Chapter

Hydrogen Sulfide as a Factor of Neuroprotection during the Constitutive and Reparative Neurogenesis in Fish Brain

Evgeniya V. Pushchina, Anatoly A. Varaksin and Dmitry K. Obukhov

Abstract

The H_2S-producing systems were studied in trout telencephalon, tectum, and cerebellum at 1 week after eye injury. The results of ELISA analysis have shown a 1.7-fold increase in the CBS expression at 1 week post-injury, as compared to the intact trout. In the ventricular and subventricular regions of trout telencephalon, CBS+ cells, as well as neuroepithelial and glial types, were detected. As a result of injury, the number of CBS+ neuroepithelial cells in the pallial and subpallial periventricular regions of the telencephalon increases. In the tectum, a traumatic damage leads to an increase in the CBS expression in radial glia with a simultaneous decrease in the number of CBS immunopositive neuroepithelial cells detected in intact animals. In the cerebellum, we revealed neuroglial interrelations, in which H_2S is probably released from the astrocyte-like cells with subsequent activation of the neuronal NMDA receptors. The organization of the H_2S-producing cell complexes suggests that the amount of glutamate produced in the trout cerebellum and its reuptake is controlled with the involvement of astrocyte-like cells, reducing its excitotoxicity. We believe that the increase in the number of H_2S-producing cells constitutes a response to oxidative stress, and the overproduction of H_2S neutralizes the reactive oxygen species.

Keywords: hydrogen sulfide, traumatic eye injury, oxidative stress, radial glia, excitotoxicity, reparative neurogenesis, adult neuronal stem cells, neuroepithelial cells, astrocyte-like cells, teleost fishes, CBS expression

1. Introduction

Hydrogen sulfide (H_2S) was initially considered as a gasotransmitter with antioxidant properties [1]. To date, the vasodilating, neuromodulating, and anti-inflammatory properties of H_2S have also been identified [2, 3]. In studies of the cardiovascular system, H_2S was assumed to act as a protective factor [4]; nevertheless, the effects of H_2S in the central nervous system (CNS) during stress or injury remain poorly understood. The involvement of H_2S, as well as other gaseous intermediaries such as NO, CO, and H_2, in the traumatic brain injury is now intensively investigated [5], but this question has not been

completely clarified thus far. H_2S, like nitric oxide (NO), is known to mediate posttranslational modification of proteins by adding additional sulfur to reactive cysteine residues. This modification, referred to as S-sulfhydration, is required to activate or inactivate many classes of proteins, including the ion channels, such as the ATP-dependent potassium channels, TRPV3, TRPV6, TRPM [6], enzymes, and the transcription factors NF-kB and Nrf2 [7]. Modulation of ion channels, as well as the inflammatory and the antioxidant transcription factors, using H_2S after traumatic brain injury, can play a significant role in reducing edema and inflammation [8].

Recently, the involvement of H_2S in cerebral ischemia, traumatic brain injury (TBI), and decrease in reactive oxygen species in the H_2S-dependent mechanisms has been studied using different models [8–10]. The use of monoclonal antibodies against cystathionine β-synthase (CBS) in immunohistochemical (IHC) detection of the H_2S-producing complexes in the brain of juvenile trout showed an increase in hydrogen sulfide production in different parts of the brain and CBS induction in the radial glia cells after the damage of the optic nerve [11]. It was shown that the toxic and/or neuroprotective effects of hydrogen sulfide depended on concentration: lower concentrations play a physiological role, while very high concentrations cause cell death [12, 13]. Although hydrogen sulfide is considered a gasotransmitter, there is uncertainty about the total concentration of this volatile gas or highly active anionic particles (SH-) in both plasma and central nervous system tissues [14].

The progress in studies of the hydrogen sulfide biology has led to a conclusion that polysulfides are more significant sources of intermediate sulfhydration of proteins than H_2S [3]. The H_2S reactions with many signal mediators, transcription factors, and channel proteins in neurons and glial cells are known both in vivo and in vitro [7, 10]. However, still little is known about interaction of the H_2S intercellular communication and its consequences in the case of a traumatic cerebral injury. Such information is necessary to determine the cytoprotective or cytotoxic effects of H_2S in the brain injury and/or cerebral ischemia.

