP) on smooth muscle cells. During this process, a modification of specific S-sulfhydrated cysteine residues of proteins of $_K$ATP channels occurs (Wang et al., 2014). After the injury, H_2S counteracts the effects that result from distribution of glutamate excitotoxicity by preparing astrocytes to receive a larger amount of glutamate from extracellular space and by increasing the glutathione synthesis in neurons.

We previously found that a proliferative response arises (Pushchina et al., 2016) and the expression of transcriptional factors—Pax2 in the optic nerves and Pax6 in the brain (Pushchina et al., 2018)—is enhanced in the integrative centers (tectum and cerebellum) of the brain of trout, *Oncorhynchus mykiss*, as a result of mechanical eye injury. After a mechanical damage to eye, we recorded the emergence of radial glia (RG) cells in the optic tectum expressing the proliferating cell nuclear antigen (PCNA) and Pax6, an increase in the number of proliferating cells in the parenchyma of tectum and cerebellum, as well as the emergence of the reactive neurogenic niches in the regions of brain both having direct retinal inputs and not having any direct connections with the retina (Pushchina et al., 2016, 2018). The involvement of hydrogen sulfide in antiapoptic, antioxidant, and anti-inflammatory functions in cerebral ischemia has recently been shown for mammals (Palencia et al., 2015). It has been established that H_2S attenuates inflammatory reactions (Sodha and Sellke, 2015). However, despite data of clinical studies and in vitro experiments indicating the possibility of using H_2S for the treatment of brain injury and/or organ ischemia-reperfusion (Wu et al., 2015), the basic mechanisms of these phenomena remain poorly understood.

After a mechanical injury of the telencephalon in *Danio rerio* and juvenile masu salmon *Oncorhynchus masou*, active replacement of lost neurons occurs due to the proliferative activity of neuroepithelial cells and RG in the periventricular zone (Ogino et al., 2016; Pushchina et al., 2017). However, it still remains unclear which signaling mechanisms are involved in the activation of stem cells after injury (reactive proliferation) and in the production of new neurons (regenerative neurogenesis) from precursor cells. In our previous studies, we showed that CBS is actively expressed in the proliferative zones of fish brain (Pushchina and Varaksin, 2011). The presence of H_2S-producing enzyme in the proliferative brain zones is probably associated with the involvement of H_2S in constitutive neurogenesis.

An in vitro study of CNS cell properties in trout (Pushchina et al., 2016) and masu salmon (Pushchina et al., 2015b, 2015c) showed their capability of proliferation and neuronal differentiation. Cells from different divisions of trout brain in culture expressed PCNA and formed neurosphere-like complexes (Pushchina et al., 2016). In studies on masu salmon, it was found that PCNA+, HuCD+, and CBS+ populations appear among brain suspension cells within 3–4 days of cultivation (Pushchina et al., 2015c). According to data on cultivation of brain cells of the brown ghost knifefish *Apteronotus leptorhynchus* (Hinsch and Zupanc, 2007), after the addition of embryonic serum to the culture medium, the cells isolated from the matrix zones of cerebellum become capable of differentiation into GFAP+ astrocytes, vimentin+ cells, HuCD+ neurons, and MAP2+ neurons (microtubulin-associated protein 2). In a study on brown ghost knifefish, it was found that cells of the cerebellum and the dorsal zone of telencephalon can form neurospheres (Hinsch and Zupanc, 2007).

Taking into account the fact that reparative neurogenesis in the brain of fish, unlike mammals, is quite intense (Kyritsis et al., 2012; Ogino et al., 2016; Pushchina et al., 2016a), we used the previously developed eye damage model to assess variations in the level of CBS expression in the proliferative zones of the brain integrative centers in adult trout, *Oncorhynchus mykiss*, before and after traumatic injury. A study of properties of trout brain cells in vitro, in particular, the ability of primary cultures of brain cells to proliferate and synthesize CBS, was one of the goals of the present work.

MATERIALS AND METHODS

In this work, we used 70 adult individuals of the rainbow trout *Oncorhynchus mykiss*, aged 15–18 months. The animals were provided by the Ryazanovka Experimental Production Fish Hatchery, Russia, in 2018. The animals had a body weight of 280–350 g and a body length of 30–36 cm. For adaptation, the trout

were kept in tanks with fresh water at a temperature of 16–17°C and fed once a day. The light/dark cycle was 14/10 h, and the dissolved oxygen content of the water was 7–10 mg/dm^3, which corresponds to the normal saturation level. All experimental manipulations with the animals were carried out in compliance with the rules prescribed by the Statute of the National Scientific Center of Marine Biology, Far East Branch, Russian Academy of Sciences (NSCMB FEB RAS) and by the Ethical Commission of NSCMB FEB RAS regulating the humane treatment of experimental animals. The animals were anesthetized in a tricaine methanesulfonate MS222 (Sigma, United States) solution for 10–15 min.

Mechanical eye injury and preparation of material for IHC study. The mechanical injury of the right eye of trout was designed in accordance with the previously described technique (Pushchina et al., 2016a, 2016b, 2018). Using a sterile needle (Carl Zeiss, Oberkochen, Germany), a mechanical damaging impact was applied to the eye area to a depth of 1 cm, at which the cornea and the mucous membrane of the eye, the retina, the lens, and the optic nerve head with the adjacent tissues were damaged. The contralateral optic nerve was used as a control. After the application of mechanical damage, the animals were kept in a tank with fresh water for recovery and further monitoring.

Immunohistochemistry. Immunoperoxidase labeling on frozen, free-floating sections of the brain was performed to investigate the localization of H_2S-producing cells and fibers in the telencephalon, tectum, cerebellum and brainstem of trout. An assessment of CBS activity was carried out at 1 week postinjury.

To identify H_2S-producing cells and fibers in the telencephalon, tectum, cerebellum and brainstem of trout, the method of indirect avidin-biotin-peroxidase labeling (ABC method) was used (Pushchina et al., 2011). Immunolabeling of CBS was conducted using both intact animals and individuals at 1 week after the mechanical damage. The brain of five intact and five injured fish was fixed in a 4% paraformaldehyde solution prepared on 0.1 M phosphate buffer (pH 7.2) for 2 h at 4°C. The material was washed for 1 day in a 30% sucrose solution. Cross-sections with a thickness of 50 μm were cut on a cryostat (Cryo-Star HM 560 MV; Germany). To block the endogenous peroxidase activity, the sections were incubated in a 1% hydrogen peroxide solution on 0.1 M phosphate buffer for 30 min. The 50-μm-thick brain sections were incubated in situ with monoclonal mouse antibodies against cystathionine β-synthase (Abcam, ab54883, Great Britain) diluted to 1 : 200 at 4°C for 48 h. The sections were then incubated with diluted biotinylated secondary horse antimouse immunoglobulin antibodies (Vector Labs, Burlingame, CA, United States) for 2 h at room temperature with subsequent washing in three changes of 0.1 M phosphate buffer for 5 min per each change. Immunohistochemical reaction was developed using the standard avidin–biotin visualization system ABC (Vectastain Elite ABC Kit, Vector Labs, Burlingame, CA, United States). To detect reaction products, the sections were incubated in a peroxidase detection substrate (VIP Substrate Kit, Vector Labs, Burlingame, CA, United States) while controlling the process of color development under a microscope; the sections were washed and mounted on slide glasses, dehydrated according to a standard technique, and embedded in the BioOptica medium (Milano, Italy).

To assess the specificity of the immunohistochemical reaction, Western blot and the negative control method were applied. The brain sections were incubated with 1% solution of nonimmune horse serum instead of primary antibodies for 1 day and then processed as the sections with primary antibodies. No immunopositive reaction was observed in all the control experiments.

The IHC labeling of CBS revealed fibers, cell bodies, and their processes, which made it possible to categorize CBS-immunopositive elements in accordance with the previously described neurochemical classification (Pushchina et al., 2011). Based on this classification, five cell types were identified: type I includes very large multipolar cells with 1–5 primary dendrites, having a body size of 40 μm and more (were not found in trout); type II, large and medium-sized cells with three or more dendrites, body size 25–40 μm; type III, medium-sized cells with 1–3 processes without varicose thickenings, body size 15–25 μm; type IV, small cells with a rounded, oval, or bipolar shape, body size 6–15 μm. The cells whose body size did not exceed 6 μm were classified as extra small and referred to type V.

For comparative characterization of CBS labeling intensity in the brain of fish of the control group and after damage, we measured optical density of the products of IHC labeling of CBS. Optical density measurements were performed using the Axiovision software for the Axiovert Apotome 200 M inverted microscope. Based on the data of densitometric analysis, different levels of CBS activity in cells were determined. These data, along with the morphometric parameters of cells (size characteristics of cell body), were used to classify and typify cells newly formed in the course of reparative neurogenesis in the proliferative zones, as well as in the definitive brain centers. Level of optical density in CBS+ cells was assessed on the following scale: high (160–130 ODU), medium (130–100), moderate (100–80), weak (80–50); its initial value was measured on control mounts.

Western blot. For Western blot, ten intact adult trout individuals and ten fish after mechanical eye injury were used. The damaged ipsilateral and contralateral optic nerves were extracted from the animals, separating the fibers of the optic nerves from adjacent tissues, and placed in 0.01 M Tris-HCl buffer (pH 7.2). The brain of the animals was extracted from the skull in 0.01 M Tris-HCl buffer (pH 7.2), and the telen-

cephalon, optic tectum, brainstem, and cerebellum were carefully separated.

The samples of the telencephalon, tectum, cerebellum, and brainstem of the control animals, as well as the samples of the same divisions of trout brain collected at 1 week postinjury, were quickly cooled and homogenized in a triple volume of ice-cooled buffer in a Potter-Elvehim PTFE glass tissue grinder (Sigma-Aldrich, United States). The homogenization buffer contained 20 mM Tris-HCl buffer (pH 7.2) supplemented with 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, and the following protease inhibitors: 2 mM PMSF, 50 mg/mL leupeptin, 25 mg/mL aprotinin, 10 mg/mL pepstatin, and 2 mM dithiothreitol. Samples of trout brain homogenates were centrifuged at 15000 *g* for 15 min in a Beckman Coulter Ti50 rotor. CBS content was determined in the homogenates of cerebellum, telencephalon, tectum, and brainstem. A 50-mg aliquot of homogenates was applied to the protein band and separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) on a 10% polyacrylamide gel. After electrophoresis, the isolated protein was gently transferred onto a nitrocellulose membrane and left overnight in 0.01 M Tris-HCl buffer (pH 8.0) supplemented with 0.15 M NaCl, containing 4% bovine serum albumin (Sigma, United States). The membranes were washed in distilled water and incubated with mouse monoclonal primary antibodies against CBS (Abcam, ab54883, United Kingdom; diluted 1 : 100) in 0.01 M Tris-HCl buffer containing 1% BSA and 0.2% Tween 20 for 3 h at room temperature. The membranes were then washed with agitation in a 0.01 M Tris-HCl buffer containing 0.2% Tween-20 and incubated with horse antimouse secondary antibodies (Vector Labs, Burlingame, CA, United States) in the same buffer for 1 h. After triple washing for 10 min each, the membranes were placed in a 0.01 M Tris-HCl buffer (pH 7.2). The immunohistochemical reaction was developed using the avidin–biotin complex (ABC) for visualization (Vectastain Elite ABC Kit, Vector Labs, Burlingame, CA, United States). To detect the reaction products, a substrate that produces red color (VIP Substrate Kit, Cat. no. SK-4600; Vector Labs, Burlingame, CA, United States) was used. After development of color, the membranes were washed in distilled water and dried. For quantitative assessment, the resulting blots were scanned on a Bio-Rad GS 670 densitometer (United States).

The molecular weight of CBS containing 551 amino-acid residues was compared with the prestained molecular weight markers (Sigma, United States) and it corresponded to 63 kDa.

