Chapter 7

Adult and Reparative Neurogenesis in Fish Brain

Evgeniya V. Pushchina, Anatoly A. Varaksin,
Mariya E. Stukaneva and Eva I. Zharikova

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/67951

Abstract

The fish brain has a unique feature of vertebrates—it grows with the growth of body over a lifetime. In this regard, fishes are a convenient model for the study of embryonic and postembryonic development of the central nervous system and of the influence of different factors on these processes. Currently, the mechanisms of adult brain morphogenesis of fish, which retain larval stage for a long time, are poorly understood. This is particularly true for participation of radial glia during morphogenesis of the brain, as well as the presence and distribution of the proliferative zone in the adult fish brain. Another interesting and little known aspect is the posttraumatic ability of fish to form active neurogenic niches. Investigation of the structural organizations of neurogenic niches and special conditions of the extracellular environment, as well as the interactions between neighboring cells in a neurogenic niche, is interesting and relevant direction in the study of the neuronal stem cells biology. Injury of fish brain creates special conditions for the implementation of genetic programs aimed at strengthening the proliferation of progenitor cells, as well as the activation and proliferation activity in the neuronal stem cells.

Keywords: adult neurogenesis, neurogenic niche, radial glia, reparative neurogenesis, proliferation, migration, neuroal differentiation, teleost fishes, regeneration, matrix areas of brain, apoptosis, neuroprotective factors, neural stem cells

1. Introduction

Among vertebrates, fishes are known to be able to effectively restore the structure of cells and fibers after damage of the central nervous system (CNS). 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 [1]. 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 [2, 3]. In contrast to the mammalian brain, numerous proliferative regions have been found in adult fish. The presence of such regions was described in *Apteronotus leptorhynchus* [4], *Sparus aurata* [5], *Gasterosteus aculeatus* [6], *Danio* sp. [7, 8], and *Austrolebias* sp. [9].

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 [10, 11]. 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 the toxic effects of glutamate and other inflammatory mediators, and further pathological changes associated with processes of secondary inflammation and involve massive cell death [12, 13]. 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 [14]. In the fish brain, the cellular response to the trauma develops in a different scenario. Apoptosis is observed 5 min after the injury, which progresses in the next few days [7]. 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 [15]. 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 zones. The high regenerative potential in the central nervous system of fish is provided by the activation of specific regenerative factors [1] and the effect of neuroprotective factors protecting damaged cells and providing long-term survival of cells formed as a result of reparative neurogenesis.

2. Adult neurogenesis and neural regeneration in fish brain

Neuroregenerative properties were investigated in various parts of the fish brain: retina [16], optical tectum [17], spinal cord [11], and cerebellum [17]. According to the «Lesion paradigm» formulated by Zupanc and his colleagues [10], a high regenerative potential of the central nervous system of fish is determined by a number of different processes, including the response of the central nervous system after a damaging effect.

The first few reports related to the development of this theory have been derived from studies on European carp *Carassius vulgaris* after injury of the spinal cord at the cervical/thoracic levels and monitoring of structural recovery within 2 weeks from the date of damage. In these studies, however, the histological studies of crossed pieces of the spinal cord have not been conducted, but the conclusions were made for the first time on the functional repair and restoration of motor activity (ability to swim) after application of the damaging effects [11, 18].

Significant progress in understanding the basic signs of successful regeneration after amputation of the caudal portion of the spinal cord has been made in research on electric fish *A. leptorhynchus* [2]. In animal studies, it was demonstrated by the successful restoration of the lost fragments of the spinal cord, along with the amputated part of the caudal fin. One of the initial stages of the repair process, resulting from an injury is the rapid destruction of damaged cells via apoptosis. The first cells with signs of apoptosis occur in an area of damage within 5 min after the injury, and then the number of cells gradually increases, reaching a maximum value within a few hours.

On the second day, the number of cells gradually declined, reaching the background level after approximately 3 weeks. During this period, only some cells underwent necrosis. An elimination of damaged cells by apoptosis in the brain of fish differs significantly from that of mammals [15]. In contrast, the main process of elimination of damaged cells to the injured area of mammals is necrosis [19]. Apoptosis also affects the small part of cells in the areas surrounding injury. The prevalence of necrosis in the mammalian brain after injury is one of the causes of subsequent secondary inflammation in the lesion [14], which in turn causes a further increase in response of necrotic injuries, resulting in the formation of larger cavities deprived of cells. These cavities are usually restricted area of reactive astrocytes, creating both mechanical and biochemical barriers that impede the growth of nerve fibers and cell migration into the damaged area. Unlike necrosis, apoptotic cells characteristically show overall compression, condensation of the nucleus, and the formation of vesicles, which are subsequently destroyed by the macrophages/microglia [20].

Initially, the numbers of phagocytes in the area of damage were small, but after about 3 days of injury, the number of macrophages begins to increase in the area of injury and in the adjacent areas [21]. The main side effects of necrosis, associated with inflammation of the surrounding tissue, are completely absent in apoptotic "clean" method of elimination of cells. Thus, the prevalence of processes of "clean" cell death for the destruction of damaged cells is a key feature underlying the regenerative capacity of the adult fish brain.

An important aspect of promoting successful regeneration in the brain of the fish is the detection of specific neuroprotective factors which play a key role in maintaining the viability of neurons in the affected areas and prevent further cell death after injury [22]. Such factors are being considered as different substances, in particular some of the calcium binding proteins, such as calbindin-28 and parvalbumin. Expression of these calcium-binding proteins in the cells briefly increases in granular layer of the cerebellum of *A. leptorhynchus* between 16 h and 7 days after injury [23]. It is assumed that the calcium-binding proteins have a protective effect by the buffering of free calcium, the level of which increases considerably after injury. Another neuroprotective factor is the enzyme glutamine synthetase (GS), which conversed synaptically released glutamate in the neutral glutamine. It is known that as a result of damage to the brain cells, the extracellular medium receives a large amount of glutamate, creating hyperexcitation of glutamate receptors and the excitotoxicity [12]. To dispose of glutamate, there arises a necessity of a sufficient amount of the enzyme glutamine synthetase excreted by astroglial cells, carrying out the reuptake of glutamate and converting it into glutamine. According to studies, after traumatic injury of fish, levels of glutamine synthetase significantly

increased [24], whereas in the mammalian brain, conversely, decreased [13, 25]. Increased synthesis of glutamine synthetase in the brain of the fish is likely to provide an important mechanism for reducing the neurodegenerative process caused by neurotoxic effects of glutamate. Such differences in the expression of GS in fish's brain and in mammals are certainly interesting because they determine significant limitations of regenerative activity of the brain tissue of mammals in comparison to the fish brain.

3. Apoptosis and cell migration after injury of cerebellum

We observed the apoptosis and migration of cells in the young masou salmon *Oncorhynchus masou* after mechanical injury of the cerebellum. Two days after injury in adjacent zone, we detected significant change in cell composition in molecular and in the granular layers [26]. The most characteristic phenomenon was the emergence of large areas of cell migration from the area of regional neurogenic niches and the largest area of the secondary neurogenesis, located in the dorsomedial part of cerebellar body (**Figure 1A**).

