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Morphological Differences between the Effects of Different Preconditioning Regimes Correcting Damage to Hippocampal Neurons Due to Exposure to Severe Hypobaric Hypoxia

M. O. Samoilov, A. V. Churilova, and T. S. Glushchenko

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Studies on five groups of rats (six animals per group) addressed changes in neurons in hippocampal fields CA1 and CA4 seven days after severe hypobaric hypoxia (180 mmHg, 3 h) using different numbers (1, 3, or 6) of sessions of preconditioning (PC) with moderate hypobaric hypoxia (360 mmHg, 2 h, 24 h before severe hypoxia). Single-session PC was found not to prevent damage to neuron structure with neuron death by day 7 after severe hypoxia. At the same time, six and especially three sessions of PC induced protective mechanisms preventing neuron damage. Use of six sessions of PC, in contrast to three, resulted in moderate chromatolysis in hippocampal neurons, which may be a consequence of prolonged hypermetabolic activity of neurons and may be evidence of their functional overloading.

Keywords: hippocampus, neurons, hypobaric hypoxia, preconditioning, neuroprotection.

Hypobaric (altitude) hypoxia occurs widely in nature (particularly in mountainous areas) and can provide a convenient (barochamber) experimental model for identifying the mechanisms of action of different degrees of oxygen deficit associated with reduced atmospheric pressure at the molecular-cellular, organ, and whole-body levels. Severe hypobaric hypoxia (SHH) on elevation in the barochamber to 6500–10000 m evokes a multitude of functional impairments to behavior and cognitive processes (memory, learning), as well as severe structural damage to neurons in the most vulnerable brain areas (neocortex, hippocampus) [10, 15, 18].

Studies in the last 10 years have yielded evidence that moderate hypobaric hypoxia in pre- and postconditioning regimes can suppress SHH-triggered molecular cell death programs in neurons, significantly preventing the development of structural and functional brain damage [4, 14, 15]. Protective effects were observed in these studies using three sessions of preconditioning (PC). In addition, there is great theoretical and practical interest in experimental comparative analysis of the actions of other PC regimes with different numbers of sessions. Previous studies showed that single- and multiple-session PC have different effects in terms of correcting structural damage to rat hippocampal neurons induced by SHH by 3 days after exposure (i.e., in the early period) [8]. The aim of the present work was to analyze changes in hippocampal neurons in the long-term period after SHH, when the morphological consequences of this treatment and PC regimes with different numbers of sessions in non-preconditioned rats are clear.

Materials and Methods

Experiments were performed on adult male Wistar rats weighing 200–250 g, kept in standard animal-house conditions. Experiments were performed in compliance with the requirements formulated by the Directives of the Council of the European Community (89/609/EEC) regarding the use of animals for experimental studies. Experimental protocols were approved by the Commission for the Humane Treatment of Animals, Pavlov Institute of Physiology, Russian Academy of Sciences.

SHH was produced in a flow-type barochamber at an atmospheric pressure of 180 mmHg for 3 h. In PC regimes,

Laboratory for the Regulation of Brain Neuron Function, Institute of Physiology, Russian Academy of Sciences, St. Petersburg, Russia; e-mail: samoilov@pavlov.infran.ru, annch05@mail.ru.

