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EXPERIMENTAL ARTICLES

The Acetylation of Histone H3 at Lys24 Is Accompanied by Delayed Expression of Neuroprotective Proteins Bcl-2 and BDNF in the Neocortex of Rats Exposed to Severe Hypoxia: the Effect of Postconditioning

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Abstract—Recent studies imply that epigenetic mechanisms may play a key role in the pathogenesis of severe neurological diseases. We have previously shown that acetylation of histone H3 at Lys 24 (H3acK24) is involved in the formation of acute adaptive response of the brain to hypoxia. Here, using an immunohistochemical technique, we compared the effects of severe hypoxia and severe hypoxia followed by neuroprotective postconditioning using mild hypoxia on the expression of the antiapoptotic Bcl-2 protein, neurotrophin BDNF, and the level of H3acK24 in the neocortex of rats in delayed period (4 days). The delayed upregulation of Bcl-2, BDNF, and H3acK24 was observed in the sensorimotor cortex of rats subjected to severe hypoxia, suggesting late induction of the pro-adaptive neuronal processes. Postconditioning by three episodes of mild hypoxia returned the levels of H3acK24 to the control level and partially abolished the upregulation of Bcl-2 and BDNF. The findings demonstrate an important role of H3 acetylation at Lys24 in the regulation of apoptosis and neuroplasticity in response to hypoxia.

Keywords: severe hypoxia, postconditioning, brain, histone acetylation (Lys24), Bcl-2, BDNF
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INTRODUCTION

One of the important fundamental problems of neurobiology is the elucidation of the endogenous mechanisms of the resistance of the mammalian brain to injurious impacts, namely, to the severe forms of hypoxia and ischemia. A large amount of data demonstrate that molecular and cellular mechanisms that underlie impairments of animal behavior and learning ability are involved in the processes of neuronal damage under severe hypoxic conditions. Correction of these mechanisms to induce neuroprotection and restoration of brain functioning after severe pathological impacts is an important topic in neurology and neurorehabilitation. The problem is even more complex, since the existing drugs demonstrate insufficient effectiveness in this respect [1]. However, it has been convincingly shown that a non-pharmaceutical method, that is, mild triple hypobaric hypoxia in the postconditioning (PostC) regime attenuates the impairments in brain functioning induced by severe hypobaric hypoxia (SH) [2]. An important role in the neuroprotective effect of PostC was attributed to stimulation of expression of anti-apoptotic factors (Bcl-2), neurotrophins (BDNF), and modification in some transcriptional factors, namely HIF-1 (hypoxia induced factor), in the hippocampus [3, 4]. The protective effect of mild hypoxia in individuals after SH occurs as prevention of cell death and normalization of lipid peroxidation (LP) and behavioral patterns [5, 6].

In recent years, many studies have been focused on epigenetic mechanisms in the regulation of neuronal response to various pathological impacts of the environment [7–9], including hypoxia and ischemia [10–16]. These studies are mainly related to the activities of the enzymes that maintain the modification of the epigenetic code, such as histone deacetilases and acetyl tranferases, as well as DNA-methyl transferases [10, 17–19]. The key role of genome-dependent processes in mechanisms of brain damage after severe hypoxia/ischemia is well documented [20–23]. In turn, expression of the genes, including adaptive ones, is activated by transcription factors that bind to definite elements within promoter gene regions; however,

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access to these regions depends on epigenetic status and, accordingly, is controlled by epigenetic mechanisms. Likewise, it has been shown that the hypoxia-induced factor HIF-1 interacts with acetyl transferases p300, CBP [24], PCAF, SRC-3 [25–27], and with deacetylases [28], which indicate an important role of acetylation in the brain response to adaptogenic hypoxia in pre- and post-conditioning regimes. Recently, we demonstrated a change in the amount of the H3 histone acetylated at lysine positions 9 and 24 in response to preconditioning with mild hypoxia [29, 30]. Thus, it has been shown that acetylation of H3 histone at lysine 24 is involved in the maintenance of the neuroprotective effect of hypoxic preconditioning via potentiated activation of the main adaptive genes [30].

The aim of this study was to perform a comparative analysis of the H3 histone acetylation at lysine 24 and changes in the expression of the antiapoptotic protein Bcl-2 and the neurotrophic factor BDNF in the neocortex of rats exposed to severe hypoxia or severe hypoxia followed by hypoxic post-conditioning.

