THE INFLUENCE OF CHRONIC PRENATAL HYPOXIA ON THE FUNCTIONAL ACTIVITY OF BRAIN NEURON-GLIAL NETWORKS AND THEIR ADAPTATION TO ACUTE OXYGEN DEFICIENCY *IN VITRO*

T.A. Mishchenko[1*‡], *E.A. Guseva*[1‡], *A.O. Kustova*[1], *E.A. Emelyanov*[2], *M.I. Krivonosov*[3], *M.V. Vedunova*[1]

[1] Institute of Biology and Biomedicine, National Research Lobachevsky State University of Nizhny Novgorod, 23 Gagarin ave., Nizhny Novgorod, 603022, Russia;
[2] Moscow Institute of Physics and Technology, 9 Institutskiy per., Dolgoprudny, 141701, Russia;
[3] Institute of Information Technology, Mathematics and Mechanics, Department of Applied Mathematics, National Research Lobachevsky State University of Nizhny Novgorod, 23 Gagarin ave., Nizhny Novgorod, 603022, Russia.

‡ These authors are contributed equally to this work
* Corresponding author: saharnova87@mail.ru

Abstract. In-depth studies of the etiology and consequences of brain damage induced by chronic prenatal hypoxia are now urgently needed to improve the therapeutic strategies and reinforcing the adaptive capacity of nerve cells to oxygen deficiency. Here, we analyzed the characteristics of spontaneous calcium activity of primary hippocampal cultures obtained from mouse embryos exposed to chronic prenatal hypoxia in a late period of development in vitro (DIV 15 – DIV 21) and the adaptive capabilities of neuron-glial networks to the acute hypoxic injury. Chronic hypoxic stress caused several delays in the functional development of primary hippocampal cultures. On DIV 15, the cultures exhibited the spontaneous calcium activity with a decreased number of active cells and duration of Ca^{2+} oscillations. The reduced values of network characteristics (DIV 15) in the presence of partial destruction of the functional architecture of neuron-glial networks with a reduced force of correlated connections between cells during entire observation period were also shown. Chronic hypoxic stress altered the functional culture's response to acute oxygen deficiency. On day 7 after acute hypoxia modelling, against the background of significant decrease in the number of functionally active cells, the frequency and duration of Ca^{2+} oscillations did not differ from the intact values. Nevertheless, significant decrease in the network characteristics accompanied by substantial rearrangements in the functional architecture of neuron-glial networks indicate the death of significant part of the network elements and increase the risk of aggravation the synaptic transmission impairments leading to loss of network functionality in more distant post-hypoxic period.

Keywords: prenatal hypoxia, acute hypoxia, primary hippocampal cultures, neuron-glial networks, functional network activity, calcium imaging.

List of Abbreviations

CNS – central nervous system
DIV – day of culture development *in vitro*

Introduction

Brain hypoxia remains one of the most pressing problems for global health that needs to be solved. The human brain is the most sensitive organ to adverse hypoxic effects due to its the highest consumption rate of oxygen and nutrients for maintaining its normal physiological functioning and cells viability as well as a limited range of antioxidant enzymes and compensatory capacity (Suresh *et al.*, 2021). Deteriorations in a morpho-functional integrity of brain neuron-glial networks not only occurs as a result of direct hypoxic exposure, but also during subsequent reoxygenation, which aggravates the damaging effects. The launch of hypoxia-induced pathological mechanisms is manifested in mitochondrial dysfunction (Wang *et al.*, 2019), impaired synaptic transmission which leads to weakening of synaptic plasticity (Zhuravin *et al.*, 2019), activation of inflammatory and apoptotic reactions (Mukandala *et al.*, 2016) accompanied by violations in a brain vascular system and integrity of the blood-brain barrier (Dunn, Isaacs, 2021). The consequences of hypoxic damage resulting in the loss of significant elements of neuron-glial networks im-

pairing the brain functioning which multiply the risks of the development of neurodegenerative and neoplastic processes (Domènech *et al.*, 2021; Lestón Pinilla *et al.*, 2021; Burtscher *et al.*, 2021; Yeo, 2019).

Chronic prenatal hypoxia is the most severe forms of hypoxia which etiology varies from annually gradual increase of anthropogenic load to maternal stress, poor nutrition, smoking or drug abuse during pregnancy and obstetric complications (Piešová & Mach, 2020). This type of hypoxia is leading the list of causes of infant severe disability and mortality (Piešová & Mach, 2020; Riljak *et al.*, 2016; Lawn *et al.*, 2005). Deteriorations in oxygen supply during onthogenesis is the main trigger for the development of gross malformations and congenital anomalies (Wang *et al.*, 2021; Coq *et al.*, 2016; Vasilev *et al.*, 2016) as well as the severe neurological deficit and psychopathologies (Giannopoulou *et al.*, 2018), impairments in cognitive functions and learning abilities (Piešová & Mach, 2020; Nalivaeva *et al.*, 2018), and may be involved in the development of neurodegenerative processes and epileptiform activity in infancy and adulthood (Kobylarek, 2019; Zhuravin *et al.*, 2019; Zhang *et al.*, 2013). Over the past decade the efforts of scientists and clinicians are aimed at discovering the specificity of molecular mechanisms of chronic prenatal hypoxia and the features of further development of the systemic adaptive response to oxygen deficiency. Studies carried out at the level of neuron-glial networks open a possibility to analyze the tiny mechanisms of development the functional architecture of the networks under hypoxic state, as well as the contribution of each element of the network to the development of adaptive functional restructuring to the additional stress factor. Such approach may act as a significant fundamental basis for improving the methods of diagnosis and treatment of hypoxic damage of the central nervous system.

Herein, using primary hippocampal cultures obtained from mice embryos exposed to chronic prenatal hypoxia with a complex of Ca^{2+}-imaging technique and original algorithm of data analysis we characterized the features of spontaneous calcium activity of hypoxia-induced neuron-glial networks in a late period of development *in vitro* and assessed their adaptive capabilities to the episode of acute hypoxic injury.

Materials and Methods

Research object

Primary hippocampal cell cultures obtained from 19-day-old embryos of hybrid mice of two lines C3H and C57Bl6 (C3H+C57Bl6) crossed according to a scheme described in Mishchenko et al., 2022a were used in this study. Pregnant mice were housed in a certified SPF vivarium of the Lobachevsky State University of Nizhny Novgorod. All experimental procedures were approved by the Bioethics Committee of Lobachevsky University and carried out in accordance with Act 708n (23 082010) of the Russian Federation National Ministry of Public Health, which states the rules of laboratory practice for the care and use of laboratory animals, and the Council Directive 2010/63 EU of the European Parliament (22 September 2010) on the protection of animals used for scientific purposes. Pregnant mice were sacrificed by cervical vertebra dislocation, and the embryos are removed from the uterus were decapitated before brain isolation.

Experimental design

Pregnant mice of the C57BL/6+C3H hybrid line were exposed to chronic hypobaric hypoxia from days 14 to 18 of the gestational period. A control group consists of pregnant hybrid mice was not subjected to hypoxia modeling. Primary hippocampal cultures were prepared from 19-day-old embryos and then cultured for 21 days. On day 14 of primary hippocampal cultures development *in vitro* (DIV), a part of cultures was simulated with an acute normobaric hypoxia (Fig. 1).

