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MECHANISMS OF CELL PROLIFERATION AND DIFFERENTIATION

Reparative Neurogenesis in the Brain and Changes in the Optic Nerve of Adult Trout *Oncorhynchus mykiss* after Mechanical Damage of the Eye

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Abstract—Reparative proliferation and neurogenesis in the brain integrative centers after mechanical eye injury in an adult trout *Oncorhynchus mykiss* have been studied. We have found that proliferation and neurogenesis in proliferative brain regions, the cerebellum, and the optic tectum were significantly enhanced after the eye injury. The cerebellum showed a significant increase in the proliferative activity of the cells of the dorsal proliferative zone and parenchymal cells of the molecular and granular layers. One week after the injury, PCNA-positive radial glia cells have been identified in the tectum. We have found for the first time that the eye trauma resulted in the development of local clusters of undifferentiated cells forming so called neurogenic niches in the tectum and cerebellum. The differentiation of neuronal cells detected by labeling cells with antibodies against the protein HuC/D occurred in the proliferative zones of the telencephalon, the optic tectum, cerebellum, and medulla of a trout within 2 days after the injury. We have shown that the HuC/D expression is higher in the proliferative brain regions than in the definitive neurons of a trout. In addition, we have examined cell proliferation, migration, and apoptosis caused by the eye injury in the contra- and ipsilateral optic nerves and adjacent muscle fibers 2 days after the trauma. The qualitative and quantitative assessment of proliferation and apoptosis in the cells of the optic nerve of a trout has been made using antibodies against PCNA and the TUNEL method.

Keywords: proliferation, teleosts, reparative neurogenesis, apoptosis, radial glia, neurogenic niche, secondary proliferative region, PCNA, HuCD, TUNEL-labeling

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INTRODUCTION

Among vertebrates, fish are known to be able to effectively restore the structure of cells and fibers after damage of the central nervous system. They have the ability to restore the number of damaged cells by production of new cells in the matrix areas of the brain and neurogenic niches, and the ability to restore the structure of damaged axons of neurons in the spinal cord pathways (Zupanc and Sirbulescu, 2013). However, it is currently unknown how this process is related to the neurogenesis in the adult brain and what elements of the matrix areas of the brain are involved in the reparative neurogenesis in fish. The evolutionarily ancient animal groups are often used as a convenient model for neurogenic studies in adults. The brain of such animals has a large number of periventricular proliferative zones and active zones of secondary neurogenesis (Zupanc, 2009; Pushchina et al., 2014). In contrast to the mammalian brain, numerous proliferative regions have been found in adult fish. The presence of such regions was described in apteronotus *Apteronotus leptorhynchus* (Zupand and Horschke, 1995), *Sparus aurata* (Zikopoulos et al., 2000), three-spined stickleback *Gasterosteus aculeatus* (Ekstrom et al., 2001), *Danio sp.* (Zupanc et al., 2005; Grandel et al., 2006), and *Austrolebias* sp. (Fernandez et al., 2011).

We investigated the change in the cellular composition of proliferative brain regions in response to eye damage in an adult trout. The regenerative processes in the brain of fish after the damaging impact are determined by a number of factors, which distinguish the dynamics of this process from that in other vertebrates, particularly mammals and humans (Zupanc et al., 1998; Becker, C.G. and Becker, T., 2008). It is known that the brain injury in the mammalian brain results in a number of pathological changes associated with the development of an inflammatory response to

Abbreviations: PCNA, proliferating cell nuclear antigen; PCNA-ip, PCNA-immunopositive; PCNA-in, PCNA-immunonegative; HuC/D, neuronal protein Hu; HuC/D-ip, HuC/D-immunopositive; HuC/D-in, HuC/D-immunonegative.

the toxic effects of glutamate and other inflammatory mediators, and further pathological changes associated with processes of secondary inflammation and involving massive cell death (Palmer et al., 1994; Grosche et al., 1995). As a result of CNS trauma, the mammalian cells are exposed to severe necrosis, and only a small part of them is eliminated via apoptosis (Kerr et al., 1995). In the fish brain, the cell response to the trauma develops in a different scenario. Apoptosis is observed 5 minutes after the injury, which progresses in the next few days (Zupans et al., 2005). The elimination of damaged cells is carried out by phagocytes (microglia/ macrophages), which remove damaged cells very effectively and provide a "clean" cell death without the remaining damaged cellular material and the development of secondary inflammation (Vajda, 2002). The replacement of the large amounts of dead cells resulted from the damage in the fish brain appears from various sources: the radial glia, centers of primary and secondary proliferation, and neurogenic niches.

The high regenerative potential in the central nervous system of fish is provided by the activation of specific regenerative factors (Zupand and Sirbulescu, 2013) and the effect of neuroprotective factors protecting damaged cells and providing the long-term survival of cells formed as a result of reparative neurogenesis.

The aim of this study was to investigate the proliferation in the integrative centers of the brain (cerebellum and tectum) and reparative neurogenesis in the adult brain of a trout *Oncorhynchus mykiss* after the mechanical eye trauma.

MATERIALS AND METHODS

The study was carried out on 20 *Oncorhynchus mykiss* trout individuals, aged 12–18 months. Animal's body weight and length were 280–350 g and 30–36 cm, respectively. Animals were obtained from the Ryazanovskii experimental fish hatchery in 2014. For adaptation, the trout were kept in tanks with fresh water at 16–17°C, with a single feeding. The ratio of the illuminated and dark periods was 14/10 hours a day. The concentration of dissolved oxygen in water was 7–10 mg/dm^3, which corresponded to the normal saturation. All experimental manipulations with the animals were performed in accordance with the regulations of the Institute of Marine Biology (Resource Center, Far East Branch, Russian Academy of Sciences) and the Ethics Committee, which regulates the humane treatment of experimental animals. Animals were anesthetized with a 0.1% solution of tricaine methanesulfonate MS222 (Sigma, United States) for 10–15 min.

Damage of the optic nerve and preparation of material for IHC study. After the fish were anesthetized, 0.1 M phosphate buffer (pH 7.2) containing 4% paraformaldehyde was administered in the intracranial cavity of immobilized animals. After the prefixation, the brain was removed from the intracranial cavity and fixed in paraformaldehyde solution at 4°C for 2 hours. The brains were washed five times in a 30% sucrose solution at 4°C for 48 hours for cryoprotection. Serial frontal and transversal sections of the brain were made on a Cryo-Star HM 560 MV freezing microtome (Germany).

The mechanical impact on the eye was applied using a sterile needle inserted in the eye region at the depth of 1 cm, which damaged both structures of the eye and the optic nerve with its adjacent tissues. Immediately after the application of the mechanical damage, the animals were released into the tank with fresh water for the recovery and further monitoring.

Immunohistochemistry. To study the proliferative activity in the optic nerve and integrative centers of the brain (the optic tectum and cerebellum) of the trout, the immunoperoxidase labeling of proliferating cell nuclear antigen PCNA on frozen free-floating sections of the optic nerve and the brain was carried out. Evaluation of proliferative activity was performed 1 week after the application of the mechanical eye damage. Brain and optic nerve sections 50 microns thick were incubated in situ with primary mouse monoclonal antibody against PCNA (PC10; Novus Biologicals, United States, 1 : 3000) at 4°C for 48 h. To detect the newly formed neurons 2 days after a traumatic exposure, monoclonal antibodies against the neuronal protein HuC/D (clone: AD2.38; Chemicon, Billerica, MA, United States; 1 : 4000) were used. To visualize IHC labeling, a standard Vectastain Elite ABC kit (Vector Laboratories, United States) was used according to the manufacturer instructions. The red substrate (VIP Substrate Kit, Vector Labs, Burlingame, United States) in combination with counterstaining with Brachet's methyl green (Merkulov, 1969) was used for detection of reaction products. The development of the IHC staining was monitored under the microscope. The slices were dehydrated by a standard protocol and embedded in the medium (Bio-Optica, Italy). To assess the specificity of the immunohistochemical labeling, the method of a negative control was used. Brain sections were incubated with 1% sodium nonimmune horse serum instead of primary antibodies for 1 day and then were treated as the sections with the primary antibodies. Immunopositive reaction was absent in all control experiments.

The densitometric study of the PCNA and HuC/D labeling in cells of the optic nerve and the brain of a trout was conducted using the Axiovision program based on inverted microscope Axiovert Apotome 200. Based on the results of densitometric analysis, various levels of PCNA and HuC/D activity were distinguished. These data, along with morphometric parameters of cells (dimensional characteristics of cell bodies), were used for the classification and typing of the newly formed cells during reparative neurogenesis in the proliferative regions and definitive centers of the brain.

Morphological parameters and the optical density of PCNA immunolabeling in cells of the optic nerve of a trout *Oncorhynchus mykiss* after the traumatic impact

Cell types	Lagre diameter, μm	Small diameter, μm	Optical dencity of the PCNA labeling, OD units	
1. Large	12.1 ± 0.8	5 ± 0.4	Min	Max
2. Elongated	8.4 ± 0.2	4.2 ± 0.5	108	124
3. Round	8.6 ± 0.3	6.1 ± 0.3	107	118
4. Small	6.9 ± 0.06	5 ± 0.02	97	120

TUNEL-labeling of apoptotic cells. The detection of apoptosis in the optic nerve of a trout was conducted 2 days after the injury by identifying the fragmented DNA with immunoperoxidase TUNEL-labeling. After the two-hour fixation in 4% paraformaldehyde phosphate buffer (0.1 M, pH 7.2) solution, the fragmented damaged and intact parts of the trout optic nerve were washed in 0.1 M phosphate buffer for one day. The tissues were kept in 30% sucrose in 0.1 M phosphate buffer solution for the cryoprotection until the total immersion. Horizontal longitudinal 50 μm sections were made on a Cryo-Star HM 560 MV freezing microtome (Germany). To identify the TUNEL-positive structures, a standard Apoptag Peroxidase In Situ Apoptosis Detetion Kit (Chemison International Inc., United States) was used according to the manufacturer's instructions. To visualize the reaction products, brain sections were incubated in a medium for the detection of peroxidase (VIP Substrate Kit; Vector Labs, United States) under the microscopic control of the color development and then washed in three changes of phosphate buffer and mounted on glass slides. The cell nuclei were counterstained with Brachet's methyl green. The slices were dehydrated by a standard protocol and embedded in the Bio-Optica medium (Italy).

Morphometric processing was carried out using the software of the Axiovert 200 M inverted microscope with the ApoTome module and Axio Cam MRM and Axio Cam HRC digital cameras (Carl Zeiss, Germany). Measurements were made at 400× magnification in five randomly selected fields of view for each area of research. Proliferation index (PI) and apoptotic index (AI) was determined per 1 mm^2 of the slice by the following equations:

$$\mathrm{PI}=\frac{n\,\text{PCNA-positive}\times 100\%}{\text{total } n \text{ nuclei}};$$

$$\mathrm{AI}=\frac{n\,\text{TUNEL-positive}\times 100\%}{\text{total } n \text{ nuclei}}.$$

Quantitative analysis of the PCNA and TUNEL IHC labeling was performed with the Statistica 12 and Microsoft Excel 2010 programs. The results were estimated using ANOVA-test; data are presented as mean ± standard error of the mean ($M \pm m$). The values $P < 0.05$ and $P < 0.001$ were considered statistically significant.

RESULTS

Investigation of the Response of the Optic Nerve Cells to the Injury. Morphology and TUNEL Labeling

After the mechanical impact on the trout eye, the morphology of optic nerve at the side of the damage and on the contralateral side was studied. The morphological structure of the trout optic nerve on the contralateral side is shown in Fig. 1a. In this region, four morphological cell types were identified among the optic nerve fibers (see table). The number of cells per field on the contralateral side was 54.5 ± 3.5. Mean apoptosis index in this region was 4.35% (Fig. 2a). On the contralateral side, single TUNEL-labeled bodies were identified in the form of small granules of different sizes and more condensed apoptotic cells (Fig. 1b). In our studies, TUNEL-labeling gave positive results in the selective detecting of apoptotic cells with the signs of DNA fragmentation (Figs. 1b–1f). After the mechanical eye damage, the distribution of TUNEL-positive elements in the optic nerve was uneven (Fig. 1c). These elements prevailed on the side of damage, and their number gradually increased towards the region of the injury. The intense labeling of the nuclei of apoptotic cells showed signs of DNA fragmentation: dark peroxidase-labeled granules (apoptotic bodies), which sometimes formed half rings and homogeneous conglomerates (Figs. 1c, 1e). These structures were located at the nucleus site and evenly distributed in the cytoplasm, were shifted to the cytoplasmic membrane, or were grouped in one of the poles of the cell soma. An intact cell membrane and absence of the inflammatory infiltration were typical for the TUNEL-positive elements.

