DOI 10.1007/s11055-014-0029-9
Neuroscience and Behavioral Physiology, Vol. 44, No. 9, November, 2014

Responses of Rat Brain Interneuronal Synapses to Hypoxia in the Early Neonatal Period

V. A. Otellin, L. I. Khozhai, and T. T. Shishko

UDC 611.018.86:612.273.2:612.648:599.323.4

Translated from Morfologiya, Vol. 145, No. 1, pp. 7–12, January–February, 2014. Original article submitted September 23, 2013.

The responses of forming synapses in the rat neocortex to the actions of hypoxia in the early period of neonatal life (day 2) were studied. Immunocytochemical studies were used to detect synaptophysin and these results, along with electron microscopic studies, addressed the sensorimotor cortex in rat pups at 3, 5, and 10 days of postnatal development (using groups of 6–10 individuals) in an experimental group and a control group (intact animals). Immunocytochemical studies of control animal showed significant differences in the quantitative distribution of synaptophysin-positive structures in different layers of the neocortex during the early neonatal period of development (day 5). Perinatal hypoxia decreased the optical density of the immunocytochemical reaction product more than twofold, and this was accompanied by reductions in the density of synaptophysin-positive granules in all layers of the neocortex. In addition, electron-dense terminals, providing evidence of degenerative processes, were seen. The neuropil of the neocortex showed a sharp decrease in the number of growth cones, small processes, and forming synapses, along with a significant increase in the electron density of synaptic elements, especially postsynaptic membranes and densities. In experimental animals, increases in the numbers of growth cones and forming synaptic structures were seen only by postnatal day 10. Thus, the consequences of hypoxia during the early neonatal period, inducing impairments to synaptogenesis, persisted throughout the study period.

Keywords: neocortex, neuron, synapse, synaptophysin, growth cone.

Studies of the dynamics of CNS structures at the early stage of ontogeny and the establishment of the mechanisms whereby adverse environmental factors influence them are among the current areas of interest in CNS studies.

Contemporary neonatology makes use of the concept of the perinatal stage of development, which includes the prenatal, delivery, and postnatal (following birth) periods. This stage determines the child's subsequent physical, neuropsychological, and intellectual development. The functions required for the independent existence of the newborn's body mature during this time. The brain in mammals at this time is characterized by a high level of sensitivity to the actions of adverse environmental factors. The common condition of hypoxia-ischemia is an important member of this group of factors, and has a variety of causes. It impairs the processes of structural and functional establishment of the brain, leading to motor disorders, seizures, mental development disorders, and other signs of cerebral impairment during subsequent ontogeny.

Recent years have seen reports of various experimental approaches to studying the effects of hypoxia-ischemia on the brains of term and preterm infants. The majority of these studies have used in-life investigations (MRI scans) allowing the dynamics of the development of the white and gray matter of the brain to be studied, which is relevant and in clinical demand. However, the low resolution of this method prevents it from assessing the state of cell and tissue processes.

It should be noted that experimental studies of the pathogenesis of hypoxic CNS injury in fetuses and neonatal children involve significant ethical, methodological, and

Nervous System Ontogeny Laboratory, Institute of Physiology, Russian Academy of Sciences, 6 Makarov Bank, 199034 St. Petersburg; e-mail: votellin@mail.ru; astarta0505@mail.ru.

methodical limitations. An alternative is therefore provided by the strategy of further pursuing studies of the pathogenesis of posthypoxic reactions in animal models with consideration of the species and neurobiological characteristics of the developing brain.

Modeling of perinatal cerebral pathology most often makes use of rats and mice, as brain structure and the responses of the brain to ischemia-hypoxia during the perinatal period of ontogeny in rodents are largely similar to those in higher mammals [1, 15]. One seminal report [4] established that the extent of differences in the neural elements of the brain, like the structure of the white matter, in Wistar rat pups on day 2 of postnatal development correspond in structural-functional terms to the brains of severely premature infants [5]. Thus, the experimental behavior of rat pups in the early postnatal period can be used to study the posthypoxic reactions of the brain of term animals and can provide a model for investigating the actions of hypoxia on the brains of preterm children.