The following groups of primary hippocampal cultures were analyzed in this study:

1. Intact – cultures obtained from mouse embryos not subjected to chronic prenatal hypoxia; the cultures were not exposed to acute normobaric hypoxia modeling *in vitro*;

2. Hypoxia chronic – cultures obtained from mouse embryos subjected to chronic prenatal

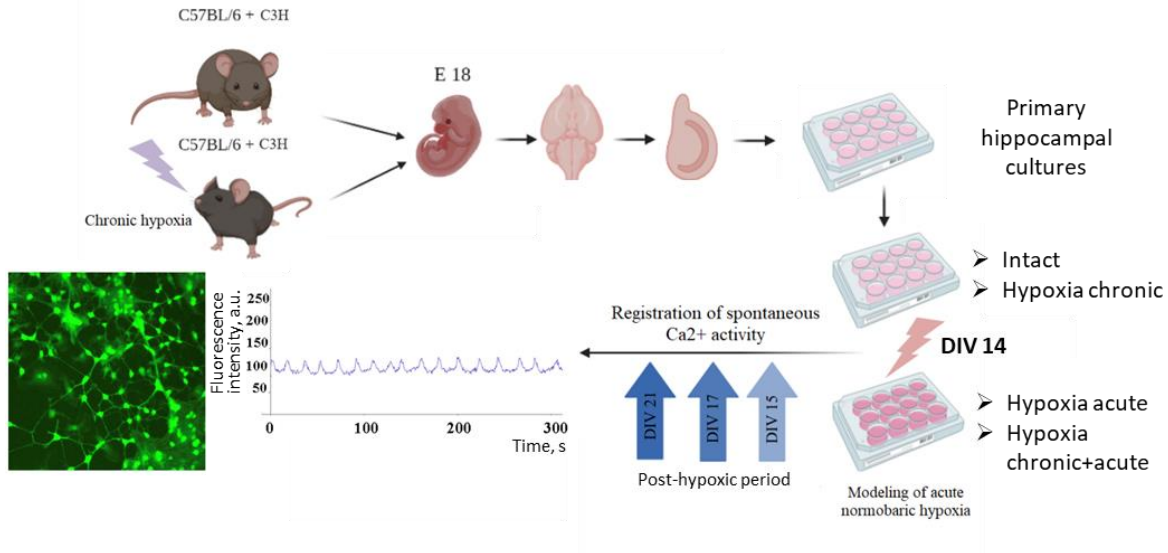

Fig. 1. Scheme of the experiment (the illustration was created using the BioRender platform)

hypoxia; the cultures were not exposed to acute normobaric hypoxia modeling *in vitro*;

3. Hypoxia acute – cultures from mouse embryos not subjected to chronic prenatal hypoxia; the cultures were exposed to acute normobaric hypoxia on DIV14;

4. Hypoxia chronic + acute – cultures obtained from mouse embryos subjected to chronic prenatal hypoxia; the cultures were exposed to acute normobaric hypoxia on DIV14.

Registration of spontaneous calcium activity of primary hippocampal cultures and the assessment of its main parameters and the network characteristics were performed on days 1, 3 and 7 of the post-hypoxic period.

Chronic prenatal hypoxia model in vivo

Chronic prenatal hypoxia was modeled by 2-hour stay of pregnant mice in a vacuum flow-type chamber which maintained a pressure of 177-218 mm Hg (≈ 6500-7000 m above sea level) (Mishchenko *et al.*, 2022a; Shchelchkova *et al.*, 2020; Urazov *et al.*, 2018). The outside air temperature was 20-22 ºC. The ascent to the simulated height was performed from the 14th day till 18th day of gestational period.

Isolation and long-term cultivation of primary hippocampal cultures

Hippocampal tissue was surgically isolated from hybrid mouse embryos (19 day of gestation) according to the previously developed protocol (Loginova *et al.*, 2021; Mishchenko *et al.*, 2019) and underwent dissociation with 0.25% trypsin-ethylenediaminetetraacetic acid (Thermo Fisher Scientific, USA) for 20 min. The suspension of dissociated hippocampal cells was centrifuged at 1000 rpm for 3 min. After that, the cell pellet is immediately resuspended in Neurobasal medium (ThermoFisher Scientific, USA) supplemented with 2% B27 (ThermoFisher Scientific, USA), 0.5 mM L-glutamine (ThermoFisher Scientific, USA) and 5% fetal bovine serum (Biosera, France) and was seeded in 25 µl droplets covering the center of the coverslips placed tentatively in 24-well culture plate. The initial cell density is approximately 4500 cells per cm^2. For improving cell adhesion, the coverslips are pretreated with polyethyleneimine solution (1 mg/mL) (Sigma-Aldrich, Germany) for 30 min in a Binder C150 incubator (BINDER GmbH, Germany) and then washed three times in a deionized water. After the cells had adhered on coverslips forming a dense monolayer, the culture plate was refilled with culture medium and grown overnight. After 24 hours, one-third of the culture medium are replaced by a complete growth medium containing 0.4% fetal bovine serum. Thereafter, the culture medium is replaced once every two days.

The viability of primary cultures was maintained under constant conditions of 37°C, 5% CO_2 and a humidified atmosphere in a CO_2-in-

cubatior. The control of culture's morphology was assessed on the days of the culture medium replacement using an inverted fluorescence microscope ZEISS Observer A1 (Carl Zeiss, Germany).

Acute normobaric hypoxia model in vitro

An episode of acute normobaric hypoxia was modeled on day 14 of cultures development *in vitro* using a previously developed protocol (Loginova *et al.*, 2021; Vedunova *et al.*, 2015). The conditioned culture medium was replaced with a medium with a low oxygen supply for 10 min followed by a reverse replacement of the complete growth medium with a normal oxygen content. The hypoxic medium was created by passing argon gas through the Neurobasal^{TM} medium in a sealed chamber at a pressure of 1–1.5 MPa for 10 min. The cultures from the «Intact» and «Hypoxiachronic» groups were subjected to total replacement of the culture medium by a complete growth medium with normal oxygen content.

Functional calcium imaging

Registration of spontaneous calcium activity of the primary hippocampal cultures in the post-hypoxic period was performed using a Ca^{2+}-imaging technique according to the previously developed protocol (Mishchenko *et al.*, 2022b; Saviuk *et al.*, 2022; Loginova *et al.*, 2021). The cultures were stained with a fluorescent calcium-sensitive dye Oregon Green 488 BAPTA-1 AM (0.4 mM, ThermoFisher Scientific, USA) preliminary dissolved in DMSO with 4% pluronic F-127 (ThermoFisher Scientific, USA). After 20 min of incubation in the CO_2-incubator, the samples obtained was examined in a LSM 800 confocal laser scanning microscope (Carl Zeiss, Germany). The Oregon Green fluorescence was excited at 488 nm, and the emission was recorded in the range of 500–530 nm. The time-series of confocal images at the registration rate of two frames per second were recorded. The recording time was 10 min; at least 3 fields of view in each culture were assessed.

The following main parameters of spontaneous calcium activity were analyzed: percentage of functionally active cells (%); the duration (time from the beginning to the end of an oscillation, s) and frequency (average number of oscillations per min) of Ca^{2+} oscillations.