Two days after the damaging impact, areas of mass cell migration and a dramatic increase in the number of cells of the first and second types were found on the ipsilateral side (Fig. 1d). These cells formed longitudinal migratory flows surrounded by cells of type 3, whose number was also significantly increased (Fig. 1d). The average number of cells on the ipsilateral side was 2.28 times higher than that on the contralateral side. The diagram (Fig. 2a) shows the ratio of cells at the contra- and ipsilateral sides (54.5/124.5 cells per field, respectively). In addition to the patterns of cell migration on the ipsilateral side, the massive accumulation of apoptotic cells and numerous TUNEL-labeled elements were identified (Figs. 1c, 2a). The mean size of

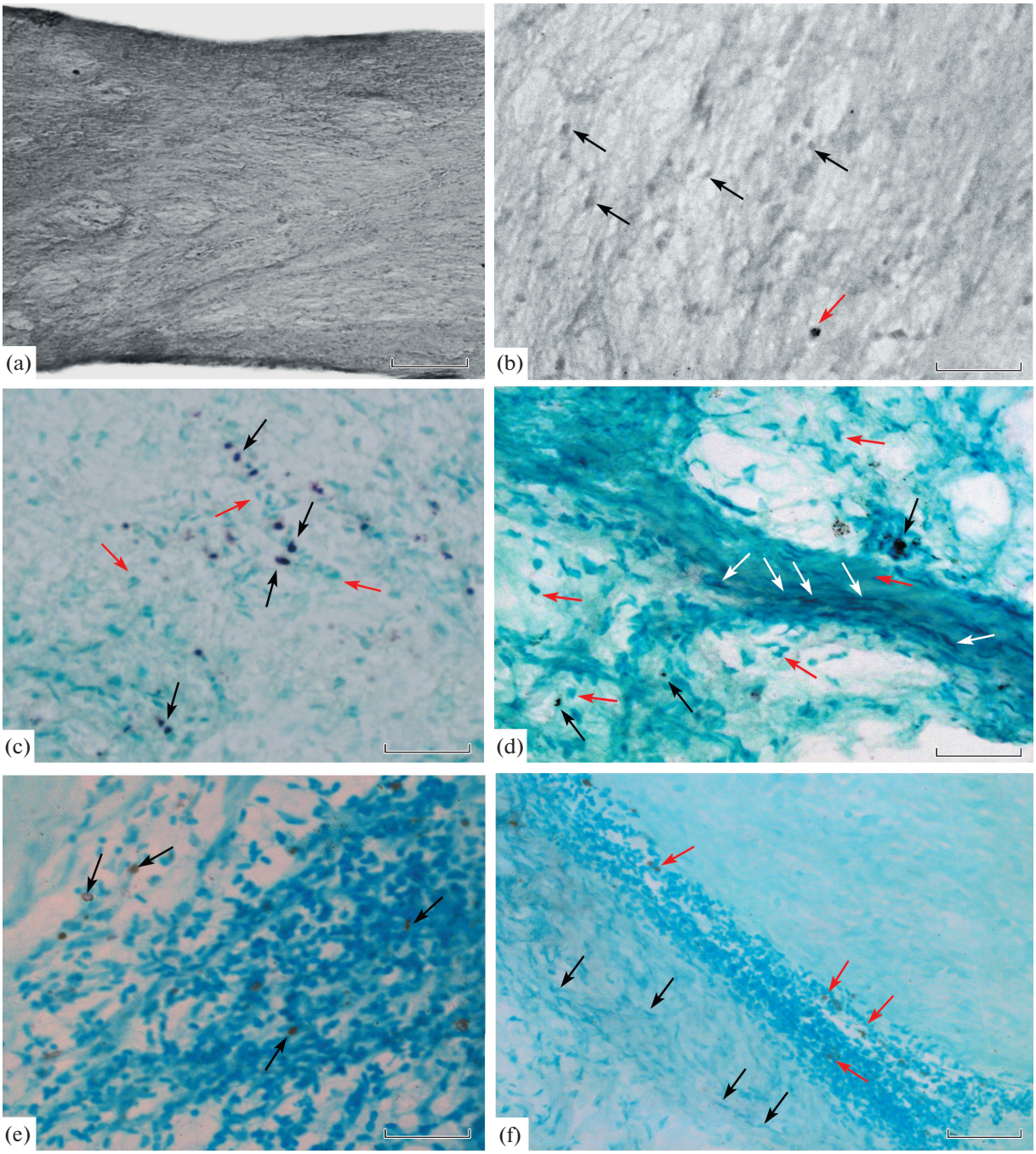

Fig. 1. Morphological structure and apoptosis in the optic nerve of a trout *Oncorhynchus mykiss* 2 days after the mechanical injury. (a) general view of the contralateral nerve; (b) cells (black arrows) and TUNEL-labeled granules in the contralateral nerve; (c) accumulation of apoptotic bodies (black arrows) and cells (red arrows) in the proximal ipsilateral optic nerve red arrows; (d) patterns of cell migration (cells of type 1 are shown with white arrows) in the ipsilateral optic nerve (the rest of the symbols are the same as in (b)); (e) mass accumulation of the cells of the third and fourth types in the epineurium of the ipsilateral optic nerve, white arrows indicate the different types of TUNEL-labeled cells; (f) general view of the proximal part of the ipsilateral optic nerve, black arrows show migrating cells type 1, red arrows show TUNEL-labeled apoptotic bodies. Immunoperoxidase TUNEL labeling with Brachet's methyl green counterstaining. Scaling: (a) 200 μm, (b–f) 50 μm.

the cells in the areas of mass localization of apoptotic cells were 5.3 ± 0.7/3.9 ± 0.5 μm, and mean size of apoptotic bodies in these regions was 5.9 ± 0.9/3.9 ± 0.5 μm (Fig. 1e). The mean number of apoptotic cells per field at the contra- and ipsilateral side was 2.5/22.5 elements respectively (Fig. 2a). Along with apoptotic bodies, the cell degranulation patterns representing the earlier stages of the apoptotic process and

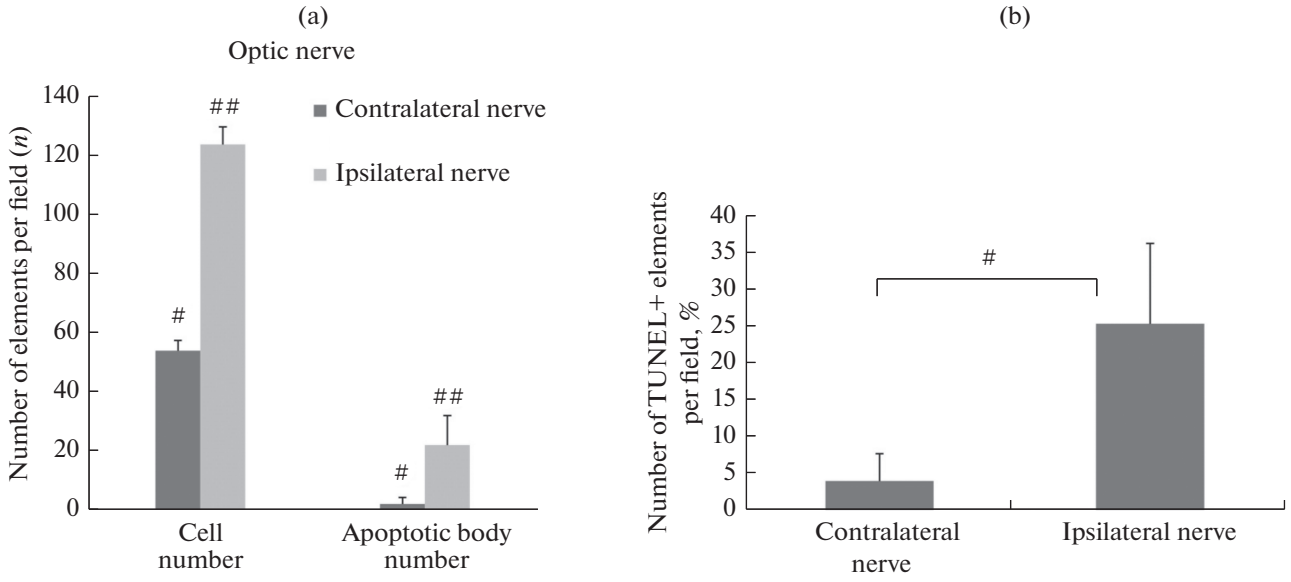

Fig. 2. Comparison of the number of TUNEL-labeled elements in the optic nerve of a trout *Oncorhynchus mykiss* 2 days after the mechanical injury. (a) the number of methyl green stained cells and TUNEL-labeled bodies ($M \pm m$) per field in contra- and ipsilateral nerves ($n = 5$ in each group; # a significant difference at $P < 0.05$ in the ipsi- and contralateral nerves; ## a significant difference at $P < 0.001$ in the ipsi- and contralateral nerves); (b) number of TUNEL-labeled elements per field in the ipsi- and contralateral nerves ($n = 5$ in each group; # a significant difference at $P < 0.05$ in the ipsi- and contralateral nerves).

TUNEL-labeled fragments of crescent-shaped degranulating chromatin were identified (Fig. 1e). These patterns were typical of degranulating cell clusters and indicated the massive cell apoptosis 2 days after the damage of the trout optic nerve. A significant increase in the cell number was observed in the areas separating the individual optic fiber bundles—mesaxons (epineurium) (Fig. 1f). TUNEL-labeled apoptotic cells were also identified in these areas (Fig. 1f). The ratio of apoptosis indexes at the contra- and ipsilateral side 2 hours after the mechanical injury was 4.3/25.7%, respectively (Fig. 2b).

Considering the complex nature of the mechanical damage affecting different membranes and structures of the eye, the optic nerve, and the adjacent oculomotor muscles together with the connective tissue, the adjacent muscle fibers of the oculomotor muscles were investigated along with the optic nerve (Fig. 3a). The postinjury study of the morphological structure of TUNEL-labeled and stained with Brachet's methyl green muscle bundles revealed a large number of cell types 1–4 in the connective tissue surrounding the muscle bundles, previously detected in contra- and ipsilateral optic nerves (Fig. 3a). These cells were also detected along radial fibers in the area between the optic fibers and adjacent oculomotor fiber bundles (Fig. 3b). We consider that these cells appearing in the region of the optic nerve damage represent the regional group of connective tissue macrophages migrating to the site of injury to remove the fragments of the damaged optic nerve and adjacent muscle cells.

The study of the morphological structure of individual muscle bundles showed the initial stages of the apoptotic process, the fragmentation of chromatin appearing as numerous diffuse TUNEL-labeled fragments in muscle fibers after the injury (Figs. 3a, 3c). However, we do not exclude the presence of necrotic and inflammatory process in these areas, since disordered chromatin condensation without its binding to the nuclear membrane and karyolysis were sometimes observed in the muscle fibers, which is not typical for apoptosis (Fig. 3c). We observed the sites of inflammatory infiltration around the muscle bundles (Fig. 3a). The maximum accumulation of TUNEL-positive apoptotic cells was detected in regions where ipsilateral optic fibers adjoined to the muscle fibers (Fig. 3d). The connective tissue surrounding muscle fiber bundles contained apoptotic bodies and 1–4 types of cells (Fig. 3d).