MATERIALS AND METHODS

The animals for the study were taken from the Collection of laboratory mammals of different taxons biocollection of the Pavlov Institute for Physiology of the Russian Academy of Sciences, which was sponsored by the program for bioresource collections of FASO, Russia. The experiments were performed with male Wistar rats at ages of 80–90 days that weighed 230–260 g. To obtain SH conditions the rats were placed in a flow-type pressure chamber at a temperature of 20–25°C, and the pressure was gradually decreased to 180 mm Hg (5% O_2 for 3 h). The mortality of rats in the chamber was 50% [31].

The PostC procedure was performed by a triple 2-hr exposure of rats to mild hypobaric hypoxia (360 mm Hg, 10% O_2). Mild hypoxia sessions were performed starting 24 hrs from the SH with 24-h intervals between the sessions. During the entire experiment the rats were under an artificial light–dark cycle (12 : 12 h) at a temperature of 20–23°C and with free access to food and water.

The animals were decapitated 4 days after the SH and, accordingly, one day after the last PostC session (the SH + 3PostC group). The animals of the control group were sacrificed in a parallel. For each group, $n = 6$. After decapitation, the skull was opened, the brain was removed, the cerebellum was separated, and the brain put into a fixative. The brain samples were then processed according to the standard histologic protocol: fixation in the FineFix molecular fixative mixture (28 mL of fixative + 72 mL of 96° ethanol, Milestone, Italy) for 24 hrs at +4°C. The samples were then washed under running water for 2 hrs, dehydrated in ethanol of increasing grades (50° → 70° → 80° → 96° → 96°, 1 h each) and left in butanol overnight. The samples were then processed in two vials of xylene (30–40 min), placed in paraffin (two batches for 1 hr in each) in a thermostat at +56°C, and paraffin blocks were prepared. Serial coronal 7 μm-thick brain sections were cut at the level between –2.80 and –3.60 mm caudal to the bregma on a rotation microtome (Reichert, Austria). The resulting sections were mounted on polylysine-coated glass slides.

The sections were deparaffinized in xylene (two batches, 5 min each) and rehydrated in alcohol (96° → 96° → 96° → 70°, 5 min each). The immunohistochemical method was used to assess the level of H3 Lys24 acetylation (H3acK24), as well as expression of Bcl-2 and BDNF. Briefly, we proceeded through the following steps: (1) incubation with polyclonal rabbit antibodies (Santa Cruz Biotechnology, Inc, United States, dissolved in PBS 1 : 50 – 1 : 200) to H3acK24 (sc-34262), Bcl-2 (sc-783) or BDNF (sc-20981); (2) incubation with secondary biotinylated antibodies (Vectastain ABC kit, Santa Cruz Biotechnology, Inc, United States, dissolved in PBS 1 : 200); (3) incubation with a complex of avidin and biotinylated peroxidase (ABC, Vector Laboratories, Inc, United States, the A and B reagents were dissolved at 1 : 100); and (4) visualization of the reaction with a diaminodenzidine kit (DAB substrate kit for peroxidase, Vector Laboratories, Inc, United States, buffer (one droplet), 3,3'-diaminobenzidine (two droplets), H_2O_2 (one droplet) per 2.5 mL of H_2O).

The sensorimotor cortex is among the brain regions that are most sensitive to the harmful effects of hypoxia. In addition, there are vast data that demonstrate the difference in the response to hypoxia between the neocortical layers II and V, which determined the choice of these structures for our current research.

Analysis of the samples was performed using a morphometric setting that consisted of a Jenaval light microscope (Carl Zeiss, Germany), a Baumer CX05c digital camera (Baumer Optronic, Germany), and an IBM personal computer with VideoTest master Morfologiya software (developed by Video Test Ltd., Russia). The cells were counted within neocortical layers II and V inside the 460 × 340 μm vision field (40× objective). Using optical density, all immunopositive cells were divided into two classes: weakly and strongly stained; the numbers of immunopositive and strongly stained cells were analyzed.

A total of four histological samples were analyzed for each animal, and the resulting values were averaged across all fields of vision of a certain structure. The results were analyzed using Statistica 7.0 software, Stat Soft, Inc., and Microsoft Excel 2003, using the nonparametric Mann–Whitney U-test. The changes were considered significant at $p < 0.05$. All results were presented as the mean ± SEM and shown as a percentage of the control value.

