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COMPARATIVE AND ONTOGENIC PHYSIOLOGY

Prenatal Hypoxia Modifies Working Memory and the Activity of Hippocampal Polyphosphoinositide System in Rats

E. I. Tyul'kova, L. A. Vataeva, O. V. Vetrovoi, and D. Yu. Romanovskii†

Pavlov Institute of Physiology, Russian Academy of Sciences, St. Petersburg, Russia
E-mail: anoxia@pavlov.infran

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Abstract—The aim of the present study was to explore spatial learning abilities in the Morris water maze (working memory) as well as hippocampal levels of phosphatidylinositol 4,5-diphosphates (TPI), phosphatidylinositol 4-phosphates (DPI), phosphotidylinositols (MPI), and expression of the type 1 inositol 1,4,5-trisphosphate receptor (IP3R1) in rats exposed to severe hypobaric hypoxia (ascent to 11 km, 3 h) on prenatal days 14–16 (group 1) or 17–19 (group 2). Exposure to severe hypoxia led to significant elevation of TPI and DPI hippocampal levels in juvenile and adult rats in group 1, however these changes were more pronounced in juvenile rats than in adults. In group 2, hypoxia upregulated TPI and DPI hippocampal levels in juvenile rats, but in adult animals of this group only a small TPI level upregulation was detected. Activation of IP3R1 expression was found to occur in the hippocampus both of juvenile and adult rats in groups 1 and 2. These data are consistent with our findings on impaired spatial learning ability in the Morris water maze indicative of a working memory deficit in the rat offspring exposed to hypobaric hypoxia during the first half of the last week of pregnancy.

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Key words: prenatal hypoxia, hippocampus, polyphosphoinositides, type 1 inositol 1,4,5-trisphosphate receptor, working memory.

INTRODUCTION

At present, a brain injury due to perinatal hypoxia-ischemia is one of the main causes of death and severe neurological disorders not only in neonates but also at later postnatal stages.

Modern concepts about the mechanisms of the effect of hypoxia-ischemia on the brain and treatment of its consequences are based mainly on the data obtained on adult animals. It is generally accepted that deep hypoxia or hypoxia-ischemia lead to the development of insufficiency of metabolic energy supply. It was established that under conditions of oxygen deficit neurons suffer first because their energy demands are higher than of any other cell type. Hypoxic exposure leads to an energy deficit and the development of acidosis mediated by glutamate excitotoxicity. During this process there occur the following events: activation of endogenous phospholipases, hydrolysis of membrane phospholipids, increase in membrane fluidity and permeability resulting in a loss of K^+ and compensative Na^+ and Ca^{2+} overload [1–3].

However, it is obviously improper to directly transfer the data obtained on adult animals to perinatal development disregarding the specific mechanisms of hypoxic brain injury inherent to this period. Only due consideration of these

mechanisms can help solve the issue of the severity of perinatal hypoxic pathology, specifically, fetal brain and general neonatal tolerance to hypoxia. At present, the idea that the immature brain is tolerant to the damaging effect of hypoxia and hypoxia-ischemia is widespread. However, numerous experimental and clinical studies showed that in pre- and perinatal ontogenesis there exist certain periods that are characterized by a higher susceptibility of the brain to various damaging factors. Perinatal hypoxia can be causal to long-persistent modification of the neuronal activity as well as to death of neurons. The effect of hypoxia or hypoxia-ischemia on the immature brain can disrupt its further development and promote later on the development of persistent pathology.

Tolerance to hypoxia depends on many factors. Among other things, sensitivity of the developing brain to hypoxia is determined by the lipid composition of neuronal membranes and lipid peroxidation-to-antioxidant protection ratio, development and modulation of the receptors of excitatory amino acids (such as glutamate receptors), intracellular Ca^{2+} levels and calcium-dependent intranuclear processes. It is well-known that the properties of membranes and receptors as well as the activity of different intracellular mechanisms change considerably during ontogenesis.