We believe that it was mainly due to the migration process in neurogenic niches and dorsomedial area. The highest density of cells was detected in the vicinity of the puncture area, gradually decreasing with the distance from the area of injury. Near the area of injury, many TUNEL-labeled components corresponding to different stages of the apoptotic process were localized (**Figure 1B**). So, dense apoptotic bodies, which are the final stage of coarse chromatin condensation and apoptotic cell degradation, were found. The size of apoptotic bodies was about 8–10 μm. In areas of apoptotic fragments localization were found large cells with basophilic cytoplasm, the diameter of cells body is about 13 μm. These cells tend to have an irregular shape and had cytoplasmic outgrowths. Presumably, these structures correspond to regional microglia/macrophages involved in phagocytosis of apoptotic fragments and recycling. Along with individual elements, there also occurred small conglomerates, including up to three apoptotic bodies.

Another variety of apoptotic bodies were small TUNEL-labeled bodies representing degranulated fragments of damaged cells. In the most superficial parts of the molecular layer was observed a very large number of small cells lacking the morphological features of differentiation (**Figure 1C**). Such morphological pattern of surface of the molecular layer apparently reflects the intensity of the processes of cell migration from the superficial regions of the cerebellum to the zone of injury. The surface area which has been characterized by a high density of cells revealed TUNEL-labeled small elements corresponding to cell degranulation products in the area.

In our studies, some effects of the damaging of cerebellum were combined with complex morphogenetic background of the ongoing postembryonic development of the brain *O. masou*. The experimental fish was in the process of active growth, resulting in increased proliferative activity in the cerebellum and morphogenetic zones of periventricular regions of the brain (**Figure 1C**). Previously in experiments with *Danio rerio*, it has been found that damage to the

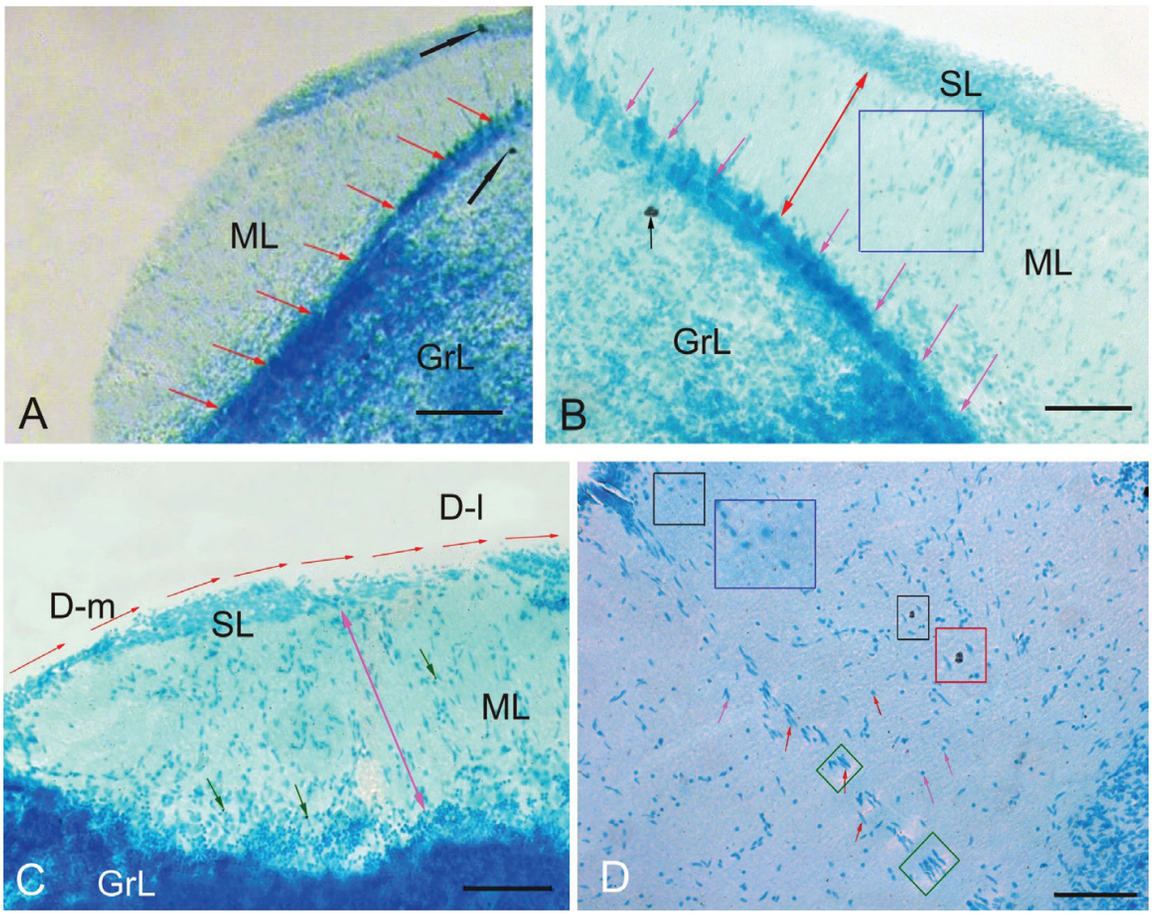

Figure 1. Patterns of cell migration and apoptosis in the cerebellum of juvenile masou salmon *Oncorhynchus masou* after injury. A—common view of the areas of trauma, small arrow shows the area of puncture, black arrows (here and below) show apoptotic bodies; B—dorsomedial part of *corpus cerebellum*, which contains migrating from the surface layer (SL) cells (contoured by rectangle), the big arrow indicates the direction of radial migration; C—lateral part of the molecular layer (ML) contained cells migrating to the area of injury, arrows show the direction of the tangential migration, big arrow—radial migration, dark arrows show TUNEL-labeling fragments of degranulated cells, D-m—dorsomedial, D-l—dorsolateral area; D—the area of median suture, small squares delineated clusters of migrating cells, in other squares are small TUNEL-labeled fragments, and apoptotic cells, arrows show the different types of cells; ML—molecular layer, GrL—granular layer. Scale bar: A—200 μm, B-D—100 μm.

cerebellum causes increased proliferation of cells in the outer regions adjacent to the meninges and also in *valvula cerebelli* and *granular eminentia* [27]. These data were obtained using experimental labeling BrdU of dividing cells [28] and other markers of cell proliferation [29]. High neurogenetic activity after traumatic injury was detected in the cerebellum of *A. leptorhynchus*. In this species belonging to the group of specialized electrical gymnotiformn fish cerebellum, it takes up 75% of total brain volume; it is the largest center for neurogenesis both during normal adult development and in terms of traumatic impact [30].

In juvenile *O. masou* was observed very high initial intensity of proliferation in the primary matrix region of brain (periventricular zone) and in zones of secondary proliferative activity detected in adult animals [31]. The corresponding data obtained by labeling both the proliferative cell nuclear antigen (PCNA) and using traditional morphological methods to assess the

mitosis in matrix areas of brain (in particular, the dorsomedial region). The intensity of cell proliferation in this region has been previously described in adults *D. rerio* [7, 32] and *A. leptorhynchus* [4] and juveniles of trout, *Salmo gairdneri* [33]. In our observations, particularly high proliferative activity has been identified in the dorsomedial area, the surface layer and the body of cerebellum, *granular eminentia* and vestibule-lateral areas of a damaged cerebellum. Thus, we can conclude that the proliferation of cells in these areas of the cerebellum, which is already very intense during normal morphogenetic activity within a given period of ontogenesis, is further enhanced after the damaging effects. The presence of newly formed cells in the superficial layer of the cerebellum associates partly with background morphogenetic activity and partly with damaging effects. As a result of damage to the cerebellum in young *O. masou*, enhanced proliferative activity was induced in both traditional areas of adult neurogenesis and the surface layer.