The study of biology of the neural stem cells, based on animal models, is becoming increasingly important, since the processes of constitutive neurogenesis occur in many areas of the animal brain [15], providing a high reparative potential of CNS. One of such models is fish, which is characterized by a high rate of reparative processes [16]. The results of preliminary studies showed an increase in proliferative activity of cells of the trout brain after damage to optic nerve [17]. To further characterize the cellular response in the trout brain after eye injury, the hydrogen sulfide-producing enzyme, cystathionine β-synthase (CBS), was analyzed using western immunoblotting, enzyme-linked immunosorbent assay (ELISA), and immunohistochemical labeling of CBS in various sections of the trout brain at 1 week after the traumatic eye injury.

2. Evaluation of CBS expression in the intact trout brain and after eye injury by the western blot analysis and ELISA immunosorbent assay

The cystathionine β-synthase enzyme has a tetramer binding with two substrates (homocysteine and serine) and three additional ligands (the coenzyme pyrodoxal 5′-phosphate, the allosteric activator S-adenosylmethionine, and heme). An assessment of CBS content by Western blot analysis showed the presence of protein with a molecular weight of 63 kDa in all the divisions of the trout brain. The quantitative CBS content in different divisions of the intact trout brain and after the mechanical eye injury is shown in **Figure 1A**. The maximum level of CBS

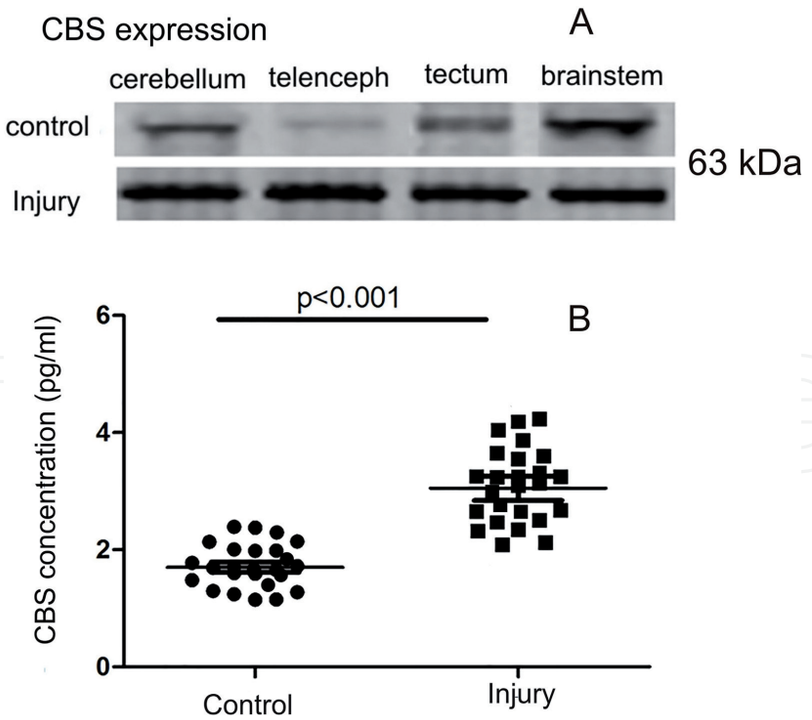

Figure 1.
Representation of western blots of cystathionine β-synthase content in the brain of the trout Oncorhynchus mykiss. *(A) the single protein band corresponding to a molecular weight of 63 kDa was present in the trout cerebellum, optic tectum, telencephalon, and brainstem in the control (intact) animals and at 1 week after the optic nerve damage. (B) ELISA assay of CBS in the rainbow trout brain at 1 week after UEI vs. control (intact) rainbow trout. Student's t-test was used to determine significant differences between the trout at 1 week after UEI vs. control (intact) fish (## P < 0.01); n = 20 in each group.*

expression in the intact animals was found in the brain stem, while the minimum was in the telencephalon. The cerebellum and tectum showed a medium level of CBS expression. A significant increase in the level of CBS expression was observed in all the brain divisions after the mechanical eye injury (**Figure 1A**). According to the enzyme immunosorbent assay, it was 1.86 ± 0.03 pg./mL and 3.12 ± 0.26 pg./mL after unilateral eye injury (UEI) ($P < 0.001$). Thus, within a week after the eye injury, the concentration of CBS increased 1.7 times compared with the control animals (**Figure 1B**).