In our previous studies we investigated the influence of severe hypoxia at different terms of prenatal development. We showed that the last week of this period in rats is characterized by a substantial heterogeneity concerning the effects of hypoxic stress on the development of behavior and learning ability in postnatal ontogenesis [4, 5]. The intracellular regulatory systems play an important role in mediating the effects of hypoxia on the brain [3, 6, 7]. Specifically, it was shown that one of the consequences of hypoxia consists in a stable modification of the phosphoinositide system known to regulate metabolism and the functional activity of neurons. Activation of the polyphosphoinositide (PPI) signaling system, which leads to Ca^{2+}release from the intracellular calcium stores [8–10], plays an important role in regulation of cell survival and death via apoptosis [11]. In our previous studies we discovered the significant changes in neocortical metabolism of polyphosphoinositides and their ability to respond to the effect of glutamate in rats that exposed to hypobaric hypoxia at different stages of their prenatal development [12]. Considering the learning disability that we and other researchers observed, it is important to investigate the status of the PPI system in the hippocampus of rats exposed to prenatal hypoxia. The aim of this study was to determine the levels of PPI and IP3-receptor expression in the hippocampus of rats exposed to severe hypobaric hypoxia on days 14–16 and 17–19 of prenatal development.

With reference to the available literature data on the persistent changes in the activity of the PPI system in brain cells of patients with Alzheimer's disease and schizophrenia [13], we conducted a number of experiments to study the features of working memory in the offspring of rats exposed to hypoxia at the beginning and at the end of the last gestation week.

MATERIALS AND METHODS

All experiments were implemented in compliance with the demands formulated in Directives 89/609/EEC on using animals for experimental research. Experimental protocols were approved by the commission on humane treatment of animals at Pavlov Institute of Physiology.

150 Wistar rats obtained from the vivarium at Pavlov Institute of Physiology were used in this study. The day of birth was considered the first day of life. On day 2 after birth the number of neonate rat pups was equalized in the brood to 8. Every female with rat pups was kept in a separate cage (54×34×19 cm) at a constant temperature (20–22°C) in the vivarium room at 12 h/12 h light/dark cycle. Food ration was semi-synthetic.

To simulate prenatal hypoxic stress, female rats (weight 250–300 g) were placed on gestation days 14–16 or 17–19 into a flow-type pressure chamber with pressure maintained at 180 torr (corresponding to 10000-m ascent or 5% of oxygen under normobaric hypoxia). Each hypobaric sessions lasted 3 hours a day during 3 days running. Rats of the same age as the experimental ones whose mothers were also placed into a pressure chamber at certain stages of pregnancy without hypobaric hypoxia were used as a control.

PI were extracted from the hippocampal tissue

of juvenile (14-day-old) and adult (90-day-old) rats and separated into different fractions by paper chromatography [14]. PI level was evaluated by the phosphorus content (μg) per 1 mg of protein. Presented in this study are the data referring to changes in the levels of TPI (phosphatidylinositol-4,5-diphosphates), DPI (phosphatidylinositol-4-phosphates) and MPI (phosphatidylinositides).

Immunohistochemical investigations. At the due stages of postnatal development animals were decapitated and their brain was excised. Then the brain was fixed by immersion using MolFix according to manufacturer's recommendations and processed according to the standard histological protocol. Serial transverse slices, ~6–7 μm thick, were cut approximately 2.8 mm from bregma and mounted pairwise on slides covered with ovalbumine to prevent slice detachment.

To evaluate the expression of inositol 1,4,5-trisphosphate receptor in the rat hippocampus, immunocytochemistry along with computer image analysis were applied. For this purpose, after standard procedures of paraffin removal, rehydration and antigen retrieval, the slices were incubated with primary anti-IP3R1 (type 1) rabbit polyclonal antibody at +4°C overnight (Santa Cruz Biotechnology, USA). For visualization of the primary antibody binding sites, biotinylated donkey secondary antibodies (Jackson Lab (USA), ultrasensitive streptavidin-peroxidase polymer (Sigma, USA) and diaminobenzidine/peroxidase kit (Vector diagnostic, USA) were used.

Diaminobenzidine was used for visualization of the reaction product. After dehydration, the sections were embedded in gelatin and analyzed quantitatively for neuronal immunoreactivity using a setup composed of Jenaval light microscope (Carl Zeiss, Germany), digital camera Baumer CX05c (Baumer Optronic, Germany) and computer IBM PC with Video Test Master Morphology software. A semi-quantitative analysis of IP3R1 expression in the neurons of the hippocampal CA1 area according to a paradigm of immunohistochemical analysis that we developed previously. To exclude any artifacts associated with changes in light intensity, all digital images were obtained sequentially during a single working day. The hippocampal areas 500 μm and 350 μm long were analyzed in adult and 2-week-old rat pups, respectively. All neuronal images exhibiting visualizable immunoreactivity were digitalized. Labeling intensity, evaluated by optical density, was computed by averaging the data obtained from each individual slice. The neurons with labeling intensity higher than average were considered as neurons with heightened IP3R-1 expression. The number of these neurons was normalized to the total number of immunoreactive neurons.