Counting the number of cell nuclei stained with methyl green, which was carried out on the surface layer of the cerebellum, indicates the presence of a large number of undifferentiated cells with high nuclear-cytoplasmic ratio. Such cells based on morphological criteria can be referred as proliferating population and cell population at the early stages of differentiation and/or migration.

We believe that the proliferative response to damage the cerebellum in young *O. masou* should be interpreted taking into account the relatively high background level of activity of the matrix areas of brain [34]. The process of apoptotic cell death accompanies the "normal" adult neurogenesis [35], and, at the same time, it is a physiological response of the nervous system to injury of *O. masou* cerebellum. Apoptosis during normal development has been described in the brain of *A. leptorhynchus* [27]. In these studies, it was found that during the proliferative activity of matrix areas, cerebellar cells formed with signs of somatic aneuploidy. This material is obviously defective, because the relevant units do not have a normal diploid number of chromosomes, and is subject to elimination of apoptotic scenario [36]. In studies on intact adult specimens of Amur sturgeon have been found high values of apoptotic index in different parts of the central nervous system, including the integrative centers of brain (*optic tectum, cerebellum*) and sensory centers of the brain stem (nucleus V and VII cranial nerves pairs) [35]. Similar phenomena are typical for continuing morphogenetic activity in the various centers of the brain of fish, where continued replenishment of new cells occurs throughout life. The resulting cells appeared *de novo* can be integrated into existing neural networks not only during embryonic neurogenesis but also in adult animals. This phenomenon, in particular the special characteristic of the sensory areas, updated with new structural elements as the growth of the animal. Apoptosis in these physiologically active developing systems may play a role of physiological filter that regulates the number of new cells and ensures elimination of "old cells".

Large TUNEL-labeled bodies (**Figure 1D**) conform to the final stage of chromatin degradation in apoptotic cells. This stage is characterized by the formation of large condensed fragments of chromatin that cannot be disposed by macrophages/microglia. These apoptotic bodies were also identified in the morphogenetic studies in mammals [37]. Other visible TUNEL-labeled elements are small weakly diffused particles, which are products of degranulation of

cells that are eliminated by apoptosis. These "remnants" of the cells were identified over large areas, located in different parts of the cerebellum. These different types of TUNEL-labeled structures in the cerebellum of *O. masou* were identified almost everywhere. Apoptotic index values that vary significantly in different parts of the cerebellum show different intensity of apoptosis in the matrix zones, areas of trauma, and in adjacent areas of intact regions of the cerebellum of young *O. masou*.

In our studies on the second day from the date of injury to the cerebellum, intensity of TUNEL-labeling of apoptotic bodies was not very high [26]. However, apoptosis in a zone adjacent to the areas of trauma has been well defined. This surely indicates a part of the mechanism in the process of disposing of damaged cells. In the mentioned period of time (2 days after injury) in the area of damage was revealed increased density of distribution undifferentiated cells. This fact indicates that the reparative process moved at a later stage. Apparently, the reparative processes of neurogenesis in juvenile salmonids implemented in earlier periods compared with what is commonly referred to in the literature [1, 27, 28]. This is likely due to the high intensity of the background morphogenetic activity in the cerebellum of young fishes as compared to that in adult animals.

After mechanical trauma of the cerebellum, patterns of tangential and radial cell migration can be observed. The zones of cell migration are best expressed in the dorsal part of cerebellar body, as well as in the areas of secondary neurogenesis. In our experimental conditions, two groups of TUNEL-labeled structures were identified: large TUNEL-labeled bodies, corresponding to the final stage of degradation of apoptotic cells, and small, weakly condensed particles, which are apparently products of cells degranulation. In the matrix areas, areas of trauma, and intact areas of the cerebellum, different levels of apoptotic activity were observed. The highest value of apoptotic index (5%) after the traumatic impact on the cerebellum was observed in the molecular layer, which is the main area of radial migration of cells. Thus, the background morphogenetic processes and physiological repair processes dominate in the cerebellum of young *O. masou* after traumatic exposure. The intensity of apoptosis vary between different areas of masu salmon cerebellum, as these areas differ considerably.

4. *In vivo* investigation of cell migration after mechanical injury

Microglia/macrophages have been identified within a few days after lesions in several divisions of the CNS of teleost fish—the cerebellum [38], the dorsal telencephalon [39, 40], and the retina [41]. We used multiphoton confocal microscopy for the *in vivo* study of early response of microglia/macrophages in the damaged midbrain of juvenile chum salmon *Oncorhynchus keta* [42]. The results obtained allow the use of injection of DiI in the area of brain injury as a method to identify a population of phagocytic cells in the brain, based on the physiological response of macrophages/microglia. Thus, the injury with injection of small particles of dye DiI causes the phagocytic response from macrophages within 30 min after the application of the damaging effects (**Figure 2A**). This allows the use of fluorescent lipophilic carbocyanine dye DiI (1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate, Aldrich, Sigma,

USA) as a vital nonspecific marker of microglia/macrophages. In mesencephalon (*tectum opticum*) of fish, thin needle containing crystals of dye puncture to a depth of 2–3 mm was applied. After that, the animal was placed in a separate aquarium with fresh water and with enhanced aeration for recovery. In 780 LSM microscope for multiphoton microscopy, we used lasers with pulse durations up to 10–13 (100 femtoseconds). Pulses follow with a high frequency (100 MHz), and the intervals between pulses is significantly shorter than the time ranking of the beam during scanning. The average radiation power at the same time may be small, of the same order as that of a single-photon excitation [42].

Animal at the beginning of the experiment was lying on the back, was submerged in the aquarium water, the surface of the skull was tightly pressed to the bottom wall of the special POC-R chamber. The brain of the animal was examined as a whole, without opening the skull and removing pigmented primary brain tunic. Thus, the substance for the study of DiI-labeled cells initially represented structurally heterogeneous environment, including the bones of the skull, cerebrospinal fluid, primary brain tunic, and brain tissue. The observation was carried out with special planar lens with built-in color correction (Advanced Correction System) at 20x magnification. The sample of cells was carried out in the middle portion of the optical section at a depth of 200 μm. Since the observations were made in *in vivo* mode (without production of brain sections), the scanning process have some aberrations.

After 30 min of exposure DiI to mesencephalon of juveniles *O. keta*, we observed local bright fluorescent cell bodies located in the midbrain *tegmentum* (**Figure 2A**). Cells were numerous, uniformly distributed on the depth of the investigated optical section and formed clearly a visible row of selective labeled components (**Figure 2A**).

As a result of optical scanning, we observed DiI-labeled elements without outgrowths, which formed local clusters (after 2 days) and were presented by individual elements (after 30 min) (**Figure 2B**). After 2 days in the optical sections of damaged *tegmentum*, the density of distribution of DiI-labeled cells was demonstrated as occurrence of cell conglomerates (**Figure 2B**). To investigate the space relationships of DiI-labeled cell conglomerates observed in the area of injury 2 days after injury with DiI-unlabeled, but intensely pigmented melanocytes of primary brain tunic, we spent the overlay of transmitted and fluorescent channels (**Figure 2C**). As a result of intensive multiphoton radiation, the majority of melanocytes in the primary brain tunic observed "light reaction" in which the outflow of melanin to the central part of the cell body was recorded (**Figure 2C**). Such melanocytes, devoid of outgrowths, were observed through a transmitted channel. DiI-labeled cells in deep layers of the midbrain *tegmentum* of juveniles *O. keta* were visualized through fluorescent channel and grouped into small conglomerates (**Figure 2C**). Thus, the overlay of transmitted and fluorescent channels made it possible to reconstruct three-dimensional (3D) picture of fluorescent cell conglomerates in the midbrain *tegmentum*, which was located in deep layers in combination with surface patterns of distribution of melanocytes in the primary brain tunic in the mode of *in vivo* imaging.