3. Telencephalon

Results of the IHC CBS-labeling in the trout telencephalon showed the presence of intensely and moderately labeled cells in the pallial and subpallial regions. CBS-labeled cells were located in the superficial periventricular and subventricular pallial layers. In deep pallial areas, the number of intensively labeled cells was elevated. The presence of a H_2S-producing enzyme in the brain cells is associated with the process of neurochemical signaling and, in particular, with the activation of NMDA receptors. Activation of neurons in the brain of vertebrates leads to release of neurotransmitters, including glutamate, activating the NMDA receptors, which, in turn, leads to an increase in the astrocytic intracellular calcium and long-term potentiation [3, 18]. Thus, the presence of two levels of CBS activity in the trout telencephalon indicates the mediator/modulatory intercellular interactions, which agrees with the previously obtained data on fish [11].

In intact trout, when labeled with polyclonal antibodies against CBS, the CBS-labeled radial glia was detected particularly in the pallial and subpallial regions

of the telencephalon, while labeling with monoclonal antibodies did not reveal similar structures [11]. The present data suggests that in the trout telencephalon CBS may label aNSCs with a glial phenotype (radial glia). Our assumption is consistent with the results of studies of the pallial neurogenic niche in adult zebrafish containing radial glia-like aNSCs with cellular bodies lining the walls of the ventricle [19]. Studies of the hydrogen sulfide biology in the mammalian brain have shown that astrocytes and glial cells constitute the main repositories of CBS in the brain [3]. In in vitro experiments, it was found that astrocytes produce 7.57 times more H_2S than the microglial cells [20]. However, in the fish brain, the detection of typical astrocytic glia gives controversial results [21, 22], and radial glia is detected frequently during attempts to identify the brain glial architectonics [23].

In the surface layer of different zones of the trout telencephalon, CBS+ cells and RG and cells of neuroepithelial type, representing a part of the constitutive matrix zones of the telencephalon, were also identified. Thus, CBS+ cells were detected in the zones of constitutive neurogenesis in the telencephalon of intact animals, which is consistent with the previously obtained data on the masu salmon and carp [24]. Studies on *D. rerio* have shown that aNSCs are associated with the ventricular system. In the fish telencephalon, aNSCs have a typical morphology of radial glial and/or neuroepithelium, which can be identified with several molecular markers of aNSCs [25, 26]. Thus, it is obvious that the CBS+ cells of the pallial and subpallial regions of the trout telencephalon are the aNSCs of the neuroepithelial and glial types. Earlier studies on trout have shown that after injury in the dorsal, medial, and lateral pallial zones, the number of the PCNA+ and HuCD+ cells significantly increases, indicating growth of proliferative and neurogenic activity in the telencephalon [17]. After the traumatic eye injury, the number of CBS+ cells increased in all areas of the telencephalon, with the exception of dorso-central zone (**Figure 2**). The number of H_2S-producing cells increases in the periventricular and subventricular regions of the telencephalon, which are characterized by intensification of proliferative processes that occur after the eye injury.

