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EXPERIMENTAL ARTICLES

Modifications of the Expression of Thioredoxins and Superoxide Dismutases in the Rat Hippocampus that Were Induced by Prenatal Hypoxia Are Preserved in Mature Animals

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Abstract—Prenatal hypoxia induces structural and functional disturbances in the brain that develop during the following postnatal ontogeny. Some cognitive and behavioral disorders that are induced by prenatal hypoxia are preserved even in adults. Oxidative stress is a key factor of damage during hypoxia. Hence, the relationship between the pro- and antioxidant systems plays an important role in the development of both pathological and adaptive processes that were induced by hypoxia. Here, using immunocytochemistry we studied the expression of four endogenous protein antioxidants (Cu,Zn-superoxide dismutase, Mn-superoxide dismutase, thioredoxin-1, and thioredoxin-2) in hippocampal neurons of adult rats that were subjected to triple hypoxia at the 14th, 15th, and 16th days of their prenatal development. We found that in a number of cases prenatal hypoxia substantially modified the expression of endogenous antioxidants in hippocampal neurons of rats that achieved an adult age (80–90 days of postnatal ontogeny). The directions of these changes, however, are different for different antioxidants and areas of the hippocampus. It seems that this multidirectionality of changes in the expression of different antioxidants in different areas reflects an inextricable connection between the pathological consequences of prenatal hypoxia and adaptation processes that are induced by this hypoxia.

Keywords: prenatal hypoxia, ontogeny, antioxidants, thioredoxin, superoxide dismutases

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INTRODUCTION

The problem of prenatal hypoxia and its postponed consequences is very challenging from the standpoint of an analysis of mechanisms of the development of different pathological states during ontogeny [1, 2]. Modern studies have shown the development of pathological changes in functional indices of the brain during the postnatal ontogeny in response to the action of severe hypoxia and other extreme factors in the prenatal period [3–10]. However, changes that are induced by prenatal hypoxia in some cases may have not only a pathological but also an adaptive character. This conclusion is confirmed by the results of our behavioral experiments [3–5, 8, 9]. Hypoxia plays an important role in the genesis of the disturbances of the development of an organism [11–13]. It is one of the most frequent causes of death of a fetus in the antenatal period and largely determines the frequency of psychic and physical diseases in postnatal ontogeny [14, 15].

Prenatal hypoxia may induce a complex of disturbances in the structure and function of the fetal brain. It seems that one of the leading factors is damage to neurons. It has also been hypothesized that hypoxia acts on neurons during the prenatal period, resulting in the formation of stable changes in these cells that are preserved after birth. In addition, hypoxia, by acting on the fetal brain, may influence not only neurons but also cause stable changes in auxiliary cells, viz., the gliocytes. Hypoxia results in the development of acidosis and oxidative stress, as well as an increase in the activity of the excitatory glutamatergic system. In addition, it is also associated with the activation of endogenous phospholipases and the decay of membrane phospholipids, as well as an increase in the fluidity of membranes and their permeability, which, as a consequence, causes the loss of K^+ ions and an overload with Na^+ and Ca^{2+} ions [15–17].

A special role in the damage of the brain neurons in hypoxia is played by oxidative stress that is related to hyperproduction of reactive oxygen species (ROS) [18, 19]. Increased vulnerability to oxidative stress of

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the immature brain, as compared to the mature one, has two causes: first, the immature brain is characterized by low activity of the systems of antioxidant defense; second, it has a high content of free ions of iron. The antioxidant defense of cells is provided by different non-enzymatic and enzymatic factors, including thioredoxins (Trxs) and superoxide dismutases (SODs), which include both cytosolic (Cu,Zn-SOD, Trx-1) and mitochondrial (Mn-SOD, Trx-2) forms. An increase in the level of hydrogen peroxide (H_2O_2) in the immature brain has a larger damaging effect due to the higher content of free iron ions as compared to the mature brain; hence, intensification occurs of Fenton's reaction, which is a chemical reaction of the non-enzymatic formation of highly reactive hydroxyl radicals from hydrogen peroxide.