The probability that neonatal infants will develop severe neurological and somatic impairments in response to the actions of adverse environmental factors (particularly hypoxia) is very high, as evidenced by physiological and clinical studies [2].

A small number of experimental studies on neonatal animals have used sometimes non-comparable conditions for hypoxic-ischemic damage and different timings for subsequent investigations. Our literature review did not identify any data on the establishment and structure of the synaptic apparatus and its responses to hypoxia during the early period of neonatal life, which prevents complete assessment of the nature and mechanisms of the posthypoxic reactions in the developing brain. The aim of the present work was to seek data of this type for the sensorimotor cortex, which takes part in organizing and executing higher nervous functions.

Materials and Methods. Experiments were performed using two groups of rats: group 1 consisted of animals subjected to hypoxia in a barochamber (experimental group); group 2 consisted of intact animals of the same age (control group). Animals were exposed to hypoxia on day 2 of postnatal development using a barochamber fitted with an automatic heater, a gas mix exchanger, and a gas flowmeter. The nitrogen-containing gas mix was prepared using a gas analyzer/exchanger (Laorg, France). Experimental animals were placed in the barochamber for 1 h. During experiments, the oxygen level in the barochamber was 7.6–7.8%, the carbon dioxide concentration was 0.15–0.20%, and the nitrogen concentration was 91.8% at $T = 21.3–23°C$ and normal total pressure. All procedures were performed in compliance with the "Regulations for Studies Using Experimental Animals."

The sensorimotor cortex was studied in rat pups on days 3, 5, and 10 of postnatal development (groups of 6–10 individuals for each time point). Histological studies of rat pup brains were performed after fixation in zinc-ethanol-formaldehyde in phosphate-buffered saline pH 7.4 for 24 h, dehydration, and embedding in paraffin using standard methods; serial frontal sections of thickness 5–7 ∝m were cut.

Immunocytochemical reactions for synaptophysin were performed using rabbit polyclonal antibodies to the integral membrane glycoprotein synaptophysin, a marker for presynaptic vesicles, from Dako Cytomation, Denmark, diluted 1:1. Antigen-antibody complexes were detected using an LSAB+2 reagent kit (Dako, Denmark). Reaction product was visualized using DAB+ chromogen (Dako, Denmark). Preparations obtained from control animals and experimental animals were processed simultaneously. After immunocytochemical reactions, some sections were counterstained with Gill's hematoxylin and embedded in Permount (Thermo, USA).

Studies were performed using a Leica DME light microscope (Leica, Germany). Images were obtained using a Leica EC3 digital video camera (Leica, Germany).

Studies used a light microscope, digital camera, and computer running the program VideoTest Master Morfologiya (Video Test, Russia) for assessment of the optical density (OD) of the reaction product in the layers of the neocortex. Data were processed by calculating arithmetic mean values and their standard errors in the groups of animals studied. Statistical analysis was performed by ANOVA (Statistica 7.0, Statsoft Inc., USA). Differences were regarded as significant at $p < 0.05$.

Neocortex (frontal, sensorimotor, and auditory areas) was prepared for electron microscopy studies by fixing cortex fragments in 2.5% glutaraldehyde solution in 0.1 M phosphate buffer pH 7.4 supplemented with sucrose, followed by additional fixation in 2% osmium tetroxide solution and embedding in Epon. Ultrathin sections were prepared on an LKB-III ultratome (Productor, Sweden), which were contrasted on grids with 1.5% uranyl acetate and lead citrate and examined under a JEM-100B electron microscope (Jeol, Japan) at an accelerating voltage of 70 kV.