Based on the analysis galleries of optical sections of the midbrain of juveniles *O. keta* was created 3D reconstruction of the spatial distribution of DiI-labeled cells in the damaged area

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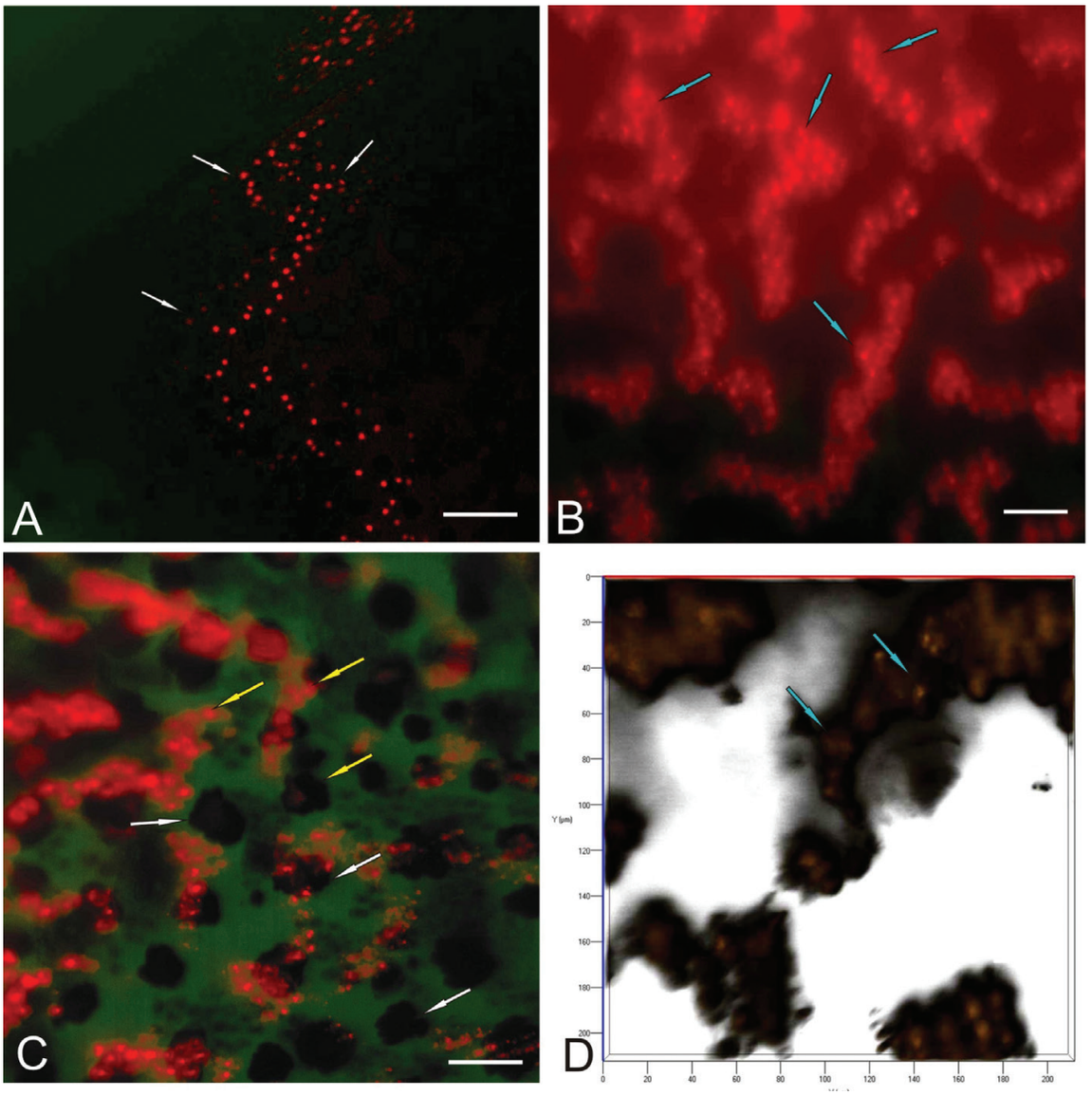

Figure 2. The results of *in vivo* monitoring at different times after injury midbrain *tegmentum* of juvenile chum salmon *Oncorhynchus keta* and injection into the area of injury carbocyanine dye (DiI). A—DiI-labeled cells (white arrows) in the surface layers of *tegmentum* 30 min after injury; B—clusters of DiI-labeled cells (arrows) 2 days after the injury; C—overly of transmitted and fluorescent channels. Transmitted channel show body of DiI-unlabeled melanocytes (white arrows) with a "light reaction." Fluorescence channel show DiI-labeled conglomerates of cells (arrows); D—3D reconstruction of 10 optical sections DiI-labeled cells in damaged *tegmentum* 2 days after injury. Multiphoton confocal microscopy. A, B, C—special planar lens (magnification 20x). Scale bar: 50 μm.

of *tegmentum* (**Figure 2D**). This 3D reconstruction shows that the distribution of fluorescent cells on day 2 after injury inflicted to *tegmentum* of juvenile *O. keta* is uneven. It revealed the formation of various DiI-labeled cell conglomerates, the number and amount of which increases toward the area of injury. Thus, for the surface layers of *tegmentum* characterized by large clusters of DiI-labeled cells (**Figure 2B**), in the deeper tegmental layers, smaller clusters of fluorescent cells were localized. Study of spatial relationships of DiI-labeled cells after 30 min of injury indicates the predominance of large accumulation of these cells in the

superficial layers of the *tegmentum* and their distribution in the form of small conglomerates in the deeper layers.

In our studies, DiI was used as a dye for vital fluorescent multiphoton confocal microscopy. It can be regarded as a possible method of identifying populations of phagocytic cells in the brain, as the effective molecular markers that allow selective identification of populations of macrophages and microglia in the brain of the fish have not been developed so far. We supposed that using multiphoton confocal microscopy *in vivo* experiments allow to have the substantial preference [42]. The results of *in vivo* monitoring in different time after injury (30 min and 2 days) suggest that as a result of midbrain injury of juvenile *O. keta* has experienced rapid cellular response and the emergence of numerous stained cells in the injuries area. It is indicating active participation of such cells in migration and phagocytosis of the dye in the area of injury.