Primary culture of trout brain cells. To study properties of the adult trout CNS in vitro, a primary culture of cells of the whole brain was obtained, with the subsequent cultivation of these cells for 4 days and the IHC assessment of PCNA expression in the cell culture. The animals were euthanized by rapid decapitation. Their brain was aseptically extracted and washed in a sterile 0.1 M phosphate buffer solution (PBS). The crushed brain tissue was transferred into a sterile 15-mL tube and washed thrice in PBS. The cell suspension was obtained by disaggregating with trypsin (0.25 or 0.025%) (Sigma, United States) or collagenase (Sigma, United States) (28U or 56U). The brain tissue was incubated with enzymes in a water bath at 28°C for 15 min and then transferred into sterile 50-mL tubes. The disaggregation process was suspended by transferring the brain tissue to the Leibovitz's medium (L-15; Gibco Laboratories, United States) supplemented with 10% fetal bovine serum (Sigma, United States) and 0.4% penicillin/streptomycin cocktail (Gibco Laboratories, United States) in accordance with the earlier described protocol (Pushchina et al., 2015c, 2016a).

The resulting suspension of brain cells was collected into tubes for centrifugation for 5 min, and the clots of floating cells were then carefully extracted using a pipette. The suspension was centrifuged in a rotor (Beckman Coulter Ti50, United States) at 200 *g* for 5 min; the supernatant was separated from the precipitate. The cell density in the precipitate was 5×10^5 cells/mL. The obtained brain-cell suspension was transplanted into small plastic cultural duplex dishes (Carl Zeiss, Germany) with a diameter of 35 mm and stored in a CO_2 incubator at 28°C for 4 days. Throughout the period of cultivation, video monitoring of the state of cultured cells was carried out under an Axiovert Apotome 200 M inverted microscope of a research class (Carl Zeiss, Germany) in the transmitted light and phase contrast.

Immunocytochemical detection of CBS and PCNA in primary culture of trout brain cells. To study the ability of adult trout brain cells to form neurospheres in vitro, a primary culture of brain cells was obtained; they were monitored in phase contrast mode for 4 days, with the subsequent analysis of CBS expression in the monolayer and suspension cell fractions. After 4 days of cultivation, the brain cells were prepared for immunocytochemical analysis according to the standard protocol. The suspension and monolayer fractions were separated and treated separately. The suspension fraction cells were centrifuged in a rotor (Beckman Coulter Ti50) at 250 *g* for 5 min. The monolayer cells in specialized culture dishes (duplex dishes; Carl Zeiss; Germany) were fixed in a 4% paraformaldehyde solution on a 0.1 M phosphate buffer (pH 7.2) for 30 min at room temperature and were then gently washed in a 0.1 M phosphate buffer. To inactivate endogenous peroxidase in accordance with the accelerated protocol, the cells were incubated in a 3% aqueous solution of hydrogen peroxide for 3–5 min and then carefully washed in the buffer. To eliminate background staining, the cells were incubated in a 1% solution of normal horse serum prepared on 0.1 M phosphate buffer. They were then incubated with primary monoclonal mouse antibodies against cystathionine

β-synthase (Abcam ab54883, United Kingdom; 1 : 200) and proliferative cell nuclear antigen PCNA (Santa Cruz Biotech, United States, 1 : 300) for 1 h at 37°C with subsequent washing in 0.01 M phosphate buffer. At the following stage, the cells were incubated with secondary biotinylated antibodies (Vectastain Elite ABC Kit, Vector Labs, Burlingame, CA, United States) for 10 min and washed in the buffer solution. The following stage was the incubation of cells with the reagent Vectastain Elite ABC (Vector Labs, Burlingame, CA, United States) for 5 min with subsequent washing in the buffer. The products of immunocytochemical (ICC) reaction in cells were visualized using a substrate to detect peroxidase (VIP Substrate Kit, Vector Labs, Burlingame, CA, United States); after color development, the cells were washed in the phosphate buffer. A microscopic analysis of the immunolabeled cells was performed in conditions of aquatic immersion in the culture duplex dishes under an Axiovert 200 M specialized inverted microscope (Carl Zeiss, Germany).

The cells of the suspension population after centrifugation were placed on poly-L-lysine coated slides, fixed in a 4% paraformaldehyde solution for 30 min at room temperature, and then treated in accordance with the above protocol. After the procedure of ICC labeling, the suspension fraction cells were incubated in the substrate for peroxidase development (VIP Substrate Kit, Vector Labs, Burlingame, CA, United States) for 10 min with controlling the color development process under a microscope. Subsequently, the cells on the slide glasses were washed in three changes of phosphate buffer, dried at room temperature, dehydrated using the standard technique, and embedded in the BioOptica medium (Milan, Italy) under cover glass. To assess the specificity of the ICC reaction, the negative control method was used. The cells were incubated in a medium containing 1% nonimmune horse serum (instead of primary antibodies) for 2 h, and then all the above manipulations were performed in accordance with the standard procedure. No immunopositive reaction was recorded in all the control experiments.

Neurosphere formation assay. Formation of neurospheres was performed under control in independent conditions on methylcellulose (Sigma, United States). For this purpose, the primary culture brain cells were treated with trypsin-EDTA or collagenase (Sigma, United States) and then cultured in 24-well plates (duplex dishes; Carl Zeiss; Germany) at 500 cells/well on the Leibovitz's medium (L-15; Gibco Laboratories, United States) supplemented with 10% fetal bovine serum (Sigma, United States) without adding the growth factors. After 4 days of cultivation, the number and size of nonadhesive neurospheres were identified and photographed under an Axiovert 200 M microscope (Carl Zeiss, Germany). The neurospheres with sizes greater than 50 μm were independently calculated in all the variants of the experiment in at least triplicate.

Morphometric analysis. Morphometric analysis was performed using the software for an Axiovert 200 M inverted microscope equipped with an ApoTome module and Axio Cam MRM and Axio Cam HRC digital cameras (Carl Zeiss, Germany). The measurements were performed at a magnification of 20×, 40×, and 63× in five randomly selected fields of view for each study region.

Statistical analysis. Quantitative processing of the morphometric data of IHC labeling of CBS was carried out using the Statistica 12 and Microsoft Excel 2010 software packages. For quantitative assessment of the results, one-way analysis of variance (ANOVA) was used; data were expressed as mean ± standard deviation ($M \pm SD$).

RESULTS

1. Evaluation of cystathionine β-synthase expression in the trout brain in normal conditions and after eye injury by the Western blot method. The cystathionine β-synthase enzyme is a tetramer binding two substrates (homocysteine and serine) and three additional ligands (the coenzyme pyrodoxal 5'-phosphate, the allosteric activator S-adenosylmethionine, and heme). The assessment of CBS content by Western blot analysis showed the presence of protein with a molecular weight of 63 kDa in all the divisions of trout brain. The quantitative CBS content in different divisions of the trout brain under normal conditions and after the mechanical eye injury is shown in Fig. 1. The maximum level of CBS expression in the intact animals was found in the brainstem, while the minimum in the telencephalon. The cerebellum and tectum showed a mean level of CBS expression. A significant increase in the level of CBS expression was observed in all the brain divisions after the mechanical eye injury (Fig. 1).

2. Unilateral mechanical eye injury. The traumatic eye damage resulted in significant functional and morphological rearrangements in various anatomical regions of the optic nerve: the head, the intraorbital segment, and the proximal and distal parts. After the traumatic eye injury, a significant increase in the number of Pax2+ astrocytes (Pushchina et al., 2018) and neuroline-expressing cells (Pushchina and Varaksin, 2019) was observed in the damaged optic nerve. The changes in the histological structure of the adjacent oculomotor muscle fibers, the data of IHC labeling of PCNA in the optic nerve cells, as well as the identification of cells with signs of apoptosis in the damaged optic nerve, indicating an alteration of its structure due to a mechanical injury, were discussed earlier (Pushchina et al., 2016b).

3. IHC of cystathionine β-synthase in the trout brain. In the cerebellum of an intact trout, a moderate initial level of CBS activity was recorded (Fig. 1). Separate thin CBS+ fibers were found in the basal part of the granular layer (Table 1; Fig. 2a). A few small gran-

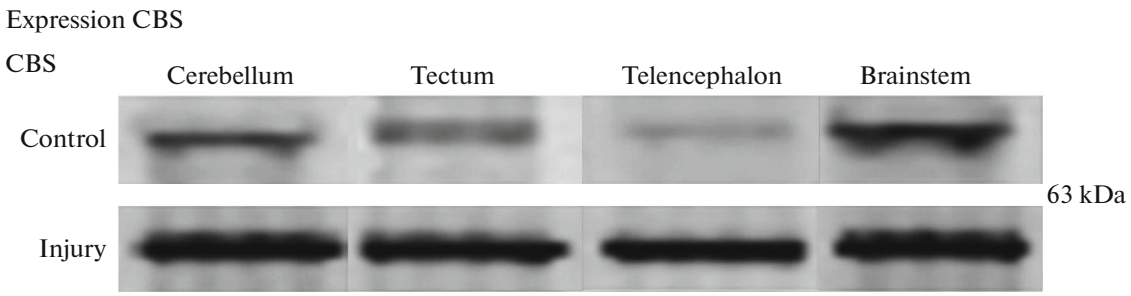

Fig. 1. Representation of Western blots of cystathionine β-synthase content in the brain of the trout *Oncorhynchus mykiss*. The single protein band corresponding to a molecular weight of 63 kDa was present in the trout cerebellum, optic tectum, telencephalon, and brainstem in the control (intact) animals and at 1 week after the optic nerve damage.

ular CBS+ cells, single or forming small aggregations, were also observed here (Table 1; Fig. 2a). In the dorsal matrix zone (DMZ) and in the molecular layer of the cerebellum of the intact trout, no CBS-immunopositivity was detected.

After the damage, a significant CBS activity in the trout cerebellum was determined in the molecular and granular layer of *valvula* (Table 1; Fig. 2b). In the molecular layer, CBS was detected in cells and fibers (Table 1; Fig. 2c). More intense immunolabeling of the neuropile was determined in the molecular layer. The distribution of CBS+ cells exhibited a gradient pattern: a more intense immunolabeling was observed in the dorsal part of *valvula*, while a less intense one in the ventral part (Fig. 2b).

In the granular layer of *corpus cerebellum*, we identified intensively labeled CBS+ cells without processes spaced at a considerable distance from each other or forming dense clusters (Fig. 2d). The intensity of fiber labeling in the granular layer varied from weak to moderate (Table 1; Fig. 2c).

Data on the frequency distribution of CBS+ cells in the test field after the unilateral eye damage are shown in Fig. 2e. The most numerous cells (with a body diameter of 7 and 9 μm) were referred to type IV; their mean dimensions were 8.4 ± 1.0/5.2 ± 0.8 μm (Fig. 2e). Less numerous cells of other types corresponded to type 3 (15.9/11.7 μm), type 4 (11.5/5.2 μm), and type 5 (5.9/3.8 μm).

The results of the distribution analysis of the minimum and maximum optical density (OD) values for CBS immunolabeling in the molecular layer cells are presented in Fig. 2f; the mean range of maximum OD is 2151 ± 244 ODU; the mean range of minimum OD is 1435 ± 257 ODU. The diagram (Fig. 2f) shows matching of the OD distribution to the normal distribution (red and blue curves) for each of the analyzed ranges. Thus, the distribution of CBS activity in the molecular layer after the damaging impact varied within quite a wide range. The mean OD value for the molecular layer of *valvula* was 1793 ± 158 ODU.

In the control trout, according to the results of Western blot, a low level of CBS activity was detected in the tectum (Fig. 1). In the marginal layer, weakly CBS labeled, small, oval and rounded cells, as well as rare weakly labeled radial glia fibers, were identified (Table 1; Figs. 3a, 3b). In the central gray layer (SGC) and the central gray and white layer (SGAC), CBS-immunopositivity was detected in the perinuclear zone of type 4 and 5 small cells and in radial fibers (Table 1; Fig. 3a).

After the traumatic damage, a high CBS immunopositivity was determined in RG cells located in the marginal layer (SM) (Table 1; Fig. 3c). RG cells were intensely CBS immunolabeled, and, thus, their morphology was determined as in the case of Golgi staining (Fig. 3d). The density of distribution of CBS+ RG in trout tectum after the eye injury significantly increased compared to that in the intact animals (Figs. 3b, 3d).