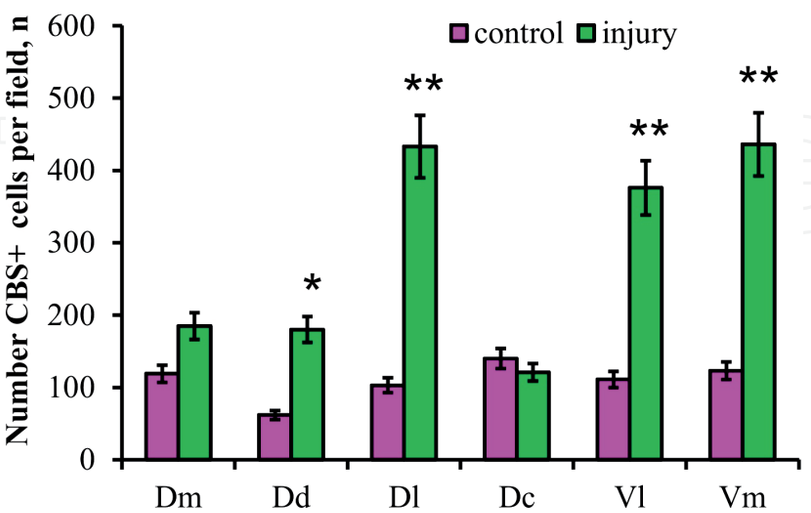

Figure 2.
Density of CBS+ cells in the telencephalon of the intact trout Oncorhynchus mykiss *and at 1 week post-injury. The one-way analysis of variance (ANOVA) followed by the student–Newman–Keuls post hoc test was used to determine significant differences between the control trout and fish after UEI (n = 5 in each group; * P < 0.01, ** P < 0.05 significant differences* vs. *the control group). Dm, Dd, Dl, and Dc are the medial, dorsal, lateral, and central parts of the dorsal telencephalic area; Vl and Vm are the lateral and medial parts of the ventral nucleus of telencephalon.*

4. CBS in the telencephalon parenchyma

An increase in another type of CBS+ cells, which are intensively labeled, having no processes, and adjacent to large moderately labeled neurons, in the telencephalon parenchyma suggests intercellular neuron/glial or neuron/microglial interactions associated with release of H_2S from intensively labeled astrocyte-like cells and/or microglia [18, 27]. In addition, after the injury, the patterns of distribution of the CBS+ radial glia cells in the telencephalon are retained, which indicates an additional production of H_2S in aNSCs of the glial phenotype. Studies showed that after ischemic brain damage, the additional production of H_2S is provided by sulfhydration [2]. In more recent studies, it has been shown that polysulfides are 300 times more active than H_2S in the TRPA1 receptor activation [3]. Polysulfides activate NMDA receptors, which is accompanied by the H_2S-dependent reduction of cysteine disulfide in extracellular domain of the receptor [3]. In this context, activation of the NMDA channels by H_2S is probably a detrimental condition arising from the excitotoxicity of glutamate causing calcium influx, which, in turn, leads to neuronal toxicity and cell death [1, 28].

The results of experimental in vitro studies have shown that the glutamate toxicity during a traumatic injury (ischemia) is attenuated by the effect of H_2S on ATP/K^+ and CFTR/Cl^- channels [29] and activation of the GLT1 transporters [30]. However, there is currently no consensus on the dual role of H_2S in the glutamate toxicity. Neurons that are formed in the matrix periventricular zones of the trout telencephalon represent the immature cell forms that migrate from the periventricular to subventricular layers of the brain. Such undifferentiated cells can express an incomplete set of glutamate NMDA receptors, and, therefore, those cascade processes that trigger apoptosis in mature neurons in immature cells cannot cause death. On the other hand, it is known that H_2S is metabolized by mitochondria through participation in the oxidation process of the H_2S-producing enzymes [13, 31]. The stress-induced H_2S production in mitochondria and a subsequent increase in the ATP production were demonstrated [32].

Changes in the mitochondrial membrane potential activate caspase-3 and then become attenuated with NaHS in the neuronal cell culture, which protects neurons from apoptosis [33]. Thus, the controversial role of H_2S in the mammalian brain neurons raises some questions about whether the excessive production of H_2S causes death of mature neurons in the trout brain as a result of eye injury. It is likely that H_2S has a protective effect on the immature telencephalic cells after the injury. What phenotype (glial or neuronal) does correspond to cells that produce H_2S after UEI in the trout brain? Considering our previous evidence that the cells of these zones in the trout telencephalon are HuCD+ [17], it is fair to assume that the H_2S-producing cells in the trout telencephalon can represent immature neurons. However, the detection of CBS expression in the radial glia cells indicates a glial phenotype. Thus, it can be concluded that as a result of UEI in the trout telencephalon, CBS expression is activated in both the neuronal and glial cell population.

5. Optic tectum

As a result of UEI in the trout tectum, the number of the CBS+ cells increases dramatically, the appearance of the dense CBS+ cell groups in different layers of the tectum is diagnosed, and the CBS expression is induced in the RG cells (**Figure 3A**). The present results of CBS labeling with polyclonal antibodies in the tectum of older trout confirm the IHC labeling data for a younger age group