Network characteristic assessments in the primary hippocampal cultures

Analysis of network characteristics in the primary hippocampal cultures was performed in accordance with a previously developed algorithm for the detection of fluctuations in the intracellular calcium level in primary brain cells cultures (state registration of computer software no 2021612870 from 25 February 2021 and no 2022667580 from 22 September 2022; Kustikova *et al.*, 2018). The algorithm assesses a collective and coordinated functional activity of both neurons and astrocytes in a neuron–glial network with its representation as an oriented graph, the nodes of which correspond to individual cells, and the edges connect the corresponding nodes and indicate a significant correlation between pairs of cells ($p > 0.3$). The spread of calcium signals between cells results in detecting time delays in the increase in Ca^{2+} concentration.

The following parameters were analyzed: mean correlation level between cells, the ratio of the available connections in the culture to the maximum possible number of connections in the culture and the signal speed propagation.

Statistical analysis

The obtained results were processed statistically in a GraphPad Prism (v. 8.0.1) using two-way ANOVA. The data were expressed as box and whisker plots with Tukey's method. Differences between groups were considered significant if the corresponding p-value was less than 0.05. At least three independent biological replicates were used for all experiments.

Results

Analysis of peculiarities of spontaneous calcium activity of primary hippocampal cultures induced by chronic and acute hypoxic injury

We have previously shown that 14-21 days of developmental period of primary hippocampal cultures *in vitro* is characterized by the presence of neurons and glial cells in an approxi-

mate ratio of 1:2 accompanied by a predominant population of mature chemical synapses with mature axo-dendritic and axo-spiny asymmetric contacts ensuring a stable functional activity of neuron–glial networks with a complex characteristic pattern (Mitroshina *et al.*, 2021; Mishchenko *et al.*, 2019; Shirokova *et al.*, 2013).

Analysis of the main parameters of spontaneous calcium activity revealed that the «Intact» group of cultures showed stable calcium activity profile with a high percentage of functionally active cells (15 DIV 76.4 [39.5; 89.8]%, 17 DIV 86.2 [43.0; 96.1]%, 21 DIV 75.5 [43.0; 94.6]%) throughout the observation period (Fig. 2).

Several alterations in the development of primary cultures obtained from hippocampal tissue of mouse embryos exposed to chronic prenatal hypoxia were shown (Fig. 2). On DIV 15, the number of cells that exhibited Ca^{2+} activity in the «Hypoxia chronic» group (20.4 [13.5; 53.9]%) was significantly lower than in the «Intact» group, whereas Ca^{2+} oscillations had, on average, a frequency of 0.06 [0.02; 0.59] osc/min and a duration of 20.5 [11.9; 24.5] s which were formed a calcium activity profile closed to that of the Intact cultures (Ca^{2+} oscillations frequency 0.2 [0.06; 1.4] osc/min and duration 14.1 [5.7; 29.3] s). On the following days of observation, the duration of the Ca^{2+} oscillations was significantly reduced relative to the «Intact» group.

An episode of acute hypoxia negatively affects the spontaneous calcium activity of primary hippocampal cultures. In the «Hypoxia acute», a significant increase in the number of functionally active cells accompanied by a reduction in the frequency and a gradual increase in the duration of Ca^{2+} oscillations in the post-hypoxic period were observed (Fig. 2). Such changes in the spontaneous calcium activity are supposed to be associated with the loss of elements in the neuron-glial networks and pronounced rearrangements of the network architectonics leading to the failure of network properties and functionality.

Preliminary exposure to chronic hypoxia altered the functional calcium activity response to acute episode of oxygen deficiency. In the «Hypoxia chronic+acute» group, against the background of significant decrease in the number of functionally active cells (26 [7.4; 65.8] %), the frequency (0.09 [0.01; 0.4] osc/min) and duration (25.8 [12.2; 39.9] s) of Ca^{2+} oscillations did not differ from the intact values on day 7 of the post-hypoxic period *in vitro* (Fig. 2). Moreover, the duration of Ca^{2+} oscillations in the «Hypoxia chronic+acute» group was significantly lower than the values of the «Hypoxia acute» group throughout the observed post-hypoxic period. Nevertheless, significant changes in the calcium activity profile of primary hippocampal cultures exposed to chronic and acute hypoxia may have an impact on the network characteristics of cells, which we analyzed further.

Features of the functional neuron–glial network interactions in primary hippocampal cultures induced by chronic and acute hypoxic injury

The assessment of network characteristics is an equally important steppingstone in the complex analysis of the functional state of primary hippocampal cultures during development and the effects of stress factors. Significant changes in the network parameters increase the risk of significant rearrangements in the functional architectonics of neuron-glial networks which in turn can potentially lead to simplification and pronounced impairments of their functioning. Potential loss of network elements can be identified by the lack of correlation in the profile of Ca^{2+} oscillations between functionally active cells (Mishchenko *et al.*, 2022b; Saviuk *et al.*, 2022).

Here, we showed that most cells in the neuron-glial network in the «Intact» cultures of DIV 15 – DIV 21 developmental period work simultaneously and are connected to each other (Fig. 3).

By DIV 21, the percentage of correlated connections from the mathematically calculated maximum possible number of connections was 68.6 [63.4; 90.5]% (Fig. 3B) accompanied by a high signal speed propagation (15.2 [8.9; 35.4]μm/s) (Fig. 3A) and average level of correlation between cells of 0.5 [0.7; 0.2] (Fig. 3C).