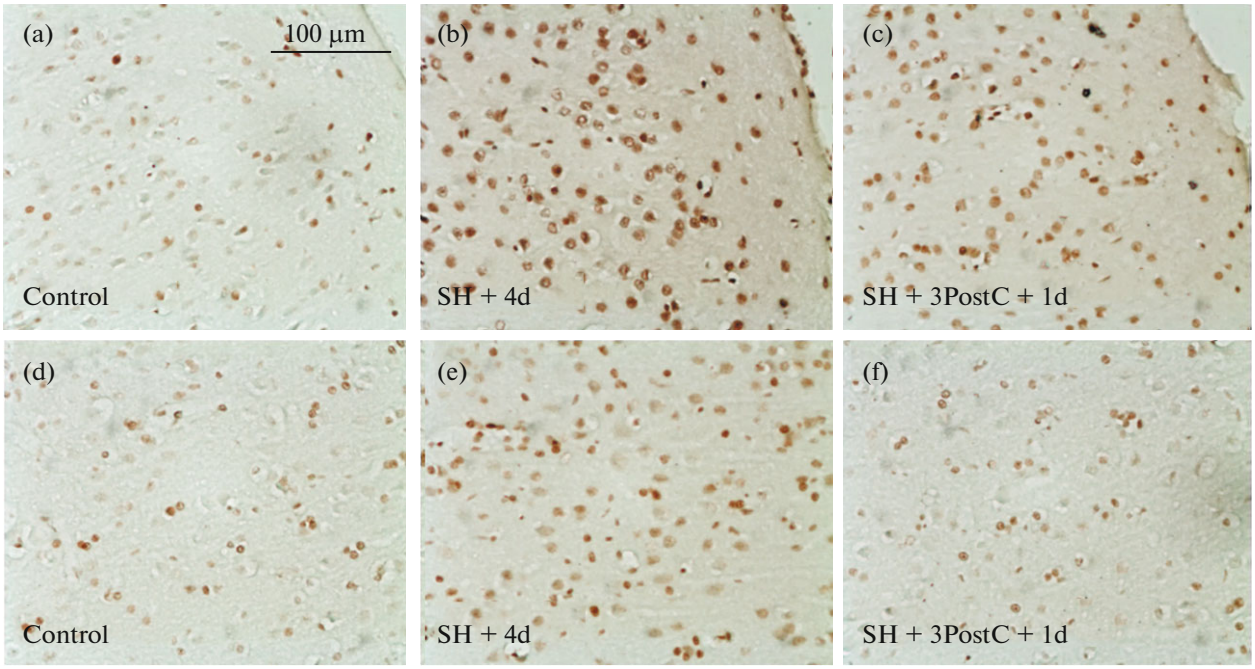

Fig. 1. Microimages (40×) of neocortical layer II (a, b, c) and V (d, e, f) of the control rats (control, a, d), rats on the 4th day after severe hypoxia (SH + 4d, b, e), and on the next day after severe hypoxia with triple postconditioning with mild hypobaric hypoxia (SH + 3PostC + 1d, c, f). Immunohistochemical staining for H3acK24. Scale bar 100 μm.

RESULTS

In separate series of experiments, we analyzed the influence of SH or the consecutive influence of SH and triple mild hypobaric PostC hypoxia on acetylation of the H3 histone at Lys24, the contents of the anti-apoptotic protein Bcl-2, and the BDNF neurotrophin in the cells of neocortical layers II and V.

The influence of SH and SH + 3PostC on the level of H3acK24 in the rat neocortex. We found specific changes in the content of the H3 histone acetylated at lysine 24 (H3acK24) in the cells of the rat neocortex 4 days after SH and on the next day after the last session of PostC (Figs. 1 and 2). Up to the 4th day after the impact, SH led to a negligible increase in the number of H3acK24-immunopositive cells in neocortical layers II and V (up to 117% of the control value) (Fig. 1) and to a significant increase in the number of strongly stained H3acK24-immunopositive cells (up to 200% and 158.9% in neocortical layers II and V, respectively) (Figs. 2a, 2b).