Direct action of oxygen insufficiency and hyperproduction of free radicals is an important but, possibly, not the only factor of the damage of brain cells during and after severe hypoxia. Another important factor of damage may be disturbance of the balance of hormones and neuromediators that interact with cell receptors, including an elevated level of stress hormones in the blood of the fetus which, in turn, may be a consequence of the action of stress responses of the maternal body. The relationship between these two simultaneously acting mechanisms of action is under discussion for the mature and, especially, for the embryonic brain because anaerobic processes play a more substantial role in the metabolism of the latter than in the mature brain [20].

The effect of hypoxia strongly depends on the period of prenatal development when it occurred [3, 4, 10, 21, 22]. In our previous studies, we showed that hypoxia on the 14–16th days (and to a lesser extent, hypoxia on the 17–19th days) of prenatal development results in disturbances of signal transduction and, as a consequence, to long-term changes in a number of behavioral characteristics and the ability to learn [3–5, 8–10, 23, 24].

Here, we studied the postponed consequences of severe hypoxia on the 14–16th days of the prenatal development on the expression of antioxidant proteins (thioredoxins and superoxide dismutases) in neurons of the hippocampus of rats that achieved adult age.

MATERIALS AND METHODS

Pregnant Wistar females, which were grown in the vivarium of the Pavlov Institute of Physiology of the Russian Academy of Sciences, were subjected to severe hypobaric hypoxia three times for 3 h per day on the 14th, 15th, and 16th days of gestation at a pressure of 180 mmHg, which corresponds to elevation to an altitude of 11000 meters or a decrease in the oxygen content to 5% of normobaric hypoxia. Hypobaric hypoxia was induced in a flowing decompression chamber at a temperature of 20–25°C [25] following the standard protocol [26–27].

The altitude of 11000 m was chosen as critical because in previous experiments the death rate of adult male rats after 3-h stay in the decompression chamber was 50% [28]. The death rate of pregnant females was substantially lower, approximately 15% on average.

Rats that were subjected to hypoxia on the 14–16th days of their prenatal development were examined on the 80–90th days of their postnatal life, i.e., after reaching their adult state. The study was performed with four animals that were subjected to prenatal hypoxia and four control animals of the same age. The experiments were performed in accordance with the Directives of the Council of the European Commission (89/609/EEC) on the use of animals for experimental studies and Directive of the European Union 2010/63/EU on experiments with animals. The protocols of the experiments were approved by the commission on the humane treatment of animals of the Pavlov Institute of Physiology of the Russian Academy of Sciences.

The perfusion and decapitation of the animals, as well as fixation and storage of the brain samples, have been described in detail in previous works [26]. For complete cryoprotection, the brain samples were incubated before analysis in a mixture (1 : 1) of 20% sucrose in PBS and Tissue-Tek for 2 days. The samples were then frozen in Tissue-Tek® O.C.T™ Compound (Sakura Finetek Europe B.V.), and 11 μm frontal brain slices were immediately made using a cryocut at a temperature of –20°C at the level of the hippocampus and baso-lateral amygdala, i.e., approximately 2.8 mm from the bregma [29].

The level of expression of antioxidants was determined in the hippocampal structures by immunocytochemical analysis using the standard avidin–biotin method. The slices were placed on poly-L-lysine-coated (Sigma) slides and preincubated in a 1% solution of bovine serum albumin (BSA, Boehringer Mannheim GmbH) for 15 min. Each of the four groups of slices were then incubated overnight with one of the primary polyclonal affinity-purified rabbit antibodies (dissolved in a phosphate buffer PBS that contained 1% BSA and 0.3% Triton X-100 at +4°C) to one of the following antioxidant proteins: (1) mouse cytosolic thioredoxin-1 (antibodies were kindly provided by Yumika Nishinaka and Yunji Yodoi, Department of biological responses of the Institute of Viral Research in the Kyoto University, Japan; 1 : 500) [30], (2) rat mitochondrial thioredoxin-2 (the antibodies were kindly provided by Yannis Spirau, Department of Biological Sciences of the Carolinska Institute, Sweden; 1 : 250) [31]; (3) human mitochondrial Mn-SOD (StressGen Biotechnologies Corp; dilution 1 : 2000); (4) bovine cytosolic Cu,Zn-SOD (the antibodies were kindly provided by Ling-Yi L. Chang, Department of medicine of National Center for medicine and research, Denver, Colorado, United States; 1 : 200) [32]. The sequence of further washes, incubation with goat anti-rabbit secondary antibodies and the avidin–