Results

Immunocytochemical reactions for synaptophysin showed that on day 3 of postnatal development, layer I of the sensorimotor cortex in intact animals showed a high reaction product OD (0.154 ± 0.015), with lower levels in layers II–III. Immunopositive granules were located singly or in small groups of 2–6 granules on rare processes both in the neuropil and the plasmalemma of the bodies of isolated neurons. In the deep layers (V–VI) of the neocortex, synaptophysin reaction product OD increased sharply compared with that in layers II–III and was essentially identical in these layers, though it was slightly greater than the OD in layer I (0.176 ± 0.011). Numerous groupings of immunopositive granules were seen in the neuropil and on the plasmalemma of neuron bodies.

On day 3 of postnatal development (one day after hypoxia), experimental animals showed a lower synaptophysin reaction product OD in almost all layers of the neocortex than controls. Layer I showed both a decrease in

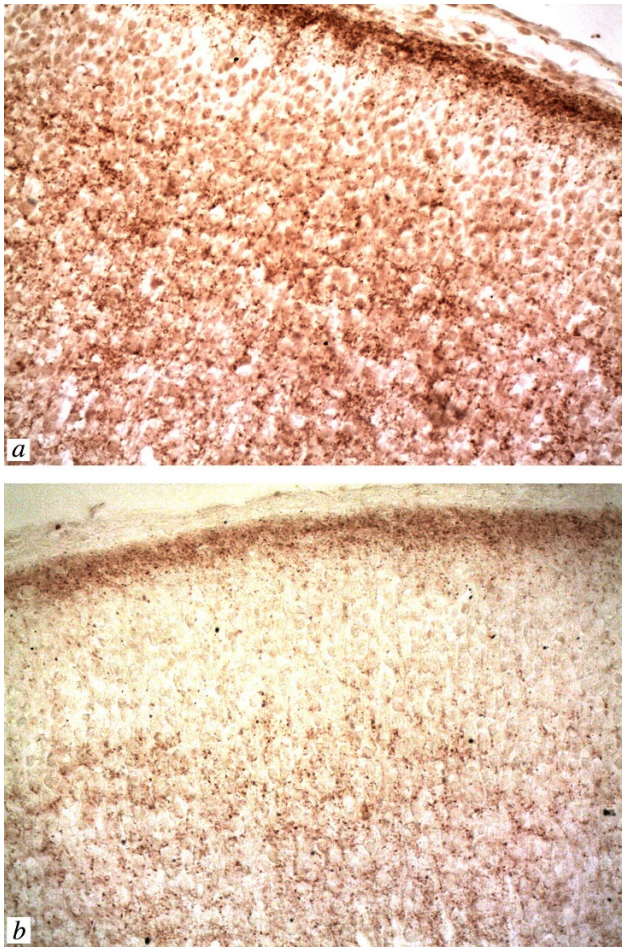

Fig. 1. Neocortex of a rat on day 3 of postnatal development. *a*) Rat of the control group; *b*) decreased intensity of immunocytochemical reaction for synaptophysin one day after exposure to hypoxia. Objective · 100, ocular · 10.

reaction product OD – about twofold (0.077 ± 0.016) – and a reduction in the density of synaptophysin-positive granules (Fig. 1, *a*, *b*). In layers II–III, only the neuropil contained isolated immunopositive granules, while granule density in layers V–VI of the neocortex was essentially identical, though synaptophysin reaction product OD was significantly lower – by a factor of 2.8 (0.063 ± 0.011) – than in controls.

On day 5 of postnatal development, all layers of the study areas of the neocortex in control animals showed marked increases in density of synaptophysin-positive granules.

On day 5 of postnatal development (three days after exposure to hypoxia), as at the previous time point, synaptophysin reaction product OD and the density of synaptophysin-positive granules in experimental animals were somewhat lower.