The analysis of frequency of occurrence of RG cells with different morphological parameters is presented in Fig. 3e; after the traumatic impact, the body diameter of the labeled RG cells was 5–12 μm and that in the control animals was 7–9 μm (Table 1). The frequency of occurrence of RG cells after the injury was approximately the same, except for cells with a soma diameter of ca. 9 μm. Such cells dominated the tectum sections (Fig. 3e). The comparative analysis of the RG parameters in the control and after the eye injury allows for a conclusion that the CBS-immunopositivity in the trout tectum after eye injury is manifested as a morphologically heterogeneous population of RG cells.

The OD parameters of CBS labeling in RG of the trout tectum after the damage are shown in Fig. 3f. The densitometric study showed that the ratios of minimum to maximum OD values in tectum cells have a more complex pattern than those in cells of the cerebellum molecular layer (Figs. 2f, 3f). Thus, the range of minimum OD values in the tectum had several expressed peaks of distribution (Fig. 3f) compared to the range of maximum OD values showing only one peak, and better matching to the normal (Gaussian) distribution. The mean value of the maximum OD range was 26.9 ± 5.7 ODU; the mean value of the minimum OD, 14.3 ± 6.6 ODU. The quantitative analysis

Table 1. Morphometric parameters of CBS+ cells ($M \pm SD$) in the proliferative zones and deep layers of the brain of trout *Oncorhynchus mykiss* in intact individuals and after a unilateral eye injury

Brain division Cell type	Intact animals		Optic nerve damage		
	cell size, μm	labeling intensity	cell type	cell size, μm	labeling intensity
Cerebellum					
Granular layer fibers	2.8 ± 1	+		2.8 ± 1.2	++/+++
Granular layer cells	6.2 ± 0.7/6 ± 0.5 IV 5.2 ± 0.6/5 ± 0.6 V	+++ ++	IV	6.2 ± 0.7/4.7 ± 0.9	+++
Molecular layer cells	–	–	III IV IV V	15.9 ± 1.5/11.7 ± 0.5 11.5 ± 2.2/5.2 ± 0.4 8.4 ± 1/5.2 ± 0.8 5.9 ± 0.5/3.8 ± 0.6	+++ +++ ++++ ++
Optic tectum					
Marginal layer Radial glia	7.2 ± 0.8/5.8 ± 0.5 9.4 ± 0.5/6.2 ± 0.7	+ +	Radial glia	5.4 ± 0.7/3.7 ± 0.5 7.6 ± 1.2/5.6 ± 0.8 9.2 ± 0.8/6.3 ± 0.6 12.1 ± 1.2/8.4 ± 0.7	++ ++ +++ +++
Central gray layer	6.4 ± 0.6/5 ± 0.9 IV 4.8 ± 0.4/4.2 ± 0.7 V	+ +	Radial fibers, cell nuclei	1.5 ± 0.6 3.3 ± 0.3/2.8 ± 0.4	++ +++
Central gray and white layer	6.1 ± 0.3/5.2 ± 0.4 IV 5.2 ± 0.4/4.6 ± 0.3 V	+ +	Radial fibers, cell nuclei	1.5 ± 0.6 2.8 ± 0.5/2.4 ± 4 ± 0.7	++ +++
Brainstem					
Reticular formation	21 ± 3/8.2 ± 2.9 III 18.8 ± 3.5/10.5 ± 3.1 III 8.2 ± 0.5/6.5 ± 1 IV	++++ +++ +++	II III IV	26.7 ± 1.8/16.8 ± 1 16.3 ± 2.6/14.1 ± 0.5 9.3 ± 1.4/7.4 ± 0.8	+++ ++++ +++
Dorsal tegmentum nuclei	35.7 ± 21.8/21.2 ± 7.8 II 16.7 ± 0.3/12.5 ± 0.6 III 5.8 ± 0.6/4.7 ± 0.7 V	+++ ++++ +++	II III IV	26.2 ± 1.5/14.8 ± 1.2 18.3 ± 0.9/16.1 ± 1.5 12.2 ± 1.2/11 ± 0.8	+++ +++ ++++
Telencephalon					
Central aggregation of dorsal region	8.2 ± 1.2/4.9 ± 1 IV 5.7 ± 1.3/3.4 ± 0.8 V	+ ++	V IV	5.3 ± 0.9/3.8 ± 0.7 10.2 ± 2/6 ± 0.9	+++ +++
Dorsal aggregation of ventral region	–	–	V	7.1 ± 1.2/4.1 ± 0.4	+++
Ventral aggregation of ventral region	–	–	IV V	8.9 ± 1.1/7 ± 1.6 5.9 ± 0.5/5.4 ± 0.4	+++

of CBS labeling in RG cells indicates the morphological and densitometric heterogeneity of OD values of labeled cells. The mean OD value in the trout tectum RG after the damage was 22 ± 2.8 ODU.

Western blot revealed a more intense CBS expression in the brainstem of the intact animals compared to other parts of the trout brain (Fig. 1). Intensive and moderate CBS labeling was determined in reticular formation (RF) cells of the intact trout brainstem (Table 1; Fig. 4a). Cells of the periventricular region of the trout mesencephalic tegmentum also showed a high intensity of CBS labeling (Table 1; Fig. 4b). In neurons of the tegmental region, the CBS activity was high or moderate (Table 1; Fig. 4b).

After the damage, the number of CBS-labeled cells in the dorsal tegmentum increased significantly (Fig. 4c). In the periventricular zone cells, on the contrary, CBS was not expressed after the injury (Fig. 4c). A part of

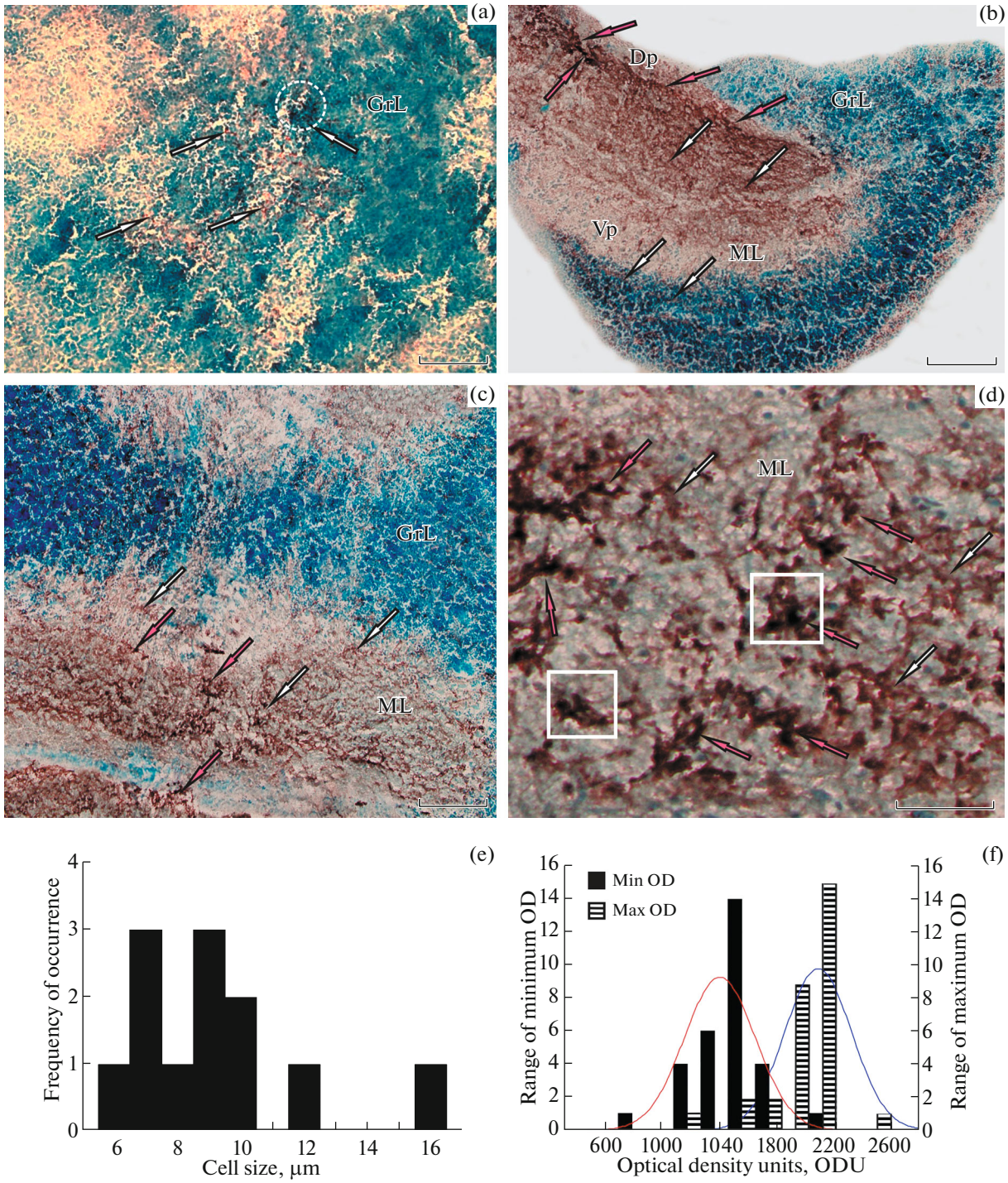

Fig. 2. Cystathionine β-synthase in the cerebellum of (a) intact trout *Oncorhynchus mykiss* and at (b–d) 1 week after eye injury. (a) In the granular layer (GrL) of *corpus cerebellum*: white arrows—CBS+ fibers; dotted line—an aggregation of CBS+ cells. (b) In *valvula* (longitudinal projection): pink arrows—CBS+ cells in the molecular layer (ML); Dp—dorsal part; Vp—ventral part. (c) In a deep layer of *valvula* (transversal projection). (d) In the granular layer of *corpus cerebellum*: white squares outline aggregations of CBS+ cells. Immunoperoxidase labeling of cystathionine β-synthase in combination with methyl green staining according to Brachet. Scale bars: (a–c) 200 μm, (d) 50 μm. (e) Distribution range of occurrence frequency of CBS+ cells with different sizes of cell somae in a test field in the cerebellum molecular layer after injury. (f) Ranges of the minimum and maximum frequency of optical density (OD) values of CBS immunolabeling in cells of the cerebellum molecular layer after eye injury.