5. Cell proliferation, neural stem cells and neuronal differentiation after injury

A common feature of any regeneration-competent CNS system examined thus far is that cells lost to injury are replaced by new cells that differentiate into various cell types, including neurons. After the stab-wound lesion to the cerebellum of juvenile *O. masou*, processes of proliferation and migration of cells were amplified compared with the intact brain (**Figure 3A**). However, these processes have properties of spatial specificity, so the most proliferative activity characteristic for the dorsal matrix zone (DMZ). In this zone, proliferative activity was observed in normal (intact) conditions and we associate it with intensive persistent neurogenesis in the cerebellum of young *O. masou*. After stab-wound lesion, we verified other areas with neurogenic activity located in the dorsal part of the molecular layer, the lateral and basal regions. The emergence of neurogenic zones is attributed to the intensification of genetic programs in the proliferative neural stem cells (NSC) and the formation of local neurogenic niches (**Figure 3A, C**). Additionally to markers of neuronal differentiation and proliferation, after stab-wound lesion to the cerebellum *O. masou*, expression of doublecortin (Dcort) was detected in cells and the fibers of molecular layer. Dcort is a specific marker of migrating stem cell population, and its expression was found in neurogenic niches of molecular layer 2 days after injury. The size and location of neurogenic niches in the molecular layer of the cerebellum containing Dcort-ip cells are differed. The largest accumulation of Dcort-ip cells were found at dorsal and dorsolateral areas. Additionally to neurogenic niches, in the infraganglionic plexus of cerebellum, single Dcort-ip NSC were revealed; in thickness of the molecular layer, Dcort-ip radial glial cells were identified. So, we observed the proliferative activity in neurogenic niches combined with differentiation of some cells and their subsequent migration to the area of injury.

In the DMZ of juvenile *O. masou*, after stab-wound lesion, four types of cells labeled by PCNA have been identified. These were small, round, intensely labeled cells, or elongated ones, which are able to migrate and form the tangential and radial rows (**Figure 3B, D**). DMZ has

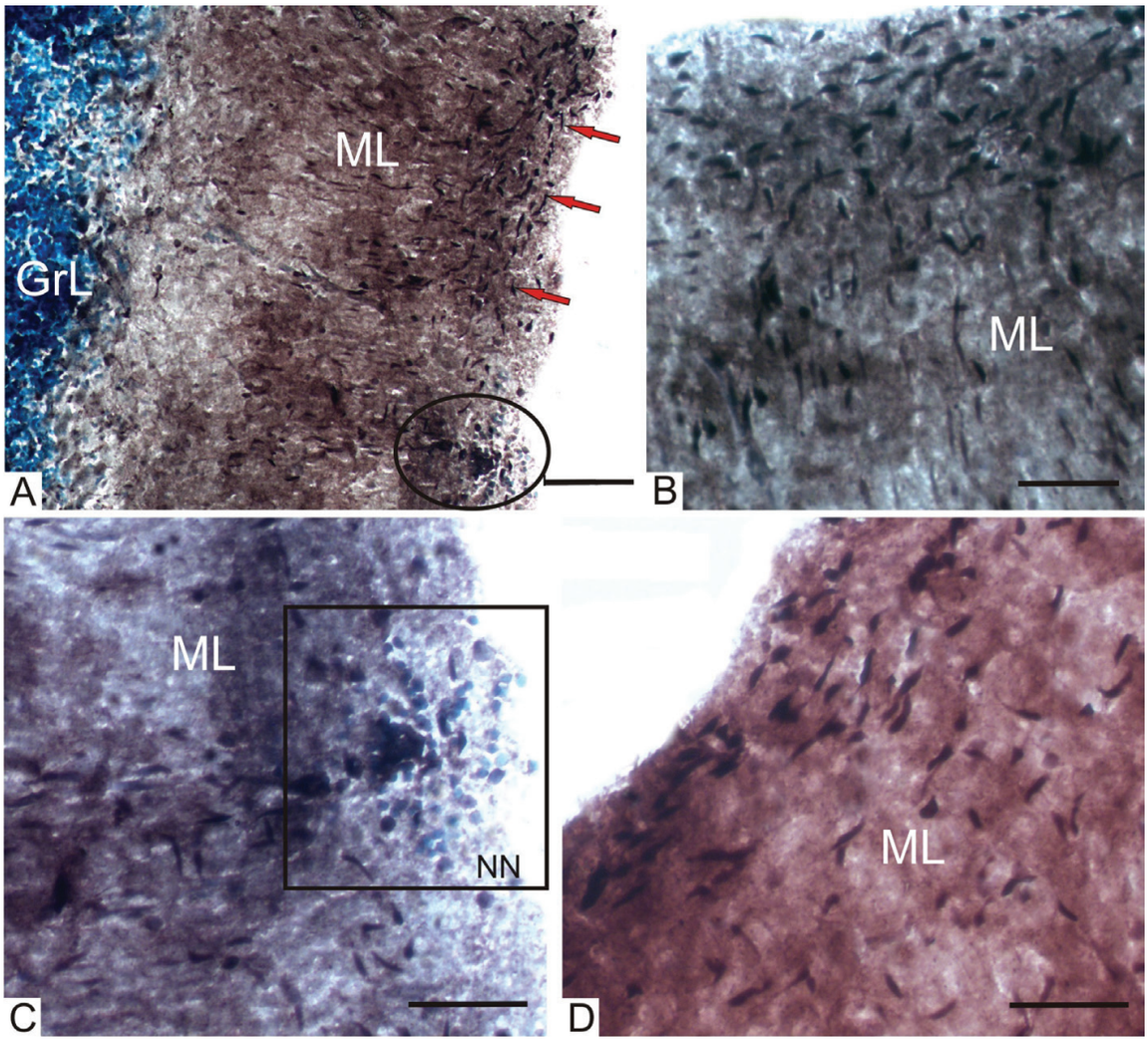

Figure 3. Localization of proliferative cell nuclear antigen (PCNA) in the cerebellum of *Oncorhynchus masou* 2 days after stab-wound injury of cerebellum. A—patterns of tangential migration (arrows) and neurogenic niche (in an oval) in the molecular layer of cerebellum; B—radial migration of PCNA-ip cells; C—neurogenic niche (in square); D—tangential migration of PCNA-ip cells. Scale bar: A—100 μm, B–D—50 μm.

been previously identified in other species of fish, in particular, *D. rerio* [28], *A. leptorhynchus* [32], *Oncorhynchus mykiss* [43]. Different species of fish do not have similar rates of proliferation in the DMZ; for example, the cerebellum of *A. leptorhynchus* contains 75% of the cells of the brain. These cells are formed mainly in the DMZ as well as granular eminentias.

After stab-wound damage to the cerebellum of juvenile *O. masou*, some neurons were eliminated by apoptosis and replaced by new cells. In the first 10 days in the damaged area, the rate of cell proliferation was increased by several times compared with other parts of the cerebellum. Experiments with BrdU labeling showed that the cells formed 2 days before the injury participate in the regeneration process [30]. This observation suggests a direct connection between the continuous cell proliferation in the intact brain and restoring of the damaged area. After injury to the fish's brain, rate of cell proliferation is much higher than in normal conditions. It is believed that some young cells develop into definitive granule neurons and

subsequently most often eliminated by apoptosis. Retrograde tracing in combination with BrdU labeling S-phase of mitosis has shown that new granule neurons project to the molecular layer [30]. This fact suggests that these neurons are integrated into the existing neural network of the cerebellum.