To evaluate the proliferative activity in the injured optic nerve, IHC of PCNA-labeling was carried out 1 week after the injury. The results of PCNA-labeling showed a high level of proliferative activity of cells in the ipsilateral optic nerve (Fig. 4a). Among immunopisitive cells, the cells of 2, 3 and 4 types were found, but the activity of PCNA was also identified in migrating cells type 1 (Figs. 4a, 4b). The distribution of PCNA-ip cells was uneven; the cells of types 3 and 4 often formed local clusters (Fig. 4a). Frequently migrating cells of type 3 were arranged in a longitudinal series in the surface layers of the individual optic nerve bundles (Fig. 4c); in this case, the immunonegative central area was clearly distinguishable in indi-

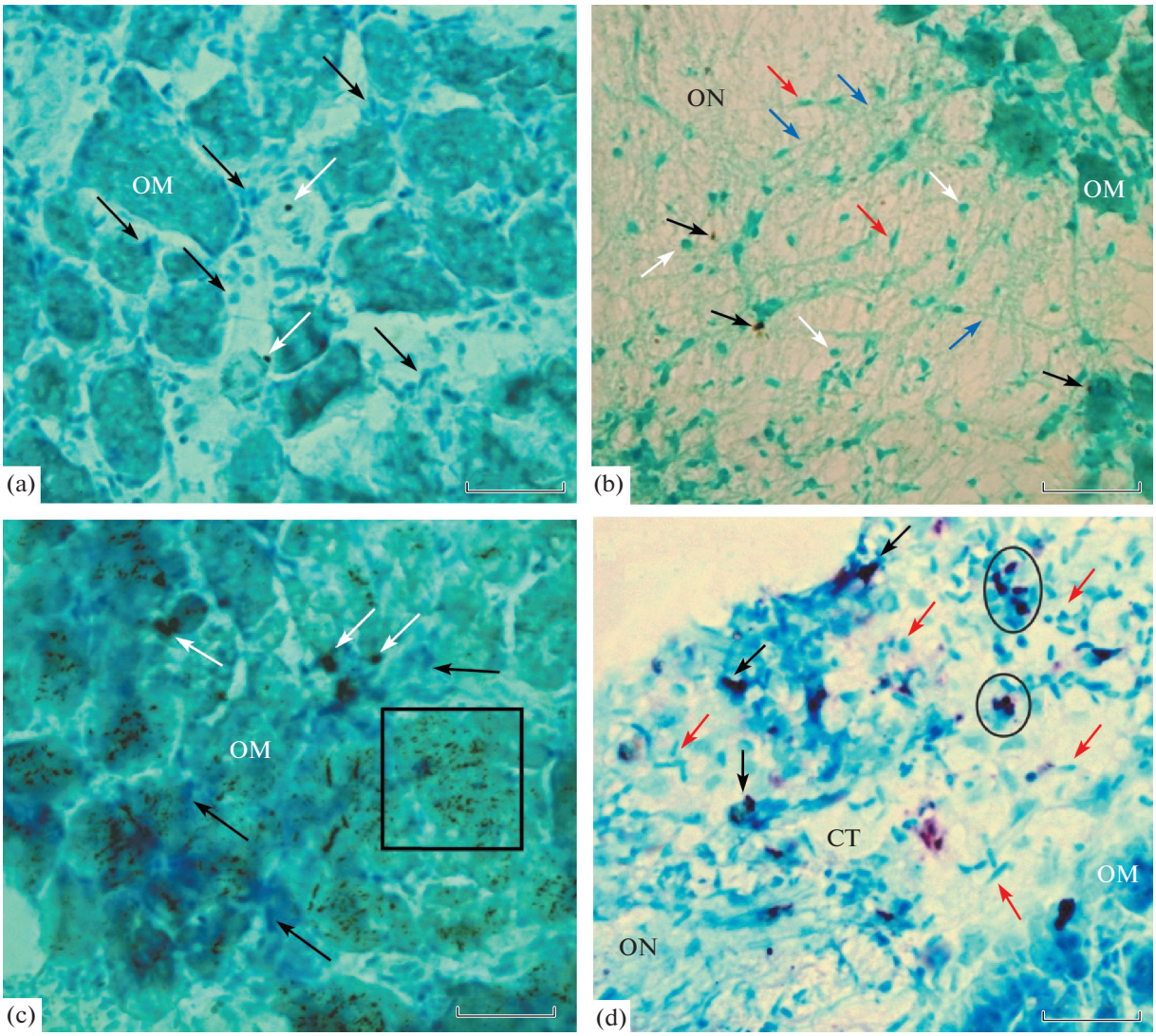

Fig. 3. Morphologic structure and TUNEL-labeling of the oculomotor muscles adjacent to the damaged nerve of a trout 2 days after the injury. (a) cross-section of the extraocular muscle fibers (MF), white arrows indicate TUNEL-labeled apoptotic bodies, black arrows cells infiltrating the space between the muscle bundles; (b) reconstruction of the transition fragment, the damaged optic nerve, and the adjacent muscle tissue containing cells of types 1–4 (cell type 3 isindicated with white arrows, type 1 red arrows), apoptotic cells (black arrows) and radially oriented fibers (blue arrows) directing the cells; (c) cross-section of the eye muscles, a square contoured fragment contains diffuse TUNEL-labeled material, dense apoptotic bodies are indicated by white arrows, cells of the intermuscular space are marked with black arrows; (d) fragment of the damaged optic nerve (ON) and a peripheral part of the oculomotor muscle surrounded by the connective tissue (CT), conglomerates of apoptotic bodies are contoured with ovals, numerous cells of types 1–4 are marked by the red arrows. Immunoperoxidase TUNEL labeling in combination with Brachet's methyl green staining. Scale bar: 50 μm.

vidual cells, and the activity level of PCNA was moderate (Fig. 5c). In some cases, it was possible to distinguish layers containing large labeled cells of type 1 forming the surface migration flows and the deeper layers of the optic nerve containing PCNA-ip cells of type 4 with high activity of PCNA (Fig. 4d). The cells of type 2 also formed the increased density regions (Fig. 4b). The ratio of the total number of cells, apoptotic cells, and PCNA-ip cells per the field 1 week after the injury is shown in Figs. 5a and 5b. The results of the densitometric analyzes indicated that there were differences in the optical density in cells of types 2, 3, and 4 (Fig. 5c, table). The densitometric analysis showed that the difference between the minimum and maximum optical density in the cells was 22–30%. The ratio of PCNA immunopositive cells at the contra- and ipsilateral trout nerves 1 week after the injury differed significantly (Fig. 5d), and the proliferative activity of cells in the optic nerve was more than 3 times increased on the damaged side.

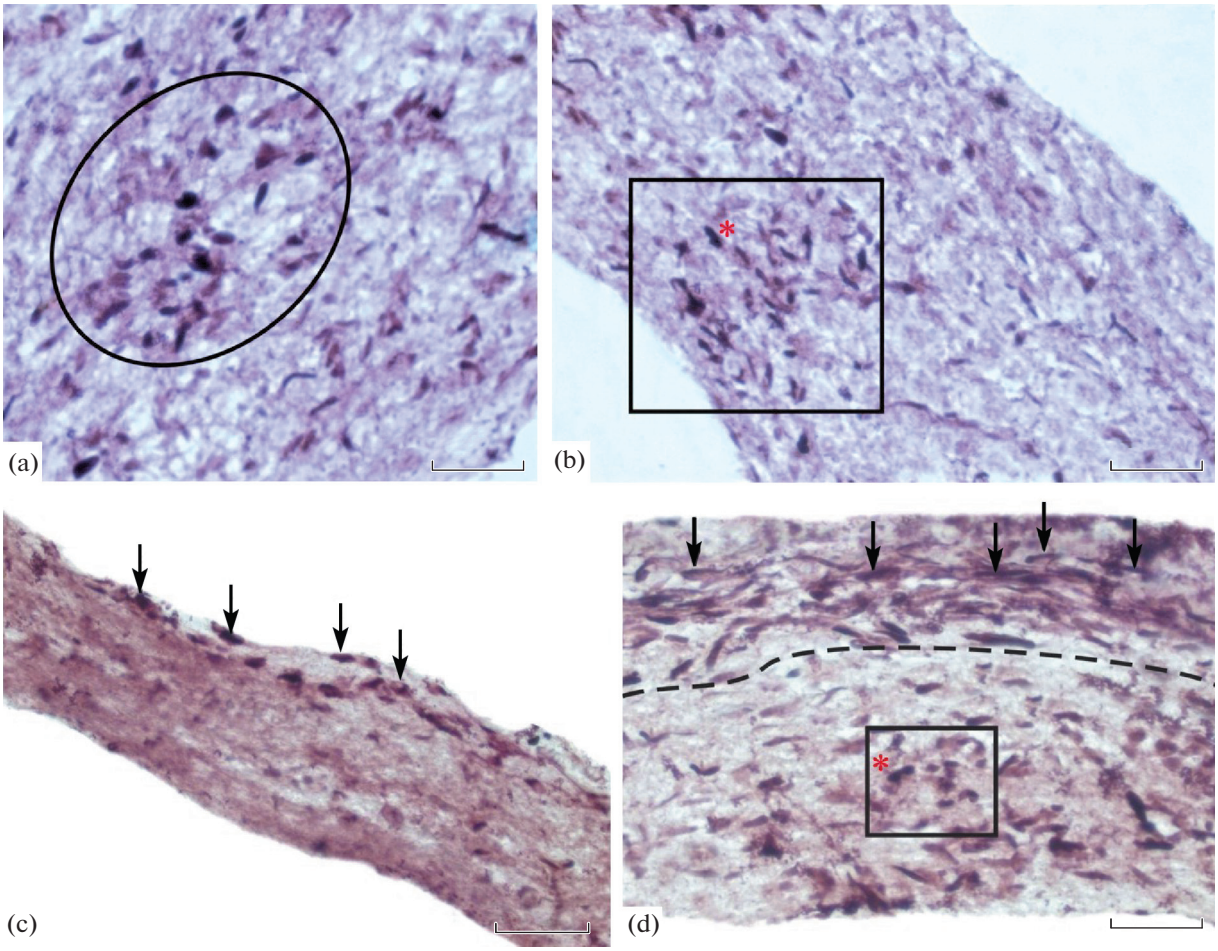

Fig. 4. Localization of proliferating cell nuclear antigen (PCNA) in the ipsilateral optic nerve of a trout 1 week after the injury. (a) clusters of intensively labeled cells of types 2 and 3 (contoured with an oval) in the deep layers of the damaged nerve; (b) accumulation of elongated immunopositive cells (contoured with a square) in the surface layers of the damaged nerve, a red star here and in (d) shows immunolabeled cells out of mitosis; (c) flow of moderately labeled cells migrating in the surface layers of the optic nerve (shown by black arrows); (d) stratification of migrating moderately immunolabeled cells of type 1 (shown by the black arrows) and highly immunogenic cell of type 4 (contoured with a square), migration zone is outlined by dotted lines. Immunoperoxidase labeling of PCNA. Scale bar: 50 μm.

Investigation of the Cell Response to the Damage in the Trout Brain Structures

After the trout eye damage, the proliferative activity of cells in the integrative center of the brain—the cerebellum and the optic tectum—was examined by IHC PCNA labeling on histological sections.

PCNA immunoreactivity was found in the dorsal and ventral regions of the body of the trout cerebellum. In the dorsal region located in the dorsomedial part of the cerebellum, a large accumulation of PCNA-ip cells forming the dorsal proliferative region (DPR) was found above the granular layer, as described in other species (Fig. 6a). Multiple cells in a state of radial and tangential migration were identified over DPR (Fig. 6b). Inside the molecular layer of the dorsal region, radially migrating cells prevailed; tangentially migrating cells were identified in the surface layers (Fig. 6b). An accumulation of PCNA-immunonegative cells with high-density distribution was found near the DPR (Fig. 6b). A similar cluster of immunonegative cells of irregular shapes was also found directly under the DPR (Fig. 6b). We believe that these cell formations are the regional neurogenic niches formed as a result of the traumatic impact. Inside the molecular layer of the DPR, PCNA-immunonegative rounded cells with increasing distribution density in the dorsal direction were also localized (Fig. 6b). PCNA-immunopositive elements of DPR included four cell types: round and oval highly immunogenic cell of $8.3 \pm 0.9/6.4 \pm 1.4$ μm, and elongated spindle-shaped and rod-shaped elements of $10.5 \pm 0.5/6.9 \pm 1.2$ μm in size (Fig. 6a). The data on the optical density of PCNA labeling of cells of different DPR types and quantitative evaluation of PCNA-ip cells in the cere-