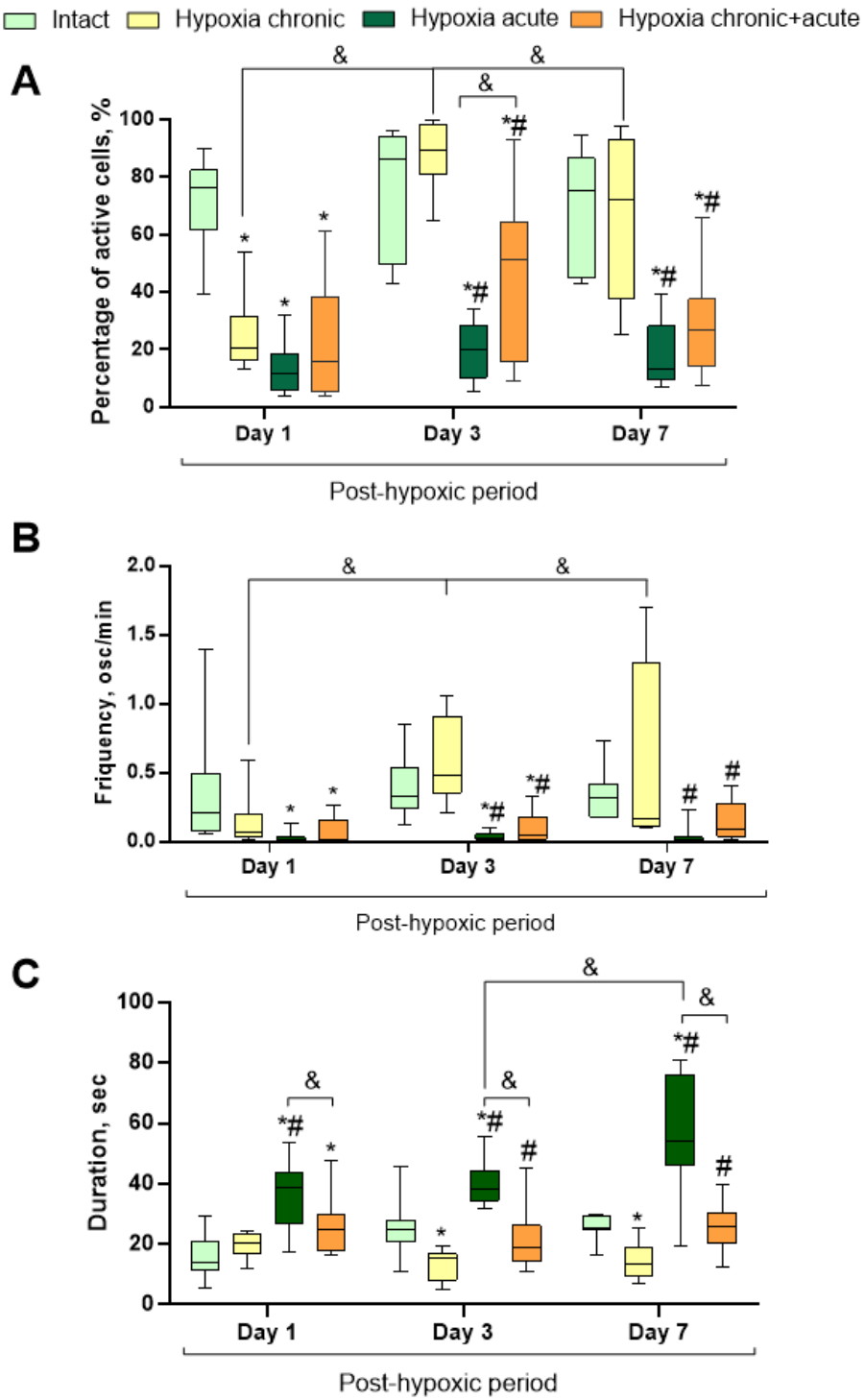

Fig. 2. Main parameters of spontaneous calcium activity of primary hippocampal cultures during 7 days after acute hypoxic injury. A – proportion of cells exhibiting Ca^{2+} activity; B – number of Ca^{2+} oscillations per min; C – duration of Ca^{2+} oscillations.
* – vs. «Intact», # – vs. «Hypoxia chronic», and &, $p < 0.05$; two-way ANOVA with Tukey multiple comparison test

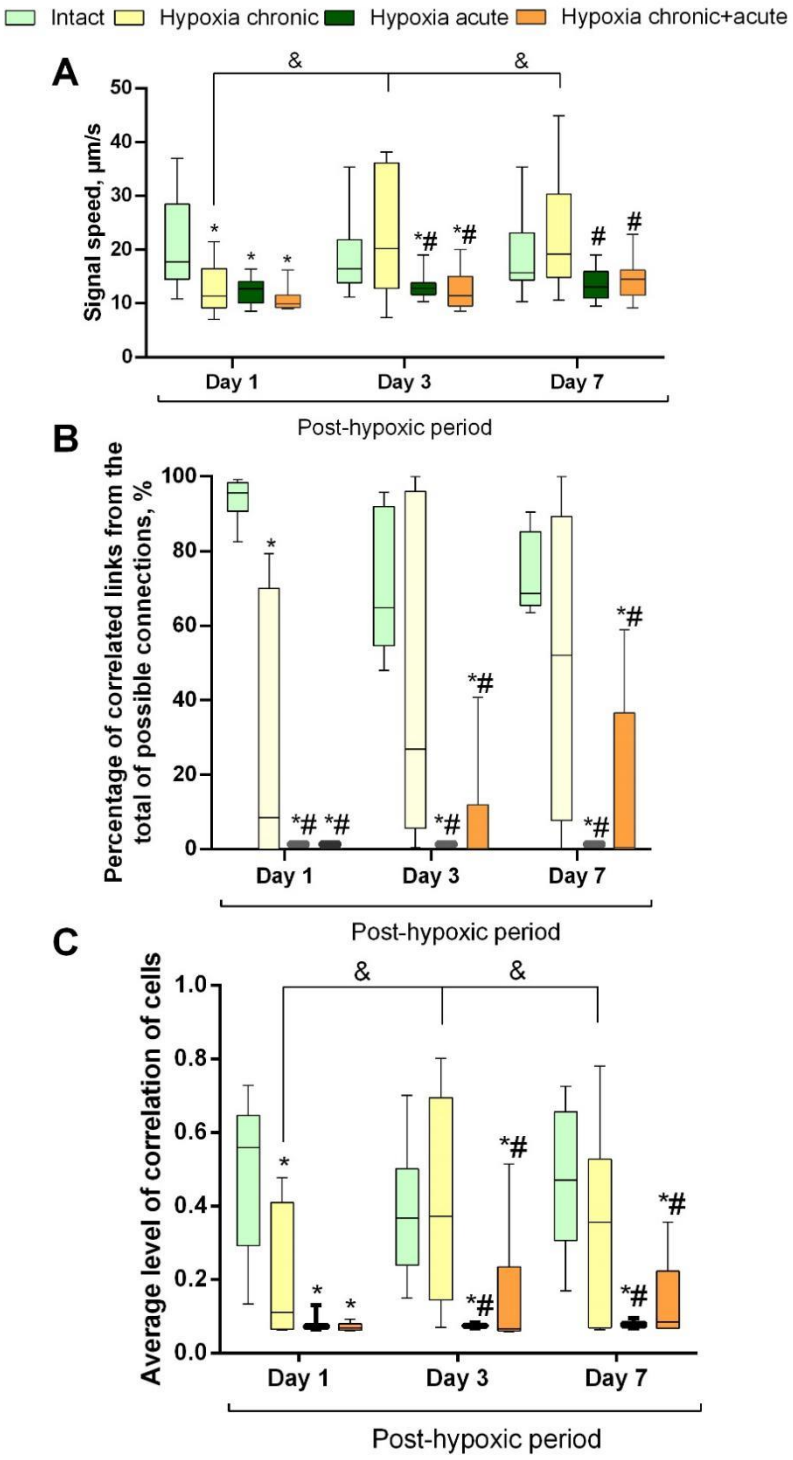

Fig. 3. Network characteristics of spontaneous calcium activity of primary hippocampal cultures during 7 days after acute hypoxic injury. A – signal speed, μm/s; B – percentage of correlated links from the total number of possible connections, %; C – mean correlation level of cells.
* – vs. «Intact», # – vs. «Hypoxia chronic», and &, $p < 0.05$; two-way ANOVA with Tukey multiple comparison test

