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Prenatal Hypoxia Leads to Impaired Formation of Nervous Tissue in the Entorhinal Area of the Cerebral Cortex in Rats

D. S. Vasilev,[1] N. L. Tumanova,[1] and D. S. Kalinina[1,2]

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Prenatal hypoxia impairs brain formation, leading to the development of cognitive deficit in the postnatal period. The probable causes of this deficit may be linked with derangement of the functioning of the dorsal hippocampus and areas of the neocortex involved in its afferentation, particularly the entorhinal cortex, whose projection neurons innervate field CA1. The aim of the present work was to assess the effects of prenatal hypoxia on formation of nervous tissues in the entorhinal cortex during early ontogeny in rats subjected to prenatal hypoxia on days 14 or 18 of embryogenesis. In vivo labeling of neuroblasts formed in embryos at the moment of exposure to hypoxia with 3'-ethinyl-5-deoxyuridine showed that depending on the time of exposure, hypoxia impaired the formation and migration of neuroblasts to the lower (hypoxia on day 14 of pregnancy) or upper (hypoxia on day 18) layers of the entorhinal cortex. Hypoxia on E14 but not E18 led to a reduction in the number of neurons in the entorhinal cortex in rat pups in the first month of postnatal ontogeny. This reduction is evidence of neuron death. Impairment to the process of neurogenesis affected the formation of projection pyramidal neurons but had no effect on the population of inhibitory interneurons. Electron microscopy revealed pathological changes to neurons in the entorhinal cortex of rat pups on postnatal day 20 (cytoplasmic organelle lysis or hyperchromatosis). Hypoxia on day 18 of pregnancy produced no changes in cell composition or pyramidal neuron death in the postnatal period in the offspring. Prenatal hypoxia on day E14 evidently leads to impairment to the radial migration of neuroblasts in the entorhinal cortex and increases elimination of projection neurons in early postnatal ontogeny. Projection neuron death in the entorhinal cortex can lead to impairment to afferentation of hippocampal neurons. The selective action of prenatal hypoxia on the excitatory neuron population of the entorhinal cortex of the rat cerebral cortex may lead to disturbance of the balance between excitatory and inhibitory processes during subsequent development.

Keywords: entorhinal cortex, prenatal hypoxia, embryogenesis, rat, neurogenesis, neuroblast migration.

Prenatal hypoxia is the commonest cause of impaired nervous system development in mammals, though the molecular and cellular mechanisms of this action remain somewhat unclear. Differences in the vulnerability of different parts of the brain during embryogenesis result from differences in the times at which cell populations form [1]. Thus, in rats, precursors of projection neurons in various parts of the brain form on E14, while at later time periods (E18) these areas mainly form future interneurons [2]. The hippocampal rudiment starts to form later than that of the lower layers of the neocortex, starting from E16, and generation of hippocampal neurons continues to early postnatal ontogeny [3–5]. However, our previous studies have shown that hypoxia on E14 alters cell composition and causes death in certain neuron populations in both the parietal cortex [6, 7], which is laid down during this period of embryogenesis, and the hippocampus [8], which forms significantly later. Data have also been obtained showing degradation of mushroom-type dendritic spines on the apical dendrites of neurons in the py-

[1] Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, St. Petersburg, Russia; e-mail: dvasilyev@bk.ru.
[2] St. Petersburg State University, St. Petersburg, Russia.

ramidal layer and impairment to long-term potentiation in the hippocampus of rat pups subjected to hypoxia on E14 [9], which is somewhat unexpected considering the time at which this part of the brain is formed. Analogous changes in axospinous contacts have been described by other authors in conditions of impaired afferentation of the hippocampus from the cerebral cortex [10]. This suggests that corticohippocampal interactions ay be deranged as a result of prenatal hypoxia, though this suggestion requires experimental testing. The entorhinal area of the cortex is known to participate in the afferentation of hippocampal field CA1 [11], where the sequelae of prenatal hypoxia are most marked [8]. One likely cause of derangement of hippocampal structure and function in hypoxia on E14 may therefore be pathological changes in the entorhinal cortex. We have previously obtained data evidencing impairments to neuroblast migration in the parietal area of the cerebral cortex in rat pups born to females subjected to hypoxia on day 14 of pregnancy [7], which explains impairment to its further development. Considering data showing that the formation of the cortical cellular layers in different areas of the cerebral cortex in rodents occurs relatively synchronously [11], analogous impairments might be expected in other areas of the cortex, particularly the entorhinal area. The literature lacks data on pathological changes in this area of the cortex in animals subjected to hypoxia during embryogenesis, and this determined the aim of the present work.