Electron microscope studies in rat pups on day 2 of life (baseline controls) showed that the neuropil in the superficial layers of the neocortex contained large numbers of mainly small processes, among which dendritic tubule characteristics identified dendrites and a multitude of axon and dendrite growth cones, as well as cytoplasmic pseudopodia belonging to neurons with large numbers of large and differently sized light vesicles (Fig. 2, *a*). These vesicles were typical of growth cones and their presence in pseudopodia may represent an initial stage in axon or dendrite development directly from nerve cell bodies (see Fig. 2).

At this stage of ontogeny, only the initial stages of formation of interneuronal synapses were seen. The picture was dominated by the early stages, at which the electron densities of the opposing axon and dendrite membranes and the membranes between axons and cell bodies increased, with some widening of the clefts between them (see Fig. 2, *c*). Axon terminals contained small numbers of light synaptic vesicles, with different extents of concentration close to the presynaptic membrane or in the center of the bouton, along with occasional light mitochondria with an undeveloped system of cristae. Postsynaptic dendrites, in terms of size and the number of dendritic tubules, were intermediate and large, without spines. These pictures were typical of the superficial layers of the sensorimotor cortex, while layer V contained relatively small numbers of synapses forming larger boutons with more differentiated dendrites and clear postsynaptic densities.

At one day after hypoxia (of day 3 of life), the sensorimotor cortex showed a sharp increase in the electron density of the of some presynaptic processes, which were virtually filled with synaptic vesicles, mainly of a single size, along the presynaptic membrane, along with small numbers of large vesicles at the opposite pole. In the synapses formed by these processes, the postsynaptic densities (on neuron bodies or dendrites) were less marked and were rarer than in controls. These electron-dense terminals could represent dark-type reactions or the onset of a degenerative process (see Fig. 2, *d*). The neuropil showed a sharp decreases in the numbers of axon and dendrite growth cones and the number of synapses at the early stages of maturation.

On day 5 of postnatal development (three days after exposure to hypoxia), the ultrastructural reaction of the neocortex was generally little different from that described above. However, as compared with the changes one day after hypoxia and, especially, controls, there was a notable delay in the differentiation of the protein-synthesizing apparatus in the cytoplasm of neurons, which was combined with significant decreases in the numbers of axon and dendrite growth cones. Many presynaptic boutons were characterized by increased axoplasm electron density without any change in the number and size of light synaptic vesicles (see Fig. 2, *e*). The initial stages of synapse formation were essentially absent. The densities in the pre- and postsynaptic membranes of already-formed synapses persisted.

On day 10 of postnatal development (7–8 days after exposure to hypoxia, the neuropil continued to be dominated by the early phases of synapse formation; axon terminals