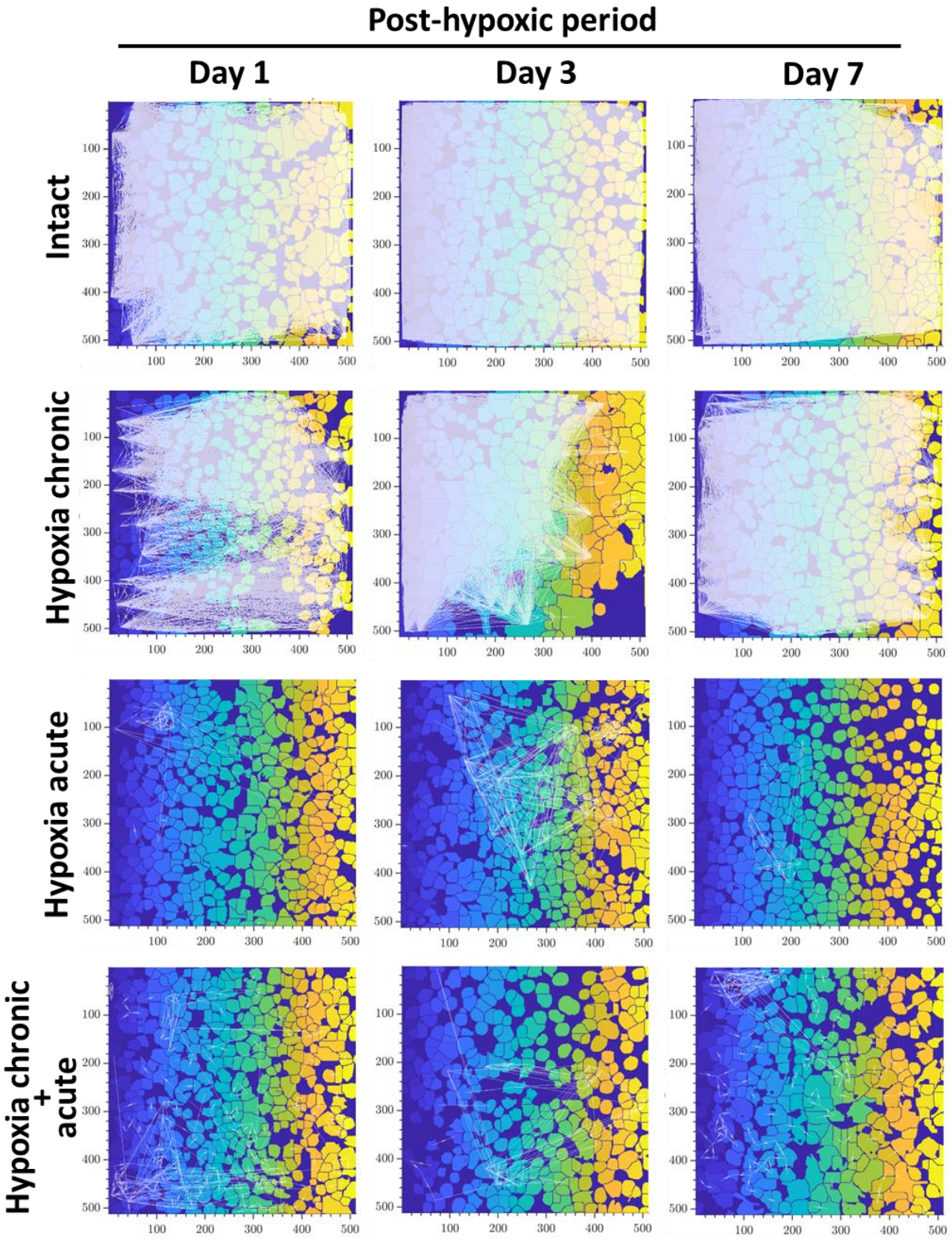

Fig. 4. Representative correlation network graphs of primary hippocampal cultures in the period after acute hypoxic injury. Each white line schematically represents the connection between cells the correlation of which exceeds the empirically calculated value 0.3. The colors represent the individual cells for better detection of cell boundaries

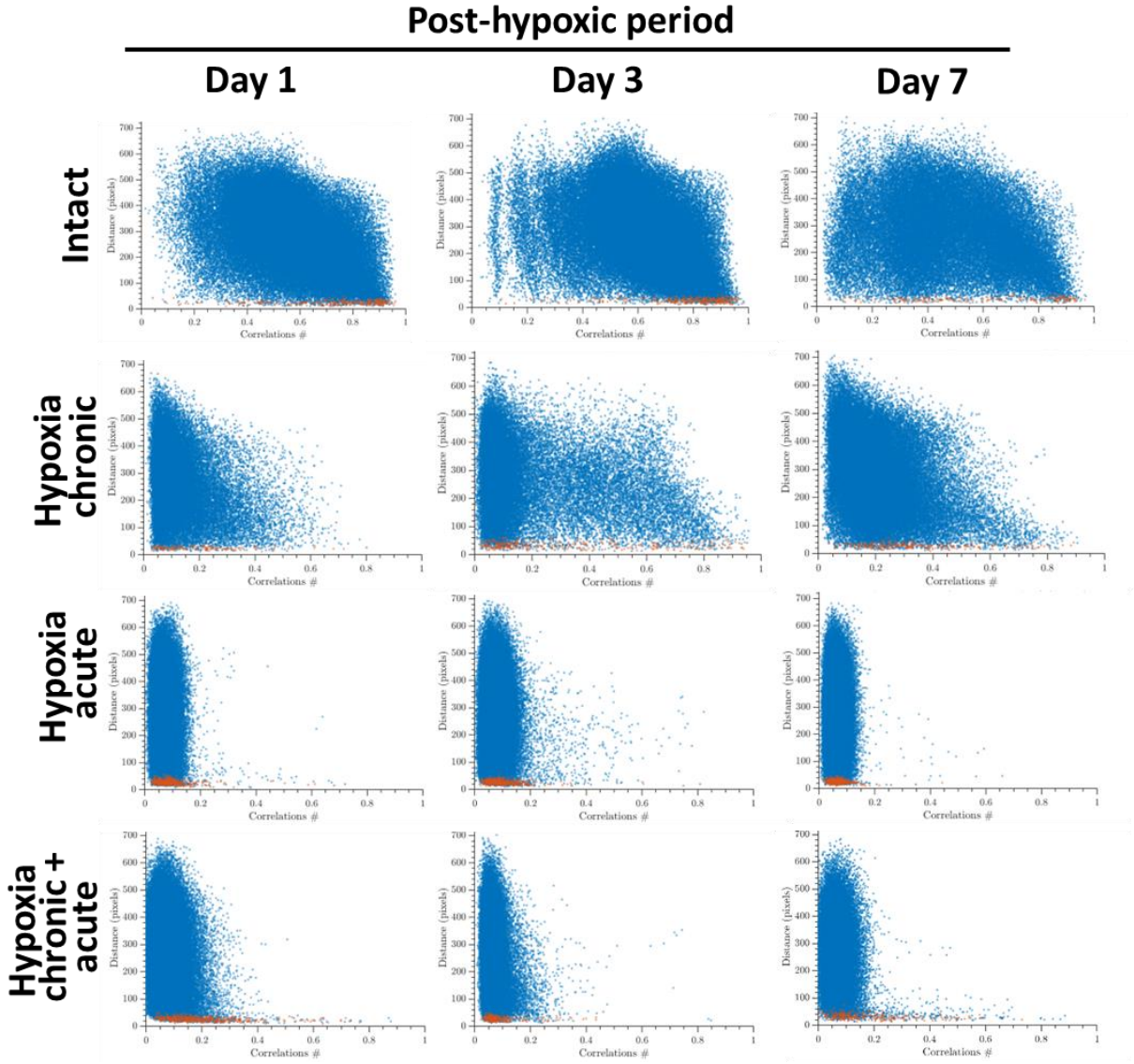

Fig. 5. Dependence between the correlation level and the distance of cells pairs in primary hippocampal cultures in the period after acute hypoxic injury. The adjacent cell pairs with their soma in direct contact are highlighted in red, and distant cell pairs in blue

A delay in the development of functional architecture of the neuron-glial networks was observed in the primary hippocampal cultures obtained from mouse embryos exposed to chronic prenatal hypoxia. On DIV 15, all network parameters examined in the «Hypoxia chronic» group were significantly lower compared to those in the «Intact» group. The network characteristics were normalized by DIV 21; no significant changes from the intact values were shown (Fig. 3).