Methods. All experiments were carried out in compliance with the protocols for working with laboratory animals of the Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, which are based on the European Communities Council Directive 86/609 for the Care of Laboratory Animals. Female Wistar rats were subjected to normobaric hypoxia on day 14 of pregnancy (7% O_2, 3 h). Control animals were kept at a normal oxygen concentration. Entorhinal cortex tissue structure was analyzed in rat pups born to control and experimental females. Four groups of rat pups were studied: group 1 were controls (labeling of proliferating cells on E14), $n = 14$; group 2 were exposed to hypoxia on E14 (cell labelling on E14), $n = 12$; group 3 were controls (cell labeling on E18), $n = 9$; group 4 were exposed to hypoxia on E18 (cell labeling on E18), $n = 10$.

In vivo labeling of proliferating cells on E14 and E18 and analysis of their distribution in the entorhinal cortex. Neuroblasts forming in embryos at the moment of exposure to prenatal hypoxia were labeled with 3'-ethinyl-5-deoxyuridine (EdU). Pregnant females of the experimental and control groups received the DNA synthesis marker 3'-ethinyl-5-deoxyuridine (EdU) on day 14 or 18 of pregnancy (four females in each group). Pregnant females of the experimental groups were subjected to hypoxia 1 h before label administration. A detailed description of the in vivo EdU labeling methods has been published elsewhere [7].

Rat pups were decapitated on postnatal day 5. Cerebral cortex tissues containing the entorhinal cortex was fixed for two weeks in 10% formalin solution in 0.1 M PBS (pH 7.4). Tissue blocks containing the entorhinal cortex were immersed in 20% sucrose solution for cryoprotection; frontal sections of the entorhinal cortex (thickness 15 μm) were cut on a Leica CM 1510S cryostat (Leica Microsystems, Germany) and examined under an ImagerA fluorescence microscope (Zeiss, Germany). Quantitative comparison was performed using series of sections of thickness 15 μm, the first section of the series being selected at random and the distance between successive sections in the series being 30 μm. EdU was visualized using an EdU Alexa Fluor® 488 Click-iT® kit (Invitrogen, USA). Some brain sections were background-stained with the nonspecific nuclear stain Hoechst 33342. Morphometric analysis of the numbers and distributions of EdU-positive cells formed on E14 and E18 was carried out on cross-sections of the entorhinal cortex of width 500 μm at a level 4.5 mm from the bregma (Paxinos and Watson [12]; see Fig. 3, *A*). The mean number of EdU-positive cells was determined for each entorhinal cortex area studied, and mean values were determined for nine sections for each animal.

Light optical studies of entorhinal cortex tissues in postnatal ontogeny. Animals were decapitated on postnatal days 20, 35, and 60 (P20, P35, P60). Brains were fixed by transcardiac perfusion with 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4) with postfixation in the same solution for 4.5 days; specimens were then placed in 20% sucrose solution for one day. Frontal sections of thickness 15 μm were cut on a Leica CM 1510S cryostat and stained by the Nissl method. Entorhinal cortex tissue was studied under an AF7000 microscope (Leica, Germany) in strips of width 500 μm at a level of 4.5 mm from the bregma (Paxinos and Watson [12]) (Fig. 3, *A*); images were digitized with a DFC495 camera (Leica). Quantitative comparisons were performed using series of 15-μm sections, the first being selected at random and the distance between successive sections being 30 μm. Tissue cell composition was analyzed in Videotest Master Morphology software (VideoTest, Russia). For each animal, nine sections were used to calculate mean values for the total number of cells and the numbers of pyramidal and nonpyramidal neurons. The pyramidal neurons group included cells with extended bodies, body areas of >25 μm^2, and cell body long:short axis ratios of >2. The nonpyramidal neuron group included round cells with body surface areas of >25 μm^2 and cell body long:short axis ratios of <2. A detailed description of the classification of rat cerebral cortex cells used in this study has been published [6, 7].