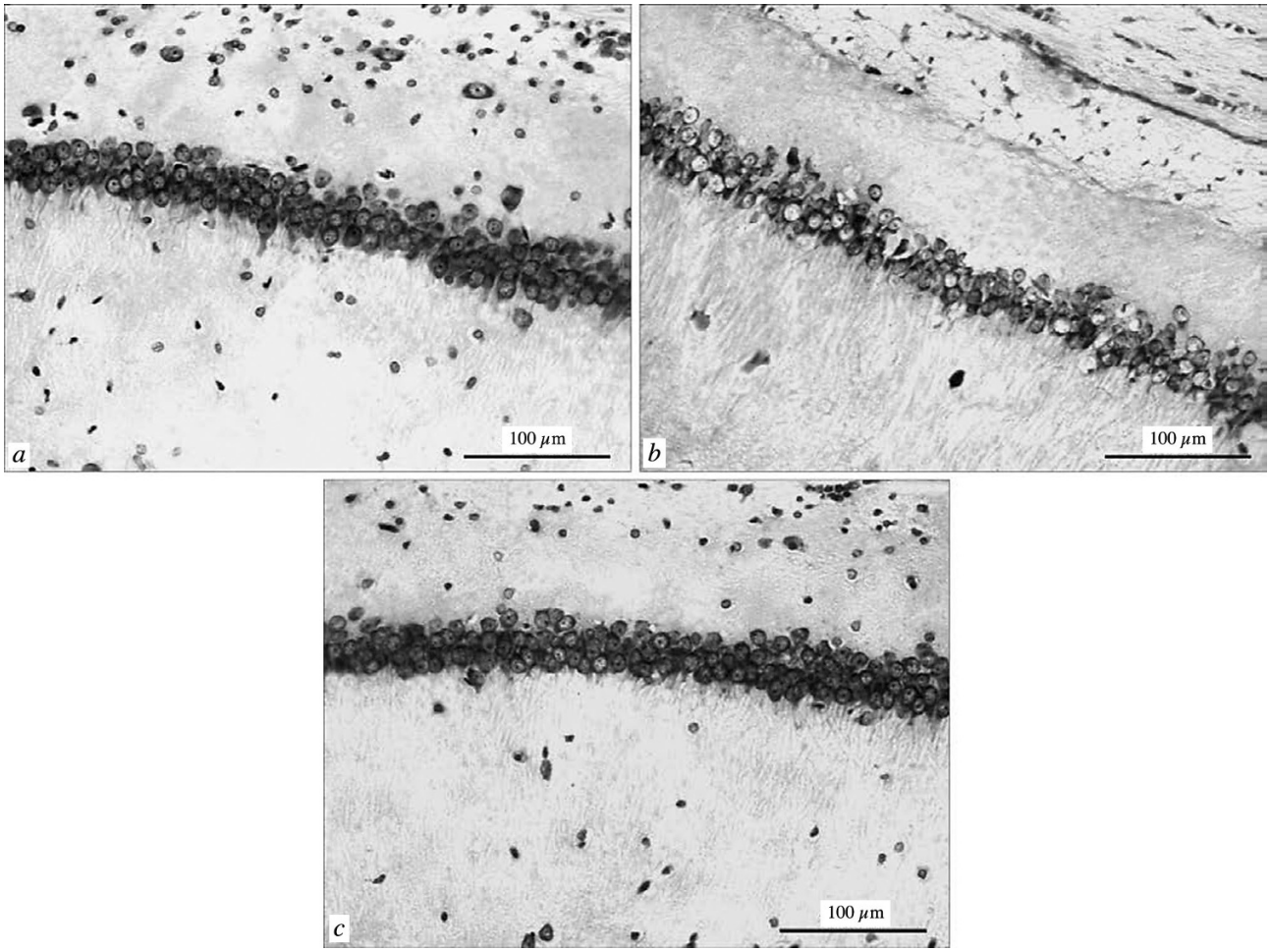

Fig. 1. Neurons in hippocampal field CA1 of control rats (intact, *a*) non-preconditioned rats (*b*), and rats exposed to three sessions of preconditioning (*c*) seven days after severe hypobaric hypoxia. Stained with toluidine blue.

rats were subjected to moderate hypobaric hypoxia (the barochamber pressure was 360 mmHg) for 2 h 24 h before exposure to severe hypoxia. Experiments were performed on five groups of rats (each of six animals). Animals of group 1 served as controls and were placed in the barochamber for 2 h but without any change in atmospheric pressure; rats of group 2 were subjected to severe hypoxia; animals of groups 3, 4, and 5 were exposed to one, three, and six sessions of PC prior to severe hypoxia. Intervals between sessions in groups 4 and 5 were 24 h. Rats were decapitated seven days after SHH and brains were extracted, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for 24 h, and embedded in paraffin using a standard protocol. Serial frontal brain sections of thickness 7 μm were cut at the level of –2.8 mm from the bregma, mounted on slides, and stained in 0.1% toluidine blue by the Nissl method. Sections were examined using a morphometric apparatus consisting of an Olympus CX31 light microscope (Olympus, Japan), a ProgRes CT1 digital camera (Jenoptic, Germany), and an IBM PC running VideoTest Master Morphology 5.2 (developed by Video Test, St. Petersburg, Russia). The computer program VideoTest Master Morphology 5.2 was used to count neurons in hippocampal fields CA1 and CA4 in rats of all groups, over distances of 450 μm, whose optical densities were in the range 0.15–0.45 brightness units relative to the background brightness of the preparation concerned.