Acute hypoxic injury caused a pronounced negative effect on the functional architecture of neuron-glial networks of both groups of primary cultures obtained from hypoxia-induced and non-hypoxic-induced hippocampal tissue. All network parameters of the «Hypoxia acute» and «Hypoxia chronic+acute» groups were significantly reduced compared to those of the control groups throughout the analyzed post-hypoxic period (Fig. 3). The observed changes potentially indicate the death of a part of the functionally active elements of neuron–glial networks, which increases the risk of the development of significant destructive rearrangements of networks leading to the impairments

in synaptic transmission, and, consequently, the loss of their function.

Analysis of correlation network graphs of primary hippocampal cultures strengthened our above-described observations. In the «Intact» group, the presence of high-correlated neuron-glial networks with a large number of functional connections between cells in the period of DIV 14 - DIV 21 (Fig. 4) was shown. On the other hand, a partial destruction of functional connections between cells of neuron-glial networks was characteristic for cultures obtained from hypoxia-induced mice embryos during whole observation period. The episode of acute hypoxia led to significant destruction of functional architectonics of neuron-glial networks. A similar dynamic of changes was observed in the «Hypoxia chronic+acute» group of cultures (Fig. 4).

Next, we analyzed the relation of the distance between cells and the level of correlation of Ca^{2+} oscillations (Fig. 5). In the period of DIV 14 - DIV 21 of the «Intact» culture's cultivation, complex network interactions with many correlated connections between distance and adjacent cells were shown. Moreover, most of the points reflecting the relationship between adjacent cell pairs with their soma in direct contact was in the correlation range from 0.8 to 1 (Fig. 5).

The cultures obtained from hypoxia-induced mice embryos showed many correlated connections between distance and adjacent cells, however the level of correlation between cells was reduced significantly. The point cloud of direct contacts of the adjacent cell pairs was shifted to the beginning of coordinates, and the correlation level was in the range from 0 to 0.4 (Fig. 5). Acute hypoxic injury led to a dramatic decrease in the level of correlation between cells. In the «Hypoxia acute» group, the point cloud was mainly concentrated in the range from 0 to 0.2, which is considered to be a lack of correlation (Fig. 5). Low correlation level between cells in the «Hypoxia chronic+actute» group was also shown. The point cloud reflected the correlated connections between distance and adjacent cells was located beginning of coordinates to 0.4.

Discussion

Chronic prenatal hypoxia is one of the leading damaging factors affecting fetal development, and for many years it has been a cornerstone of global health (Piešová & Mach, 2020; Nalivaeva *et al.*, 2018). The diversity of the causes of the development of fetal hypoxia, the sensitivity to the gestational period of the onset of its manifestation, the duration and intensity of hypoxic injury diminish the abilities in timely diagnosis and therapy as well as significantly challenge the prognosis of newborn's survival (Suresh *et al.*, 2021). The brain is the most vulnerable organ to oxygen supply disturbances resulting in a number of changes in its morphology and functional properties that in turn aggravates the functioning of other organs and systems in the human body. The overlapping effects cause the development of different pathologies of varying severity degrees, which determine the life expectancy and life quality of newborns in infancy and adulthood (Yeo *et al.*, 2019; Vasiliev *et al.*, 2016; Oechmichen *et al.*, 2006; Erecińska *et al.*, 2001).

In addition to the formation and increase the frequency of chromosomal aberrations mainly accompanied by hypoxic influence in the I-II trimester of pregnancy which leads to gross malformations, an oxygen deficiency can significantly affect the expression of a variety of genes, resulting in changes in a mRNA signature and protein expression with posttranslational modifications, including protein misfolding and clearance (Merelli *et al.*, 2021). Hypoxia-induced violations in the structure of synaptic apparatus and disturbances in the synaptic transmission prevents the formation of new contacts between cells and the signal speed propagation, primarily in the cerebral cortex and hippocampus, which play important roles in learning and memory, higher cognitive functions and development of neurodegenerative processes (Yan *et al.*, 2022). It is also worth noting a different level of gender adaptive capacity and plasticity of the nerve tissue, which determines the effectiveness of resistance to chronic prenatal hypoxia (Mishchenko *et al.*, 2022; Netto *et al.*, 2017; Sanches *et al.*, 2015). Therefore, studies of short- and long-term con-

sequences of prenatal hypoxia is extremely relevant in terms of the mechanisms of development of various pathological states and ways to stimulate the adaptive capacities of the CNS to hypoxic injury.

Addressing this issue at the level of neuron-glial networks, the main functional units of the CNS responsible for the implementation of processing, storage, and transmission of information, (Mishchenko *et al.*, 2022b; Yuste, 2015), is of particular interest. Current progress in methods of neurobiology and mathematical data analysis allow to assess systematically the tiny mechanisms of morpho-functional rearrangements in the neuron-glial network architectonics under hypoxic damage, as well as the contribution of each element of the network to the development of the functional network response to the stress factor (Mishchenko *et al.*, 2022b). This opens a new avenue to fully understand the hypoxia-induced pathological processes in the CNS and make a breakthrough in therapeutic efficiency in near future.

In this study, we assessed the features of functional activity of neuron-glial networks of primary hippocampal cultures derived from mouse embryos exposed to chronic prenatal hypoxia in a late period of development *in vitro* and assessed their adaptive capabilities to the episode of acute hypoxic injury. The mice embryos were exposed to chronic prenatal hypoxia in the gestation period of days 14-18 corresponding to the II-III trimester of pregnancy, which is accompanied by the risk of neonatal mortality and hypotrophy, developmental disorders, neurodegeneration and accelerated aging and alterations in brain functions in the postnatal period (Mishchenko *et al.*, 2022a; Shchelchkova *et al.*, 2020; Urazov *et al.*, 2018).

We have shown the pronounced signs of developmental delays in the functional activity of neuron-glial networks of primary cultures obtained from embryonic hippocampal tissue exposed to chronic hypoxia. On day 15 of primary hippocampal cultures development *in vitro*, which, in normal state, is characterized by a stable morphologically formed neuron–glial networks providing a complex functional pattern of their activity (Mitroshina *et al.*, 2021; Mishchenko *et al.*, 2019; Shirokova *et al.*, 2013), the spontaneous calcium activity of primary cultures from the «Hypoxia chronic» group is associated with a reduced number of functionally active cells, followed by changes in a calcium activity profile formed by Ca^{2+} oscillations with reduced duration. The observed changes in the calcium activity profile indicate impaired calcium fluxes across the plasma membrane of nerve cells, suggesting altered structure of synaptic contacts and disturbances in the synaptic transmission (Schneider & Miller, 2019). In addition, the network characteristics of primary cultures obtained from hypoxia-induced mice embryos is represented by a reduced number of correlated connections accompanied by both decreased signal speed propagation and average level of correlation between cells in a neuron-glial network, which are normalized to the intact values at a later stage of cultivation period (DIV 21). However, a partial destruction of the functional architecture of neuron-glial networks characterized by a reduced force of correlated connections between distance and adjacent cells in a network persisted throughout the entire observation period. The observed changes potentially indicate to the impairments in synaptic transmission affecting on the synaptic plasticity followed by a reduction of adaptive capabilities of nerve cells to external stress factors.