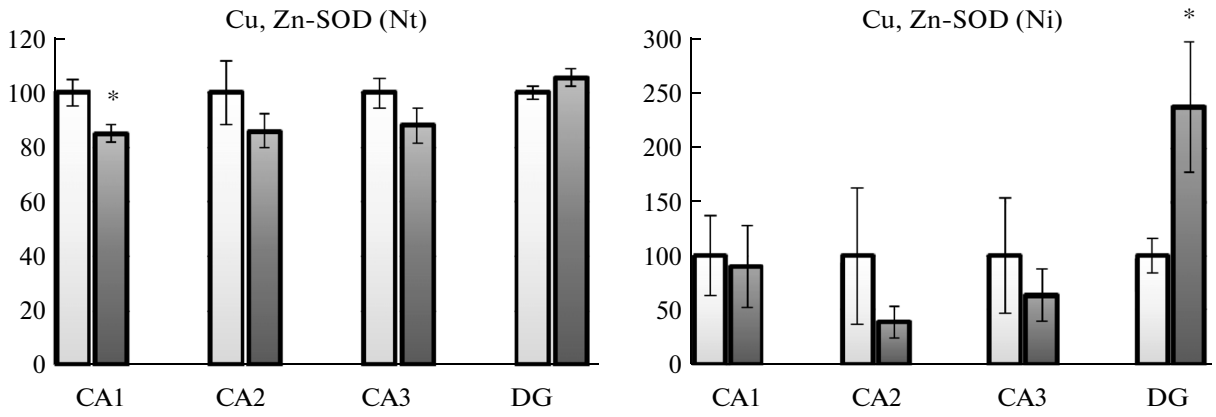

Fig. 1. The total number of Cu,Zn-SOD-expressing neurons (Nt) and the number of neurons that intensely express Cu,Zn-SOD (Ni) in different areas of the hippocampus of adult rats (80–90th days after birth) that were subjected to threefold hypoxia on the 14–16th days of prenatal development expressed as a percentage of control. CA1, CA2, CA3, and DG are areas of the hippocampus. *, significantly different ($p < 0.05$) as compared to the control.

biotin complex, visualization of the immune reaction using diaminobenzidine, dehydration, and the mounting of slices in balm was in accord with that in previous studies [26].

Quantitative analysis of immunoreactive neurons was performed using a system of a microscope (Nikon-Microphot-FXA), SensiCam digital camera (PCO Computer Optics GmbH), and an IBM PC computer with Image-Pro Plus (Media Cybernetics) and Morphix software. The level of expression of the four studied antioxidant proteins was determined in CA1, CA2, CA3, and dentate gyrus (DG) neurons of the hippocampus. Analysis of the images was performed in a 500 μm region using Morphix software, which we developed for this analysis [33]. The level of immunoreactivity of neurons to each of the antioxidant proteins in the studied brain areas was evaluated in six slices from each brain. The intensity of staining in the digital images was expressed in conventional units of optical density from 0 (absolute white) to 100 (absolute black). Immunoreactive cells were subdivided into two conditional classes: weakly stained (1–10 conventional units above the background) and intensely stained (over 10 units above the background).

The level of immunoreactivity was evaluated using two criteria: the total number of immunoreactive cells, expressed as a percentage of the control (Nt) and the number of intensely stained cells, which is also expressed as a percentage of the control (Ni). Statistical analysis of the data was performed using one-factor dispersion analysis (ANOVA) and the results were analyzed using the non-parametric Mann–Whitney-Wilcoxon U-test.

RESULTS

It was shown that hypoxia during prenatal period modified, in some cases significantly, the expression of the studied antioxidant proteins in the hippocampal neurons of rats that reached adult age (80–90 days of postnatal ontogeny).

The total number of Cu,Zn-SOD-positive neurons (Nt) in rats that were subjected to prenatal hypoxia was 85 ± 3% in CA1, 86 ± 6% in CA2, 88 ± 6% in CA3, and 106 ± 3% in the DG, as compared to the control (Fig. 1). The number of intensely expressing Cu,Zn-SOD neurons (Ni) was 90 ± 38% in CA1, 64 ± 24% in CA3, and 238 ± 60% in the DG (Fig. 1). Thus, it may be seen that the expression of Cu,Zn-SOD in neurons of rats that were subjected to prenatal hypoxia has a trend to a decrease in CA1, CA2, and CA3, but had a trend to an increase in the DG, as compared to the control animals. In some cases, Nt in CA1 ($p = 0.03$) and Ni in the DG ($p = 0.04$), the changes were significant.