Statistical analysis was carried out using the Student's *t*-test. The differences were considered significant at $p < 0.05$.

Training in the Morris water maze. Experiments were conducted on adult Wistar male rats. The animals were born both from intact females and those experienced severe hypobaric hypoxia on days 14–16 and 17–19 of prenatal development. Testing in the Morris water maze was conducted according to a protocol developed for the demonstration of specific disorders of working memory. The water maze represented a round swimming pool, 2.0 m in diameter and 0.7 m in depth. The water with added chalk powder lost its transparency. The water temperature was 23–24°C. The level of water was 20 cm lower than the pool brim. The maze was separated into four sectors. Animals were trained to look for a platform hidden under the water (the distance between the water surface and the platform was 2 cm, the platform diameter—12 cm). In testing, an animal was placed in one of the four maze sectors. If the rat could not find the platform during 60 s, it was aided by pushing gently toward the platform. The rat dwelt on the platform for 15 s. Then the animal was withdrawn from the cylinder, dried with a towel and placed back to a cage. After 15 s of testing in the trial 1, the animal was returned to the maze for the trial 2. The whole testing procedure included 4 pairs of trials separated by 4-min intervals. Between each pair of trials the position of the platform hidden under the water was changed by moving it to another maze sector. Search time during which rats spotted the platform (latent period for learned escape) in both trials was recorded. Based on the results obtained in the trials 1 and 2, the deficit of working memory was evaluated in the experimental animals.

Learning behavior of animals was recorded by

Content of TPI, DPI and MPI (μg P/mg of tissue protein) in the rat brain hippocampus

14-day-old			90-day-old		
TPI	DPI	MPI	TPI	DPI	MPI
0.512	0.288	0.740	0.174	0.171	0.712
±0.0211	±0.0215	±0.0466	±0.0139##	±0.0053##	±0.0387

Statistical significance: ##—$p < 0.01$ ($n = 8$–10) adult animals relative to 14-day-old animals.

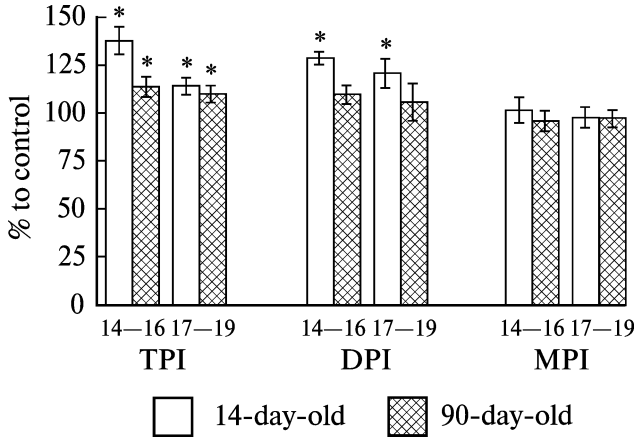

Fig. 1. Changes in the content of phosphatidylinositol-4,5-diphosphate (TPI), phosphatidylinositol-4-phosphate (DPI) and phosphatidylinositol (MPI) content in the hippocampus of juvenile (14-day-old) and adult (90-day-old) rats exposed to severe hypoxia on days 14–16 and 17–19 of prenatal development as compared with control. *—$p \le 0.05$; $n = 8$.