Preliminary exposure to chronic hypoxia altered the functional calcium activity of primary hippocampal culture's response to acute episode of oxygen deficiency. Against the background of significant decrease in the number of functionally active cells, the frequency and duration of Ca^{2+} oscillations did not differ from the intact values on day 7 after acute hypoxic stress modelling. Interestingly, the duration of Ca^{2+} oscillations was significantly reduced in comparison with the «Hypoxia acute» group during the post-hypoxic period examined. It can be assumed that a more stable calcium profile is associated with the activation of adaptive processes aimed at maintaining a delicate balance in the glutamatergic system preventing the development of pronounced excitotoxicity in nerve cells (Dong *et al.*, 2009). An equally im-

portant aspects that could be addressed in further research is a study of possible maintenance of mitochondrial respiration activity and HIF-regulated pathways which can be important players in weakening the negative effects induced by acute hypoxic stress (Mitroshina *et al.*, 2021; Fuhrmann & Brüne, 2017).

Nevertheless, significant decrease in the values of network characteristics accompanied by substantial rearrangements in the functional architectonics of neuron-glial networks indicate the death of a part of the functionally active network elements and increase the risk of aggravation of synaptic transmission impairments leading to loss of network's functionality. Future studies of the functional activity and network characteristics of primary hippocampal cultures in more distant post-hypoxic period may shed light on our assumptions.

Conclusion

Primary hippocampal cultures derived from mouse embryos exposed to chronic prenatal hypoxia demonstrate several delays in the development of their functionality *in vitro*. Alterations in the spontaneous calcium activity of primary hippocampal cultures are associated with a reduced number of cells that exhibiting Ca^{2+} activity, and changes in a calcium activity profile formed by Ca^{2+} oscillations of reduced duration. Partial destruction of the functional architecture of neuron-glial networks is represented by a reduced network parameters (DIV 15) with a persistent reduced force of correlated connections between distance and adjacent cells in a network at a later stage of development (DIV 21).

Preliminary exposure to chronic hypoxia altered the functional calcium activity of primary hippocampal culture's response to acute episode of oxygen deficiency. Against the background of significant decrease in the number of functionally active cells, the frequency and duration of Ca^{2+} oscillations did not differ from the intact values on day 7 after acute hypoxic stress modelling. Nevertheless, significant decrease in the values of network characteristics accompanied by substantial rearrangements in the functional architectonics of neuron-glial networks indicate the death of a part of the functionally active network elements and increase the risk of impairments in synaptic transmission leading to loss of network's functionality in more distant post-hypoxic period.

Acknowledgements

This research was funded by grant from the Russian Science Foundation (RSF), project no. 18-75-10071-p. This research was carried out using The Core Facilities «Molecular Biology and Neurophysiology».

References

BURTSCHER J., MALLET R.T., BURTSCHER M. & MILLET G.P. (2021): Hypoxia and brain aging: Neurodegeneration or neuroprotection? *Ageing Res Rev* **68**, 101343.

COQ J.O., DELCOUR M., MASSICOTTE V.S., BAUD O. & BARBE M.F. (2016): Prenatal ischemia deteriorates white matter, brain organization, and function: implications for prematurity and cerebral palsy. *Dev Med Child Neurol* **58** (Suppl 4), 7–11.

DOMÈNECH M., HERNÁNDEZ A., PLAJA A., MARTÍNEZ-BALIBREA E. & BALAÑÀ C. (2021): Hypoxia: The Cornerstone of Glioblastoma. *Int. J. Mol. Sci.* **22**, 12608.

DONG X., WANG Y. & QIN Z. (2009): Molecular Mechanisms of Excitotoxicity and Their Relevance to Pathogenesis of Neurodegenerative Diseases. *Acta Pharmacol Sin* **30**, 379–387.

DUNN J.F. & ISAACS A.M. (2021): The impact of hypoxia on blood-brain, blood-CSF, and CSF-brain barriers. *J Appl Physiol (1985)* **131**(3), 977–985.

ERECIŃSKA M. & SILVER I.A. (2001): Tissue oxygen tension and brain sensitivity to hypoxia. *Respir Physiol* **128**(3), 263–276.

FUHRMANN D.C. & BRUNE B. (2017): Mitochondrial composition and function under the control of hypoxia. *Redox Biology* **12**, 208–215.

GIANNOPOULOU I., PAGIDA M.A., BRIANA D.D. & PANAYOTACOPOULOU M.T. (2018): Perinatal hypoxia as a risk factor for psychopathology later in life: the role of dopamine and neurotrophins. *Hormones* **17**, 25–32.

KOBYLAREK D., IWANOWSKI P., LEWANDOWSKA Z., LIMPHAIBOOL N., SZAFRANEK S., LABRZYCKA A. & KOZUBSKI W. (2019): Advances in the Potential Biomarkers of Epilepsy. *Frontiers in Neurology* **10,** 685.

KUSTIKOVA V., KRIVONOSOV M., PIMASHKIN A., DENISOV P., ZAIKIN A., IVANCHENKO M., MEYEROV I. & SEMYANOV A. (2018): CalciumCV: Computer Vision Software for Calcium Signaling in Astrocytes. In *Proceedings of the Lecture Notes in Computer Science (including subseries Lecture Notes in Artificial Intelligence and Lecture Notes in Bioinformatics)*; Vol. 11179 LNCS. 7th International Conference - Analysis of Images, Social networks and Texts (AIST 2018), Moscow, Russia, 5–7 July 2018.

LAWN J.E., COUSENS S. & ZUPAN J. (2005): Lancet Neonatal Survival Steering Team. 4 million neonatal deaths: when? Where? Why? *Lancet* **365**(9462), 891–900.

LESTÓN PINILLA L., UGUN-KLUSEK A., RUTELLA S. & DE GIROLAMO L.A. (2021): Hypoxia Signaling in Parkinson's Disease: There Is Use in Asking «What HIF?». *Biology* **10**, 723.

LOGINOVA M., MISHCHENKO T., SAVYUK M., GUSEVA S., GAVRISH M., KRIVONOSOV M., IVANCHENKO M., FEDOTOVA J. & VEDUNOVA M. (2021): Double-Edged Sword of Vitamin D3 Effects on Primary Neuronal Cultures in Hypoxic States. *Int J Mol Sci.* **22**(11), 5417.

MERELLI A., REPETTO M., LAZAROWSKI A. & AUZMENDI J. (2021): Hypoxia, Oxidative Stress, and Inflammation: Three Faces of Neurodegenerative Diseases. *J Alzheimers Dis.* **82**(s1), S109–S126.

MITROSHINA E.V., LOGINOVA M.M., SAVYUK M.O., KRIVONOSOV M.I., MISHCHENKO T.A., TARABYKIN V.S., IVANCHENKO M.V. & VEDUNOVA M.V. (2021): Neuroprotective Effect of Kinase Inhibition in Ischemic Factor Modeling In Vitro. *Int J Mol Sci.* **22**(4), 1885.

MITROSHINA E.V., SAVYUK M.O., PONIMASKIN E. & VEDUNOVA M.V. (2021): Hypoxia-Inducible Factor (HIF) in Ischemic Stroke and Neurodegenerative Disease. *Frontiers in Cell and Developmental Biology* **28**, 9.

MISHCHENKO T.A., MITROSHINA E.V., USENKO A.V., VORONOVA N.V., ASTRAKHANOVA T.A., SHIROKOVA O.M., KASTALSKIY I.A. & VEDUNOVA M.V. (2019): Features of Neural Network Formation and Their Functions in Primary Hippocampal Cultures in the Context of Chronic TrkB Receptor System Influence. *Front Physiol.* **9**, 1925.