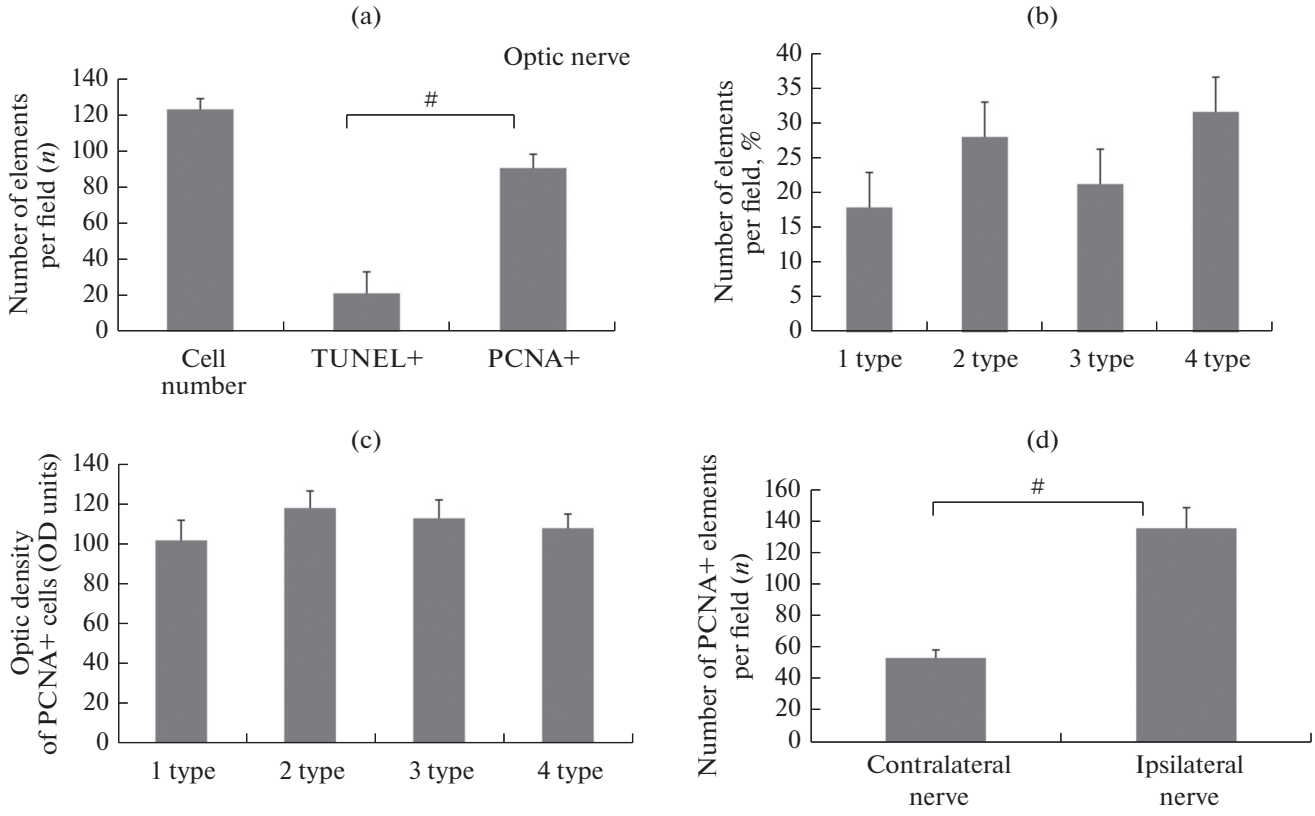

Fig. 5. Evaluation of the number of proliferating and apoptotic elements in the optic nerves of a trout 1 week after the injury. (a) number of TUNEL+ and PCNA-ip elements and Brachet's stained cells per field in the damaged nerve ($n = 5$ in each group; # $P < 0.05$ significant difference between PCNA+, TUNEL+ groups); (b) percentage of PCNA+ cells of types 1–4 in the damaged nerve ($M \pm m$); (c) optical dencity of the PCNA immunolabeling in the cells of type 1–4 in the damaged nerve ($M \pm m$); (d) ratio of PCNA-ip cells in the contralateral and ipsilateral nerves per field ($n = 5$ in each group; # $P < 0.05$ significant difference between ipsi- and contralateral nerves).

bellar DPR are shown in Figs. 7a and 7b. The optic density of immunolabeling slightly increases in all cell types after the damage (Figs. 7a, 7b). The number of PCNA-labeled cells of types 1, 2 and 4 in the DPR 1 week after the mechanical damage significantly increased compared to controls ($n = 5$; $p < 0.05$), and the number of cells of type 3 was significantly increased ($n = 5$; $p < 0.001$).

In the ventral region of the cerebellar body, the concentration of PCNA-ip elements was much lower (Fig. 6c). Single PCNA-ip parenchymal cells were identified in the molecular layer (Fig. 6c). In the upper granular layer, single and/or forming small clusters PCNA-ip parenchymal cells of 7.4 ± 1.6/5.7 ± 0.9 μm in size were present. PCNA-ip cells often belonged to populations of small granule cells. On the dorsal side of the granular layer, the number of PCNA-ip cells was much higher (Fig. 6c). In this region, PCNA-immunogenicity was also present in small parenchymal cells of the infraganglionar plexus (Fig. 6d). Small clusters of PCNA-ip and negative cells forming clusters with high cell density were sometimes found in the infraganglionar plexus region (Fig. 6d). We believe that these clusters are regional neurogenic niches, whose proliferative activity, as in a niche adjacent to the DPR, was induced by the mechanical trauma.

PCNA immunolabeling in the tectum. After mechanical trauma of the eye, numerous immunopositive cells were identified in the inner tectum layers (Fig. 6f): the surface fibrous and cellular layer (SFCL), the central gray layer (CGL), the central white layer (CWL), and the periventricular layer (PVL). The size of PCNA-ip cells in all layers ranged from 6.2/3.4 to 9.5/6.1, and the mean value was 7.8 ± 1.1/4.9 ± 0.9 μm. In the marginal layer, PCNA-ip cells of radial glia were identified (Fig. 6e), the size of the bodies of the radial glial cells varied from 6.7/5.1 to 9.5/8, and the mean value was 8.4 ± 1.2/6.1 ± 1.3 μm. The distribution density of PCNA-ip radial glia was relatively low and amounted to 33 ± 7 cells per profile field at 40× magnification of the lens. The ratio of PCNA-immunolabeled radial glial cells in the marginal layer and the inner cell layers in the tectum is shown in Fig. 7c. The optical density of PCNA immunolabeling of radial glia cells varied: highly

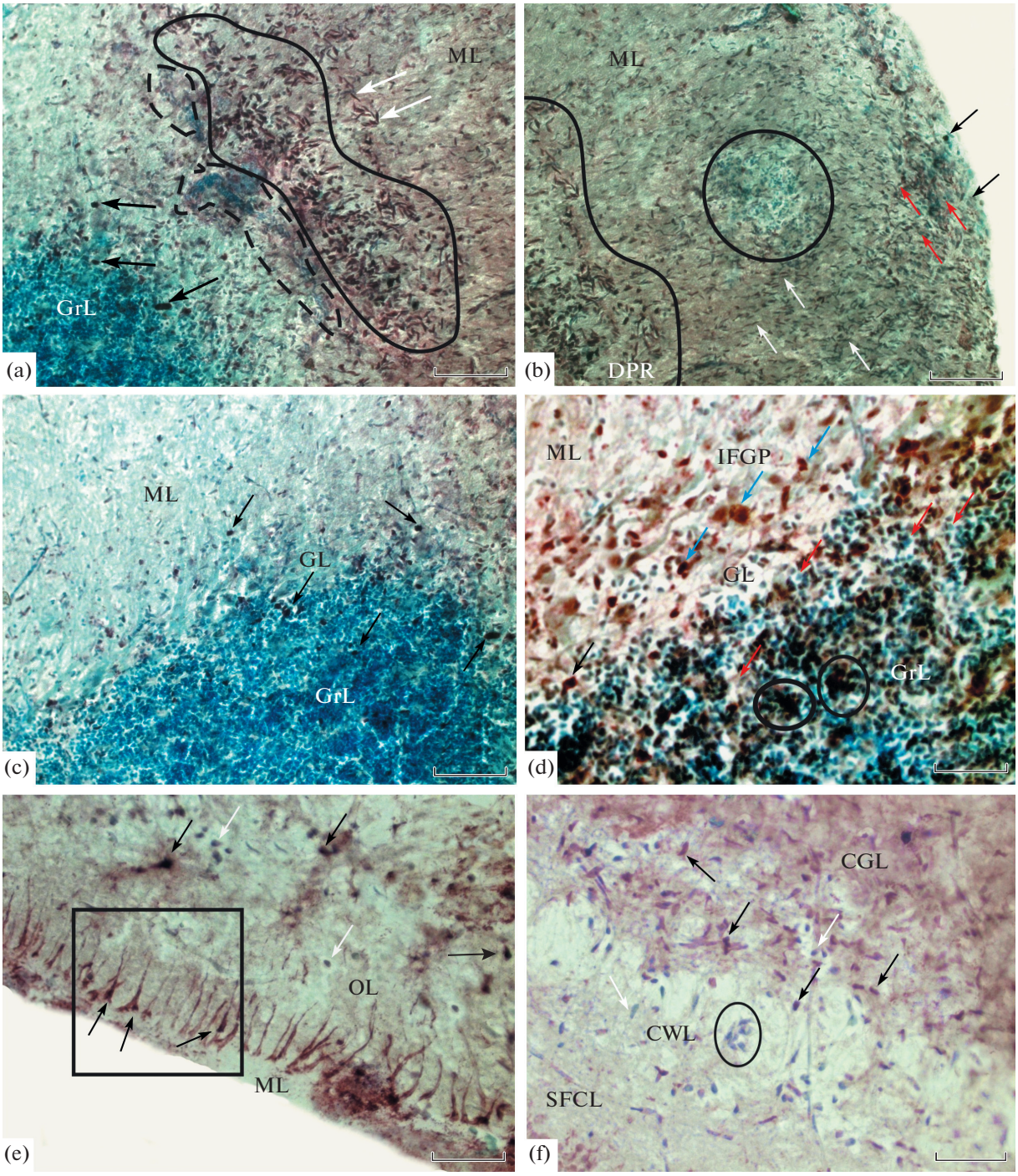

Fig. 6. Localization of PCNA in the trout brain integrative centers 1 week after the mechanical injury. (a) PCNA-ip proliferative cells in the dorsal area of the cerebellum (highlighted by a solid line), the accumulation of PCNA-in cells under the DPR is highlighted by a dotted line matrix, the black arrows mark the intensely labeled oval cells of the granular layer (GrL), white is rod-shaped migrating cells; (b) the dorsal part of the molecular layer (ML) of the cerebellum, the accumulation of PCNA-in cells is highlighted with a circle, the black arrows show the PCNA-in tangentially migrating cells, red arrows show PCNA-in small round cells, white arrows show weakly labeled radially migrating cells; (c) ventral part of the body of the cerebellum, the black arrows show the PCNA-ip cells in the molecular, ganglionar (GL) and granular layers; (d) region of the infraganglionar plexus (IFGP) in the dorsal part of the cerebellar body, the blue arrow shows the PCNA-ip IFGP cells, red arrows show PCNA-in cells in the granular layer, ovals highlight the clusters of PCNA-ip cells in the granular layer; (e) radial glia (contoured with a square) in the marginal layer (ML) of the optic tectum, PCNA-ip cells are marked with black arrows, PCNA-in are marked with white arrows, OL is optical layer; (f) PCNA-ip cells in the surface layer of fibrous and cellular layer (SFCL), the central gray layer (CGL), the central white layer (CWL), an oval highlights the accumulation of PCNA-in cells forming the neurogenic niche. Immunoperoxidase labeling of PCNA in sections in situ. Scale bar: (a–c) 100 μm, (d–f) 50 μm.