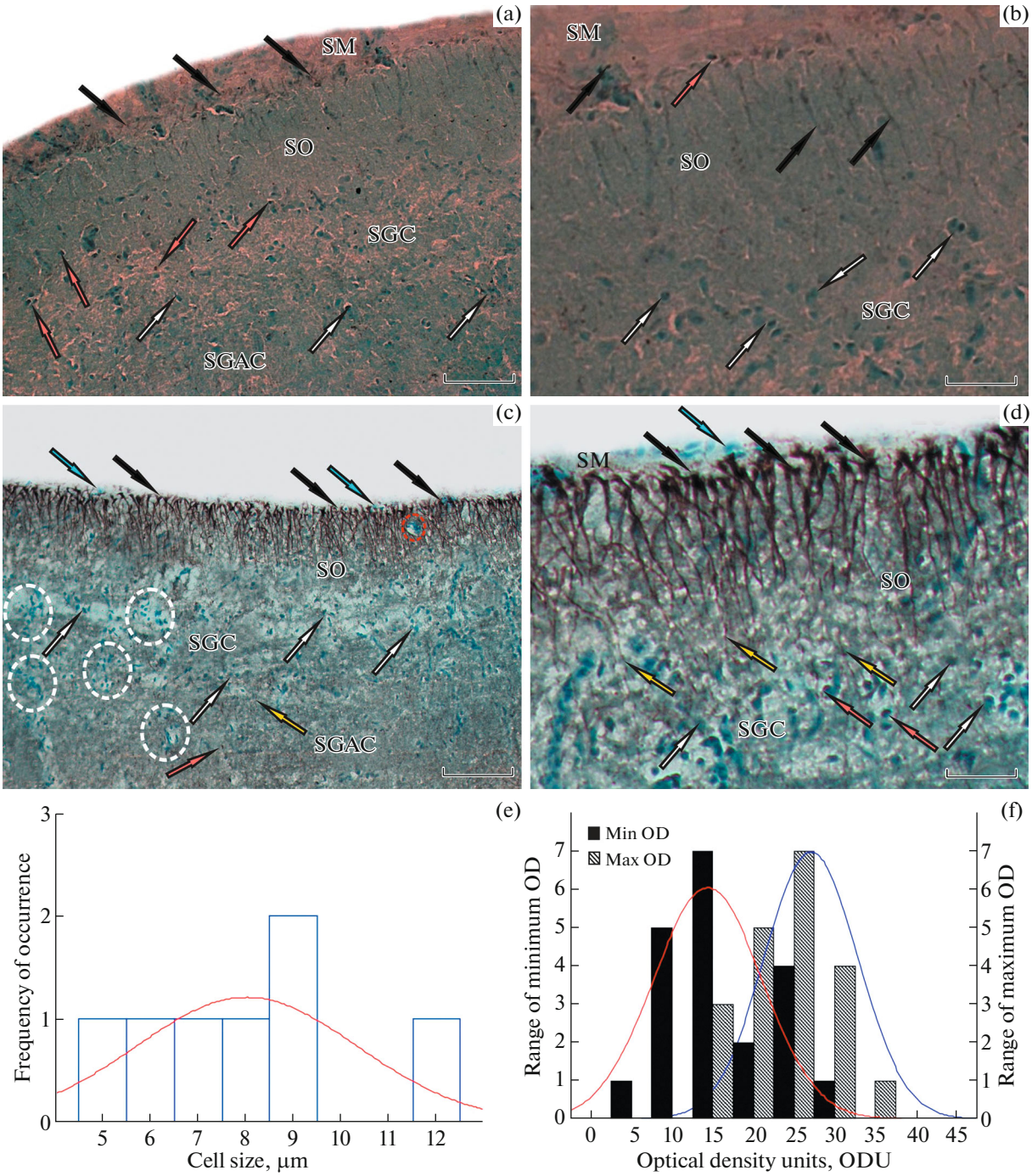

Fig. 3. Cystathionine β-synthase in the optic tectum of (a, b) intact trout *Oncorhynchus mykiss* and at (c, d) 1 week after eye injury. (a) Weak CBS immunolabeling in small cells of *stratum marginale* (SM) is indicated by black arrows; weakly labeled radial glia fibers can be observed deep in the *stratum opticum*; in *stratum griseum centrale* (SGC) and *stratum griseum et album centrale* (SGAC), CBS-immunopositivity is identified in the perinuclear zone of small cells of types 4 (white arrows) and 5 (red arrows). (b) A fragment of the tectum at higher magnification; CBS– cells are indicated by white arrows. (c) CBS+ radial glia (black arrows) and CBS-neuroepithelial cells (blue arrows) in SM: reactive neurogenic niches in SM (in red oval) and in SGC (in white ovals) contain aggregations of CBS– reactive neuroblasts; separate CBS– cells (white arrows) along with thin CBS+ RG fibers (yellow arrows) permeate the deep layers of the tectum. (d) A fragment of the tectum at higher magnification: red arrows—CBS+ fragments near the nuclear zone of cells. Immunoperoxidase labeling of cystathionine β-synthase in combination with methyl green staining according to Brachet. Scale bars: (a, c) 100 μm; (b, d) 50 μm. (e) Distribution range of occurrence frequency of CBS+ RG cells with different sizes of cell somae in a test field in the marginal layer of optic tectum after injury. (f) Ranges of the minimum and maximum frequency of optical density (OD) values of CBS immunolabeling in cells of the marginal layer of the optic tectum after eye injury.

the large neurons of the dorsomedial zone had a low intensity of CBS immunolabeling or was immunonegative (Fig. 4c). In CBS+ cells, bodies were labeled, but the processes of the cells were not determined. In case of moderate or weak immunolabeling, cell nuclei were clearly visible; with intense labeling, cell nuclei were not determined (Fig. 4d). CBS+ neurons were surrounded by numerous small immunonegative cells whose nuclei were stained with methyl green (Fig. 4d). Similar cells were also found in the periventricular region. We believe that this population contains reactive migrating cells that emerge from the periventricular proliferative zone after a damaging impact. In the periventricular zone of the trout tegmentum, there was an area free of periventricular cells corresponding to the boundary of mesencephalic neurons (Fig. 4c).

Among immunopositive tegmentum cells, three types were identified: large cells (type 2), having a size of 22.2 ± 1.5/14.8 ± 1 μm, with high CBS activity; medium-sized cells (type 3), 18.3 ± 0.9/16.1 ± 1.5 μm, with high or moderate CBS activity; and small cells (type 4), 12.2 ± 1.2/11 ± 0.8 μm, with moderate CBS activity.

A diagram of frequency distribution of CBS+ cells in the trout tegmentum after the injury is shown in Fig. 4e. The most frequently occurring cells were those of types 2 and 3 with a large diameter of 18 and 21 μm, respectively; cells with diameters of 12, 19, and 24 μm were twice as rare (Fig. 4e). The densitometric study of OD of CBS immunolabeling in the trout dorsal tegmentum showed that the cells in this region are quite heterogeneous after injury (Fig. 4f). The range of the minimum OD was relatively narrow, with a pronounced peak of 1151 ± 198 ODU; the range of maximum OD with 2276 ± 581 ODU was wider (Fig. 4f).

The results of Western blot showed that the level of CBS expression in the intact trout telencephalon was very low (Fig. 1a). In the intact animals, CBS+ cells and fibers were hardly present in the telencephalon (Fig. 5a); single small perinuclear fragments with an oval shape occurred rarely (Table 1; Fig. 5b).

After the injury, CBS-immunopositive cells in the trout telencephalon were found in the ventral subventricular region (Table 1; Fig. 5c). CBS+ cells were localized in the form of small aggregations under the layer of immunonegative cells of the periventricular zone (Fig. 5c). Intensity of cell immunolabeling was very high. Only single, small, intensely labeled cells of type 4, with dimensions of 7.1 ± 1.2/4.1 ± 0.4 μm, morphologically similar to the cells of the subventricular region, were detected in the deep inner regions of the ventral zone (dorsal nucleus) (Table 1; Fig. 5c). In the subventricular and deeper regions of the dorsal nucleus, an increased cell distribution density was detected (Fig. 5c); in some cases, it was possible to trace rows of consecutively arranged immunonegative cells (Fig. 5d). We believe that this pattern of cell distribution after injury is associated with the increased cell migration from the proliferative zone that began after the injury. The frequency of occurrence of CBS+ cells is shown in Fig. 5e. CBS+ cells are dominated by small type 4 cells with a diameter of 7 μm (Fig. 5e). The results of densitometric study of OD in immunopositive cells of the trout telencephalon after the injury are shown in Fig. 5f. The ranges of minimum and maximum OD values contained one pronounced peak each (Fig. 5f). The mean OD value in immunopositive cells of the ventral telencephalon constituted 1715 ± 188 ODU.

4. Monitoring of primary culture of trout brain cells in vitro. A study of the primary culture of trout brain cells by phase-contrast microscopy showed that, during the process of cultivation, a small part of the cells precipitate on the surface of culture dish, forming a monolayer, and some of these cells began to form processes (Fig. 6a). But most of the cells remained in suspension (Fig. 6b). A study of the condition of cells in the suspension showed that the cells were able to form conglomerates under different modes of pretreatment with enzymes (trypsin or collagenase) (Fig. 6c). According to the analysis of cell composition of the conglomerates, some of them were composed of isometric cells that could be descendants of one cell and, therefore, represent typical neurospheres (inset in Fig. 6c). Other types of conglomerates were apparently formed secondarily, by heteromorphic cells due to changes in the surface properties and adhesiveness of cells during obtaining the primary culture (Fig. 6c). In the process of cultivation, we quite frequently observed patterns of mitosis in the suspension fraction of cells (Fig. 6d). As a result of morphometric analysis of cells, it was found that the suspension fraction contains cells of types 2–5 according to the above classification (Pushchina et al., 2011).

5. IHC verification of cystathionine β-synthase in the primary culture of trout brain cells. After 4 days of cultivation of trout brain cells, we performed an IHC labeling of CBS in the monolayer and suspension cell fractions. Both CBS-positive and negative cells without processes were detected in the monolayer (Fig. 7a). The cells in the initial stage of process formation were CBS-immunonegative (Fig. 7b). CBS-positivity was found in all cell types (Table 2). The number of CBS+ cells of different types varied: most cells belonged to type 4 (38.9%) and type 2 (30.5%) (Table 2). OD of CBS in type 3 cells was 123.2 ± 26.8 ODU (Table 2) with the number of type 3 cells accounting for 18.6%. In the largest neurons of type 1 (Fig. 7c), constituting 5% of CBS+ cells, two peaks of enzyme OD were recorded: the first was 175 ODU and the second was 78.7 ODU. Clusters of two CBS+ cells with high enzyme activity formed mainly by type 3 cells were also found in the monolayer fraction of trout brain cells (Fig. 7d). Among the type 2 cells, the maximum OD was 142 ODU and the minimum was 103.1 ODU. Among other cell types, the maximum and minimum OD values of CBS labeling were also determined: these