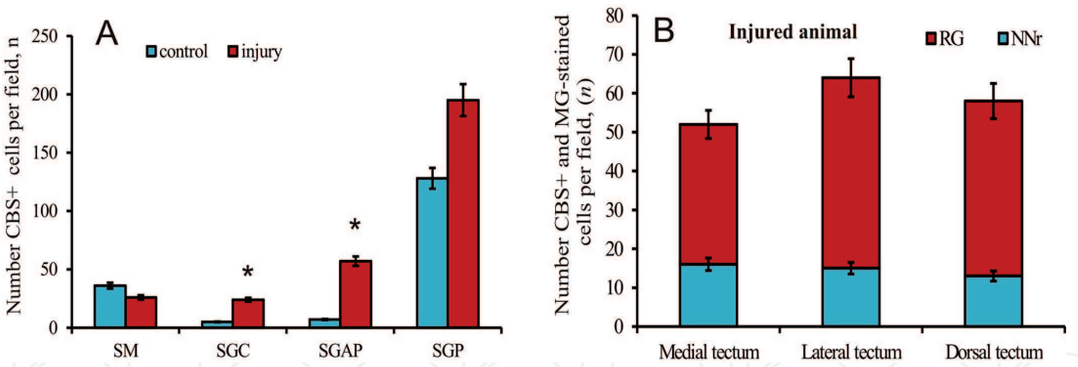

Figure 3.
Density of CBS+ cells in the optic tectum of the intact trout Oncorhynchus mykiss *and at 1 week post-injury. (A) Number of cells in the intact tectal layers and after UEI; the one-way analysis of variance followed by the student–Newman–Keuls post hoc test was used to determine significant differences between the control trout and fish after UEI (n = 5 in each group; (mean ± SD), * P < 0.05, vs. control group). SM, stratum marginale; SGC, stratum griseum centrale; SGAP, stratum griseum at album periventriculare; SGP, stratum griseum periventriculare. (B) Number of radial glia cells (RG) and reactive neurogenic niches (NNr) in different part of tectum after UEI (mean ± SD).*

of trout using monoclonal antibodies [11]. This indicates that at different periods of the trout constitutive ontogenesis, UEI leads to activation of expression in the aNSC radial glia cells against the background of a general decrease in the number of CBS immunopositive neuroepithelial cells, detected in the intact animals. Data of the quantitative analysis showed that in the lateral part of the tectum, the RG distribution density is greater than in the dorsal and medial parts, while the average number of reactive neurogenic niches in these areas remains approximately the same (**Figure 3B**). After UEI, an increase in the number of CBS+ cells is observed in all the tectum layers, reaching a maximum value in SGP (**Figure 3A**). In SGC and SGAP, a significant increase in the number of CBS+ cells ($P < 0.05$) was detected as compared with the control (**Figure 3A**).

Post-traumatic disorders of energy metabolism that occur in the trout tectum after UEI cause a number of changes resulting from the depletion of ATP. One of the main metabolic changes is glycolysis, which serves as the main factor of reduction in ATP-generating oxidative phosphorylation [4]. The loss of ATP leads to an imbalance in ionic homeostasis in the tectum cells due to the breakdown of ATPases or ATP-dependent ion transporters [9], which regulate the influx of calcium and sodium. These changes lead to an outflow of potassium due to the subsequent ATP depletion and calcium accumulation [28, 34]. The increase in intracellular calcium leads to growth of glutamate level, which increases calcium overload and activates calcium-dependent lipases and proteases [28]. Such shifts in ionic homeostasis lead to an increased production of reactive oxygen species (ROS), the opening of transitional pores of mitochondrial permeability, inflammation, and neuronal death [9, 35]. In intact animals, the astrocytes surrounding the neurons absorb extracellular glutamate and protect neurons from excitotoxicity [1, 4]. However, during the brain injury, the damaged astrocytes may exacerbate ischemic reperfusion injury due to the inhibition of the main glutamate transporter (GLT1) [1, 12].

Thus, as a result of UEI, a number of pathophysiological changes develop in the trout tectum, causing the oxidative stress. In vivo experiments indicate the important role of H_2S involved in many ways to control oxidative stress, including the glutathione cycle, activation of enzymes, and transcription factors related to the redox balance [3]. One of the pathophysiological effects in trout after UEI is assumed to be microglial polarization with appearance of clusters of activated microglia with a pro-inflammatory phenotype (**Figure 4**). Similar effects were also identified in a cell culture with exogenous administration of sodium hydrosulfide [36]. Thus, an