Statistical processing was run in SPSS Statistics 22. Normality of distributions was tested using the Kolmogorov–Smirnov test and the Levene test was used to confirm the equality of variances.

Groups were compared by one-way analysis of variance (ANOVA). A posteriori comparisons were run using the Tukey test. Differences were regarded as significant at

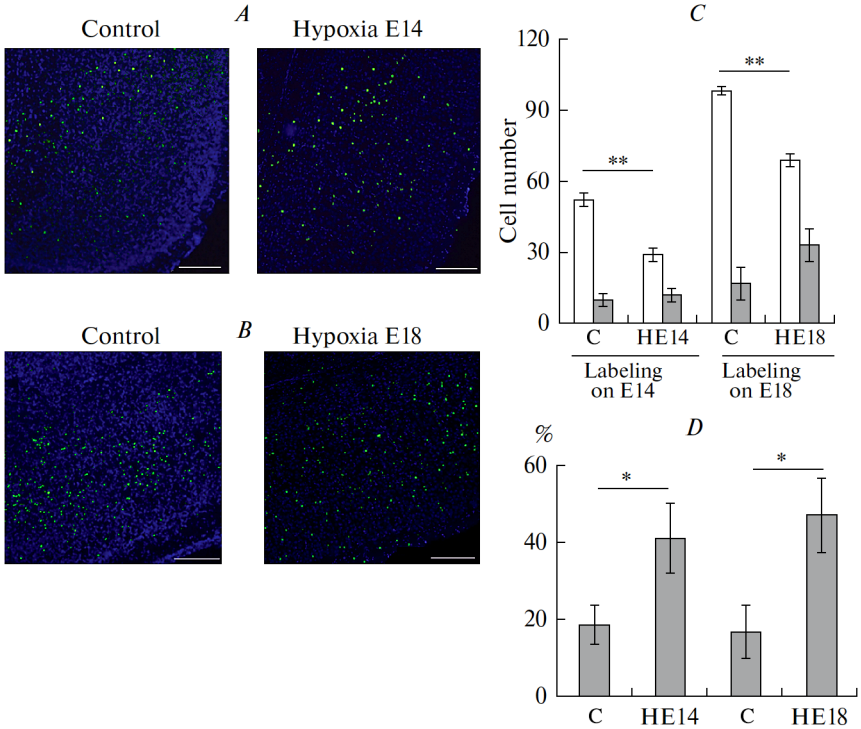

Fig. 1. Distribution of EdU-positive cells in the rat entorhinal cortex. *A*) Photomicrograph of entorhinal cortex tissue from five-day-old rat pups of the control (left) and hypoxia (right) groups. Proliferating cells were labeled on day 14 of embryogenesis. The nuclei of cells formed on E14 show green. Background staining of nuclei with the nonspecific nuclear stain Hoechst 33342 (blue). Scale bar 200 μm. *B*) Photomicrographs of entorhinal cortex tissue from five-day-old rat pups of the control (left) and hypoxia (right) groups. Proliferating cells were labeled on day 18 of embryogenesis. Background staining with Hoechst 33342. Scale bar 200 μm. *C–D*) Quantitative analysis results. White columns in the histogram in *C* show mean numbers of EdU-positive cells in the study field of the entorhinal cortex of rat pups of the control (C) and hypoxia (HE14 and HE18) groups. Results are presented as mean ± error of the mean. In histograms *C* and *D*, gray columns show mean numbers of cells outside the layers formed during the labeling period (upper layers in the case of labeling on E14, lower layers in the case of labeling on E18). These cells were eliminated during early postnatal ontogeny. In histogram *D*, the number of these cells is expressed as a percentage of the total number of EdU-positive cells. Statistically significant differences between the control and hypoxia groups: $*p < 0.05$, $**p < 0.01$ (one-way analysis of variance, a posteriori Tukey test).

$p < 0.05$. Data in the text and illustrations (except Fig. 2) are shown as means and standard errors.