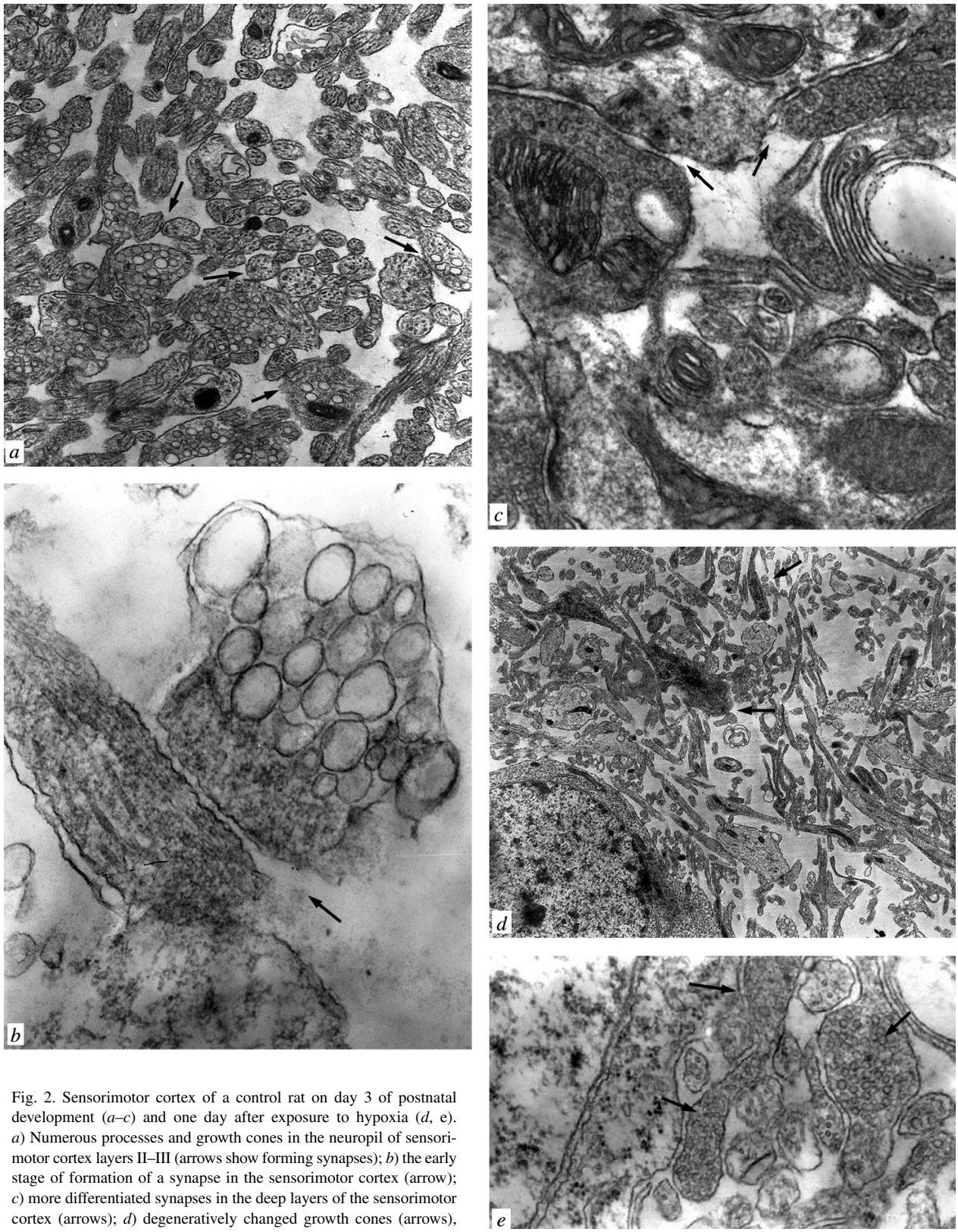

Fig. 2. Sensorimotor cortex of a control rat on day 3 of postnatal development (*a*–*c*) and one day after exposure to hypoxia (*d*, e). *a*) Numerous processes and growth cones in the neuropil of sensorimotor cortex layers II–III (arrows show forming synapses); *b*) the early stage of formation of a synapse in the sensorimotor cortex (arrow); *c*) more differentiated synapses in the deep layers of the sensorimotor cortex (arrows); *d*) degeneratively changed growth cones (arrows), absence of initial stages of synapse establishment in the upper layers of the sensorimotor cortex; *e*) presynaptic boutons with increased electron density, forming synapses (arrows) in the deep layers of the sensorimotor cortex. Magnification: *a*) · 30,000; *b*) · 60,000; *c*, *e*) · 40,000; *d*) · 10,000.

with flocculent matrix and decreased numbers of synaptic vesicles (due to destructive processes) were seen, though there was an increase in the number of nerve process growth cones. Dendrites bore occasional spines.

Thus, slowing of synaptogenesis was seen throughout the whole of the early neonatal period, along with reactions of a number of pre- and postsynaptic processes, with reductions in the number of growth cones and delays in their ultrastructural formation seen at one and five days after exposure to hypoxia. Only by day 10 was there some activation of axonal and dendritic process growth, though the number of mature synapses remained insignificant.

Discussion

At the early stages of the post-embryonic period, the brain shows continuing migration and differentiation of neurons and glial cells, and synaptogenesis is activated.