The intensity of proliferation depends on the nature of injury and the amount of damaged brain tissue. After the damaging effects in the cerebellum of *O. masou*, proliferative activity in the cells was significantly enhanced in the dorsal area (**Figure 4A**). These data are consistent with the established data for other species of fish, in particular *A. leptorhynchus* [28] indicating that the main volume of cell proliferation after injury localizes at the DMZ. At the surface layers of the lateral zones were detected neurogenic niches (**Figure 4B**), HuCD-ip individual undifferentiated cells, and patterns of cells radial migration. Our results show that in the lateral area of cerebellum, migration processes intensified to compare with the dorsal one. Nevertheless, after a damaging effect in lateral zones were detected neurogenic niches containing HuCD-in

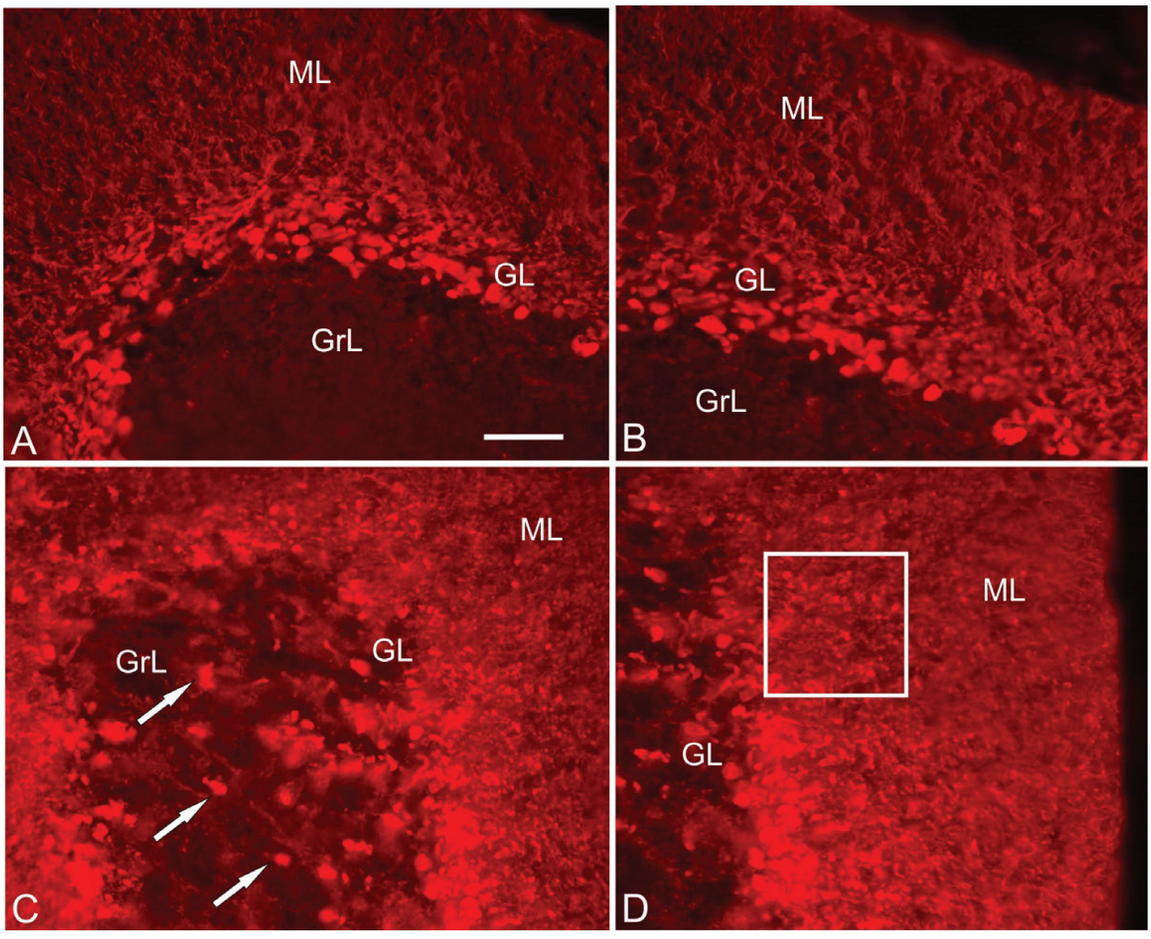

Figure 4. Immunofluorescence labeling of parvalbumin in the control (A, B) and 2 days after stab-wound injury of cerebellum (C, D) of *Oncorhynchus masou*. A—dorsal; B—lateral part of *corpus cerebellum*; Par-ip cells and fibers are present in ganglion (GL) and molecular (ML) layers, the density of Par-ip is not high; C—emergence of Par-ip cells (arrows) in the granular layer (GrL); D—increasing of density Par-ip innervation in the molecular layer (contoured by rectangle) after injury. Scale bar: 100 μm.

and Dcort-ip cells, which testifies to the existence of neural stem cells in the lateral area and enhancing their proliferative potency after injury.

In the basal area of *O. masou*, cerebellum proliferative activity of cells is high enough to control, but significantly reduced after damaging effects. The proliferative activity of the cells after the injury persists, prevailing over the processes of cell migration. A process of cell migration in basal zone is mild; however, after stab-wound lesion, the cell migration activity is increased. Thus, the proliferative activity of cells in the basal area of juvenile *O. masou* largely provides a persistent process of neurogenesis, however, after stab-wound lesion is decreased and begins to dominate the processes of cell migration.

Forebrain proliferative activity of juvenile *O. masou* revealed proliferative surface area corresponding to the periventricular area other fish, including the dorsal, lateral, and medial compartments. In addition to the large number of cell clusters in the surface zone, under conditions of normal proliferation, some cells were observed in the parenchyma of telencephalon and were identified as single or paired immunopositive PCNA-labeled cells. The presence of such cells with a high proliferative potential evidences a high level of persistent neurogenesis in telencephalon of juvenile *O. masou* salmon.

After mechanical injury in the telencephalon of *O. masou*, there has been an increase of cell density of distribution in the proliferative zone. In the deep layers of the telencephalic parenchyma of masu salmon, we observed an increase in proliferative activity: the number of single PCNA-immunopositive cells grew compared to those in intact animals. Induced neurogenesis zone appeared; it was presented by neurogenic niches and areas of secondary neurogenesis surrounded by radial glial fibers.

HuCD-immunopositive cells were identified as part external proliferative zones and in the deep layers of the telencephalon juvenile *O. masou*. We established that in juvenile *O. masou* telencephalon, HuCD protein is detected in cells being at different stages of neuronal differentiation. HuCD-immunopositive neurons were identified in the area of proliferative zone; differentiated neurons of various degrees of maturity were found in the deeper layer. The same characteristic HuCD was different levels of marking immunopositive cells. Densitometric analysis allowed to distinguish two levels of protein HuCD activity in the telencephalon *O. masou*: intense and medium. However, definitive intensely labeled neurons dominated in all areas in the control animal's brain.

We have identified four types of HuCD-labeled cells differing in morphological parameters (large and small size cell bodies) and optical density. Type 1 cells are the smallest undifferentiated cells with a high OD; Type 2 are larger oval cells with high and average value of OD; multipolar cells with high OD were the third type; and bipolar neurons with large high OD belong to the fourth type.

There are considerable changes in topography HuCD-immunopositive cells in the telencephalon *O. masou* after mechanical injury. Occurrence of neurogenic niches was registered, representing a collection of intensely labeled HuCD positive cells. Appearance of neurogenic niches was registered, representing an accumulation of intensely labeled HuCD positive cells. The density of distribution of immunopositive and negative cells increased; also, a distinct

pattern of cell migration from the surface proliferative zone to the deep parenchymal layer was observed to appear.

This evidence based on HuCD and PCNA marking shows us the intense persistent neurogenesis in proliferative zone of dorsal region telencephalon *O. masou*. Distribution of neurogenic activity of deeper layers of the parenchyma appears after injury. The main sources of new neurons in the process of reparative neurogenesis are neurogenic niches.