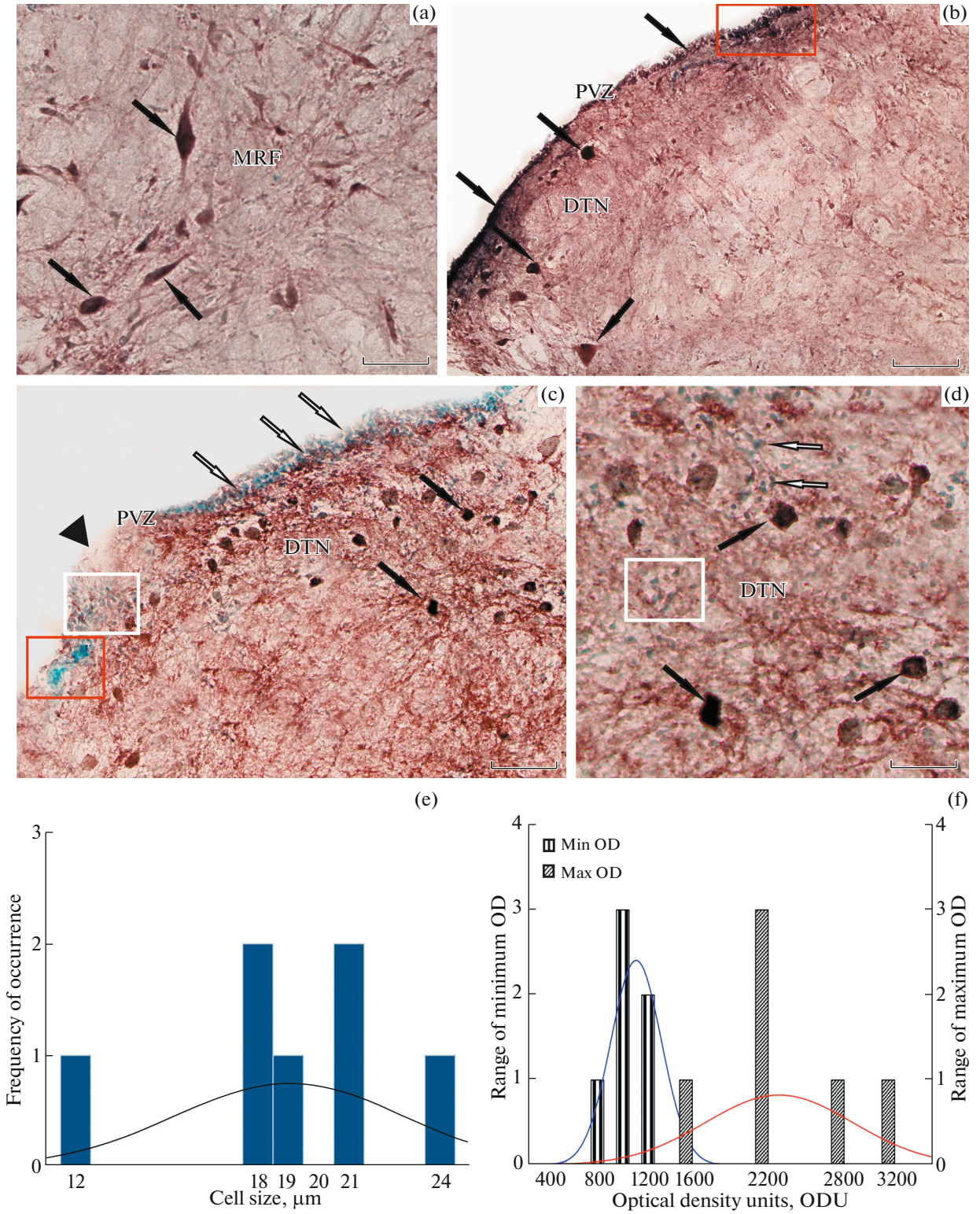

Fig. 4. Cystathionine β-synthase in the brainstem of (a, b) intact trout *Oncorhynchus mykiss* and at (c, d) 1 week after eye injury. (a) CBS+ cells (black arrows) within the mesencephalic reticular formation (MRF). (b) In dorsal tegmentum nuclei (DTN): in red rectangle—a fragment of the periventricular zone (PVZ) containing CBS+ cells. (c) In DTN: white arrows—CBS– cells of PVZ; black arrowhead—a CBS– fragment corresponding to the boundary of mesencephalic neurons; in white and red rectangles—CBS-reactive neurogenic niches. (d) A fragment of DTN at higher magnification: white arrows—reactive neuroblasts. Immunoperoxidase labeling of cystathionine β-synthase in combination with methyl green staining according to Brachet. Scale bar: (a, d) 50 μm; (b, c) 100 μm. (e) Distribution range of occurrence frequency of CBS+ RG cells with different sizes of cell somae in a test field in DTN after injury. (f) Ranges of the minimum and maximum frequency of optical density (OD) values of CBS immunolabeling in cells of DTN after eye injury.

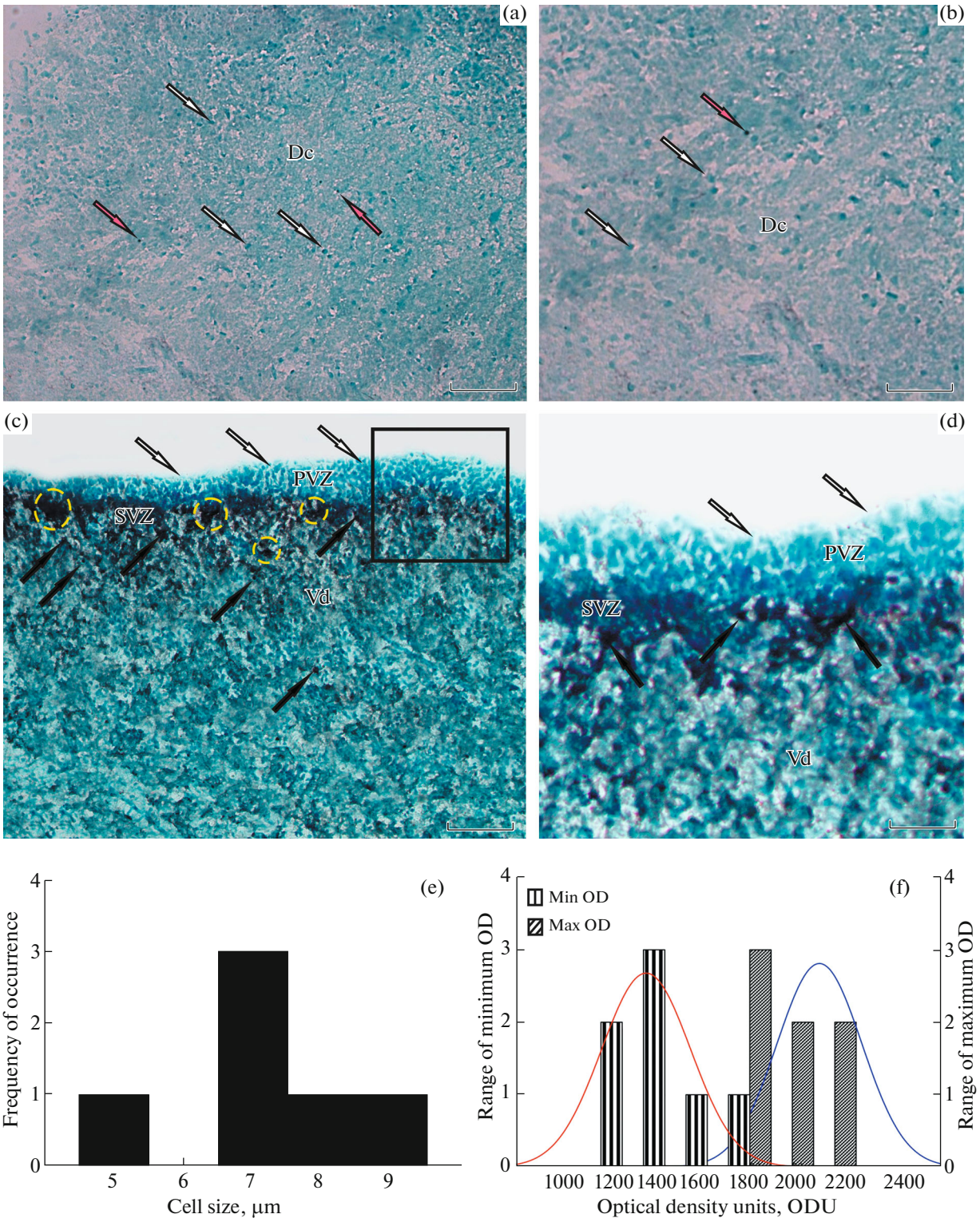

Fig. 5. Cystathionine β-synthase in the telencephalon of (a, b) intact trout *Oncorhynchus mykiss* and at (c, d) 1 week after eye injury. (a) CBS+ cells (pink arrows) and CBS– cells (white arrows) in the central zone of the dorsal region (Dc). (b) A fragment of Dc at higher magnification. (c) In the dorsal zone of the ventral region (Vd): in yellow ovals—aggregations of CBS+ cells in the subventricular zone (SVZ). (d) The fragment outlined by a black square in (c) at a higher magnification. Immunoperoxidase labeling of cystathionine β-synthase in combination with methyl green staining according to Brachet. Scale bar: (a, c) 200 μm; (b, d) 50 μm. (e) Distribution range of occurrence frequency of CBS+ RG cells with different sizes of cell somae in a test field in the Vd of telencephalon after injury. (f) Ranges of the minimum and maximum frequency of optical density (OD) values of CBS immunolabeling in cells of the Vd of telencephalon after eye injury.

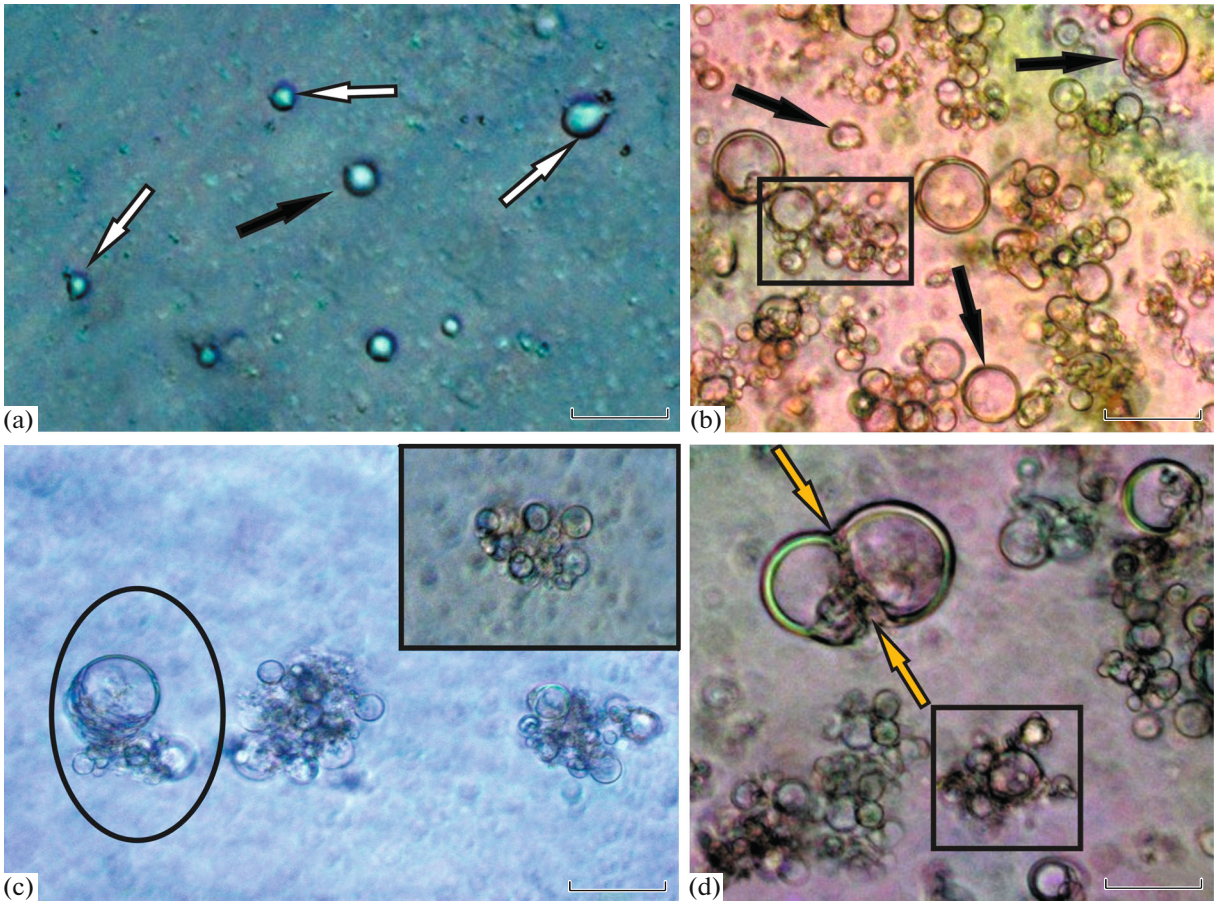

Fig. 6. Primary culture of brain cells of trout *Oncorhynchus mykiss*: (a) black arrows—cells in the monolayer, white arrows—cells that begin to form processes; (b) cells of the primary culture in the suspension with a conglomerate of cells (in rectangle); (c) conglomerates of cells in the primary culture (in oval), including a conglomerate of isomorphic cells within a neurosphere (in inset); (d) cells of the suspension fraction in the state of mitosis (yellow arrows) and those with a cell conglomerate (in rectangle). Phase-contrast microscopy. Scale bar: 50 μm.

were 142.3 ODU (max) and 77.4 ODU (min) in type 3 cells, respectively; 103.2 ODU (max) and 73 ODU (min) in type 4 cells; and 100.5 (max) and 75.3 ODU (min) in type 5 cells.

The IHC analysis of suspension fraction cells revealed CBS expression in type 2–5 cells. The morphometric and densitometric parameters of CBS+ and CBS– cells are presented in Table 3. A large proportion of CBS+ elements (84.1%) consisted of type 5 cells with the highest OD values, 112.3 ± 19.6 ODU (Table 3). The proportion of type 4 cells with OD 99.7 ± 26.4 ODU accounted for 14.8%; type 2 and 3 cells with OD 65.0 and 67.2 ODU were 1.0 and 1.9%, respectively (Table 3). Immunonegative elements were represented by type 2 and 5 cells with similar proportions equal to 18.5%; type 3 cells constituted 29% and type 4 cells were 33.3% (Table 3). The CBS labeling in the monolayer revealed larger clusters formed by isomorphic cells of types 4 and 5 resembling neurospheres (Fig. 8a).