Establishment of interneuronal connection systems serves as a measure for both the differentiation of neurons and the development of the whole CNS and its multifarious functions. Data have been obtained on changes in the electrophysiological characteristics and measures of synaptic transmission in pyramidal cells in hippocampal field CA1 after ischemia-hypoxia during the neonatal period in rats [9]. Studies using a fluorescence/electron microscopy method have shown that perinatal asphyxia induces early changes in postsynaptic cytoskeletal F-actin, combined with modifications to other components of the neuron cytoskeleton in the rat striatum [10].

Attention to the problem of synaptogenesis comes from the fact that the development of interneuronal contacts and the onset of their functioning reflect the rates of maturation of the brain, the extent of the differentiation of its cellular elements, and the formation of genetically determined systems of connections supporting its various functions. The mature synapse is an indicator of a high level of neuron differentiation.

An immunocytochemical method for detecting synaptophysin-positive structures provides objective evaluation of the level of brain maturation, demonstrating not only progressive increases in the number of synaptic vesicles, but also development of the shape and size of the presynaptic structures containing them [11]. Synaptophysin is an integral synaptic vesicle membrane component and is expressed not only in mature synapses, but also in axon growth cones [7].

Our light microscope immunocytochemical studies identified significant differences in the layer-by-layer and quantitative distributions of synaptophysin-positive structures, which is consistent with data from laboratory and animals reported by several authors [7, 9, 11–13]. In response to hypoxia, ischemia, and asphyxia, these indicators in fetuses, as in our experiment, show decreases in the number and density of presynaptic boutons [14]. Our observations provide evidence that during the process of establishment of synapses in controls and, to a significantly greater extent, after exposure to perinatal hypoxia, there were significant changes in the electron density of synaptic and especially postsynaptic membranes and densities. These responses to conditions and parameters of experimental hypoxia different from those used here are attracting the interest of increasing numbers of investigators. Staining specimens for electron microscopy studies with phosphotungstic acid after intrauterine hypoxia, the authors found disorganization of synapses and increases in the widths of postsynaptic densities in the neostriatum and hippocampus in young rats, which was less marked in males than females [3].

Correlated fluorescence-electron microscopy studies established that perinatal asphyxia induces early changes to postsynaptic cytoskeletal F-actin, combined with intense modification of other components of the neuron cytoskeleton [10]. The authors suggested that F-actin accumulation in dendritic spines might be involved in posthypoxic neuron death, i.e., spines whose development times indicate an important role in the responses of the developing brain to hypoxia. Studies of fetal and postnatal synaptogenesis in mice led to the suggestion that synaptogenesis correlates with the development of spines and synaptophysin expression, i.e., maturation of synapses is synchronized with the differentiation of dendritic spines [8].

Considering current views on the role of dendritic spines in synaptogenesis and their absence in our specimens, we can be more confident in identifying that the synaptic apparatus is immature throughout the whole of the early neonatal period.

Despite the fact that neonatal rat pups showed a predominance of interneuronal contacts which did not reach maturity, they nonetheless functioned in the developing brain. In field CA1 of neonatal rats, exposure to hypoxia-ischemia was followed immediately by changes in the electrophysiological characteristics of neurons and measures of synaptic transmission involving the glutamate transporter, various receptors, and synaptophysin [9]. It remains to be seen which functions involve the nerve centers studied.

Recent years have seen the beginning of the accumulation of data on the relationship between impaired synaptogenesis and the development of various neuromuscular diseases and syndromes (autism, epilepsy, learning and behavioral difficulties, etc.) [6]. The present study may contribute to this direction; apart from its basic aspect, it provides the opportunity to test pharmacological agents regulating brain development processes in preterm infants and to correct the effects of adverse environmental conditions on CNS structures and functions in subsequent ontogeny.

This study was supported by the Russian Foundation for Basic Research (Grant No. 13-04-00160) and a grant from the “Basic sciences – medicine” Program of the Presidium of the Russian Academy of Sciences.