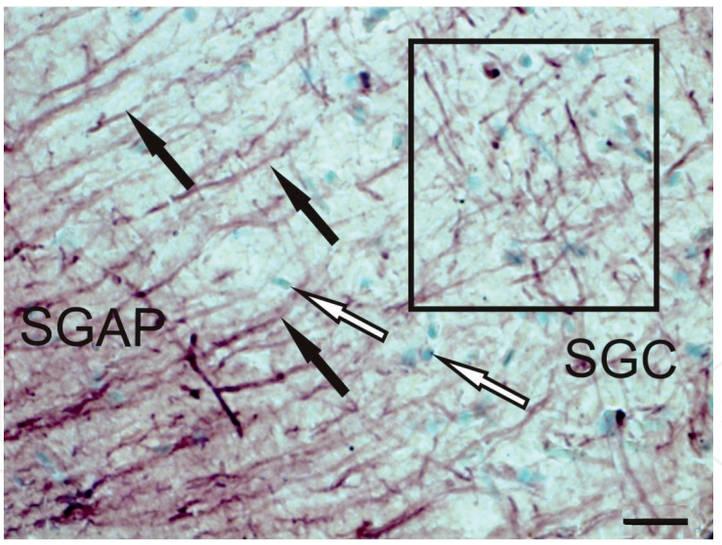

Figure 4.
CBS in the optical tectum of the trout Oncorhynchus mykiss *at 1 week after UEI. Clusters of tangentially located CBS+ reactive astrocytes (in black rectangle), radial glia (black arrows), and CBS- cells (white arrows). SGC, stratum griseum centrale; SGAP, stratum griseum at album periventriculare. Immunoperoxidase labeling of CBS in combination with methyl green staining. Scale bar: 20 μm.*

increase in the H_2S production in the trout brain after UEI should be considered in terms of maintaining cerebrovascular homeostasis, implying anti-apoptotic, anti-inflammatory, and antioxidant effects, and reducing the level of secondary neuronal damage that results from oxidative stress.

6. Cerebellum

The CBS localization was studied in the cerebellar body and granular eminences of trout. Earlier studies on juvenile trout using monoclonal antibodies against CBS showed the presence of H_2S-producing complexes in the granular layer and *valvula cerebelli* [11]. The data from this study allows a suggestion that the hydrogen sulfide production differs significantly between different neuroanatomical regions of *corpus cerebelli* (CCb), in particular, among granular cells. The highest number of CBS+ cells was found in the dorsolateral (DLP) and dorsomedial parts (DMP) of CCb, while in the basal part, the number of immunopositive cells was lower. In DLP, CBS+ Purkinje cells prevailed over negative cells; in the basolateral part (BLP), *vice versa* (**Figure 5**). In the medial zones of the trout CCb, dorsal and basal, typical CBS+ eurydendroid neurons (EDC) were revealed. The proportions of the CBS-positive and CBS-negative Purkinje cells (PC) in the basomedial part (BMP) and DMP were almost the same, and the number of CBS+ EDCs was higher in BMP (**Figure 5**). Thus, in the intact trout CCb, a heterogeneous population of PC was identified, some of which are CBS-positive and others are CBS-negative. The distribution of the CBS+ and CBS– Purkinje cells in CCb is characterized by a certain spatial specificity: most of the CBS+ PCs are localized in DLP. In the basal and dorsal parts of CCb, CBS+ eurydendroid neurons were found forming extracerebellar projections.

An essential feature of CBS-immunopositivity of projection cells in the trout ganglionic layer (GL) is their relationship with the CBS+ glial-like cells. Similar patterns of colocalization of the moderately labeled PCs and EDCs with small, intensely labeled cells were characteristic for all CCb areas and were also found in the adjacent areas of the granular layer (GrL) and molecular layer (ML). In most cases, we identified small, intensely labeled astrocyte-like cells and/or microglia