After SH, exposure to three sessions of mild hypobaric hypoxia (PostC regime) resulted in reduction of the number of both H3acK24 immunopositive and intensively stained cells down to the control level in neocortical layers II and V (Figs. 1 and 2a, 2b).

The influence of SH and SH + 3PostC on the anti-apoptotic protein Bcl-2 expression pattern in the rat neocortex. The representative microimages of Bcl-2 immunopositive cells in the neocortex (layers II and V) of the control rats and rats exposed to SH and SH + 3 PostC are shown in Fig. 3. The total number of Bcl-2-immunopositive cells increased on the 4th day after SH in layer II (but not in layer V) by 29.1% compared to the control. The number of strongly stained Bcl-2 cells was essentially increased in both examined brain areas (to 376 and 191% of the control level in layers II and V, respectively, Figs. 3a, 3b).

At the same time point, animals of the PostC group demonstrated a decrease, both in the total number and the number of strongly stained Bcl-2 cells, while in neocortical layer II their number remained above the control value (120 and 233%, respectively) and in layer V it decreased below the control level (23.6 and 65.6%, respectively) (Figs. 3, 4a, 4b).

The influence of SH and SH + 3PostC on the expression pattern of the proadaptive BDNF protein in the rat neocortex. On the 4th day after SH, the number of BDNF-immunopositive and strongly stained cells increased (to 142.3 and 214.2% of the control value in layer II and to 10.6 and 120.9% of the control level in layer V, respectively) (Figs. 5, 6a, 6b).

Mild hypoxia in the PostC regime led to normalization of BDNF expression in neocortical layer V but not layer II, where the number of strongly stained cells greatly exceeded the number in the control group (735.7% compared to the control) (Figs. 5 and 6a, 6b).

DISCUSSION

We described the characteristic features of severe hypobaric hypoxia at a postponed period and its effect in

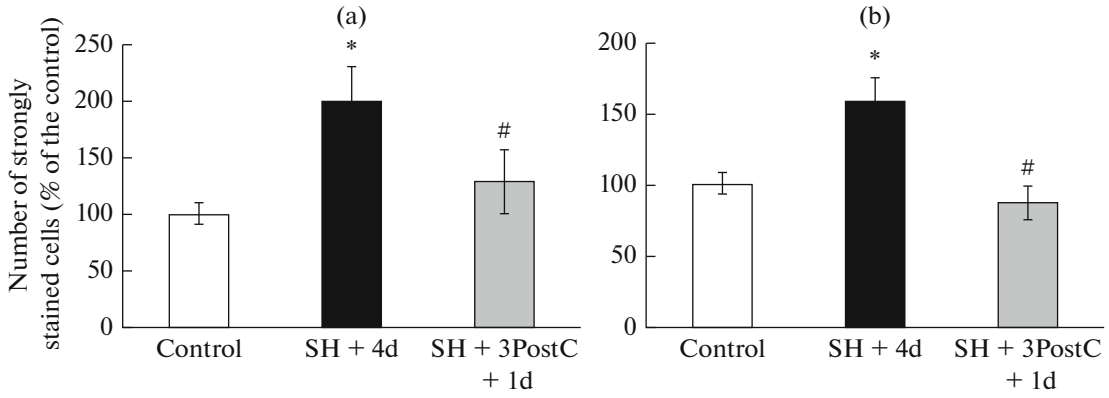

Fig. 2. The number cells that were strongly immunopositive to H3acK24 in neocortical levels II (a) and V (b) of the control rats (Control), 4 days after severe hypoxia (SH + 4d), and on the next day after severe hypoxia in combination with triple postconditioning with mild hypobaric hypoxia (SH + 3PostC + 1d). X-axis: experimental groups; Y-axis: the number of strongly positive cells presented as the percentage of the control group. *, significant difference from control, $p \le 0.05$; #, significant difference from severe hypoxia, $p \le 0.05$.