Results

Significant differences in neuron structure were seen seven days after SHH in non-preconditioned rats, as well as in rats exposed to one, three, and six sessions of PC, in both the dorsal (CA1) and ventral (CA4) parts of the hippocampus. SHH induced extensive morphological damage in fields CA1 and CA4 and produced selective neuron death (up to 30–40% of control) (Figs. 1, *a*, *b* and 2). In rats exposed to single sessions of PC, the response to SHH consisted of similar changes (see Fig. 2). In contrast, three and six sessions of PC prevented loss of neurons in fields CA1 and CA4 following SHH (see Figs. 1, *c* and 2).

SHH induced clear structural damage to hippocampal neurons, with the appearance of significant quantities of hy-

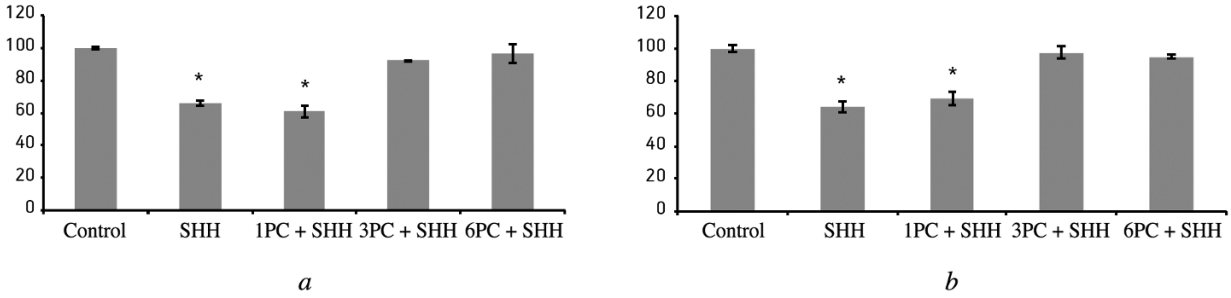

Fig. 2. Relative contents of neurons in rat hippocampal fields CA1 (*a*) and CA4 (*b*) seven days after severe hypobaric hypoxia. On the horizontal axis: SHH – severe hypobaric hypoxia; 1PC + SHH, 3PC + SHH, and 6PC + SHH – one, three, and six sessions of PC before SHH, respectively; the ordinate shows changes compared with controls, taken as 100%. *Significant differences compared with controls, $p \le 0.05$. Vertical bars show standard errors.

perchromic pyknotic cells and pericellular edema. In addition, many neurons showed global chromatolysis, with cytoplasmic vacuolization and nuclear and nucleolar degradation (Fig. 3, *a*, *b*). When single sessions of PC were used, SHH was followed by a similar picture of structural damage to neurons in fields CA1 and CA4 (see Fig. 3, *c*). In contrast, neuron structure was close to normal after SHH in rats exposed to three sessions of PC (see Fig. 3, *d*). At the same time, in animals exposed to six sessions of PC, most neurons in fields CA1 and CA4 showed moderate chromatolysis after SHH, with degradation of large clumps of chromatophilic material (ribonucleoprotein granules) and its scattering in the cytoplasm, as well as decomposition of the nucleolus (see Fig. 3, *e*).

Discussion

The studies reported here established that by post-SHH day 7 there was significant damage to neuron structure in the dorsal (CA1) and ventral (CA4) parts of the hippocampus. Our previous studies [8] identified clear morphological changes to neurons in these areas by post-SHH day 3, which in the later period (day 7) became significantly more severe, to the level of death of 30–40% of cells. Many of the surviving neurons showed signs of apoptotic and necrotic damage. Pre-SHH PC regimes with different numbers of sessions were found to have specific actions directed at preventing this damage. One PC session was ineffective in this regard. Six and especially three sessions of PC induced protective mechanisms preventing neuron damage.