The total number of Mn-SOD-positive neurons (Nt) in rats that were subjected to prenatal hypoxia was 91 ± 5% in CA1, 93 ± 5% in CA2, 81 ± 3% in CA3, and 110 ± 5% in the DG (Fig. 2). The number of intensely stained Mn-SOD-positive neurons (Ni) was 62 ± 14% in CA1, 63 ± 11% in CA2, 78 ± 15% in CA3, and 244 ± 63% in the DG (Fig. 2). Thus, it is possible to see the same direction of changes as in the case of Cu,Zn-SOD: the expression of Mn-SOD in neurons of rats that were subjected to prenatal hypoxia also had a trend to a decrease in CA1, CA2, and CA3 but also a trend to an increase in the DG, as compared to the control. These changes were significant for Ni in CA2 ($p = 0.045$), Nt in CA3 ($p = 0.01$), and Ni in the DG ($p = 0.04$).

The total number of Trx-1-positive neurons (Nt) in rats that were subjected to prenatal hypoxia had a trend to decrease in all hippocampal areas and comprised, as compared to the control, 75 ± 4% in CA1, 92 ± 6% in CA2, 83 ± 6% in CA3, and 84 ± 7% in the DG (Fig. 3). This decrease was significant in CA1 ($p = 0.00003$) and, in addition, it was close to significant in the DG ($p = 0.058$). The number of intensely express-

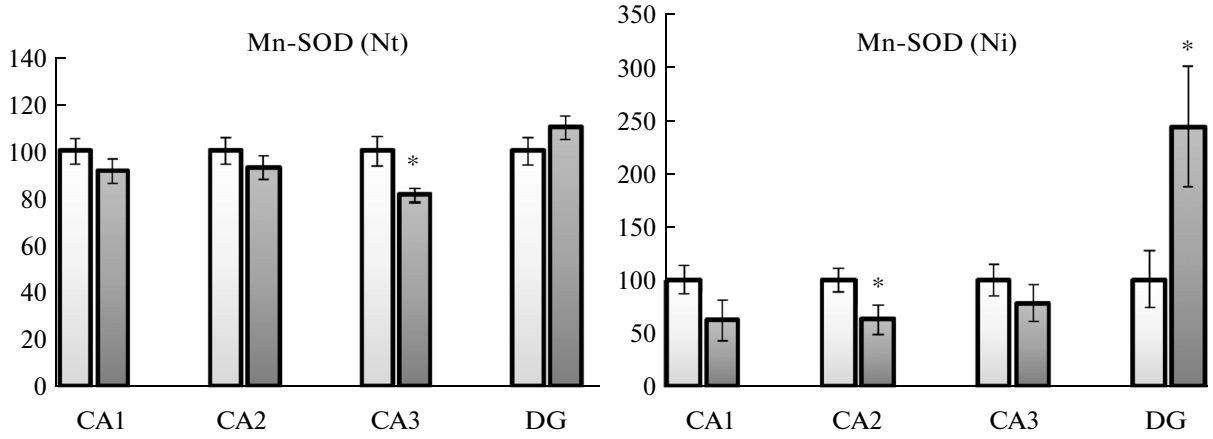

Fig. 2. The total number of Mn-SOD-expressing neurons (Nt) and the number of neurons that strongly express Mn-SOD (Ni) in different areas of the hippocampus of adult rats (80–90th days after birth) that were subjected to threefold hypoxia on the 14–16th days of prenatal development expressed as a percentage of the control. CA1, CA2, CA3, and DG are the areas of the hippocampus. *, significantly different ($p < 0.05$) as compared to the control.