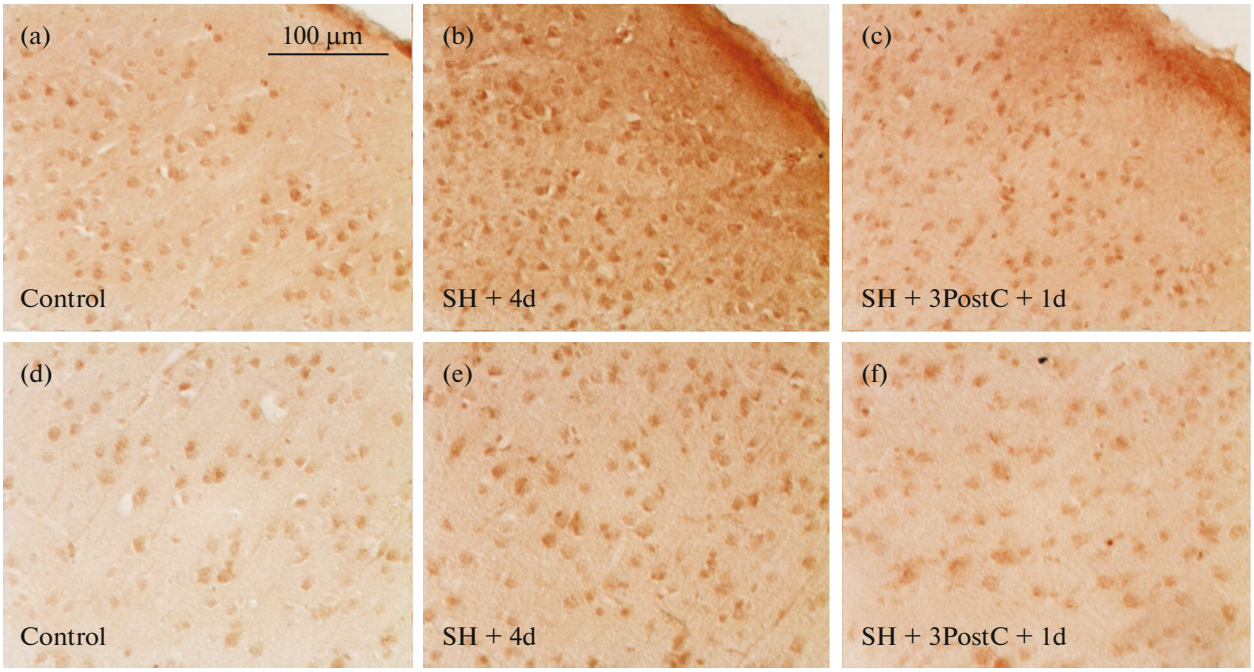

Fig. 3. Microimages (40×) of neocortical layer II (a, b, c) and V (d, e, f) of the control rats (control, a, d), rats on the 4th day after severe hypoxia (SH + 4d, b, e), and on the next day after severe hypoxia with triple postconditioning with mild hypobaric hypoxia (SH + 3PostC + 1d, c, f). Immunohistochemical staining for Bcl-2. Scale bar, 100 μm.

combination with mild hypobaric PostC on acetylation of the H3 histone at Lysine 24, as well as on the expression pattern of the anti-apoptotic protein Bcl-2 and adaptive protein BDNF in the neocortical cells of rats.

We found that SH leads to postponed significant elevation of H3acK24 in neocortical cells. These data correspond well to our previous results on the influence of hypoxia at different regimes on the expression of the proteins that are responsible for neuronal survival and neuroplasticity, namely, neurotrophins (BDNF) and anti-apoptotic factors (Bcl-2) in the hippocampus and neocortex of rats [32, 33]. Thus, it was shown that SH resulted in the expression of these factors in the postponed period (3 days after SH). However, Bcl-2 and BDNF expression in the neocortex either did not change or dropped in the early period (3–24 h) after the severe impact; this correlated with the H3acK24 decrease in the hippocampus [30]. The pool of data obtained in our study indicates that both early and delayed SH-induced changes in the amounts