6. Neuroprotective factors in damaged fish brain

Calbindin-D28k has been postulated to exert a neuroprotective function by buffering intracellular free Ca^{2+}. This hypothesis is supported by the findings that calbindin-D28k-expressing neurons exhibit a relative resistance to neurotoxicity induced by glutamate, calcium ionophore, or acidosis [44] and that the rate of survival of neurons can be increased after various types of insults by overexpression of the gene for calbindin-D28k [45, 46].

Other calcium-binding protein like parvalbumin may also be involved in the neuroprotective properties of fish nervous system. The results of studies on young *O. masou* showed that cerebellum after injury can significantly increase the level of expression of parvalbumin (**Figure 4A, C**). In control animals, immunofluorescence of parvalbumin was detected in cells of ganglionic layer of the cerebellum (pear-shaped Purkinje neurons) and fibers of infraganglionic plexus (**Figure 4A, B**). After mechanical injury of the cerebellum, immunofluorescence of parvalbumin was found in the cells of the granular layer and multiple synaptic terminals in the molecular layer (**Figure 4C, D**).

According to Grosche et al. [13], glutamine synthetase (GS) is a specific glial protein performing the conversion of toxic glutamate into a non-toxic amino acid, glutamine. In normal conditions, this mechanism prevents accumulation of glutamate neurotoxicity in nerve tissue, protecting neurons from cell death. But after a brain injury in a mammalian brain, volume of synthesized GS is insufficient to neutralize the toxic effects of glutamate. This determines such effects as the development of primary and secondary inflammations and progressive neurodegenerative processes observed following injury of the CNS in mammals and human glutamine synthetase [13]. In fish, the increased activity of GS is likely to provide an important mechanism for reducing the neurodegenerative effects caused by glutamate neurotoxicity. This assumption indicates the presence of certain ways that determine such strong differences in the regenerative potential of the two taxa of vertebrates [47].

The results of immunohistochemical analysis of GS indicate significant differences between the distribution of the enzyme in normal conditions and after stab-wound lesion to the cerebellum (**Figure 5A, C**). In both cases, enzyme activity was identified in cells and fibers. Densitometric analysis of enzyme activity in cells has shown that there are two levels of activity: intensive and moderate. The results of the morphological analysis and some literature data [48] indicate that cells containing the GS represent the population of astrocytes. Morphological studies of GS-ip cells in the cerebellum in young *O. masou* show the presence of a heterogeneous population of cells in control (**Figure 5A**). A maximal number of GS-ip

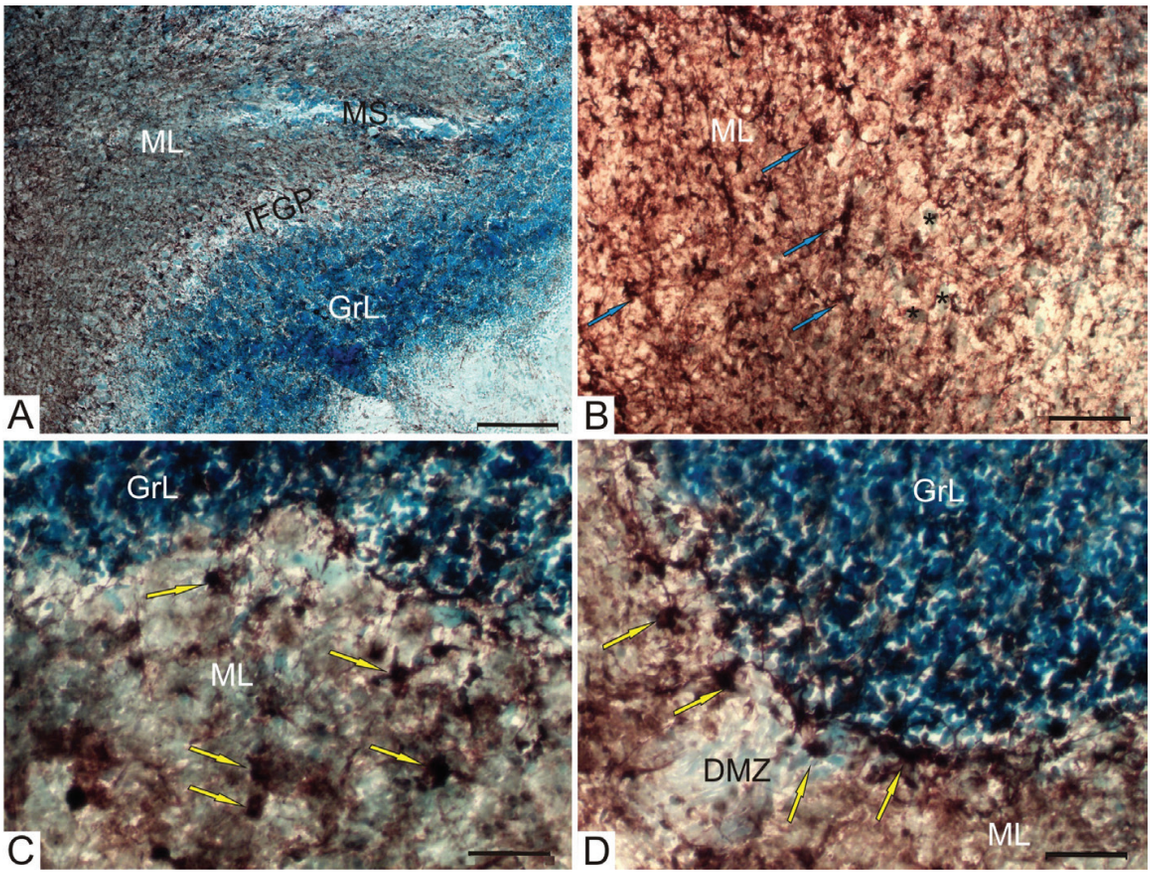

Figure 5. Immunocytochemistry of glutamine synthetase in cerebellum of *Oncorhynchus masou*. A—general view of GS-ip element distribution in the area of median suture (MS) of *corpus cerebellum*; B—GS-ip cells (arrows) in molecular layer (ML) and infraganglionic plexus (IFGP), the body of Purkinje cells indicated by asterisk. Distribution of Glutamine synthetase in the cerebellum of *Oncorhynchus masou* 2 days after stab-wound injury; C—GS-ip cells in molecular layer (arrows); D—in the dorsal matrix zone (DMZ). Scale bar: A—200 μm; B—50 μm; C, D—100 μm.

cells have been identified in the molecular layer (**Figure 5B**). The density distribution of GS-ip cells in the molecular layer in control was quite high, which indicates a high level of GS activity and, possibly, its particular impact on the processes of persistent neurogenesis.

After stab-wound lesion to the cerebellum, maximal number of GS-ip cells has been identified in the granular layer of cerebellum (**Figure 5C, D**). Redistribution of cells synthesizing GS, from the molecular to the granular layer, was revealed.