Type 4 and 5 cells also prevailed among the suspension CBS+ cells (Fig. 8b). The PCNA labeling of suspension fraction cells showed that the proliferating cells belong to types 4 and 5 (Table 3; Fig. 8c). In most cases, such cells formed neurospheres (Fig. 8d). Data of the quantitative analysis of PCNA+ and CBS+ cells are represented in the diagram of Fig. 8e. The ratios of PCNA+ to CBS+ cells in the trout brain coincided quantitatively (Fig. 8e). Thus, the results of cultivation of trout brain cells indicate that cells of various types retain the ability to proliferate and express CBS in vitro. Another important finding is the ability of small proliferating cells in the trout brain to form neurospheres and express CBS in vitro.

DISCUSSION

The fish brain is capable of synthesizing cystathionine β-synthase (CBS), an enzyme of hydrogen sulfide synthesis, in various functional complexes of the adult animal brain (Pushchina et al., 2011). In differ-

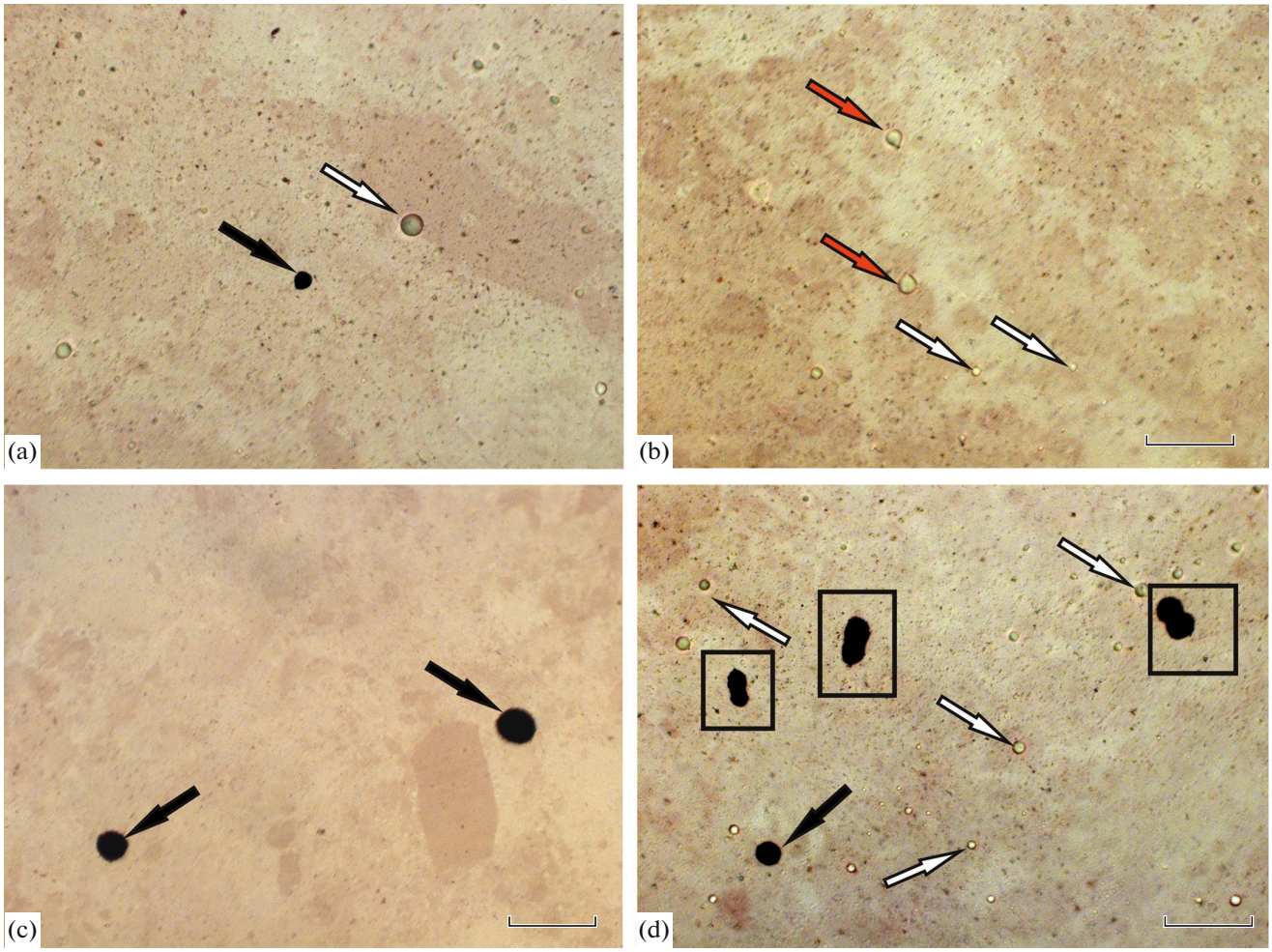

Fig. 7. Immunohistochemical labeling of cystathionine β-synthase in the primary culture of brain cells of the trout *Oncorhynchus mykiss*: (a) CBS+ in the monolayer (black arrow) and CBS– cells without processes (white arrows); (b) CBS– cells in the initial stage of the formation of processes (red arrows); (c) CBS+ large neurons of type 1 (black arrows); (d) CBS+ clusters of two cells of different types (in black rectangles). Immunoperoxidase CBS labeling in primary culture of trout brain cells. Scale bar: 50 μm.

ent parts of the brain and spinal cord, CBS-producing neurons and fibers can be spatially conjugated with parvalbumin- and GABA-producing systems (Pushchina and Varaksin, 2011; Pushchina et al., 2011). The periventricular zones of the fish brain, located along the rostrocaudal axis of the brain, are in the state of constitutive neurogenesis and contain proliferating PCNA-positive cells involved in the construction of multicomponent structures of the reticular formation in the brainstem controlling the locomotor activity and innervating the body–tail muscles, the volume of which in salmonids grows throughout life (Pushchina et al., 2017). In the region of the diencephalon, the periventricular proliferative zones are involved in

Table 2. Morphometric parameters of CBS+ cells in the primary culture of trout brain in the monolayer

Cell types	Greater and lesser diameters of cell bodies, μm	Proportion of cells, %	Optical density, ODU
1	44 ± 2.07/31.61 ± 6.82	5	126.8 ± 68
2	24.78 ± 4.44/16.57 ± 5.34	30.5	110.7 ± 15.6
3	16.70 ± 1.28/11.63 ± 2.26	18.6	123.2 ± 26.8
4	12.32 ± 1.28/9.27 ± 2.21	38.9	85.4 ± 15.8
5	8.91 ± 0.66/6.97 ± 1.24	6.7	86 ± 11.4

Table 3. Morphometric parameters of PCNA+, CBS+, and CBS– cells of the primary culture of trout brain in the suspension

CBS– cells			CBS+ and PCNA+ cells		
cell types	greater and lesser diameters of cell bodies, μm	proportion of cells, %	greater and lesser diameters of cell bodies, μm	proportion of cells, %	optical density, ODU
1	–	–	–	–	–
2	24.9 ± 4.9/17.2 ± 3.6	18.5	23.1 ± 2.4/17.3 ± 1.6	1	65 ± 2.7
3	17.5 ± 1.5/13.5 ± 3.2	29.6	18.4 ± 0.9/16.5 ± 3.1	1.9	67.2 ± 2.2
4	12.7 ± 1.2/9.4 ± 1.2	33.3	11.7 ± 1.5/9.5 ± 1.7	14.8	99.9 ± 26.4
			11.7 ± 1/9.2 ± 1.8*	14.4*	120.8 ± 15.5*
5	8.5 ± 1.2/7.1 ± 1.2	18.5	6.8 ± 1.7/5.6 ± 1.5	84.1	112.3 ± 19.6
			6.9 ± 1.6/5.9 ± 1.4*	88.4*	113.2 ± 8.4*

* Parameters of PCNA+ cells.

recruitment to the cellular composition of large sensory projective centers of the thalamus, such as the nuclei of the preglomerular and pretectal complexes (Puschina, 2012; Pushchina et al., 2012a). Along with proliferating cells, the presence of gas transmitters, such as NO and H_2S, was also detected in the proliferative zones (Pushchina and Varaksin, 2011; Pushchina et al., 2012b). Other morphogenetic factors were also shown to be present in the proliferative zones of the fish brain: the transcription factor Pax6 (Pushchina et al., 2012a, 2018) and the classical neurotransmitters and GABA (Pushchina et al., 2017).

CBS labeling in the intact trout brain. The results of the present study have shown that the basic level of H_2S synthesis in the integrative brain centers in intact adult trout—the cerebellum, tectum, telencephalon, and brainstem—varies, but CBS is expressed in all the brain centers, which agrees with the previously obtained data on the masu salmon *O. masou* and the common carp *Cyprinus carpio* (Pushchina et al., 2011). Thus, the results of the present study confirm the previously obtained data on interspecies differences in CBS activity in the fish brain. We suggest that such differences may correlate with some age-related features: for instance, CBS labeling in the cerebellum, tectum, and telencephalon was more intense in juvenile masu than in trout. The intensity of CBS immunolabeling in the carp brain agreed to a great extent with the results of immunolabeling of intact trout; however, interspecies features have also been identified in these species.

In general, the results of the Western blot analysis and IHC labeling of CBS in the intact trout brain indicate a pronounced expression in the brainstem (large- and giant-celled part of the RF) and a moderate activity in the cerebellum and optic tectum, which corresponds to the data of IHC labeling of CBS in juvenile masu (Pushchina et al., 2017).

The results of CBS immunolabeling in the telencephalon of intact trout, vice versa, showed a low level of enzyme expression in the cells of the central zone (Dc) and the lack of immunolabeling in the telencephalon periventricular zone, which is also confirmed by Western blot data indicating the low CBS expression in the trout telencephalon. The data on trout differ significantly from the results of immunolabeling in the masu telencephalon, which revealed medium- and small-size CBS+ bipolar and oval neurons of in the dorsal zone (Pushchina et al., 2011, 2017). We believe that the differences in the CBS labeling in intact trout telencephalon may be caused by a decrease in the intensity of constitutive neurogenesis in the matrix zones of adult trout telencephalon and more intense processes of postembryonic neurogenesis in juvenile masu. In this regard, it is relevant to assume that the features of CBS expression in the brain of growing juvenile masu salmon differ from the conditions of H_2S synthesis in the adult trout brain. In general, the reduction in constitutive neurogenic processes in the fish brain correlates with a decrease in the CBS expression.

CBS labeling in the trout brain after traumatic eye injury. After the unilateral eye damage, the data of Western blot and IHC labeling in the integrative centers of the trout brain indicate a significant increase in CBS expression in all the brain regions. As a result of traumatic injury, the total number of CBS+ cells and fibers significantly increases, and the number of labeled cells in the matrix zones of brain that are absent in intact animals grows multifold.