The authors would like to express their deep thanks to Doctor of Biological Sciences E. A. Rybnikova and Master of Biological Sciences K. A. Baranova, Neuroendocrinology Laboratory, for assistance in developing methods for measuring the optical density of immunological reaction products.

REFERENCES

1. S. V. Lebedev, A. V. Karasev, S. O. Rogatkin, et al., "Problems and perspectives in the experimental modeling of hypoxic-ischemic damage to the central nervous system," *Vestn. Ros. Adak. Med. Nauk.*, No. 2, 21–26 (2009).
2. I. B. Ushakov and V. P. Fedorov, *Oxygen. Radiation. The Brain.*, Nauch. Kniga, Voronezh (2011).
3. E. Cebral and C. F. Loidl, "Changes in neostriatal and hippocampal synaptic densities in perinatal asphyctic male and female young rat: role of hypothermia," *Brain Res. Bull.*, **84**, No. 1, 31–38 (2011).
4. B. Clancy, R. B. Darlington, and B. L. Finlay, "Translating developmental time across mammalian species," *Neuroscience*, **106**, No. 1, 7–17 (2001).
5. J. M. Dean, M. D. Moravec, M. Grafe, et al., "Strain-specific differences in perinatal rodent oligodendrocyte lineage progression and its correlation with human," *Dev. Neurosci.*, **33**, No. 3–4, 251–260 (2011).
6. J. J. Garcia-Penas, J. Dominguez-Carral, and E. Pereira-Bezanilla, "Abnormalities of synaptogenesis in autism. Pathogenic and therapeutic implications," *Rev. Neurol.*, **54**, Suppl. 1, 1–50 (2012).
7. L. K. Laeme, K. B. Repke, R. Hawkes, and F. L. Rice, "Synaptogenesis in the rat suprachiasmatic nucleus: a light microscopic immunocytochemical survey," *Brain Res.*, **544**, No. 1, 108–117 (1991).
8. M. Li, Z. Cui, Y. Niu, et al., "Synaptogenesis in the developing mouse visual cortex," *Brain Res. Bull.*, **81**, No. 1, 107–113 (2010).
9. H. J. Pyeon and Y. I. Lee, "Differential expression levels of synaptophysin through developmental stages in hippocampal region of mouse brain," *Anat. Cell Biol.*, **45**, No. 2, 97–102 (2012).
10. G. E. Saraceno, M. V. Avala, M. S. Badorrey, et al., "Effects of perinatal asphyxia on rat striatal cytoskeleton," *Synapse*, **66**, No. 1, 9–19 (2012).
11. H. B. Sarnat, R. N. Auer, and L. Flores-Sarnat, "Synaptogenesis in the fetal corpus striatum, globus pallidus, and substantia nigra: correlation with striosomes of Graybiel and dyskinesias in premature infants," *J. Child Neurol.*, **28**, No. 1, 60–69 (2013).
12. H. B. Sarnat and D. E. Born, "Synaptophysin immunocytochemistry with thermal intensification: a marker of terminal axonal maturation in the human fetal nervous system" *Brain Dev.*, **21**, No. 1, 41–50 (1999).
13. H. B. Sarnat, L. Flores-Sarnat, and C. L. Trevenen, "Synaptophysin immunoreactivity in the human hippocampus and neocortex from 6 to 41 weeks of gestation," *J. Neuropathol. Exp. Neurol.*, **69**, No. 3, 234–245 (2010).
14. E. Strackx, D. L. Van den Hove, H. P. Steinbusch, et al., "Fetal asphyxia leads to the loss of striatal boutons in adult rats," *Int. J. Dev. Neurosci.*, **28**, No. 3, 277–281 (2010).
15. S. J. Vannucci and H. Hagberg, "Hypoxia-ischemia in the immature brain," *J. Exp. Biol.*, **207**, No. 18, 3149–3154 (2004).