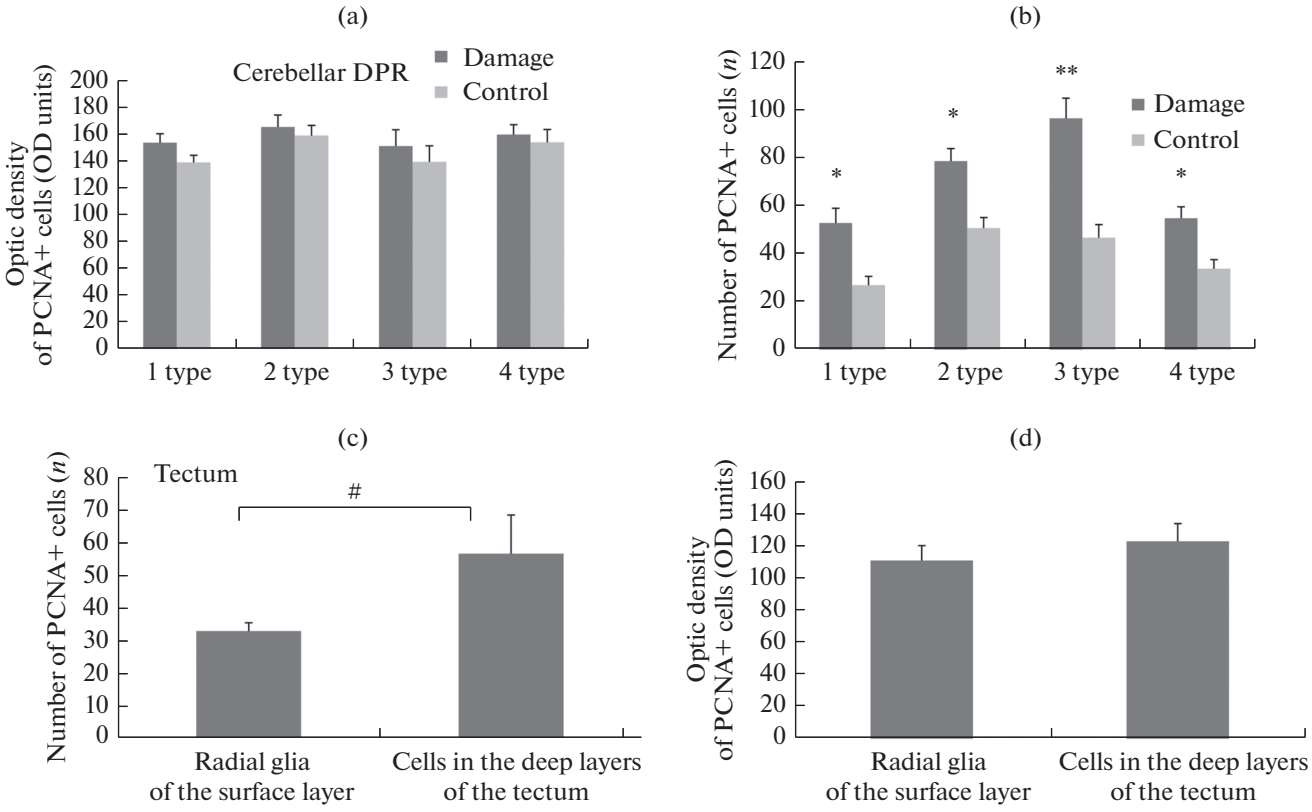

Fig. 7. Assessment of the number of proliferating cells and their densitometric parameters in the integrative centers of the trout brain 1 week after the injury. (a) optical density of PCNA labeling in the cells of types 1–4 in the cerebellar DPR ($M \pm m$); (b) number of PCNA-ip cells of types 1–4 in the cerebellar DPR ($n = 5$ in each group; * $P < 0.05$ significant difference from the controls; ** $P < 0.001$ significant difference from the controls); (c) ratio of PCNA-ip elements: radial glia and cells in the inner layers of the optical tectum ($n = 5$; # $P < 0.05$ significant difference between groups); (d) optical density of PCNA-immunolabeling in radial glia and cells of the deep layesrs of the tectum ($M \pm m$).

immunogenic cell (114.5 OD units) and less intensely labeled (104.4 OD units). In the inner layers of the tectum, the density level of PCNA-ip was higher than in the cells of radial glia and was 123 OD units on average (Fig. 7d). The maximum density level of PCNA in the cells of the tectum was 127.7 OD units and minimum was 116.2 OD units.

HuC/D Neuronal Marker Expression in the Proliferative Regions of the Trout Brain after the Mechanical Injury of the Optic Nerve

Our study showed that the neuronal protein HuC/D is expressed in the definitive centers and proliferative regions of the adult trout brain. In the **telencephalon**, regions of neurogenesis adjoined to the external borders of the dorsal and ventral regions were found. Since the telencephalon of teleosts has the everting structure of hemispheres, the proliferative region (PR) is located at the outer wall of the cerebral hemispheres. It was found that the PR in the medial part of the dorsal region of hemispheres has a laminar structure; HuC/D expression was detected in the surface layers of the region indicating a very early neuronal differentiation of cells formed after the traumatic injury (Fig. 8a). On the border of the dorsal and medial areas, the maximum number of HuC/D-ip cells and the highest level of HuC/D activity was found (Fig. 8c) among the cells formed in the post-traumatic period (Figs. 8b, 9a, 9b). In the lateral zone, the local accumulations of HuC/D-ip cells were present (Fig. 8b). However, the total thickness of the proliferative layer and the proliferative HuC/D expression in cells in the proliferative region was minimal in this region (Fig. 9c) and maximal in the dorsal region (Figs. 8c, 9a, 9c). The general scheme of the dorsal and ventral regions of the trout telencephalon and the distribution of proliferative zones are shown in Fig. 8d.

In the ventral region of the trout telencephalon corresponding to the striatal formation of other vertebrates, the HuC/D expression was prominent in the definitive nuclei: dorsal and ventral (Figs. 8e, 8f). Investigation of the expression of neuronal proteins HuC/D showed that the HuC/D expression level was significantly different in the definitive areas of the forebrain (medial, dorsal and lateral areas of the dorsal region and the dorsal and ventral regions of the ventral area), as

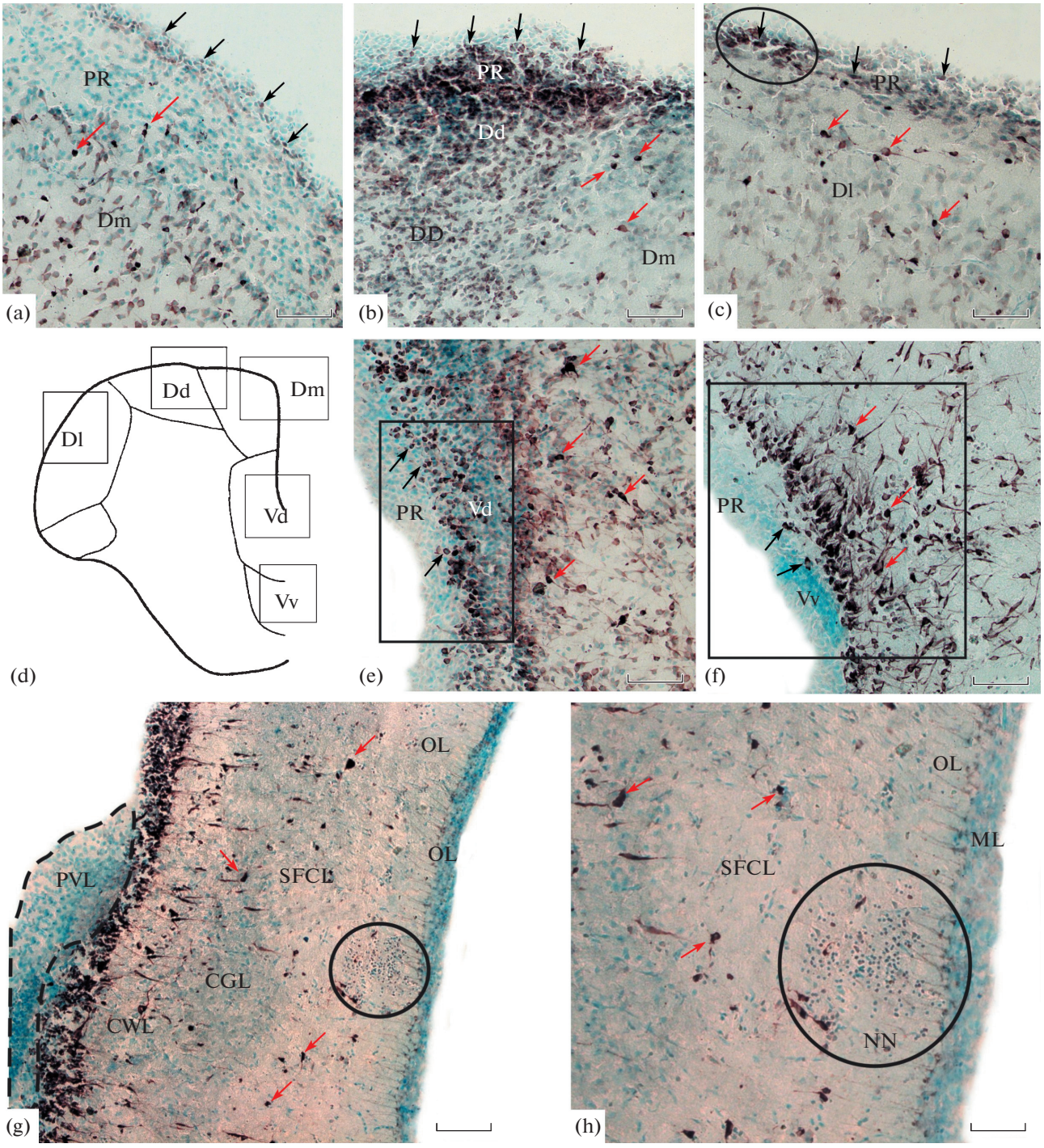

Fig. 8. Localization of neuronal protein HuC/D in the proliferative regions of the telencephalon and optic tectum 2 days after the mechanical injury. (a) HuC/D-ip cells (black arrows) in the proliferative region (PR) and deep layers (red arrows) of the medial region of the telencephalon (Dm); (b) at the border between the dorsal (Dd) and medial (Dm) regions; (c) in the lateral region (Dl), an oval marks the conglomerate of HuC/D-ip cells; (d) dorsal and ventral regions of the trout telencephalon containing ptoliferative regions from figures (a–c) and (e–f); (e) HuC/D-ip cells in PR (marked with a square) of the dorsal nucleus of the ventral part of the telencephalon (Vd); (f) ventral nucleus of the ventral region (Vv) (highlighted with a square); (g) HuC/D-immunolabeling in the optic tectum, (indications are the same as at 6e–6f), an oval contoures the neurogenic niche, the dotted line highlightes the caudal proliferative region of the tectum; (h) neurogenic niche in the tectum (NN) at larger magnification. Immunoperoxidase labeling of HuC/D cells on sections in situ. Scale bar: (a–c, e, f) 100 μm, (g) 200 μm, (h) 50 μm.

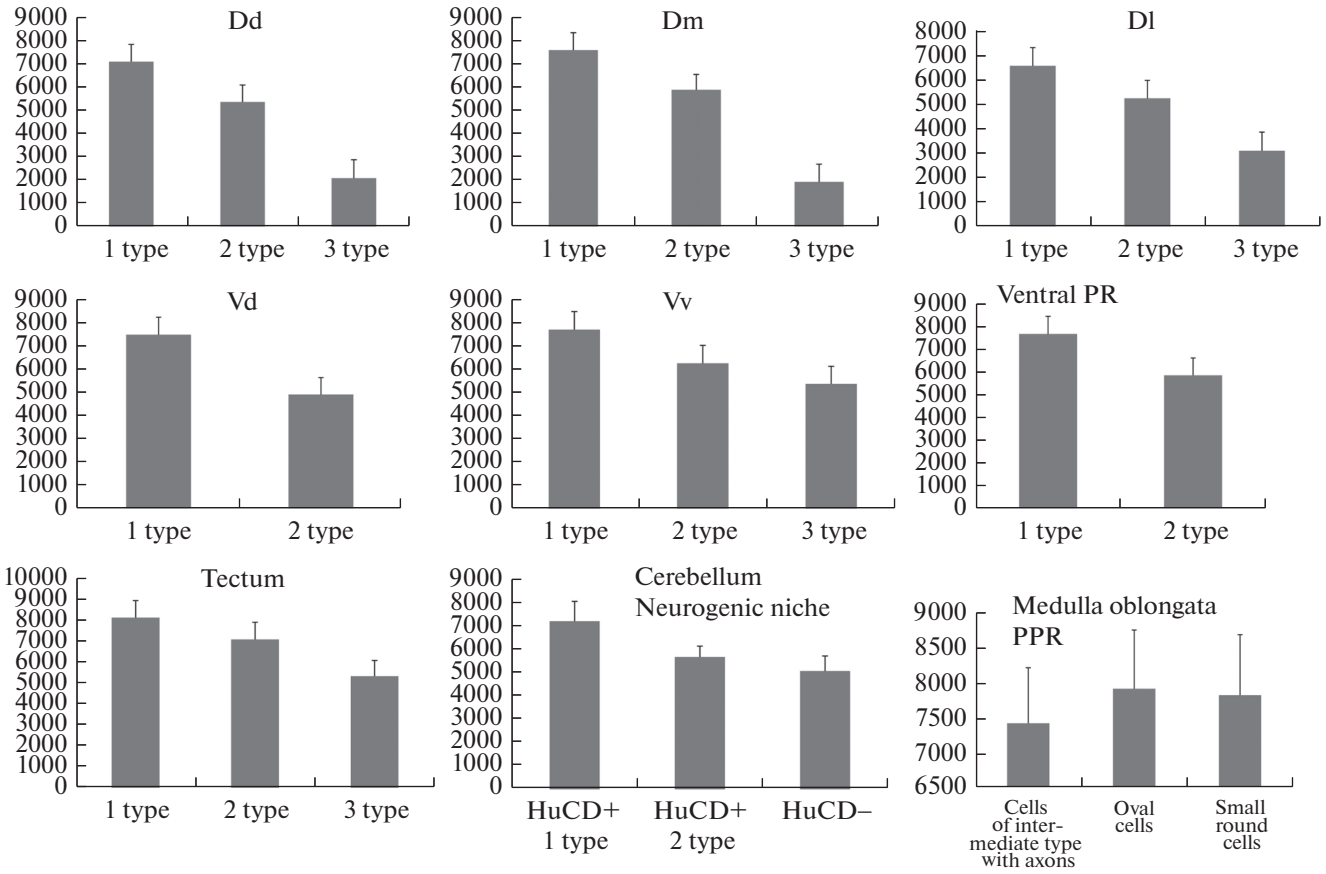

Fig. 9. Densitometric results of the neuronal protein HuC/D labeling in the integrative centers and proliferative regions of the trout brain 2 days after the mechanical trauma ($M \pm m$). Upper row shows the optical density of HuC/D immunolabeling in the neurons of types 1–3 in the dorsal (Dd), medial (Dm), lateral (Dl) regions of the dorsal telencephalon; the middle row shows that in the neurons of dorsal (Vd), ventral (Vv) nuclei, and proliferative region of the ventral telencephalon (PRV); the lower row shows that in the tectum, neurogenic niche of the cerebellum, and periventricular region (PVR) of the medulla oblongata.

well as in the PR of the ventral region (Figs. 9a–9f). Typically, the newly formed cells in the ventral part of the proliferative region had a very high level of HuC/D activity (Fig. 9f), while the neurons of definitive regions the intensity of HuC/D immunolabeling could be low.