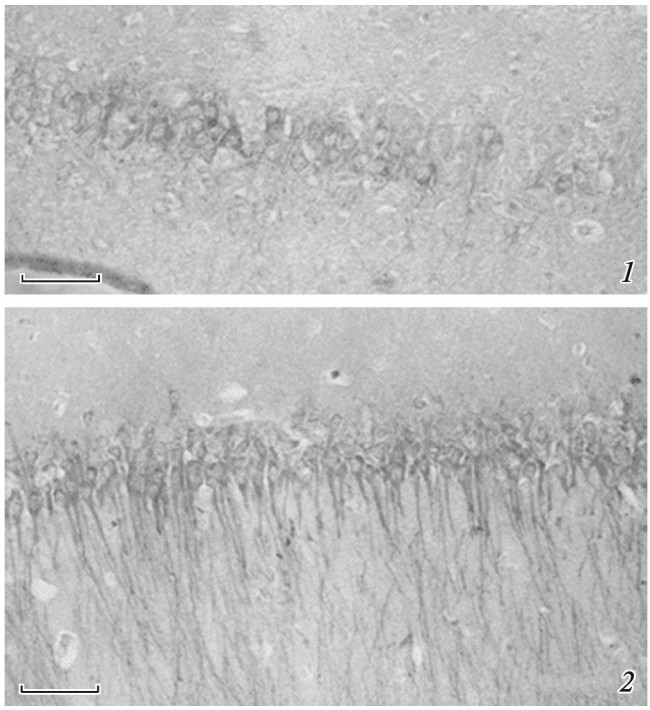

Fig. 2. Distribution of IP3R1 immunoreactivity in hippocampal CA1 area in 14-day-old (*1*) and 90-day-old (*2*) control rats (representative images). Scale: 100 μm.

a web camera. To get a soft diffused illumination in the experimental room, the light from a high-power lamp (500 W) was directed up to the ceiling which served as a secondary emitter with a large surface of emission. During the experiments, we watched animal's behavior on a computer screen. Both the observer and equipment were isolated from the experimental zone by a room partition.

RESULTS

The data on the content of some components of the hippocampal PI system of control juvenile and adult rats are presented in the Table.

From the Table it is seen that the PPI level in the hippocampus is much higher in control juvenile rats than in control adult animals.

Exposure to severe hypoxia on days 14–16 of prenatal development results in an increase in TPI and DPI levels in the hippocampus of 14-day-old rats. In adult animals exposed to severe hypoxia on days 14–16 of prenatal development, the TPI and DPI levels in the hippocampus, although to a lesser extent, were also higher than in control (Fig. 1). The MPI level in the brain of the experimental animals did not differ from the control level.

Long-term changes in the PPI content in the rat brain after prenatal hypoxia can be reflected in the state of the IP3R complex. We conducted a comparative study of the immunoreactivity of IP3R1-positive neurons in the hippocampal CA1 area of adult (90-day-old) and juvenile (14-day-old) control rats (Fig. 2), as well as rats exposed to hypoxia in the prenatal period.

We found the IP3R1 immunoreactivity in the hippocampal CA1 area of juvenile and adult animals (Fig. 2, (*1*) and (*2*)), what is consistent with literature data [15–17]. However, it was noticed that there was a significant difference in the number of immune-positive neurons between these two age groups. In the hippocampal CA1 area of the 14-day-old rats, 28.3 ± 9.1% of the total number of neurons exhibited clear-cut IP31-specific labeling, while in the respective brain area of the adult animals the number of these neurons came to 79.5 ± 12.1 % ($p < 0.05$, $n = 7$ for each group). Besides, it was shown that neurons of the juvenile animals exhibit most intensive labeling in soma and proximal axonal segments with its decrement in distal direction along the axon. In contrast, neuronal axons of the adult animals were labeled relatively uniformly along their length.

Exposure to prenatal hypoxia on days 14–16 and 17–19 of embryonic development raises the number of neurons with heightened expression of IP3R1 in the 14-day-old rats as compared with control. In the group exposed to hypoxia on days 17–19 of prenatal development the difference was significant ($p < 0.05$) and preserved in the adult animals (90-day-old rats) (Fig. 3).