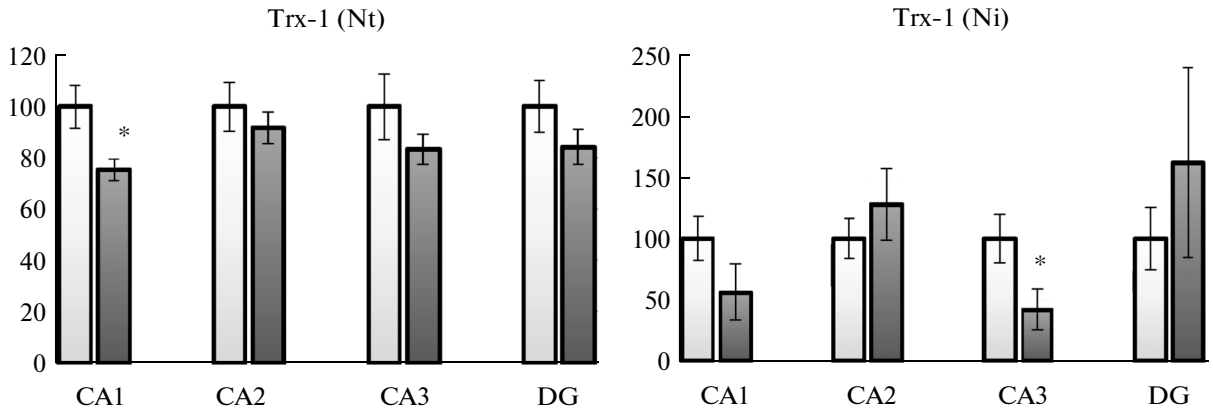

Fig. 3. The total number of thioredoxin-1-expressing neurons (Nt) and the number of neurons that intensely express thioredoxin-1 (Ni) in different areas of the hippocampus of adult rats (80–90th days after birth) that were subjected to threefold hypoxia on the 14–16th days of prenatal development expressed as a percentage of the control. CA1, CA2, CA3, and DG are areas of the hippocampus. *, significantly different ($p < 0.05$) as compared to the control.

ing Trx-1 neurons (Ni) in different areas had different directions of changes; it was 56 ± 22% in CA1, 128 ± 30% in CA2, 43 ± 17% in CA3, and 162 ± 78% in the DG (Fig. 3), as compared to the control; however, only in CA3 were these changes in Ni significant ($p = 0.04$).

The total number of Trx-2-positive neurons (Nt) in rats that were subjected to prenatal hypoxia was 116 ± 11% in CA1, 93 ± 12% in CA2, 70 ± 4% in CA3, and 106 ± 9% DG (Fig. 4), as compared to the control. Thus, a significant decrease ($p = 0.03$) in Nt of Trx-2-positive cells occurred only in CA3, whereas in other hippocampal areas changes in the total number of Trx-2-positive neurons were insignificant. The number of neurons that intensely express Trx-2 (Ni) was 200 ± 90% in CA1, 122 ± 32% in CA2, 720 ± 215% in CA3, and 1000 ± 289% in the DG (Fig. 4), as compared to the control (Fig. 4). Thus, in all of the studied areas of the hippocampus, the number of intensely expressing Trx-2 neurons to larger or lesser extent had a trend to an increase as compared to the control; in CA3 ($p = 0.04$) and the DG ($p = 0.014$) this increase was significant.

Summarizing the data, the prenatal hypoxia resulted in long-term modifications of the status of the endogenous antioxidant systems of the brain during their postnatal development, including in the adult state. These modifications include changes in the background level of the expression of antioxidant proteins. However, the directions of these changes in animals that were subjected to prenatal hypoxia were different for different antioxidants in different areas of the hippocampus.

DISCUSSION

The data we obtained indicate that prenatal hypoxia induces a stable modification of the "basal"