In the **optic tectum**, the HuC/D activity was found in mature neuronal populations of virtually all layers except the marginal layer (ML) and the optic fiber layer (OL) (Figs. 8g, 9g). HuC/D-ip cells were found in the layers of the tectum (Fig. 8g). In the surface fibrous and cellular and the central gray layers, cells of various types were found, including the most heavily labeled cells of type 3 with the size 12.3 ± 14/8.5 ± 1 μm. Another type of cells—vertical bipolar cells 29.9 ± 5.6/6.6 ± 0.7 μm in size—were located in the central gray layer and had a low level of HuC/D activity (5460 ± 437 OD units). Medium oval-shaped cells of types 3 and 4 located in the surface fiber and the central gray layers with the size of 15.2 ± 3/10.1 ± 2.1 and 13.4 ± 1.2/ 8.6 ± 1.1 μm, respectively, also had a high level of HuC/D activity.

In **the caudal tectum**, neurogenic areas of activity were identified: the periventricular layer with significantly higher thickness than that of the central and rostral area, and the appearance of **neurogenic niches** in the surface layers of the tectum (marginal and optical) (Figs. 8g, 8h). In these areas, the HuC/D activity in a centrally located cells was not found, but single HuC/D-ip cells or small groups of these cells were located on the periphery (Fig. 8h), which indicated the undifferentiated pluripotent nature of the cells of these regions in the midbrain. In the neurogenic niches, HuC/D-negative small cells 5.3 ± 0.4/4.7 ± 0.5 μm in size were found; the density level was 6810 ± 416 OD units. In the caudal prolipherative region of the tectum, the optical density of HuC/D-ip cells was 7721 ± 697 OD units, and that in the immunonegative regions was 5850 ± 689 OD units.

In the trout **cerebellum**, regions containing HuC/D-immunopositive cells were also identified in

the definitive cell molecular, granular layers, and infraganglionar plexus, as well as in areas with neurogenic activity: DPR, neurogenic niches, molecular and granular layers, and so called granular elevations 2 days after the mechanical injury (Figs. 8a–8f).

DPR was located in the dorsal-medial part of the molecular layer, just above the granular layer (Fig. 8b). PCNA-ip/HuC/D-in elongated cells were found in the DPR; and highly HuC/D-ip cells surrounded the DPR and formed localized areas of increased density distribution (Fig. 8b). The level of optical density of the HuC/D-ip cells of the DPR was high and moderate (Fig. 9h). Small, highly immunopositive cells with different optical density and the size of $8.7 \pm 0.5/7.7 \pm 1.1$ and $10.7 \pm 0.6/8 \pm 1$ μm (Fig. 9h) and larger cells sized $13.8 \pm 1.2/10.4 \pm 0.9$, 15.3/7.4, and 20.4/12.8 μm were found. In small moderately labeled cells, the density level was 5697 ± 572 OD units.

The local neurogenic niches morphologically similar to those in the tectum were found in the body of the cerebellum after the optic damage (Figs. 8c, d). The most part of the neurogenic niche was HuC/D-immunonegative, but HuC/D-ip cells were located around and within the niche, which indicated neuronal differentiation of such cells early in the post-traumatic period (Fig. 8d). The sizes of immunogenic cells were $5.9 \pm 0.4/4.8 \pm 0.6$ μm, the optical densty was 4821 ± 346 OD units. Strongly labeled HuC/D cells sized $10.7 \pm 0.6/6.9 \pm 2.1$ and $8.5 \pm 0.4/5.6 \pm 0.6$ μm were located inside the niche. The level of the optic density in such cells was 7660 ± 644 OD units. The sizes of moderately labeled cells in the neurogenic niches were $11.8 \pm 1.6/7.1 \pm 1.5$ μm and the optic density was 5213 ± 533 OD units.

The tangential and radial cell migration was found on the dorsal-lateral surface of the body of the cerebellum in the molecular layer (Figs. 8e, 8f). HuC/D-immunonegative elongated cells stained with methyl green formed multilayered series of migrating cells in the surface and inside the molecular layer (Fig. 8e). In these tangential flows, the distribution density of HuC/D-ip cells was smaller than in other parts of the molecular layer. Radial migration patterns were found in the lateral areas of the body of the cerebellum (Fig. 8f). On the lateral and dorsal surface of the cerebellar body, the intensive HuC/D-ip cells were often found in the surface layers (Figs. 8c, 8d).

In the trout **medulla oblongata**, the definitive cells were found in the reticular formation, and cells with HuC/D activity were found in the periventricular proliferative region (Figs. 8g, 8h). Definitive neurons of the reticular formation had different levels of the HuC/D activity in large, medium, and small cells (Fig. 8g). In the periventricular region, the high HuC/D expression was detected in cells of the medial, lateral, and dorsal proliferative zones (Fig. 8h). Thus, all periventricular proliferative regions of the medulla oblongata were characterized by the presence of morphologically heterogeneous but intensively HuC/D labeled cells (Fig. 9i) in contrast to the definitive areas of the reticular formation containing the largest brain cells but having heterogeneous (moderate or low) HuC/D activity.

DISCUSSION

Animals developing without external embryonic membranes (fish and amphibians) have a very significant capacity for regeneration of CNS in contrast to amniote (reptiles, birds, and mammals), which have relatively limited regenerative capacity. Since the retina and optic nerve are functional parts of the brain, accessible to experimental manipulations, the damage and regeneration of the optic nerve is often used as a model for the study of nerve regeneration in the CNS (García and Koke, 2009). The damage of the optic nerve in mice is accompanied by axonal degeneration. In fish, an active neurite sprouting occurs across the damaged axons, and the reinnervation of the brain takes place in a few days. After the cutting of the optic nerve of mammals, the number of OX42 and/or NDP-immunopositive microglia/macrophages increases in the retina and in the optic nerve (Garcia-Valenzuela et al., 2005; Cen et al., 2007; Luo et al., 2007). Microglia normally does not secrete proinflammatory factors, however, the activated microglia produces a large amount of cytokines, proteases, free radicals, and various trophic factors (Hanisch, 2002; Hailer, 2008). Due to the secretion of proinflammatory cytokines and neurotoxic substances, the appearance of microglia usually exacerbates neuronal damage and facilitates the transition of acute inflammation to chronic. In the developing retina, the nerve growth factor produced by microglial cells causes the death of retinal ganglion cells (Frade and Barde, 1998). In fish, astrocytes apparently do not form a specific barrier preventing the regeneration of the optic nerve; however, no systematic studies have been carried out in this field.

Regenerative sources in the fish brain and retina. In teleost retina, new neurons and photoreceptors emerge from two neurogenic niches (Otteson and Hitchcock, 2003). The first niche is represented by a small area localized at the periphery of the retina, in the transition region of the retina in the ciliary epithelium (Johns, 1977). This area is commonly referred to as the ciliated marginal zone. The second niche is formed by rod-shaped progenitors located in the central area of the retina. The cells of this zone in fish are capable of producing rod photoreceptors after the retinal damage (Johns, 1982). Recent studies have shown that rod progenitor cells are derived from the Muller glia, which has properties typical for stem cells (Bernardos et al., 2007).

It was found that new neurons in the adult mammalian brain develop from radial glial cells, which is the functional analogue of stem cells (Kriegstein and Alvarez-Buylla, 2009). In the adult brain of fish, sev-

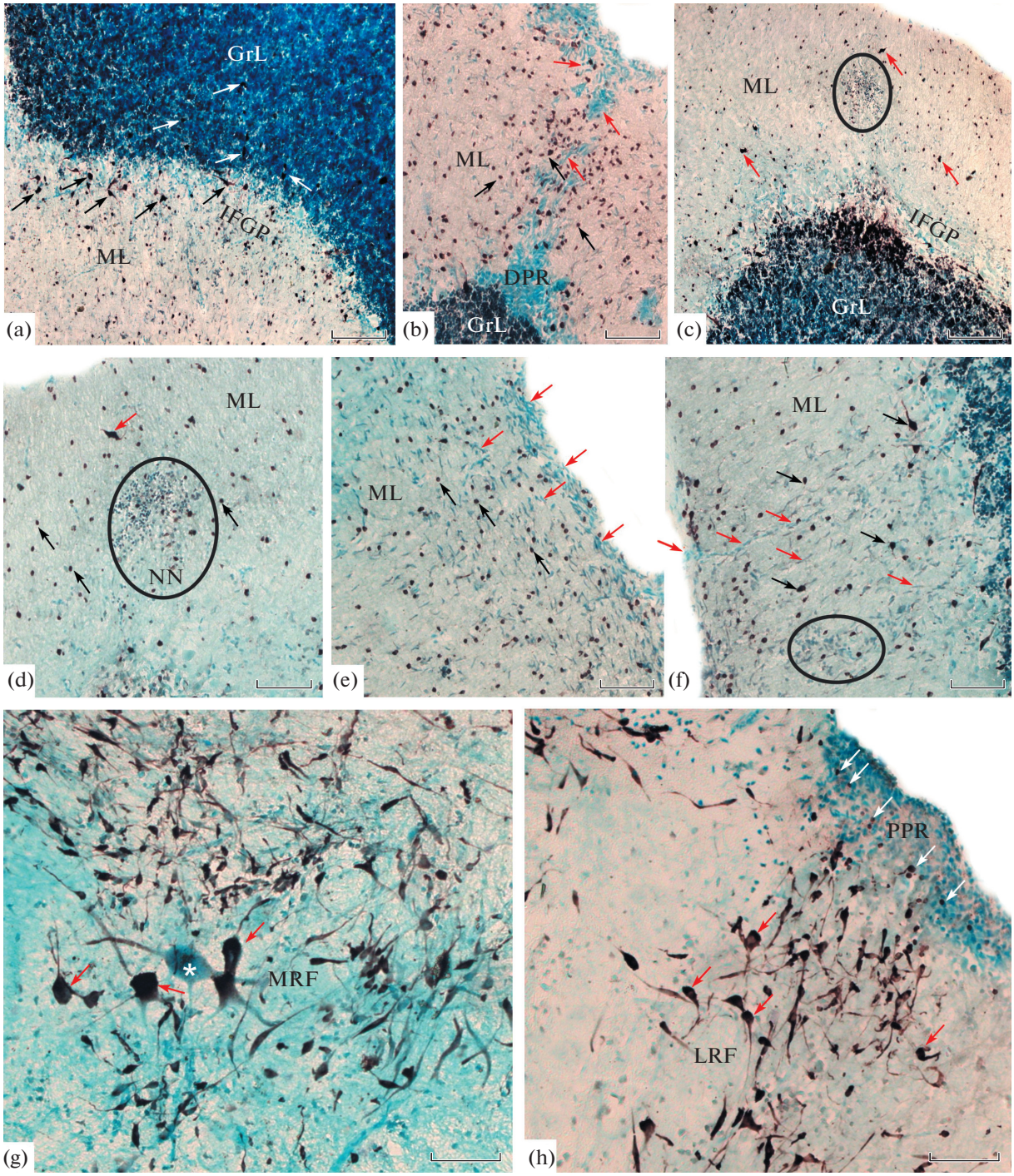

Fig. 10. Localization of neuronal protein HuC/D in the proliferative regions of the cerebellum and medulla of a trout 2 days after the mechanical injury. (a) general view of the cerebellar body, the black arrows show immunopositive differentiated IFGP cells, white is neurons of the granular layer; (b) immunolocalization in DPR, red arrows show HuC/D-in migrating cells; (c) in the dorsal part of the cerebellar body, an oval contours the neurogenic niche; (d) a fragment containing neurogenic niche in the higher magnification; (e) patterns of the tangential migration of HuC/D-in cells (red arrows) in the surface layers of the molecular layer; (f) radial migration of cells in the lateral part of the body of the cerebellum, an oval contours the neurogenic niche; (g) in the medial reticular formation (MRF) of the medulla oblongata, white star marks a large neuron with low HuC/D activity, red arrows show highly differentiated neurons; (h) in the periventricular proliferative region (PPR) and lateral reticular formation (LRF), white arrows indicate HuC/D-ip cells in the PPR. Immunoperoxidase HuC/D labeling in sections in situ. Scale bar: (a, c) 200 μm, (b, d–h) 100 μm.

eral sources of progenitor cells have been found. One of the populations of progenitor cells have the phenotype of radial glia (Pellegrini et al., 2007; Ganz et al., 2010; Rothenaigner et al., 2011; Chapouton et al., 2011). These cells are capable of both self-renewal and production of different types of cells, which is the basic characteristic of stem cells (Rothenaigner et al., 2011). The other population of progenitors is characterized by the absence of expression of typical glial proteins (Kaslin et al., 2009; Ganz et al., 2010; Rothenaigner et al., 2011). In the cerebellum of zebrafish *D. rerio*, such cells are phenotypically similar to neuroepithelial cells (Kaslin et al., 2009).