Previous studies showed that SHH induces the expression of proapoptotic factors Bax, c-Jun, and JNK, in the hippocampus, while use of three sessions of PC suppresses these and increases the expression of the antiapoptotic factors Bcl-2, Bcl-xL, and ERK-MAP [4, 6, 14].

There is great interest in the differences between the effects of PC using three and six sessions on hippocampal neuron structure seven days after SHH. The morphological picture after three sessions of PC was close to that in controls. At the same time, most neurons in hippocampal fields CA1 and CA4 in rats exposed to six sessions of PC showed moderate chromatolysis. This process is evidently linked with functional neuronal overloading due to the extreme number of PC sessions preceding SHH, which can probably lead to prolonged hypermetabolic cell activity. It is very clear that this could have adverse functional consequences. As shown previously, use of a Morris water maze test – an experimental model for studies of learning and memory – showed that SHH induced memory impairments in rats, three sessions of PC almost completely preventing these disorders [1, 2]. This may be due to increased neuron metabolism associated with moderate chromatolysis. Many studies from the middle of the last century established that moderate chromatolysis is linked with depletion of cerebral nerve cells of ribonucleoprotein in conditions of increased functional activity [3]. Prolonged functional loading due to long-lasting stimulation of neurons by various factors, apparently including an extreme number of episodes of hypoxic PC, may develop so-called "fatigue chromophobia," which leads to functional disorders. Our data should be considered in selecting the most effective neuroprotective PC regime, particularly the use of three sessions of PC consisting of moderate hypobaric hypoxia. The mechanisms of the neuroprotection against damage induced by severe forms of hypoxia/ischemia have been studied intensely in the past decade [4, 11, 19]. An important role in preventing structural and functional brain cell damage is played by activation of proadaptive genes and proteins. Hypoxic PC consisting of three sessions has been shown to induce overexpression of proadaptive early genes and their products, i.e., transcription factors (NGFI-A, c-Fos, pCREB, NF-κB, GR, MR, Hif-1b), in hippocampal and neocortical neurons, these factors controlling neuroplasticity and nerve cell survival/death [4]. It is important to note that their expression increases both before and after SHH. In addition, in contrast to this, a single session of PC has been found not to increase the expression of these transcription factors or the products of their target genes – the neuroprotective protein BDNF and Bcl-2 [4, 7, 9, 13, 16].

Recent years have seen recognition of the important role of epigenetic mechanisms regulating the expression of