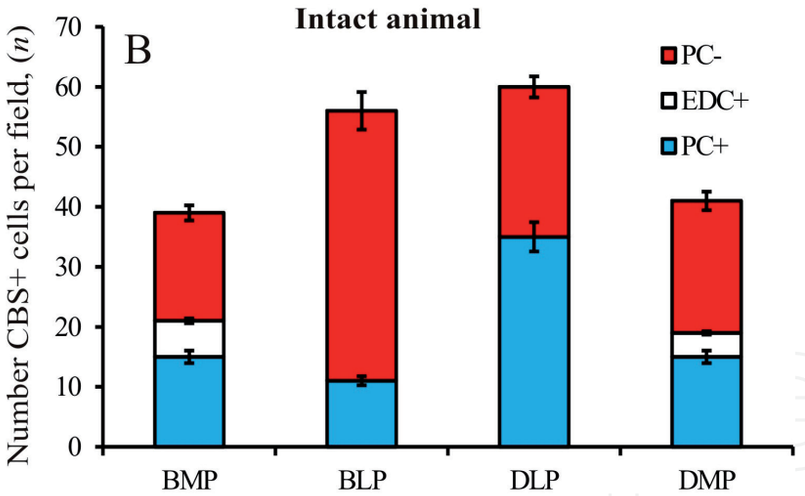

Figure 5.
Density of CBS+ cells in the cerebellum of the intact trout Oncorhynchus mykiss. *Ratio of Purkinje cells (PC) and eurydendroid cells (EDC) in different parts of the cerebellum (mean ± SD). BMP, basomedial part; BLP, basolateral part; DLP, dorsolateral part; DMP, dorsomedial part.*

attached to large EDCs or weakly or moderately labeled PC cells. Such a neuroglial/microglial construction corresponds to the established model of the intercellular relationships, in which glial cells are the main source of hydrogen sulfide for neurons [20, 27]. The relationship with neurons is a prerequisite for the release of H_2S from astrocytes and the increase in intracellular calcium in astrocytes [18]. H_2S activates the transition potential A1 channels (TRPA1) of the transition receptor, leading to an influx of calcium and activation of astrocytes by transmitting calcium waves after neuronal activation [37]. Further, D-serine is released from astrocytes, which subsequently activates the NMDA receptors [3]. Thus, the neuron-glial relationships were identified in CCb and granular eminences of the intact trout, in which H_2S is very likely to be released from the astrocyte-like cells with subsequent activation of the NMDA receptors in neurons. Such features of organization of the H_2S-producing cell complexes in trout correspond to physiological principles established in a mammalian model, according to which the astrocyte-like cells in the fish cerebellum regulate the amount of glutamate produced and its reuptake, preventing the excitotoxicity effects and providing the effective conditions for neurotransmission [2, 3].

After UEI in the trout cerebellum, the number of the CBS+ cells in ML increases dramatically, indicating a sharp activation of the ATP-dependent processes in this area. Along with the increase in the number of immunopositive cells, the number of the cellular CBS+ clusters also increases, both in the surface and deeper parts of ML. There is a reactivation of numerous neurogenic niches, with patterns of the neuron/glial/microglial colocation detected not only in GrL but also in ML. In ML, we found CBS+ fibers, which are absent in intact animals. In the neuron-glial complexes, glial cells are very intensely labeled, which indicates an increase in the CBS activity. We believe that this increase in the number of the CBS+ cells is due to the oxidative stress and the accumulation of ROS, which are neutralized by hydrogen sulfide. The sources of ROS generation in cell include mitochondria, superoxide-producing enzymes, such as xanthine oxide, NADPH oxidase, and hydrogen peroxide-producing enzymes, such as superoxide dismutase [12, 13]. ROS acceptors include antioxidants, such as glutathione, as well as enzymes superoxide dismutase and catalase. The modulation of ion channels and inflammatory and antioxidant transcription factors using H_2S after UEI may play a protective role in reducing edema and inflammation [20, 29, 30]. Similar effects have been reported

in the culture of endothelial cells and hippocampal neurons with the addition of donors of hydrogen sulfide, Na_2S (50 μM) or NaHS (50 and 250 μM), which increase the activity of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase [27]. In vivo experiments indicate that the role of H_2S to control oxidative stress is expressed in many ways, including the glutathione cycle, enzyme activation, and transcription factors related to the redox balance [38]. Astrocytes provide cysteine as an important source of production in the GSH neurons [39]. In experiments on mammalian brain, the incorporation of the hydrogen sulfide donors, NaSH (100 μM), attenuated excitotoxicity and increased intracellular GSH levels in a dose-dependent manner in a primary culture of neurons [40]. Experiments with the inclusion of a metabolic radiolabel have confirmed the incorporation of L-cysteine generated by transulfuration of CBS and CSE into glutathione in astrocytes and neurons [41]. These studies suggest that H_2S may be useful to enhance the mechanism of cellular antioxidant protection in the brain.

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