In the retina, ciliated cells of the marginal zone and Mullerian glia exhibit stem cell properties, in particular, they can have asymmetrical mitosis and are able to regenerate. The origin of the stem cells in the ciliated marginal region is not completely clear. Neuroretinal cells are supposed to be derived from stem ciliated marginal region together with the population of proliferating cells adjacent to the pigment epithelium of the common stem cells in the larval eye (Wehman et al., 2005). On the contrary, rod progenitors producing rod receptors in the intact retina originate from the slowly dividing Muller glia population scattered among differentiated cells of the retina (Bernardos et al., 2007).

After the eye damage, the Muller glia cells in the retina of fish dedifferentiate to the progenitor cells, which are the basis for the regeneration of all types of cells lost after the damage (Yurco and Cameron, 2005; Thummel et al., 2008). In case of a distinct localization of the damaged area, proliferating cells appear near the damaged area, mostly in the outer nuclear layer (Stenkamp, 2007). However, there is evidence that an increase in proliferative activity can also be observed at some distance from the site of injury, which probably indicates the existence of diffuse factors regulating this response (Yurco and Cameron, 2005). The timing of cell proliferation induced by the damage is surprisingly similar in the retina, the brain, and the spinal cord (Zupanc and Sirbulesku, 2011). For example, after administration of the toxin into the eye cavity in zebrafish, the retinal proliferating cells are randomly distributed in all three layers of the retina (Fimbel et al., 2007). Three days after the injury, proliferating cells are localized mainly in the inner nuclear layer, and their number continues to increase and reaches the maximum value by day 5.

Proliferation and Apoptosis in the Optic Nerve of a Trout after the Injury

Our results showed that both apoptosis and proliferative activity took place in the optic nerve after the mechanical damage of the trout eye. These processes prevail on the side of injury, since the number of TUNEL-labeled cells was 6.5 times higher and the number of proliferating cells was 2.8 times higher than those on the contralateral side. The structure of the optic nerve in a trout, as well as in other fish, is formed by axons of retinal ganglion cells and glia cells (Schwann cells, oligodendrocytes, and astrocytes) and has some connective tissue containing macrophages/microglia, which activate in response to a damaging impact. Presently, the overall picture of the interactions between astrocytes/ganglion cells during regeneration of ganglion cells in fish is poorly understood. For example, in studies on goldfish, the network of GFAP-immunopositive reactive astrocytes was discovered 7 days after the crush of the optic nerve (Nona et al., 1994). These cells appeared simultaneously with the growth of axons in the damaged optic nerve. However, the damaged area remained GFAP-negative, and astrocytes were excluded from the area of the damage during regeneration. Nona et al. suggested that the fibers of the optic nerve of goldfish regenerated so quickly that they were not myelinated when they reached the brain (Nona et al., 2000). It is assumed that the myelin formation in regenerating axons of the optic nerve is determined not only by glial cells but also by additional factors of the microenvironment of the optic nerve, whose origin is not clear yet.

The analysis of morphological parameters, the type of PCNA-immunopositivity, the optical density of PCNA labeling of the optic nerve cells in a trout allowed to identify four types of cells. These data indicate the heterogeneity of the population of proliferating cells in the optic nerve. We suppose that the proliferating cells may include microglia and Schwann cells. The data on the goldfish demonstrate that Schwann cells of unknown origin appear in the area of the optic nerve damage, which are absent in the damaged area prior to the myelination of axons. According Nona et al., such Schwann cells begin to proliferate after a few axons reach the brain during the regeneration; and then only a small number of cells exits the cell cycle and joins the regenerating axons by participating in the formation of myelin. Remyelination is completed 3 months after the damage of the optic nerve in a goldfish. Regenerating axons become myelinated with the participation of both oligodendrocytes and Schwann cells distally (near the brain) of the damaged area (Nona et al., 1992).

Currently, the relationship between the phagocytic cells of the retina and regeneration of axons in the optic nerve of fish remains poorly studied. The studies on gold fish showed that the expression of cell adhesion molecules is significantly increased in the non-neuronal cells (Battisti et al., 1992). It has been shown that the optic nerve of goldfish includes macroglia, microglia, and a population of monocytic cells (granular macrophages) that were considered unique to the goldfish (Battisti et al., 1995). It was found that granular macrophages and microglia in the optic nerve of goldfish are OX-42 immunopositive. One hour after the optic nerve crush, the number of OX-42 immunopositive cells increased in the region of the injury and remained at a high level until the axonal sprouting

completed, and then the number of these cells decreased. In the optic nerve of a goldfish, microglia (and not granular macrophages) exhibit phagocytic activity. Astrocytes are well distinguished morphologically in the intact nerve; 1 hour after the injury, their morphological structure is significantly altered causing the difficulties to distinguish. Oligodendroglia in the damaged nerve of a goldfish has not been reliably established before the end of the formation of myelin in regenerating fibers. It has been established that granular macrophages are capable of producing tenascin and transforming growth factor (TGF-beta 1). The presence of tenascin is associated with the presence of astrocytes and/or microglia. Thus, the rapid response of nonneuronal cells, their fast phagocytic activity, and secretion of growth factors contribute to the rapid regeneration of the optic nerve after the injury. According to our data, apoptosis and signs of massive cell migration were found after the damage of the optic nerve in the trout eye. The maximum concentration of apoptotic cells was detected in the areas of the massive cell accumulation in the epineural areas possibly indicating a prominent cell death and a subsequent increase in the phagocytic activity of microglia. The presence of cell migration identified on day 2 after the injury also indicates an increase in cell flow directed to the area of the injury. We interpret it as an invasion of macrophages. We assume that these regions of the optic nerve may correspond to the zones of reactive gliosis occurring in response to injury. The nature of this phenomenon will be investigated in detail in future studies.

We suppose that heterogeneous cell types were present among the apoptotic bodies, including optic nerve macroglia (astrocytes and Schwann cells) and the resident fibroblast-like cells appearing as a result of the damaging impact. We found no data in the available literature discussing the process of apoptosis of non-neuronal cells in the optic nerve of fish. Presently, in the studies of regeneration of the optic nerve in the lower vertebrates, the participation of retinal ganglion cells in the regeneration can be considered the most studied (Devadas et al., 2000; Zhou and Wang, 2002; Kustermann et al., 2008). Some analogues can be found between the studies of the optic nerve regeneration in mammals and fish, mainly because there are pronounced differences in gene expression found in these vertebrate groups. For example, the expression of *Bcl-2* gene may play a role in the regeneration of the optic nerve both in mice and in fish (Cho et al., 2005). Phenomenological similarities can also be associated with apoptosis of ganglion cells due to the damage of the optic nerve. Despite the fact that this phenomenon is not fully studied in zebrafish in situ, the changes in the amount and distribution of ganglion cells were found after the optic nerve damage (Zhou and Wang, 2002). In the adult zebrafish retina, there are approximately 40000 to 56000 ganglion cells whose distribution density depends on their localization. After crushing the optic nerve, apoptotic death occurs in approximately 20% of the ganglion cells during the regeneration (Zhou and Wang, 2002). Recent studies of organotypic cultures of adult zebrafish retina using the TUNEL labeling of retinal explants showed a low percentage of apoptosis (10%) of ganglion cells cultivated for 3 days in vitro (Kustermann et al., 2008). However, after 10 days of cultivation, approximately 50% of the population of ganglion cells was TUNEL-labeled, probably as a result of axotomy necessary for the preparation of retinal explants and subsequent removal of the pigment epithelium. BrdU labeling studies have shown that the loss of ganglion cells in the explants was not subsequently recovered despite the extensive proliferation of other cell types. In studies by Kusterman et al., no spatial hypertrophy of ganglion cells were found in the explants after the axotomy, which had been previously described in the goldfish retina during regeneration of optic nerve after the axotomy (Devadas et al., 2000). These differences in the results obtained in vivo and in vitro show that the signals present in the intact organism and absent in retinal explants could be crucial for the survival of the ganglion cell axons and activation of the axon growth. Presently, additional studies are needed to clarify if the axonal sprouting reflects the ganglion cell survival or ganglion cells appear *de novo* by the differentiation from the progenitor stem cells.

Significant progress in the understanding of the basics of the successful regeneration after the amputation of the caudal part of the spinal cord has been made in the research on electric gymnotiform fish *Apteronotus leptorhynchus* (Zupanc, 2009). In studies of this species, the successful recovery of the fragments of the spinal cord amputated with the part of the caudal fin was discovered. One of the initial stages of the repairing process was the significant apoptotic cell death in the area of the injury. The first cells with signs of apoptosis appeared in the damaged area 5 minutes after the injury; then their number gradually increased and reached the maximum in a few hours (Zupanc et al., 1998). On the second day, the number of such cells in the spinal cord gradually decreased and reached the initial level after approximately 3 weeks. During this period, necrosis appeared in few cells. Apoptosis, as a mechanism of elimination of damaged brain cells in fish, differs significantly from that of mammalians (Vajda, 2002). In the latter, the main process of elimination of damaged cells in the injured area is necrosis (Liou et al., 2003). Apoptosis also affects the small number of cells in the areas adjacent to the injury site. The presence of necrosis in the injured region in mammals is one of the causes of the subsequent inflammation in the area of the secondary damage (Kerr et al., 1995), which, in turn, causes the further increase in the necrosis resulting in the formation of large cavities without cells. These cavities are usually surrounded by reactive astrocytes creating both mechanical and biochemical barriers preventing the

growth of nerve fibers and cell migration into the damaged area. Unlike necrosis, there are no signs of the inflammatory response during apoptosis, and cells are subsequently destroyed by the macrophages/microglia (Elmore, 2007). Such changes in the optic nerve of a trout were observed 2 days after the injury. Initially, the number of phagocytes in the damaged area is small, but the number of macrophages begins to increase in the damaged region and adjacent tissues approximately 3 days after the injury (Nagamoto-Combs et al., 2007). These data are entirely consistent with our data on the trout optic nerve. Thus, the prevalence of "clean" cell death during the development of reparative processes in the central nervous system of fish is the most important feature underlying the regenerative capacity of the adult fish brain.

The study of **proliferative activity** in the optic nerve of a trout 1 week after the injury showed the presence of a large number of proliferating cells forming areas of increased density. These clusters are formed by oval cells of types 2 and 3, and special elongated fusiform migrating cells. PCNA labeling allows one to identify cells with an additional expression of DNA polymerase δ, which was observed during S-phase of mitosis. PCNA expression was maintained in cells for 24 hours after the end of the mitosis (Wullimann and Puelles, 1999). The investigation of the migratory and proliferative elements in the trout optic nerve showed that the formation of the increased density areas was typical of actively proliferating cells, while migratory cells were characterized by a diffuse and surface location among the fibers of the optic nerve. The densitometry of PCNA immunoreactivity revealed that high optical density was typical of oval cells of types 2 and 3, while the OD in the large cells of type 1 and small cells of type 4 was lower by 22–30%. The postmitotic cells in PCNA labeling were characterized by the reduction of the activity by 30% (Bravo and MacDonald Bravo, 1987). This suggests that both proliferating elements (we believe that these are glial cells) and postmitotic cells maintaining the decreased level of PCNA activity in comparison to the proliferating cells or cells that just came out of the mitosis were present in the optic nerve. We assume that the populations of proliferating cells are represented by glia (Schwann cells, oligodendroglia, and/or astrocytes) involved in the formation of posttraumatic reactive gliosis. Migrating postmitotic large cells probably belong to the population of resident macrophages, while small, poorly PCNA labeled cells are part of microglia; both populations of weakly labeled cells (types 1 and 4) may be the elements of the immune system mobilized in response to the damaging effects. The ratio of PCNA-immunolabeled cells in contra- and ipsilateral trout optic nerves were significantly different 1 week after the injury. More than three-fold increase in cell proliferation activity was found at the side of the lesion. We suggest that such an increase in the number of cells in the optic nerve on the side of damage was due to both the proliferation of glial cells and microglia and the resident macrophage migration from the adjacent connective tissue.