Electron microscopy. Electron microscopy studies of the entorhinal cortex of control ($n = 3$) animals and animals subjected to hypoxia on E14 ($n = 4$) were performed on P20. Tissues were fixed by transcardiac perfusion with a mix of 1% glutaraldehyde and 1% paraformaldehyde in 0.1 M PBS pH 7.4, postfixed with 1% OsO_4, contrasted with uranyl acetate, dehydrated, and embedded in Araldite using a standard protocol [13, 14]. An LKB-III ultratome (LKB, Sweden) was used to cut ultrathin (500 Å) sections which were then examined under an FEI Tecnai V2 electron microscope (FEI, USA).

Results. ***Studies of the distribution and quantity of EdU-positive neurons formed on E14 and E18.*** Most entorhinal cortex cells in five-day-old control rat pups labeled on E14 were located in the lower (V–VI) layers (Fig. 1, *A*), while cells labeled on E18 were located in the superficial layers (Fig. 1, *B*).

Prenatal hypoxia on E14. In animals subjected to hypoxia, as in control animals, most cells labeled on E14 were located in the lower (V–VI) cortical layers (Fig. 1, *A*). Most EdU-positive neurons in these layers were characterized by chromatin in the diffuse state. The number of EdU-positive cells in the entorhinal cortex in five-day-old rat pups exposed to hypoxia on E14 (Fig. 1, *B*) was 1.8 times smaller than that in the control group (a posteriori Tukey test, $p < 0.01$). This indicates that prenatal hypoxia can induce decreases in the intensity of cell proliferation during embryogenesis. In animals subjected to prenatal hypoxia on E14, the proportion of labeled neurons in the superficial layers of the entorhinal cortex was about 42% of the total number of labeled cells and was significantly larger (see Fig. 1, *D*, a posteriori Tukey test, $p = 0.02$) than in control animals (19%). Thus, prenatal hypoxia on E14 impaired neuroblast migration to layers V–VI of the entorhinal cortex.

Prenatal hypoxia on E18. Cells labeled on E18 were located in the superficial layers (Fig. 1, *B*) and chromatin in many of them was condensed. This may point to greater transcriptional activity of neurons in the lower layers of the entorhinal cortex than for cells in layers II–III. The number of EdU-positive cells in the entorhinal cortex in five-day-old rat pups subjected to hypoxia on E18 was 1.4 times lower than the level in the control group (a posteriori Tukey

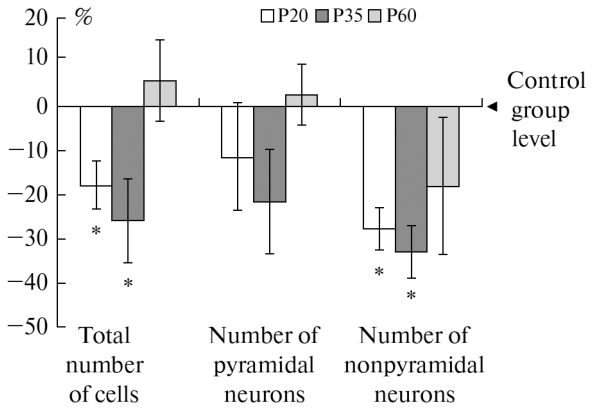

Fig. 2. Results of morphometric analysis of the number of cells in rats prenatally exposed to hypoxia (E14) at age P20 (white columns), P35 (dark columns), or P60 (light columns). Results are presented as mean ± variance and are expressed as percentages of the level in the control group (null). Statistically significant differences from controls: $*p < 0.05$, one-way analysis of variance, a posteriori Tukey test.

test, $p < 0.01$). This points to a decrease in the intensity of cell proliferation during embryogenesis. In animals subjected to hypoxia on E18, the number of EdU-positive cells located in the lower layers of the cortex wea about 48% and was greater (see Fig. 1, *D*; a posteriori Tukey test, $p = 0.03$) than that in controls (17%). Thus, as in the case of hypoxia on E14, prenatal hypoxia on E18 impaired neuroblast migration to entorhinal cortex layers II–III.