In the cerebellum, we recorded the emergence of CBS activity in the cells of the granular and molecular layers, as well as in fibers and cells of the cerebellar *valvula*, which was not determined in intact animals. Data of Western blot show a significant increase in CBS production in the trout cerebellum after injury. We believe that the overall increase in CBS synthesis in the posttraumatic period, diagnosed by the Western blot method, is associated with the intensification of the activity of the CBS-producing systems in various cells of the molecular and granular layers of the cere-

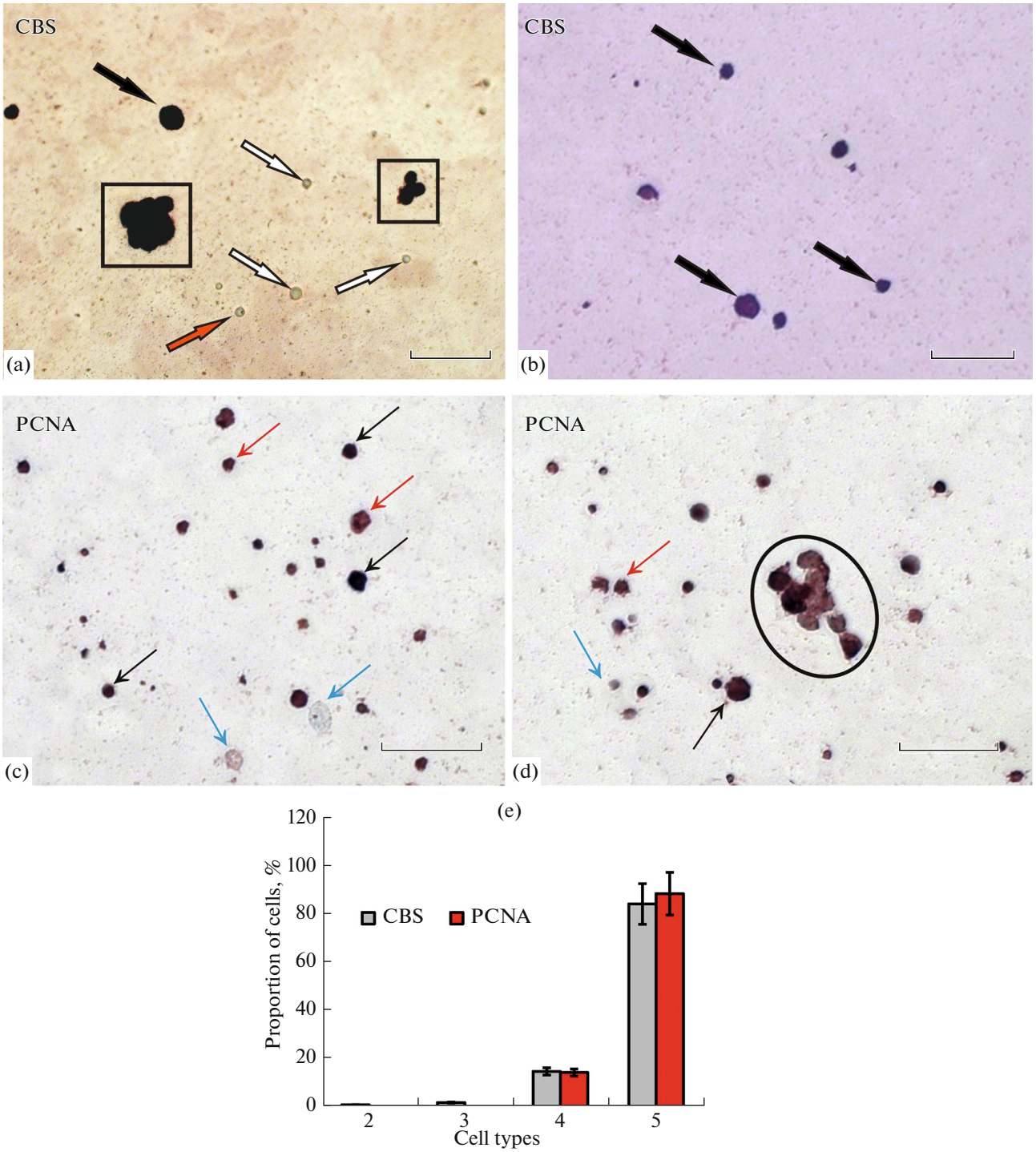

Fig. 8. Immunohistochemical labeling of cystathionine β-synthase and proliferative nuclear antigen (PCNA) in the primary culture of brain cells of the trout *Oncorhynchus mykiss*. (a) CBS+ clusters of isomorphic cells in the monolayer forming neurosphere-like complexes (in black rectangles); red arrows—cells beginning to form processes; white arrows—CBS– cells. (b) CBS+ cells in suspension (black arrows). (c) PCNA+ cells in suspension; black arrows—intensely labeled cells; red arrows—moderately labeled; blue arrow—PCNA– cells. (d) Neurosphere-like complex of PCNA+ cells (in black oval). Immunoperoxidase labeling of CBS and PCNA in the primary culture of trout brain cells. Scale bar: 50 μm. (e) Quantitative ratio of CBS+ and PCNA+ cells of different types in the suspension fraction of the primary culture of trout brain cells.

bellum as well as with the intensification of the CBS synthesis in the matrix zones. It is obvious that the appearance of numerous H_2S-producing cell complexes is associated with the development of inflammatory reaction (Sodha and Sellke, 2015). To date, it has been found that H_2S is involved in antiapoptotic, antioxidant, and anti-inflammatory functions in cerebral ischemia (Palencia et al., 2015). H_2S has been shown to attenuate inflammatory responses in rats (Sodha and Sellke, 2015). We believe that the multifold increase in H_2S production in the trout cerebellum after injury is associated with the neuroprotective and anti-inflammatory response. A traumatic eye injury induces the development of the general inflammatory response in the trout brain, particularly in the areas associated with proliferative activity.

Data of Western blot sections showed a significant increase in the level of CBS synthesis in the trout **tectum** after the injury. According to the results of IHC labeling the number of CBS+ cells with the RG phenotype dramatically increases in SM of the tectum after the traumatic eye injury. The data of morphological analysis indicate the heterogeneity of the CBS-producing RG population, which is absent in intact animals. Another important effect after unilateral damage to a trout eye is the appearance of CBS– reactive niches localized in SM and deeper layers of the tectum and absent in intact animals. The results suggest that the heterogeneous RG population, which is currently considered as NSC, begins to express CBS due to the injury. We believe that the enhanced CBS expression in the radial glia is associated with H_2S involvement in NSC activation after injury (reactive proliferation) and in the production of new neurons (regenerative neurogenesis) from precursor cells.

The results of hodological studies have shown that the optic tectum is a direct projection of the eye retina, and, according to our hypothesis, we expected the reactivation of neurogenic niches in this region of the brain, which confirms the previously obtained data (Pushchina et al., 2018; Pushchina and Varaksin, 2019). The emergence of CBS+ heterogeneous radial glia in SM of the trout tectum after an eye injury indicates the ability of reactive NSC to synthesize H_2S.

In the trout **telencephalon**, according to the data of Western blot, a significant increase in CBS expression was also revealed after the eye injury. The results of IHC analysis indicate a substantial increase in the number of CBS+ cells in the subventricular zone of the ventral region (Vd). In the posttraumatic period, dense aggregations of CBS+ cells, forming reactive niches, appeared in the subventricular zone. Thus, a significant increase in the number of CBS+ cells with the neuroepithelial phenotype, localized in the areas of reactive neurogenesis and in the neuron migration and differentiation zones and formed in the posttraumatic period, was detected in the Vd zone of the telencephalon after the optic nerve (ON) injury. In the telencephalon dorsal zone, such effects were not observed.

According to immunoblotting data, a high level of CBS expression was determined in the **brainstem** of the intact trout. Data of IHC labeling in brainstem sections showed a high and moderate intensity of labeling in cells of the RF, dorsal tegmental nuclei, and periventricular zone. The obtain results indicate that H_2S-producing neurons in the trout brainstem also provide a constitutive level for neurochemical interactions, which confirms the results of immunolabeling of GABA and parvalbumin (PA) in similar complexes of the brainstem in juvenile masu (Pushchina and Varaksin, 2011). Large CBS+, GABA+, and PA+ cells localized in the dorsomedial tegmentum nuclei were identified in the masu tegmentum. Neurons of this type represent the most rostral population of large brainstem cells involved in the organization of fast locomotor responses in fish (Braun et al., 1985). In masu salmon, unlike cyprinid fishes, a high degree of colocalization of calcium-binding protein PA and GABA was revealed in the basal mesencephalon and myelencephalon (Pushchina and Varaksin, 2011), which indicates a significant functional involvement of PA in the GABA-ergic brain systems. The results of studies on masu salmon showed that, in addition to GABA and PA, large tegmental neurons may contain H_2S. We suggest that the presence of H_2S in cells of this type can act as a modulator of GABA-ergic transmission, and the presence of PA in them indicates high levels of energy metabolism and/or electric activity.

Another important feature found in both intact trout and juvenile masu was the presence of CBS+ cells in the periventricular zone of the mesencephalic tegmentum. We associate the presence of CBS+ cells in the periventricular matrix zone of the trout brain with the participation of H_2S in the processes of constitutive neurogenesis. Similar patterns of distribution of CBS+ cells were also observed in adult carp (Pushchina and Varaksin, 2011). The results of recent studies have shown that neurons, soon after their formation from precursor cells and long before the formation of interneuronal connections and the onset of synaptogenesis, begin to secrete characteristic signaling molecules (Ugrumov, 2010). These molecules can be neuropeptides, enzymes of "classical" neuromediator synthesis, and transmembrane and vesicular transporters. Most of the signaling molecules are involved in autocrine and paracrine regulation of differentiation of target neurons, acting as morphogenetic or transcriptional factors (Pushchina et al., 2017). In mammals, the time of action of signaling molecules is limited to certain periods of ontogenesis, during which a long-term morphogenetic effect on the differentiation of target neurons and the expression of their specific phenotype is exerted (Ugrumov, 2009). In fish, the processes of constitutive neurogenesis in

the periventricular region of the brain continue throughout the lifetime (Pushchina et al., 2017).

In the trout brainstem, as in other its investigated regions, the level of CBS synthesis increases after a traumatic eye damage. The results of IHC labeling show that CBS+ cells in the periventricular region disappear after the injury, while the activity in the large cells of the dorsal tegmentum and reticular formation is maintained. Thus, in the trout brainstem, a change in CBS production in cells of the periventricular zone is observed after the ON injury. As in telencephalon, CBS+ undifferentiated cells with neuroepithelial phenotype were detected in the subventricular zone of the mesencephalic tegmentum after the eye injury. At the same time, CBS-reactive cells were detected to appear deep in the tegmentum and reticular formation. We believe that a significant rearrangement of the activity of CBS-producing systems in the trout brainstem occurs as a result of traumatic injury. These changes are associated with the reorganization of constitutive neurogenic regions and reactivation of CBS+ cells involved in the anti-inflammatory reaction in the subventricular zone.

The involvement of H_2S in antiapoptic, antioxidant, and anti-inflammatory functions in cerebral lesions has been reported for mammals (Wang, 2012; Palencia et al., 2015). The results of the present study differ from those obtained from mice, which also showed a change in CBS synthesis after a traumatic brain injury (Zhang et al., 2013). At 12 h after a brain injury, an abrupt decrease in the CBS level was observed in the cortex and hippocampus of mice, but the further dynamics (on day 3) was associated with a significant increase in CBS expression in the cortex and a less pronounced one in the hippocampus. However, within 1 week postinjury, the CBS level in the cortex increased to almost a control value and remained much lower than the control level in the hippocampus (Zhang et al., 2013). The results of a biochemical analysis show a significant increase in CBS expression in different divisions of the trout brain at 1 week postinjury compared to the control level. We suggest that the increased H_2S production creates favorable conditions for the cellular microenvironment with a pronounced neuroprotective orientation, which facilitates the reparative neurogenesis process in the matrix zones of the trout brain. It has shown that, after a mechanical eye injury in trout, an active replacement of lost neurons occurs due to the proliferative activity of neuroepithelial cells and RG in the periventricular zone of the tectum (Pushchina et al., 2016b). We assume that H_2S participates in the signaling mechanisms associated with the activation of NSC after a tectum damage (reactive proliferation) and in the production of new neurons (regenerative neurogenesis) from precursor cells in the telencephalon, cerebellum, and in the matrix zones of the brainstem.

After a brain injury, the number of microglia and leukocytes in the damaged hemisphere of the telencephalon is zebrafish (Kyritsis et al., 2012) and chum salmon (Pushchina et al., 2015a) increases significantly and remains at this level for a few days. Due to the increased expression of a number of proinflammatory cytokines, such as IL-8 and IL-1b and the tumor necrosis factor, a rapid development of the active inflammatory response can be observed after injury (Kyritsis et al., 2012). These findings confirm that fish brain injury leads to an acute inflammatory response, which is required to enhance the proliferation of neuronal progenitor cells and subsequent reparative neurogenesis.