If we draw an analogy with the process of regeneration of the optic nerve of mammals, the observations made on the goldfish lead to the conclusion that reactive astrocytes in the optic nerve can be generated from a population of Schwann cells (Nona et al., 2000). In primary cultured astrocytes obtained from newborn rats, neuregulins and their receptors are expressed, which attract Schwann cells in vivo to the axons during the development and promote myelination of peripheral nerves (Francis et al., 1999). The ganglion cells inhibit the differentiation of Schwann cells until their axons reach the brain. The mechanism of the delay in myelination of growing axons involves the Notch signaling pathway (Wang et al., 1998). In the developing rat optic nerve, oligodendrocytes express Notch1, and ganglion cells express Jagged1, which acts as a ligand of Notch. Such interactions inhibit the differentiation of oligodendrocytes until the ganglion cell axons reach the tectum (Wang et al., 1998). Astrocytes also can modulate the microenvironment of the optic nerve. It has been found that fish astrocytes are connected by tight junctions and desmosomes (Mack and Wolburg, 2006). In the study of regeneration of damaged fibers of the optic nerve in *Astatotilapia burtoni*, the expression of the proteins of tight junctions, in particular Claudin, was found in astrocytes localized near the newly formed axons of ganglion cells. The authors interpret this finding as showing the various properties of the membranes of astrocytes modifying neuroglial interactions that may ultimately determine the specific microenvironment of the optic nerve. In the mammalian CNS, astrocytes are interconnected by gap junctions, but the presence of tight junctions has not been previously shown. Recently, it was found that oligodendrocytes in the developing optic nerve of a zebrafish express contactin 1 mRNA, while the expression was observed in glial cells of the optic fiber layer of the retina in an adult fish (Schweitzer et al., 2007). Contactins are the proteins of the immunoglobulin superfamily and are supposed to participate in the cell recognition. After the optic nerve damage, the level of contactin 1 in both oligodendrocytes and the ganglion cells in adult fish is significantly higher than during the embryonic development. It is assumed that the oligodendrocytes may play an important role in the successful regeneration of the CNS, probably significantly affecting the regions near the injury site (Schweitzer et al., 2007).

Proliferative response in the integrative centers of the trout brain after the eye injury. After the damage, proliferative activity was found in the cerebellum and the optic tectum, which is the target of primary retinal projection. After the mechanical eye injury, the proliferative activity was found in the dorsal matrix area of the cerebellum of a trout, as well as among individual parenchymal cells located in the basal part of the

molecular layer, the infraganglionar plexus, and the granular layer. PCNA-in clusters of cells were found, which we identified as regional neurogenic niches activated as a result of a traumatic impact. The proliferative activity in the cerebellar DPR of a trout, which we discovered, was also observed in the cerebellum of a zebrafish *D. rerio* (Grandel et al., 2006) and apteronotus *A. leptorhynchus* (Zupanc et al., 2005). In the brain of a zebrafish, approximately 6000 cells formed during the 30 minute period, which corresponded to 0.2% of the brain cells (Hirnsch and Zupanc, 2007). Quantitative studies on the cerebellum of an apteronotus showed that approximately 100000 cells were produced during a 2-hour period in this species, which corresponded to 0.06% of the brain cells (Zupanc and Horschke, 1995). After the traumatic effect, the PCNA-in was found in three types of cells of the cerebellum of a trout, including the population of highly active oval cells (OD 116 ± 4.5 units), and less intensely labeled population of elongated cells (OD 103 ± 3.2 units). We suppose that highly immunogenic cells in the DPR are a population of cells that is in the mitotic cycle, and less intensely labeled cells are postmitotic elongated elements of DPR migrating toward the area of the injury as a part of the radial and tangential migration flows.

Another population of cells involved in the proliferative response of the trout CNS to the mechanical damage is represented by PCNA-negative local accumulations of cells found immediately above and below the DPR and in the infraganglionar plexus. We assume that these clusters of PCNA-in cells are the neurogenic niches activated in response to the injury, which is in agreement with (Zupanc et al., 1996, 2005). According to the data, in the body of the cerebellum neural stem cells (NSC) are specific proliferative zones "neurogenic niches" located in the molecular layer. The descendants proliferated cells migrate on specific highways in the granular layer, where they are distributed evenly (Candal et al., 2005; Zupanc et al., 2005). During such migration, the newly formed cells are directed by the radial glial fibers. The third population includes PCNA-ip single cells or small clusters of cells in the lower third of the molecular layer and the granular layer.

The optic tectum is another integrative center of the midbrain of a trout and is the primary retinal projection. The proliferative activity of the mesencephalic matrix areas was previously studied in *Carassius carassius* using PCNA labeling (Margotta et al., 2002). It was found that PCNA labeling allows the identification of distribution patterns of mitotically active cells in the brain, which form morphogenetic fields—the matrix zone. The PCNA-labeled matrix zones have also been identified in the midbrain of adult sturgeon *Acipenser shrenkii* (Puschina and Obukhov, 2011).

When the optic nerve is damaged, the PCNA-immunopositivity was found in the cells of the radial glia, single parenchymal cells of the inner layers of the tectum, and the periventricular region of a trout. Since the population of radial glia in the brain of the fish is known to be heterogeneous and includes fast and slow proliferating cells (Adolf et al., 2006), we assume that proliferative activity was enhanced in the slowly proliferating radial glial cells after the damage of the trout tectum. This assumption is based on the results of densitometric studies of PCNA activity in radial glial cells. The distribution density of PCNA-ip radial glia was low compared with the distribution of single parenchymal PCNA-ip cells in the deeper layers of the tectum. We suppose that only a part of the radial glia population begins to proliferate in the tectum after the eye injury. Among the radial glial cells, few cells with high PCNA activity (114 OD units) were found, which allows us to consider these cells as neuronal precursors (Malatesta et al., 2008) in a state of asymmetrical mitosis, which was previously shown in radial glia (Noctor et al., 2004). Another, more numerous population of radial glia in the trout tectum had lower optical density values (104 OD units); we considered these cells as postmitotic cells.

A particularly large accumulation of PCNA-ip small cells was detected in the caudal periventricular tectum, as observed in other species (Extröm et al., 2001; Zupanc et al., 2005).

Thus, in the integrative centers of the trout brain, the proliferative activity in the secondary matrix area of the cerebellum, in the radial glial tectum, in the primary periventricular region of the tectum, and in the single parenchymal cells was found 1 week after injury. The patterns of radial and tangential cell migration were identified in the cerebellum, including both PCNA-ip and PCNA-in elements. PCNA-in neurogenic niches were especially numerous in the dorsal part of the body of the cerebellum and tectum.

In the brain of an adult trout, the persistent neurogenesis was not intense, and an increase in the proliferation in the matrix areas of the cerebellum, the appearance of neurogenic niches, PCNA-ip of radial glia, and single proliferating cells in the parenchyma of the cerebellum and the optic tectum are a complex response of the brain to the mechanical eye injury.

Neurogenesis in proliferative brain regions of a trout induced by the damage. After mechanical injury of the optic nerve, a significant proliferative activity was found in the brain proliferative regions of a trout. The newly formed cells in these areas are differentiated into various cell types, including neurons. To estimate the number of neurons formed as a result of the mechanical injury of the optic nerve and the retina, the proliferative brain regions, including secondary proliferative and periventricular zone of the forebrain, tectum, cerebellum, and brainstem of a trout, were investigated. The results showed the intensive formation of new neurons in various centers of the brain. Neurogenesis has been reported in the dorsal and ventral regions of the

matrix everted regions of the trout telencephalon. The studies in zebrafish showed that the newly formed cells began to express neuronal protein HuC/D 3–4 days after injury in the dorsal telencephalon (Ayari et al., 2010; Kroehne et al., 2011; Kishimoto et al., 2012). These new neurons appeared near the injury site and in remote areas of the damaged regions. In the following days, the number of HuC/D-expressing cells gradually increased, especially in the area of the damage. Spatial and temporal distribution pattern of these cells showed that newly formed cells acquired phenotypic features typical of neurons during the migration to the site of injury (Zupanc and Sirbulescu, 2013).

According to our observations in a trout, the most intensive formation of new cells occurring 2 days after the injury was typical of the dorsal proliferative zone. The maximum distribution density of small, undifferentiated HuC/D-ip cells located under the HuC/D-in layer of proliferating cells has been identified in this area. Similar processes were found in other areas of the dorsal region: medial and lateral with specific structural organization, in particular, the medial PR was larger in comparison with the lateral PR. In the ventral region, a thick HuC/D-in cell layer was detected both in the dorsal and ventral nuclei. In these structures, the layered organization separating PR from definitive HuC/D-ip differentiated cells was clearly visible. In the ventral nucleus, undifferentiated HuC/D-ip cells located in the PR were found, which indicated early neuronal differentiation of cells formed in the post-traumatic period. However, in the telencephalon of a trout, we did not find the typical neurogenic niches after the damage, unlike in other parts of the nervous system—the tectum and cerebellum. Nevertheless, HuC/D-in undifferentiated cells were found in the dorsal region of the dorsal area along with HuC/D-ip undifferentiated cells in the deep zone. We consider that the appearance of these elements in the Dd region is a response to the injury. The density of distribution of HuC/D-in cells in the Dd was quite high. This could indicate the presence of specific types of neurogenic niches in the telencephalon of a trout, which was not previously found in fish.

In the tectum and cerebellum of a trout, the activation of large regional neurogenic niches occurred in the dorsal part of the body of the cerebellum (in the molecular layer) and the optical layer of the tectum, while smaller niches activated in the infraganglionar plexus and lateral areas of the cerebellar body. Another feature of the reparative neurogenesis in the tectum of a trout is the emergence of PCNA-ip radial glial cells in the surface layers of the tectum. We consider this feature to be associated with adaptive properties of the brain center, which is a direct retinal projection and has a close functional relationship with the injured region. An increase in proliferative activity in the caudal tectum of a trout is also a sign of increased proliferative potential of the brain, and we consider it as an additional repairing reserve in the trout brain. An increase in the speed of neurogenesis in proliferative zones of the trout brain, particularly in the periventricular region of the medulla oblongata, in combination with a high HuC/D activity in the cells in the periventricular area, in our opinion, is also a complex response of the CNS to the reparative stimuli.

Using the method of the retrograde tracing combined with BrdU labeling in the cerebellum of *A. leptorhynchus*, the damage in the granular layer can result in the appearance of cells phenotypically similar to those lost due to the injury (Zupanc and Ott, 1999). These new cells are directed to the injured region by the GFAP-immunopositive fibers of the radial glia (Clint and Zupanc, 2001). The studies of spinal cord injury showed that the newly formed cells develop into HuC/D-positive and serotonin-positive neurons and S100-positive and GFAP-positive ependymocytes and glial cells (Takeda et al., 2008; Sirbulescu et al., 2009).

The mentioned studies suggest that an increase in the production of new neurons in the proliferative zone of the forebrain, the optic tectum, cerebellum and brain stem in adult trout occurre 2 days after the eye and optic nerve injury, in contrast to *D. rerio*, in which a similar response occurs 3–4 days after the injury (Ayari et al., 2010). In the telencephalon of adult trout, the most intense neurogenesis is observed in the dorsal area of the Dd proliferative region. In the optic tectum, neurogenesis is significantly enhanced in the caudal periventricular proliferative region among parenchymal cells in the layers of the tectum. In the surface layers (marginal and optical) of the tectum, neurogenic niches containing proliferating PCNA-ip/HuCD-in cells and HuCD-ip cells were discovered. In the cerebellum, there was a significant increase in cell proliferation in the dorsal proliferative region among isolated parenchymal cells of the molecular and granular layers. In the lateral part of the cerebellar body, the neurogenic niches with the structure similar to those in the optic tectum. In the medulla oblongata, the high proliferative activity of periventricular region and adjacent areas of the dorsal, lateral, and medial reticular formation with high levels of HuC/D activity was identified. Thus, our results suggest that neurons formed as a result of the reparative process are characterized by a high level of expression of HuC/D neuronal marker in comparison with the cells of definitive centers of the brain. Based on the data, we assume that this peptide plays an active role in the postembryonic and reparative neurogenesis.

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