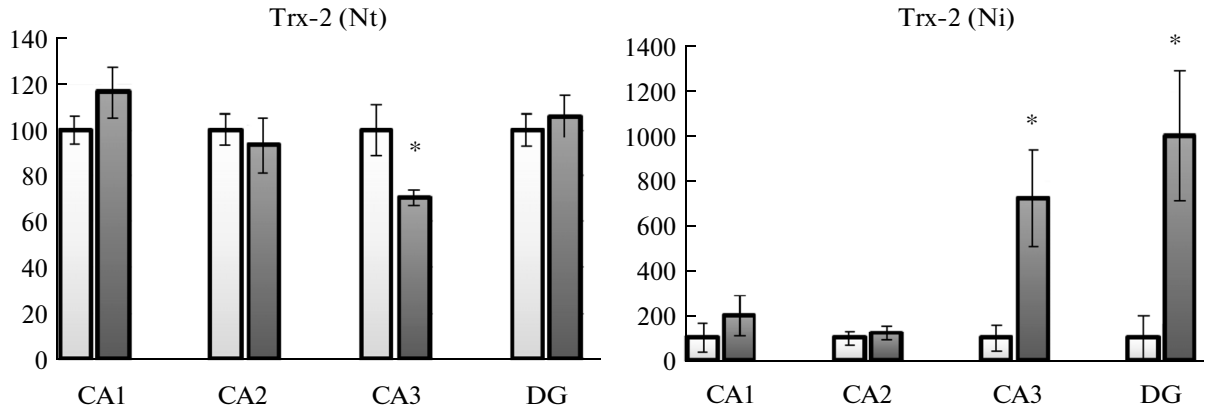

Fig. 4. The total number of thioredoxin-2-expressing neurons (Nt) and number of neurons that intensely express thioredoxin-2 (Ni) in different areas of the hippocampus of adult rats (80–90th days after birth) that were subjected to threefold hypoxia on the 14–16th days of prenatal development expressed as a percentage of control. CA1, CA2, CA3, and DG are areas of the hippocampus. *, significantly different ($p < 0.05$) as compared to the control.

level of expression of antioxidants that seems to influence the sensitivity of brain cells to ROS-mediated signaling transduction and, hence, to alter the character of their response to events that are related to the development of oxidative stress.

We note that both SODs had a general trend to a decrease in expression in CA1, CA2, and CA3 and to an increase in the DG in the animals that were subjected to prenatal hypoxia. This result may be explained by the different periods of maturation of these areas in prenatal ontogeny and, hence, a difference in periods that are critical with respect to the action of hypoxia.

We note that long-term changes in the background level of antioxidants in rats that were subjected to prenatal hypoxia influenced both of the studied parameters: both the number of intensely expressing neurons and the total number of neurons that express the corresponding antioxidants. The influence of prenatal hypoxia on the expression of antioxidants differs from its influence on the expression of inositol-1,4,5-triphosphate receptors of the first type (IP3R1); the changes in the intensity of expression of these receptors is weakly related to the number of expressing cells [34].

The character and direction of changes in Nt and Ni did not always coincide. For example, Nt of Cu,Zn-SOD significantly decreased in the CA1 area and in other areas weakly differed from the control, whereas Ni in CA1, in contrast, did not differ from the control but in CA2 decreased by a factor of 2 and increased by more than twofold in the DG. Changes in Nt and Ni of Mn-SOD were also significant in different areas of the hippocampus; in particular, in the DG Nt it practically did not differ from the control, whereas Ni increased by more than twofold. Presumably, the different characters of the changes in the two indices of the expression (the total number of immunoreactive cells and the number of intensely stained cells) reflects differences in the mechanisms of the regulation of the expression of the studied endogenous antioxidants in different areas of the hippocampus; however, current data do not allow one to propose the exact difference in the mechanisms. Nevertheless, both SODs and Trx-1 were not characterized by significant oppositely directed changes in Nt and Ni. An opposite directionality of changes in two indices of level of expression was noted only for Trx-2 in the CA3 area, where more than a sevenfold increase in Ni was associated with a significant decrease in Nt. In other words, regulation of the same antioxidant protein in the same area of the hippocampus in different neurons had the opposite directionality: in one group of neurons expression of Trx-2 decreased to or even below the level of its expression in the surrounding extracellular space (this caused a decrease in Ni), whereas in other neurons the optical density, which reflects the expression of Trx-2, was 10 arbitrary units above this level. Earlier, in a series of experiments with the subjection of adult rats to hypoxia, the opposite directionality of changes in the indices of expression was observed as a specific trait of Trx-2 that is not typical of other studied antioxidants [35–39]. The opposite direction of the changes in the total number of antioxidant-expressing neurons and neurons with a high level of its expression presumably reflects the indivisible interrelationship of adaptive and disadaptive processes that were induced by prenatal hypoxia in the studied model.

We note that the absolute number of cells that intensely expressed the studied antioxidants is small compared to the total number of immunoreactive cells; hence, the scatter of Ni values is substantially higher than the scatter of the Nt index which, however, does not depreciate the importance of this index.

It is interesting to compare the results of this study with previous data on the effects of hypoxia that occurred at different periods of prenatal development and adult age.