It has been shown that an increased proliferation of precursor cells and, as a consequence, an increase in the number of new neurons is observed in fish in induced inflammation, as in traumatic brain damage (Kyritsis et al., 2012). This suggests that the inflammatory response may be one of the molecular signals that inevitably precede the activation of RG cells that perform the NSC functions in the fish brain (Ogino et al., 2016). According to studies on fish, inflammation is necessary to enhance the proliferation of neuronal progenitor cells and the subsequent neurogenesis. This process, observed after a CNS injury, is triggered through activation of molecular programs induced by injury (Kyritsis et al., 2012). Studies on mammals have shown that acute inflammation prevents adult neurogenesis and regenerative processes (Kizil et al., 2012). Thus, unlike mammals, inflammation in fish can be considered a positive regulator of neuronal regeneration in the central nervous system.

CBS expression in trout brain cells in vitro. The results of cultivation of trout brain cells showed that cells of different types have different properties and retain the ability to express CBS when cultured in vitro. The results of our study are consistent with earlier data obtained from trout (Pushchina et al., 2016a) and juvenile masu salmon (Pushchina et al., 2015b, 2015c). Taking into account the previous data and the results of the present study, it can be concluded that salmon brain cells retain a high proliferative potential during cultivation and are capable of forming neurosphere-like complexes (Pushchina et al., 2015b, 2015c, 2016a). We previously found that a significant proliferative activity in trout is characteristic of the suspension cell fraction (Pushchina et al., 2016a). Such cells, as a rule, belonged to types 4 and 5 of small undifferentiated cells and were a part of PCNA+ neurospheres. The most intensively labeled cells were PCNA cells with a diameter smaller than 5 μm, which are considered by us as actively proliferating NSC in the matrix regions of the trout brain (Pushchina et al., 2016a).

The results of the present study of trout brain cells in the primary culture with their subsequent CBS labeling show that the cell forms of different types found in the monolayer were characterized by intense

CBS expression. Large cells of types 1 and 2 in the monolayer population were intensively CBS labeled. Such cells in vivo were most characteristic of the brainstem and found in the RF and DTN. When cultivated in vitro, they retain CBS immunopositivity. Previously, CBS+ cells were found in the primary culture of the brain and spinal cord of juvenile masu (Pushchina et al., 2015c).

Linear quantitative correlations in the monolayer were found between cells of types 4 and 5 in juvenile masu, expressing the HuCD markers of neuronal differentiation, the transcription factor Pax6, and CBS (Pushchina et al., 2015b). The proportion of type 4 cells expressing these markers in juvenile masu ranged from 15 to 20%, and that of type 5 cells was approximately 80% (Pushchina et al., 2015b). The results of the present study on trout have shown that CBS+ cells of types 4 and 5 in the monolayer are capable of forming CBS+ neurosphere-like complexes. These data extend the previously obtained results of PCNA immunolabeling in the primary culture of trout brain cells (Pushchina et al., 2016a) and suggest that the formation of neurosphere-like complexes is typical of not only small cells in the suspension fraction but also of larger cells in the monolayer. The populations of type 4 and 5 cells are capable of expressing CBS and apparently differ in properties from the suspension cell forms with proliferative activity and labeled with PCNA, which we previously identified in trout (Pushchina et al., 2016a).

In the suspension fraction of trout cells, the CBS+ cell population is heterogeneous. The analysis of the quantitative ratio of CBS+ and PCNA+ suspension cells showed a high degree of similarity in the quantitative distribution of type 4 and 5 cells expressing these markers in trout, which possibly indicates the colocalization of these markers. These results are consistent with previous data on the suspension fraction of masu spinal cord cells (Pushchina et al., 2015c). The presence of PCNA+ neurospheres with the properties of stem cells was reported earlier for the suspension fraction of cells of juvenile masu (Doe et al., 1998). Thus, the results of cultivation of trout brain cells indicate the involvement of H_2S in the constitutive neurogenesis, which agrees with the previously obtained data on juvenile masu salmon.

ACKNOWLEDGMENTS

The work was financially supported by the president of the Russian Federation (grant no. MD-4318.2015.4) and the Far East Program for Basic Research for 2015–2017, Far East Branch, Russian Academy of Sciences (project no. 15-I-6-116, section III).

REFERENCES

Braun, K., Scheich, H., Schachner, M., and Heizmann, C.W., Distribution of parvalbumin, cytochrome oxidase activity and ^{14}C-2-deoxyglucose uptake in the brain of the zebra finch. I. Auditory and vocal motor systems, *Cell Tissue Res.*, 1985, vol. 240, pp. 101–115.

Doe, C.Q., Fuerstenberg, S., and Peng, C.Y., Neural stem cells: from fly to vertebrates, *J. Neurobiol.*, 1998, vol. 36, pp. 111–127.

Hinsch, K. and Zupanc, G.K., Generation and long-term persistence of new neurons in the adult zebrafish brain: a quantitative analysis, *Neuroscience*, 2007, vol. 146, pp. 679–696.

Kizil, C., Kaslin, J., Kroehne, V., and Brand, M., Adult neurogenesis and brain regeneration in zebrafish, *Dev. Neurobiol.*, 2012, vol. 72, pp. 429–461.

Kyritsis, N., Kizil, C., Zocher, S., Kroehne, V., Kaslin, J., Freudenreich, D., Iltzsche, A., and Brand, M., Acute inflammation initiates the regenerative response in the adult zebrafish brain, *Science*, 2012, vol. 338, pp. 1353–1356.

Nagpure, B.V. and Bian, J.S., Brain, learning, and memory: role of H_2S in neurodegenerative diseases, *Handb. Exp. Pharmacol.*, 2015, vol. 230, pp. 193–215.

Ogino, T., Sawada, M., Takase, H., Nakai, C., Herranz-Pérez, V., Cebrián-Silla, A., Kaneko, N., García-Verdugo, J.M., and Sawamoto, K., Characterization of multiciliated ependymal cells that emerge in the neurogenic niche of the aged zebrafish brain, *J. Comp. Neurol.*, 2016, vol. 524, pp. 2982–2992.

Palencia, G., Medrano, J.A., Ortiz-Plata, A., Farfan, D.J., Sotelo, J., Sanchez, A., and Trejo-Solis, C., Antipoptotic, anti-oxidant, and anti-inflammatory effects of thalidomide on cerebral ischemia/reperfusion injury in rats, *J. Neurol. Sci.*, 2015, vol. 351, pp. 78–87.

Puschina, E.V., Neurochemical organization and connections of the cerebral preglomerular complex of the masu salmon, *Neurophysiology*, 2012, vol. 43, no. 6, pp. 437–451.

Pushchina, E.V. and Varaksin, A.A., Hydrogen sulfide-, parvalbumin-, and GABA-producing system in the masu salmon brain, *Neurophysiology*, 2011, vol. 43, no. 2, pp. 109–122.

Pushchina, E.V. and Varaksin, A.A., Neurolin expression in the optic nerve and immunoreactivity of Pax6-positive niches in the brain of rainbow trout (*Oncorhynchus mykiss*) after unilateral eye injury, *Neural Regen. Res.*, [serial online]. 2019 [cited 2018 Nov 21]; vol. 14, pp. 156–171. Available from: http://www.nrronline.org/text.asp?2019/14/1/156/243721.

Pushchina, E.V., Varaksin, A.A., and Obukhov, D.K., Cystathionine β-synthase in the CNS of masu salmon *Oncorhynchus masou* (Salmonidae) and carp *Cyprinus carpio* (Cyprinidae), *Neurochem. J.*, 2011, vol. 5, no. 1, pp. 24–34.

Pushchina, Ye.V., Obukhov, D.K., and Varaksin, A.A., Neurochemical markers of cells of the periventricular brain area in the masu salmon *Oncorhynchus masou* (Salmonidae), *Russ. J. Dev. Biol.*, 2012a, vol. 43, no. 1, pp. 35–48.

Pushchina, E.V., Varaksin, A.A., and Obukhov, D.K., Gaseous mediators in the brain of masu salmon *Oncorhynchus masou* (Salmoniformes, Salmonidae), *Zh. Evol. Biokhim. Fiziol.*, 2012b, vol. 48, no. 1, pp. 85–95.

Pushchina, E.V., Varaksin, A.A., Shukla, S., and Bulygyn, D.A., Multiphoton confocal microscopy (in vivo imaging) in the study of early response of macrophages/microglia in damaged midbrain of juvenile chum salmon *Oncorhynchus keta, Am. J. BioSci. Spec. Iss.: Adult Repar. Neurog: Actual Quest.*, 2015a, vol. 3, nos. 2–3, pp. 12–18.

Pushchina, E.V., Shukla, S., and Varaksin, A.A., Hydrogen sulfide in proliferating and differentiated cells in primary cultures of juvenile brain of masu salmon *Oncorhynchus masou, Adv. Biosci. Biotechnol.*, 2015b, vol. 6, pp. 539–545.

Pushchina, E.V., Shukla, S., and Varaksin, A.A., Cell proliferation and differentiation in primary cultures of the juvenile brain of the masu salmon *Oncorhynchus masou, Russ. J. Mar. Biol.*, 2015c, vol. 41, no. 6, pp. 499–502.

Pushchina, E.V., Varaksin, A.A., and Obukhov, D.K., Reparative neurogenesis in the brain and changes in the optic nerve of adult trout *Oncorhynchus mykiss* after mechanical damage of the eye, *Russ. J. Dev. Biol.*, 2016a, vol. 47, no. 1, pp. 11–32.

Pushchina, E.V., Shukla, S., Varaksin, A.A., and Obukhov, D.K., Cell proliferation and apoptosis in optic nerve and brain integration centers of adult trout *Oncorhynchus mykiss* after optic nerve injury, *Neural Regen. Res.*, 2016b, vol. 11, pp. 578–590.

Pushchina, E.V., Varaksin, A.A., Obukhov, D.K., and Shukla, S., *The Neurochemical Organization and Adult Neurogenesis in the Masu Salmon Oncorhynchus masou Brain*, New York: Nova Science Publ., Inc., 2017a.

Pushchina, E.V., Zharikova, E.I., and Varaksin, A.A., Persistent and reparative neurogenesis in the juvenile masu salmon *Oncorhynchus masou* telencephalon after mechanical injury, *Russ. J. Dev. Biol.*, 2017b, vol. 48, no. 5, pp. 307–320.

Pushchina, E.V., Varaksin, A.A., and Obukhov, D.K., The Pax2 and Pax6 transcription factors in the optic nerve and brain of trout *Oncorhynchus mykiss* after a mechanical eye injury, *Russ. J. Dev. Biol.*, 2018, vol. 49, no. 5, pp. 264–290.

Sodha, N.R. and Sellke, F.W., Attenuation of inflammatory responses by hydrogen sulfide (H_2S) in ischemia/reperfusion injury, *Methods Enzymol.*, 2015, vol. 555, pp. 127–144.

Ugrumov, M.V., Non-dopaminergic neurons partly expressing dopaminergic phenotype: distribution in the brain, development and functional significance, *J. Chem. Neuroanat.*, 2009, vol. 38, pp. 241–256.

Ugrumov, M.V., Developing brain as an endocrine organ: a paradoxical reality, *Neurochem. Res.*, 2010, vol. 35, pp. 837–850.

Wang, R., Physiological implication of hydrogen sulfide: a whiff exploration that blossomed, *Physiol. Rev.*, 2012, vol. 92, pp. 791–896.

Wang, J.F., Li, Y., Song, J.N., and Pang, H.G., Role of hydrogen sulfide in secondary neuronal injury, *Neurochem. Int.*, 2014, vol. 64, pp. 37–47.

Wu, D., Wang, J., Li, H., Xue, M., Ji, A., and Li, Y., Role of hydrogen sulfide in ischemia-reperfusion injury, *Oxid. Med. Cell. Longev.*, 2015. doi 10.1155/2015/186908

Zhang, X. and Bian, J.S., Hydrogen sulfide: a neuromodulator and neuroprotectant in the central nervous system, *ACS Chem. Neurosci.*, 2014, vol. 5, pp. 876–883.

Translated by E. Shvetsov