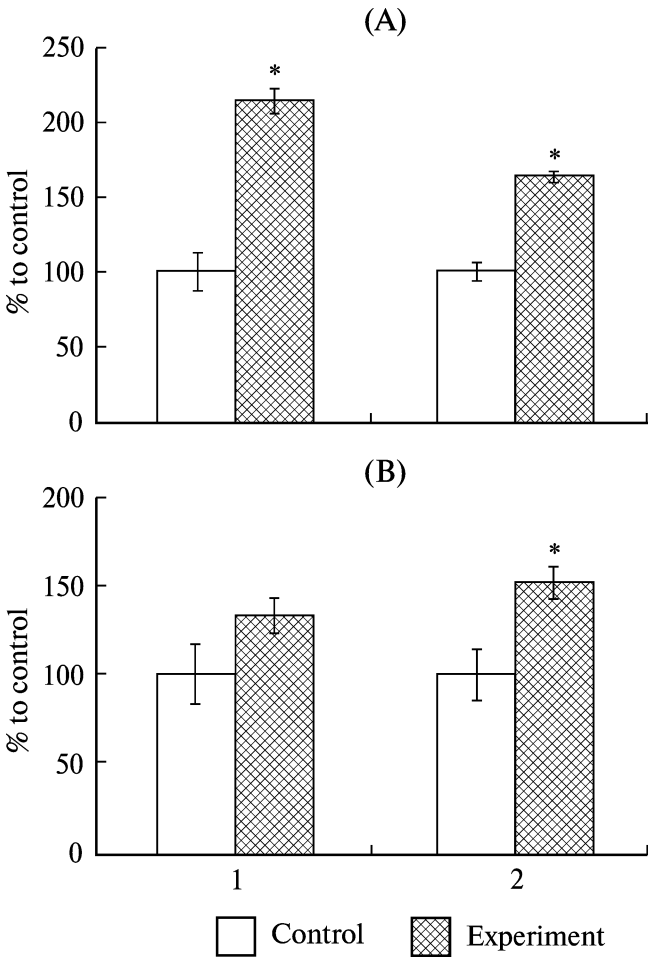

Fig. 3. The effect of exposure to severe hypobaric hypoxia on days 4–16 (1) and 17–19 (2) of rat prenatal development on the number of heavily labeled IP3R-immunoreactive neurons in hippocampal CA1 area of juvenile (A) and adult (B) rats as compared with control. *—$p \le 0.05$; $n = 7$.

In a series of experiments on the peculiarities of working memory in the offspring of rats exposed to hypoxia at the beginning and at the end of the third week of gestation, the following data were obtained. The dynamics of the spatial differentiation development in the rats of control and experimental groups in the Morris water maze showed a significant difference in the latent period for learned escape between the trials 1 and 2 in control males and those exposed to prenatal hypoxia on days 17–19 of gestation (Fig. 4). In the group of experimental animals exposed to hypoxia on days 14–16 of prenatal development, no significant differences were revealed between the platform search time in the trials 1 and 2. These findings indicate a working memory deficit in this experimental group.

DISCUSSION

As shown previously [18], the level of polyphosphoisitides in the brain cortex of 14-day-old rats was significantly higher than in adult rats. Our data showed that the TPI level in the hippocampus of the 14-day-old rats was higher than in the neocortex. Like in the neocortex, the hippocampal PPI level in adult rats was significantly lesser than in 2-week-old animals. The early postnatal period (days 1–15 of life) of rat development is known as a period of "explosive" brain growth and characterized by dendrite ramification, formation of synaptic contacts and asrtroglia proliferation. Our results support the idea of the activation of PPI-dependent processes of myelination, proliferation and differentiation of the brain cells [19] and axonal growth [20] in this period. The second week of postnatal development is a critical period

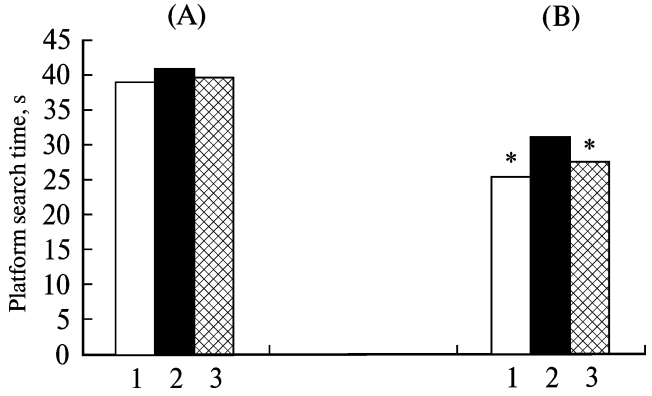

Fig. 4. The effect of severe hypoxia, experienced by female rats during gestational period, on training their offspring in the Morris water maze. (A) Platform search time in trial 1; (B) platform search time in trial 1. (*1*) Control rats, (*2*) and (*3*) rats exposed to hypoxia on days 14–16 and 17–19 of prenatal development, respectively. *—statistical significance of differences the platform search times in trials 1 and 2 at $p < 0.05$; $n = 8–10$ (criterion of learning ability).

as evidenced by many parameters of brain development [20–21].