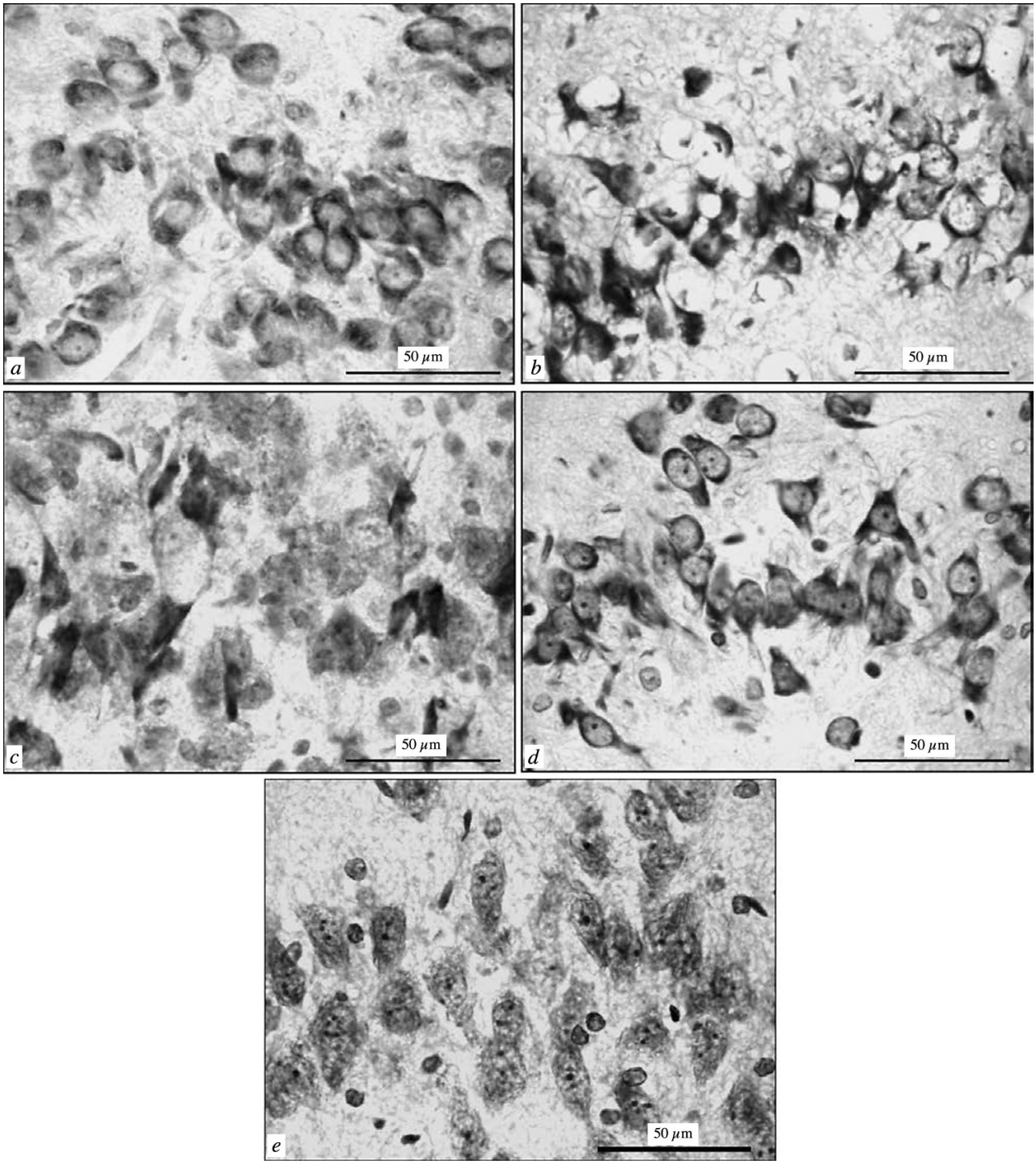

Fig. 3. Neurons in hippocampal field CA4 in control rats (*a*), non-preconditioned rats (*b*), and rats exposed to one (*c*), three (*d*), and six (*e*) sessions of preconditioning assessed seven days after severe hypobaric hypoxia. Stained with toluidine blue.

genes in the responses of cerebral neurons to hypoxia/ischemia [12, 17, 20]. Epigenetic modifications to histones (acetylation, methylation) activate or repress transcription, including that of the proadaptive genes identified above. Preliminary results have been reported [5] showing significant differences in the influences of severe hypoxia and single- and multiple-session PC with SHH on the nature of epigenetic modifications of histones in neocortical and hippocampal neurons. These studies may be of great significance for understanding the mechanisms and developing

methods of controlling the activity of proadaptive genes and proteins involved in increasing the resistance of the brain to severe forms of hypoxia/ischemia.

Thus, the studies reported here identified differences in the influences of different regimes (one, three, and six sessions) of PC consisting of moderate hypobaric hypoxia on the structure of neurons in hippocampal fields CA1 and CA4 in rats in the later period (day 7) after severe hypoxia. Single-session PC had no protective action on structural damage to neurons evoked by SHH, while multiple-session PC (three and six sessions) prevented this damage. However, after six-session PC, in contrast to three-session PC, neurons in the dorsal (CA1) and ventral (CA4) parts of the hippocampus showed moderate chromatolysis, which appears to provide evidence of prolonged cellular hypermetabolic activity linked with functional overloading due to the large number of PC sessions. This can have adverse effects on learning and memory processes after SHH. These data should be considered in the development of effective non-medication-based means of protecting brain cells in harmful conditions.

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