Previously, the effect of hypoxia on the expression of Trx-1 in the hippocampus of rats was studied in a completely identical experimental model (180 mmHg, three times for 3 h at the 14th, 15th, and 16th days of prenatal ontogeny) not only after the achievement of the adult state but also at different stages of postnatal ontogeny (the 3rd and 14th days of postnatal life) [26]. It was shown that hypoxia on the 14–16th days of prenatal ontogeny substantially influences the state of the antioxidant systems in the brain neurons from the first days of life after birth and until the adult state. This modification has a complex character and the direction of changes in the expression depends on the studied area of the hippocampus and the chosen time point in the postnatal ontogeny, as well as the antioxidant that is studied.

It is possible to compare the data on the effect of prenatal hypoxia with previously obtained data on the effects of different modes of hypoxia that were endured at an adult age on the antioxidant systems in rat neurons. Until recently, an increase in the level of antioxidants in response to hypoxia has always been considered as a marker of adaptive processes and its decrease, as a marker of disadaptive pathological states. Our studies showed that the real situation is more complex and ambiguous. On the one hand, an increase in the expression of endogenous antioxidants is a defense response but is not always sufficient (for example, in the case of non-preconditioned severe hypoxia) for the prevention of the mass apoptosis of neurons [35–39]. On the other hand, moderate hypoxia, which does not induce pathological responses and, in contrast, has a pronounced adaptogenic character, may be accompanied by a considerable decrease in the level of expression of antioxidants in neurons [27, 40–47]. This decrease may be important for the enhancement of ROS-mediated signaling transduction and the formation of tolerance to the following severe hypobaric actions. Taking these results into account, care should be taken when interpreting the data that were obtained in our study. In particular, the fact should be taken into account that a moderate decrease in the level of expression of endogenous antioxidants as a consequence of endured prenatal hypoxia is not necessarily an indication of pathology and disadaptation but, by analogy with preconditioning, may be a manifestation of a special adaptive response.

However, the paradoxical effect of preconditioning, which is associated with a decrease in the "background" level of antioxidants, does not fit well with the known "classical" cases, when an increase in the level of antioxidants during and after hypoxia correlates with the adaptation and survival of cells, and a decrease leads to irreversible disruption of the mechanisms of adaptation and cell death. One known example of this situation is the opposite directionality of processes in the infarction core and penumbra during severe focal ischemia [48–50].

These facts form the concept of the integration and continuity of the pathological and adaptive processes that are induced by any stress of sufficient strength. We note that only relatively intense stresses, which are sometimes referred to in the literature as "sublethal," have a protective preconditioning effect, whereas weak stresses have no preconditioning neuroprotective effect. Moreover, the processes of adaptation and pathology are frequently developed via the same molecular mechanisms. For example, a cascade of caspases is triggered during preconditioning, training, and learning [51–54] but also during the initiation of delayed cell death [55–58]. The same holds for the modification of the status of the antioxidant system after prenatal hypoxia, which we showed here. The results of behavioral experiments [3–5, 8, 9] indicate that the response of rats that were subjected to prenatal hypoxia under the same experimental conditions to different environmental influences may be more adaptive in some cases and less adaptive in other cases, as compared to the responses of intact animals. In particular, it was shown that if rats that endured prenatal hypoxia were subjected to the repeated action of severe hypoxia at an adult age, their viability was higher than in rats that were not subjected to prenatal hypoxia. This means that prenatal hypoxia, as mentioned, is an analogue of hypoxic preconditioning; however, the effect of preconditioning is short-term (a day or, presumably, several days after the last "conditioning"), whereas the effect of prenatal hypoxia on the viability of animals and the basal level of expression of antioxidants is maintained practically during the entire postnatal development, including at an adult age. However, in rats that were subjected to prenatal hypoxia the repeated action of severe hypobaric hypoxia at an adult age induced a substantial aggravation of cognitive functions, including learning in the Morris maze, as compared not only with control intact animals but also with "untrained" rats that were subjected to severe hypoxia for the first time (Vataeva et al., unpublished data).

It seems that the opposite directionality of the changes in the levels of different antioxidants in different hippocampal areas also reflects the indissociable unity and struggle of opposite processes, viz., the developing pathological consequences of prenatal hypoxia and adaptive compensatory responses that are induced.

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