In this study we showed that exposure to severe hypoxia during the gestation period can induce long-term changes in the content of polyphosphoinositides in the hippocampus of the rat offspring. The consequences of hypoxic exposure manifest themselves in different ways depending on the exposure time during the gestation period. When the effect of hypoxia fell on the beginning of the third week of gestation, significant changes in the PPI content in the hippocampus of both juvenile and adult rats were observed. The exposure to hypoxia at the end of the third week of gestation also induced an increase in the PPI content in the offspring hippocampus, although to a lesser extent. It is noteworthy that prenatal hypoxia does not affect the MPI content. It means that hypoxia affects the components of signaling transduction but not their precursors.

While the majority of studies of the functional role of IP3R-dependent intracellular Ca^{2+} release were fulfilled on the pyramidal neurons of the adult rat hippocampus and only few of them were implemented on rat pups at the age younger than 3 weeks [16, 22, 23], we were interested in comparing IP3R1 distribution in the hippocampus of juvenile (14-day-old) and adult (90-day-old) rats. As shown previously, the bulk of the IP3R1-specific immunoreactivity is confined to the hippocampal CA1 area [17]. No wonder that in our work we estimated the expression of IP3R1 in this specific brain area (Fig. 2). Although at the early stages of brain development IP3R1 are few, they do play an important role because of the high sensitivity of the phosphoinositide system [24]. These data are well consistent with our previous results on Ca^{2+} signaling in juvenile and adult animals [12].

Exposure to hypoxia on days 14–16 and 17–19 of prenatal development leads to an increase in the number of IP3R1-immunoreactive cells in the brain both of the 14-day-old and adult animals. The heightened expression of IP3R1 leads to an increase in Ca^{2+} release from intracellular stores and activation of the Ca^{2+}-dependent processes such as apoptosis, intracellular pH regulation and triggering gene expression [25, 26]. The data on the upregulation of IP3R1 expression by prenatal hypoxia obtained herein are consistent with our previous studies on the effect of prenatal hypoxia on the functional state of the Ca^{2+}-regulatory system in rats of the same age groups through the evaluation of Ca^{2+}-signaling mediated by the excitation of group I metabotropic glutamate receptors (ImGluR) in surviving brain slices [12].

Although a regulated elevation of intracellular calcium is necessary for Ca^{2+}-signaling, a sustained maintenance of the high intracellular Ca^{2+} level may lead to necrosis and apoptosis [27]. Among the intracellular mechanisms involved in calcium release, IP3R1 attracts special attention because it represents a unique combination of properties (including the regulatory complex with Ca^{2+} and other secondary messengers) which allows it to participate in most signaling pathways [28–32]. Undoubtedly, the IP3 receptor complex is believed to be the pivotal component of the Ca^{2+}-signaling system. It plays an important role in the regulation of such processes as gene expression, secretion, fertilization, proliferation, cell differentiation, development, signal initiation and cell death. Disregulation of the IP3R1 function may result in a pathological disruption of the Ca^{2+}-signaling system, namely, of its initiation, amplitude of Ca^{2+} signals, their frequency

and duration [33]. It was shown that the activation of IP3R1 has a negative impact on cognitive functions, while a blockade of IP3-mediated Ca^{2+} release in the prefrontal cortex increases the working memory [34–36]. Long-term activation of the IP3 receptor complex 1 in the hippocampal CA1 area (during the whole period tested) significantly changes the intracellular Ca^{2+} signaling system that may lead to memory impairment and deterioration of learning abilities resulting later on in neuronal dysfunction (associated with changes in neuronal functional activity), including Alzheimer's disease and schizophrenia [25, 32, 33].

The results obtained in our behavioral experiments using the Morris water maze demonstrate a deficit of working memory in the rats that were exposed to hypobaric hypoxia on days 14–16, but not 17–19, of prenatal development. It may relate to the above-described changes in PPI system changes described above.

Thus, exposure of rats to severe hypoxia at the last week of the gestational period causes to long-term changes in the activity of the PPI system in the rat offspring brain leading, in turn, to the disorders of behavior, memory and learning abilities. The data obtained may have a special significance for clinical practice, as they reveal the mechanisms of cognitive dysfunctions associated with hypoxia and other adverse impacts in early ontogenesis.

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