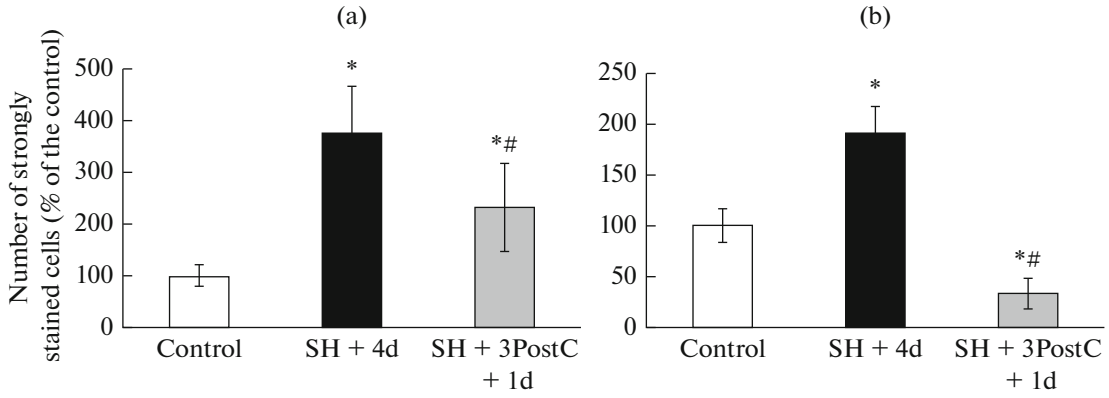

Fig. 4. The number of cells strongly that were immunopositive to Bcl-2 in neocortical levels II (a) and V (b) of the control rats (Control), 4 days after the severe hypoxia (SH + 4d), and on the next day after severe hypoxia in combination with triple postconditioning with mild hypobaric hypoxia (SH + 3PostC + 1d). X-axis: experimental groups; Y-axis: the number of strongly positive cells presented as the percentage of the control group. *, significant difference from the control, $p \le 0.05$; #, significant difference from severe hypoxia, $p \le 0.05$.

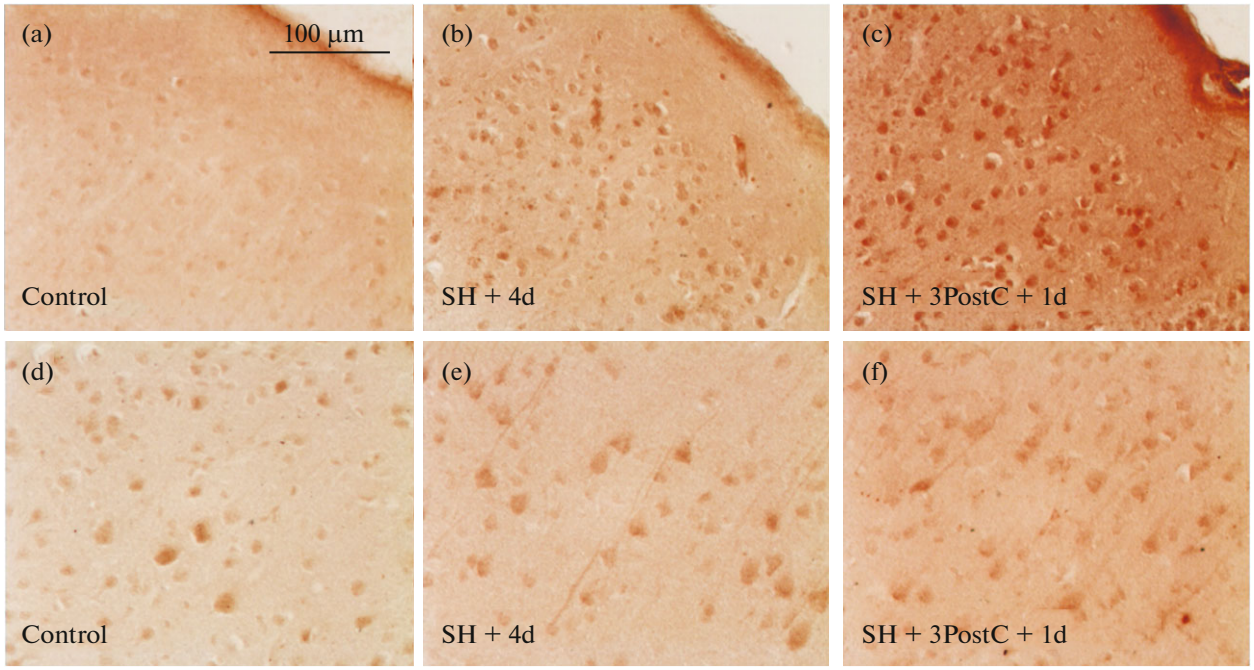

Fig. 5. Microimages (40×) of neocortical layer II (a, b, c) and V (d, e, f) of the control rats (control, a, d), rats on the 4th day after severe hypoxia (SH + 4d, b, e), and on the next day after severe hypoxia with triple postconditioning with mild hypobaric hypoxia (SH + 3PostC + 1d, c, f). Immunohistochemical staining for BDNF. Scale bar, 100 μm.