MISHCHENKO T.A., ZHIDKOVA N.M., URAZOV M.D., GOLUSHKOVA A.D., KUSTOVA A.O., LUKOVNIKOVA L.B., TERENTIEVA K.A., BABAEV A.A. & VEDUNOVA M.V. (2022): The Influence of Chronic Prenatal Hypoxia on the Functional State of Mice and Their Adaptation to Audiogenic Seizures. *Opera Med. Physiol*. **9**, 42–53. (a)

MISHCHENKO T.A., YARKOV R.S., SAVIUK M.O., KRIVONOSOV M.I., PERENKOV A.D., GUDKOV S.V. & VEDUNOVA M.V. (2022): Unravelling Contributions of Astrocytic Connexin 43 to the Functional Activity of Brain Neuron-Glial Networks under Hypoxic State In Vitro. *Membranes (Basel)* **12**(10), 948. (b)

MUKANDALA G., TYNAN R., LANIGAN S. & O'CONNOR J.J. (2016): The Effects of Hypoxia and Inflammation on Synaptic Signaling in the CNS. *Brain Sci.* **6**, 6.

NALIVAEVA N.N., TURNER A.J. & ZHURAVIN I.A. (2018): Role of Prenatal Hypoxia in Brain Development, Cognitive Functions, and Neurodegeneration. *Frontiers in Neuroscience* **12**, 825.

NETTO C.A., SANCHES E., ODORCYK F.K., DURAN-CARABALI L.E. & WEIS S.N. (2017): Sex-dependent consequences of neonatal brain hypoxia-ischemia in the rat. *Journal of Neuroscience Research* **95**(1-2), 409–421.

OECHMICHEN M. & MEISSNER C. (2006): Cerebral hypoxia and ischemia: the forensic point of view: a review. *J Forensic Sci* **51**(4), 880-7.

PIEŠOVÁ M. & MACH M. (2020): Impact of perinatal hypoxia on the developing brain. *Physiological Research* **69**(2), 199–213.

RILJAK V., KRAF J., DARYANANI A., JIRUŠKA P. & OTÁHAL J. (2016): Pathophysiology of perinatal hypoxic-ischemic encephalopathy - biomarkers, animal models and treatment perspectives. *Physiological Research* **65**, 533–545.

SANCHES E.F., ARTENI N., NICOLA F., ARISTIMUNHA D. & NETTO C.A. (2015): Sexual dimorphism and brain lateralization impact behavioral and histological outcomes following hypoxia-ischemia in P3 and P7 rats. *Neuroscience* **290**, 581–593.

SAVYUK M.O., TURUBANOVA V.D., MISHCHENKO T.A., LERMONTOVA S.A., KLAPSHINA L.G., KRYSKO D.V. & VEDUNOVA M.V. (2022): Unraveling of Functional Activity of Primary Hippocampal Neuron-Glial Networks in Photodynamic Therapy Based on Tetracyanotetra(aryl)porphyrazines. *Cells* **11**(7), 1212.

SCHNEIDER J. & MILLER S.P. (2019): Preterm brain Injury: White matter injury. *Handb Clin Neurol* **162**, 155-172.

SHCHELCHKOVA N.A., KOKAYA A.A., BEZHENAR' V.F., ROZHDESTVENSKAYA O.V., MAMEDOVA M.A., MISHCHENKO T.A., MITROSHINA E.V. & VEDUNOVA M.V. (2020): The Role of Brain-Derived Neurotrophic Factor and Glial Cell Line-Derived Neurotrophic Factor in Chronic Fetal Oxygen Deprivation. *Sovremennye tehnologii v medicine* **12**(1), 25–31.

SHIROKOVA O.M., MUKHINA I.V., FRUMKINA L.E., VEDUNOVA M.V., MITROSHINA E.V., ZAKHAROV Y.N. & KHASPEKOV L.G. (2013): Morphofunctional Patterns of Neuronal Network Developing in Dissociated Hippocampal Cell Cultures. *Sovremennye tehnologii v medicine* **5**(2), 6–13.

URAZOV M.D., ASTRAKHANOVA T.A., USENKO A.V., MISHCHENKO T.A., SCHELCHKOVA N.A., KRAVCHENKO G.A., VEDUNOVA M.V. & MITROSHINA E.V. (2018): New Aspects of Central Nervous System Adaptation to Prenatal Hypoxia. *Sovremennye tehnologii v medicine* **10**(4), 60–66.

VASILEV D.S., DUBROVSKAYA N.M., TUMANOVA N.L. & ZHURAVIN I.A. (2016): Prenatal Hypoxia in Different Periods of Embryogenesis Differentially Affects Cell Migration, Neuronal Plasticity, and Rat Behavior in Postnatal Ontogenesis. *Front Neurosci*. **10**, 126.

VEDUNOVA M.V., MISHCHENKO T.A., MITROSHINA E.V. & MUKHINA I.V. (2015): TrkB-Mediated Neuroprotective and Antihypoxic Properties of Brain-Derived Neurotrophic Factor. *Oxid Med Cell Longev* **2015**, 453901.

VICTOR S., ROCHA-FERREIRA E., RAHIM A., HAGBERG H. & EDWARDS D. (2022): New possibilities for neuroprotection in neonatal hypoxic-ischemic encephalopathy. *Eur J Pediatr* **181**(3), 875–887.

WANG X., HOU Y., LI Q., LI X., WANG W., AI X., KUANG T., CHEN X., ZHANG Y., ZHANG J., HU Y. & MENG X. (2019): Rhodiola Crenulata Attenuates Apoptosis and Mitochondrial Energy Metabolism Disorder in Rats with Hypobaric Hypoxia-Induced Brain Injury by Regulating the HIF-1α/MicroRNA 210/ISCU1/2(COX10) Signaling Pathway. J. *Ethnopharmacol.* **241**, 111801.

WANG B., ZENG H., LIU J. & SUN M. (2021): Effects of Prenatal Hypoxia on Nervous System Development and Related Diseases. *Frontiers in Neuroscience* **15**, 755554.

YAN W., FAN J., ZHANG X., SONG H., WAN R., WANG W. & YIN Y. (2021): Decreased neuronal synaptosome associated protein 29 contributes to poststroke cognitive impairment by disrupting presynaptic maintenance. *Theranostics*. **11**(10), 4616–4636.

YEO E.J. (2019): Hypoxia and aging. *Exp Mol Med.* **51**(6), 1–15.

YUSTE R. (2015): From the Neuron Doctrine to Neural Networks. *Nat. Rev. Neurosci*. **16**, 487–497.

ZHANG X., LI L., ZHANG X., XIE W., LI L., YANG D., HENG X., DU Y., DOODY R.S. & LE W. (2013): Prenatal hypoxia may aggravate the cognitive impairment and Alzheimer's disease neuropathology in APPSwe/PS1A246E transgenic mice. *Neurobiol Aging* **34**(3), 663–678.

ZHURAVIN I.A., DUBROVSKAYA N.M., VASILEV D.S., POSTNIKOVA T.Y. & ZAITSEV A.V. (2019): Prenatal hypoxia produces memory deficits associated with impairment of long-term synaptic plasticity in young rats. *Neurobiology of Learning and Memory* **164**, 107066.