In the lateral and dorsal regions of the cerebellum of *O. masou*, increased activity of GS in the fibers was observed (**Figure 5C, D**). In the control, activity of the fibers in the granular layer (granular eminence) is not high (**Figure 5A**). We identified heterogeneous population of GS-ip cells in cerebellum of *O. masou*. We believe that among these cells are present glutamatergic neurons, containing GS labeled of metabolic glutamate and astrocytes that can receive glutamate due to its reuptake of extracellular space. The observations show that 3 days after injury in the cerebellum of *O. masou*, substantial redistribution of GS activity in various parts of the cerebellum may occur. Thus, in area of injury, we showed a significant increasing number of GS-ip cells (**Figure 5C**) and reducing number of GS-ip fibers (**Figure 5D**). This spatial specific-

ity can be connected with both the toxicity, induced by stab-wound lesion, and change in the glutamatergic neurotransmission in damaged neural networks.

From other hand, the high activity of GS in control suggests the involvement of glutamate in plastic processes, including morphogenesis taking place during persistent neurogenesis in normal fish cerebellum [1, 47].

We believe that metabolic glutamate which is presumably involved in morphogenic cerebellar functions in *O. masou* can be localized in normal conditions, as in the growing neurons and in the fibers. This assumption is confirmed by the results of studies showing the high activity of GS in the cells of the molecular layer, especially in dorsal region. This region contains a DMZ of cerebellum, characterized by a high neurogenic activity (**Figure 5D**). Our results suggest that the pattern of GS activity was decreased during 3 days of injury, but we do not exclude the possibility that these changes are temporary. This hypothesis was supported by data of enzyme immunoassay (ELISA) carried out by us in the cerebellum of *O. masou* (**Figure 6**). Thus, during long-term monitoring, the GS activity was found to be increased during the initial few hours after injury (1–3 h) and then continue to increase till the 14th day, except a decrease in the 12 h after injury. However, these changes of metabolic activity of GS may only represent a local decrease in enzyme activity as shown on the third day. The results of ELISA immunoassay established that the enzyme activity after damaging effects has a complicated pattern. The increase in enzyme activity was observed during the first few hours (1–3 h) after injury and at second, fifth, and tenth days after the damaging effects (**Figure 6**). On the second day, we observed increase in activity of GS by ELISA immunoassay, which is consistent with results of IHC labeling on frozen brain sections. Thus, the decrease in activity of GS on the third day after mechanical injury of cerebellum *O. masou* can be a particular manifestation of the changes in the metabolic status. At sufficiently high intensity of persistent neurogenesis in young *O. masou*, we tend to believe that the response from the GS-producing elements

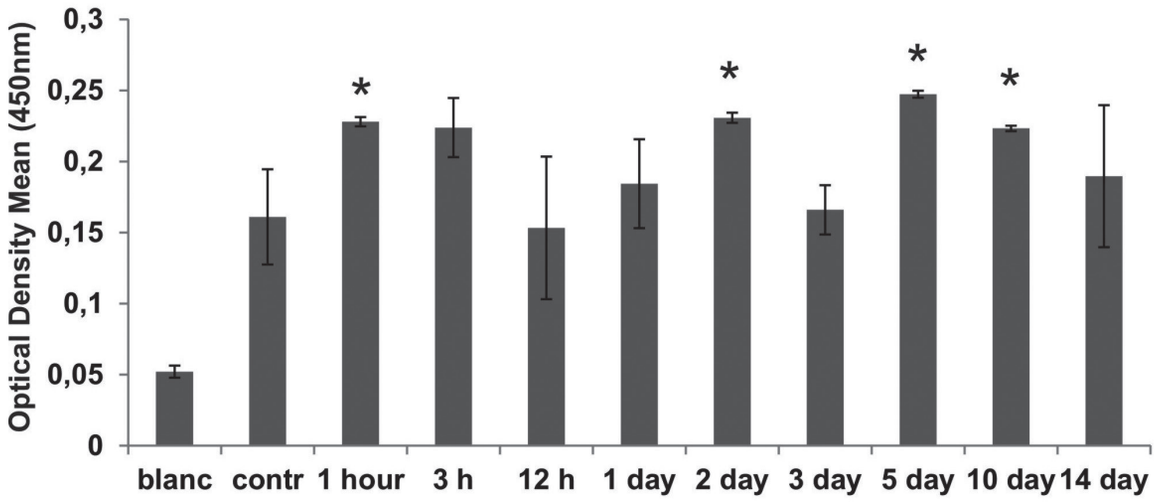

Figure 6. The immunoassay data of glutamine synthetase content in the cerebellum of *Oncorhynchus masou* at different time intervals after stab-wound injury. Protein concentration is 100 ng/ml. On the *x* axis are shown different time points after injury, whereas mean optical density is shown on *y* axis—(absorbance at 450 nm). Data are shown as mean ± S.E.M; *P < 0.05 significant differences compared to the control group (n = 5 in each group).

in the cerebellum can be complex and ambiguous. So, damaging effects can be the cause of death of a large number of cells by apoptosis, as a result of glutamate toxicity. However, the presence of increased level of GS in first, second, and fifth day indicates a rather high-level production of the enzyme whose activity can be reduced and cyclically be determined by different factors, the nature of which remains to be established.

The increase in the number of GS-ip cells in the granular layer and the high activity of GS in them on the second and third day are referred by us to as the astrocytic response observed after a damaging impact. However, the number of these cells is not high enough for response to the GS-ip cells as «reactive gliosis» appeared on central nervous system of mammals after damaging effects. In the mammalian brain, as is known, as a result of a traumatic impact, pool of reactive astrocytes formed morphological and biochemical barrier, features which significantly differ from those in normal astrocytes [47]. The cellular mechanism associated with the transformation of a population of astrocytes and isolating a subpopulation of activated glia in the brain of the fish is currently poorly understood. Unlike mammalian brain, astrocytes in a fish brain do not form the astrocytic barrier, which is characteristic for the development of posttraumatic process in the mammalian brain. Nevertheless, changing GS synthesis is an unambiguous evidence in favor of the neuroprotective properties of the enzyme and increased production in the cerebellum *O. masou*. This indicates that not only GS is the marker of cells involved in the conversion of glutamine/glutamate but it can also be considered as an effective neuroprotective factor contributing to posttraumatic reparative processes.

7. Conclusion

The fish brain has a unique feature of vertebrates—it grows with the growth of body over a lifetime. In this regard, fish is a convenient model for the study of embryonic and postembryonic development of the central nervous system and of the influence of different factors on these processes. Injury of fish brain creates special conditions for the implementation of genetic programs aimed at strengthening the proliferation of progenitor cells as well as activation and proliferation activity in the neuronal stem cells. Study of neurogenic activity, migration, and differentiation in the neurogenic niches contributes to a better understanding about how these structures operate, not only in fish but in other vertebrates as well.

Acknowledgements

This study was supported by a grant of the President of the Russian Federation (MD-4318.2015.4), Grant of Scientific Foundation (№ 10046–2016.4), and the "Far East" Program for Basic Research of the Far East Branch of the Russian Academy of Sciences 2015–2017 (project number 15-I-6-116, Section III).

We are grateful to Dr. Sachin Shukla Eye Research Centre (Hyderabad, India) for the participation in ELISA immunoassay and editing the text.

Author details

Evgeniya V. Pushchina[1,2]*, Anatoly A. Varaksin[1], Mariya E. Stukaneva[1] and Eva I. Zharikova[1]

*Address all correspondence to: puschina@mail.ru

1 National Scientific Center of Marine Biology, Far Eastern Branch, Russian Academy of Science, Laboratory of Cell Differentiation, Vladivostok, Russia

2 Bogomoletz Institute of Physiology, National Academy of Science of Ukraine, Laboratory of Stem Cell Biology, Kiev, Ukraine

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