Bcl-2 and BDNF are accompanied by recruitment of epigenetic mechanisms, namely by acetylation of histone H3 at lysine 24. Elevation of H3acK24 in the postponed period with subsequent activation of Bcl-2 and BDNF expression apparently reflects the delayed launch of adaptive processes in animals after SH; thus, it has a compensatory character and characterizes the regenerative potential of injured but surviving neurons of the brain. However, we note that it does not contribute to the structural and functional rehabilitation of the brain, since progressive neuronal loss lasts for at least 7 days [34], while behavioral abnormalities last up to 10–11 days after SH [35].

It may be assumed that effective rehabilitation requires activation of adaptive mechanisms at an earlier time point after an injuring impact. This is supported by clinical data on the treatment of post-insult pathologies, as well as by our own studies with hypoxic PostC in an original model, whose special feature is that PostC sessions begin on the next day after SH and were presented for 3 consecutive days, thus making

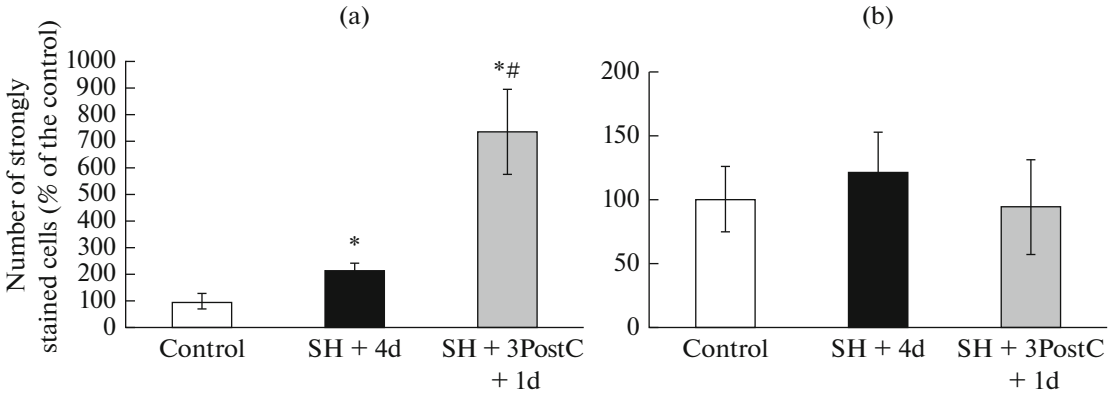

Fig. 6. The number of cells strongly immunopositive to BDNF in the neocortical levels II (a) and V (b) of the control rats (Control), 4 days after the severe hypoxia (SH + 4d), and on the next day after severe hypoxia in combination with triple postconditioning with mild hypobaric hypoxia (SH + 3PostC + 1d). X-axis: experimental groups; Y-axis: the number of strongly positive cells presented as percentage of the control group. *, significant difference from control, $p \le 0.05$; #, significant difference from severe hypoxia, $p \le 0.05$.

this procedure relatively long term. It has been previously shown that the protective effect of PostC on brain neurons occurs already after the first session [6]; by the 4th day adaptive processes begin to attenuate. This corresponds to the results of the present study that the level of the H3 histone acetylation at lysine 24 did not differ from the control 24 h after the last session of PostC (4 days after SH), while the Bcl-2 and BDNF levels decreased.

CONCLUSIONS

The data obtained in our study indicate an association between acetylation of the H3 histone at lysine 24 and neuroprotective mechanisms. Interpretation of the causal relationship that underlies this association is of potential importance for the development of new therapies for post-hypoxic and post-stroke neurological disorders, aimed at specific regulation of H3 acetylation at lysine 24, and may be useful for determination of the time windows for these therapeutic interventions.

COMPLIANCE WITH ETHICAL STANDARDS

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Conflict of interest. The authors declared no conflicts of interest.

Ethical approval. This study met all requirements listed in the Directives of the European Community Council (86/609/EEC) on the use of animals in experimental research. Protocols of experiments were approved by the Ethics committee of the Pavlov Institute for Physiology of RAS.

Informed consent. This article does not contain any studies with human participants performed by any of the authors.

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