Morphometric studies of the cell components of entorhinal cortex tissue in rats with normal and impaired embryogenesis. *Prenatal hypoxia on E14*. By the end of the first month of postnatal ontogeny, the total number of cells in the entorhinal cortex of animals subjected to hypoxia on E14 was lower than that in sibs from the control group (Fig. 2). Thus, this was 82.0% of the control level on P20 (a posteriori Tukey test, $p = 0.04$) and 74.1% on P35 (a posteriori Tukey test, $p = 0.02$). At the adult stage of development (P60), there were no differences between the control and experimental groups (a posteriori Tukey test, $p = 0.18$). The dynamics of quantitative changes in neurons of different classes were different. On P20 and P35, rat pups subjected to hypoxia on E14 showed a statistically significant decrease in the number of pyramidal neurons (a posteriori Tukey test), which was 72.1% of the control levels on P20 ($p = 0.02$) and 67.0% on P35 ($p = 0.03$). At the adult stage of development, there were no differences between the control and experimental groups (a posteriori Tukey test, $p = 0.15$). No between-group differences were seen in the numbers of nonpyramidal neurons at any age (a posteriori Tukey test, $p = 0.22$ for P20, $p = 0.07$ for P35, $p = 0.10$ for P60).

Prenatal hypoxia on E18. The cell composition and neuron distribution density in the entorhinal cortex in rats subjected to hypoxia on E18 were no different from those in controls on P20 (a posteriori Tukey test, $p = 0.08$ for the total number of cells, $p = 0.12$ for the number of pyramidal neurons; $p = 0.07$ for nonpyramidal neurons), P35 (a posteriori Tukey test, $p = 0.06$ for the total number of cells, $p = 0.09$ for the number of pyramidal neurons; $p = 0.08$ for nonpyramidal neurons), or P60 (a posteriori Tukey test, $p = = 0.08$ for the total number of cells, $p = 0.08$ for the number of pyramidal neurons; $p = 0.11$ for nonpyramidal neurons). Morphometric analysis data indicated that hypoxia on E18 had no effect on cell composition in the entorhinal cortex.

Changes in the state of entorhinal cortex neurons in rat pups subjected to hypoxia on E14. Decreases in the numbers of neurons in entorhinal cortex tissue in rats subjected to hypoxia on E14 provided evidence of their death, so these were used to investigate the possibility that cellular neurodegenerative changes took place. Entorhinal cortex neurons in rat pups subjected to hypoxia on E14 showed clear pathological changes both at the light microscopic (Fig. 3, *B*, *D*) and ultrastructural (Fig. 3, *F*, *G*) levels. Most such degenerative neurons displayed swelling of cell bodies and their processes, with the appearance of unstained areas (Fig. 3, *C*) and lysis of cytoplasmic organoids (Fig. 3, *F*), providing evidence of chromatolysis-type neurodegeneration. In addition, another type of neurodegenerative change was also noted – hyperchromatosis (Fig. 3, *D*, *G*), with shrinkage of cell and bodies and their processes and increased electron density in the cytoplasm (Fig. 3, *G*). Cell nucleus volume in these cells was decreased and nuclei were surrounded by a rim of dark cytoplasm in which it was difficult to discriminate organelles, i.e., endoplasmic reticulum and mitochondria. Glial processes were found in the neuropil of the entorhinal cortex, located around degenerating neurons (Fig. 3, *B*).

Discussion. The results obtained here provide evidence that prenatal hypoxia on E14 or E18 impaired the radial migration of neuroblasts to layers V–VI and II–III of the entorhinal cortex respectively. In addition, prenatal hypoxia was found, regardless of the time of exposure, to be able to decrease the intensity of cell proliferation. Data on the entorhinal cortex obtained in the present study are consistent with our previously published results on the parietal cortex of rats born to females subjected to hypoxia on day 14 or day 18 of pregnancy [7], which was the first experimental evidence for this action of prenatal hypoxia on the development of different areas of the neocortex. It should be noted that data on impairments to cell proliferation and radial migration in prenatal hypoxia on E14 or E18 are consistent with observations reported by other authors obtained using different models of prenatal pathology (not associated with hypoxia) at the corresponding periods of embryonic development of the brain [15–17]. A similar type of influence of various adverse factors on neuroblast neurogenesis and migration processes cannot be regarded as unexpected, though most published sources lack comparison of structural impairments in different areas of the neocortex, including the entorhinal cortex.

We have previously shown that decreases in the total number of pyramidal neurons in layers II–III and V–VI of

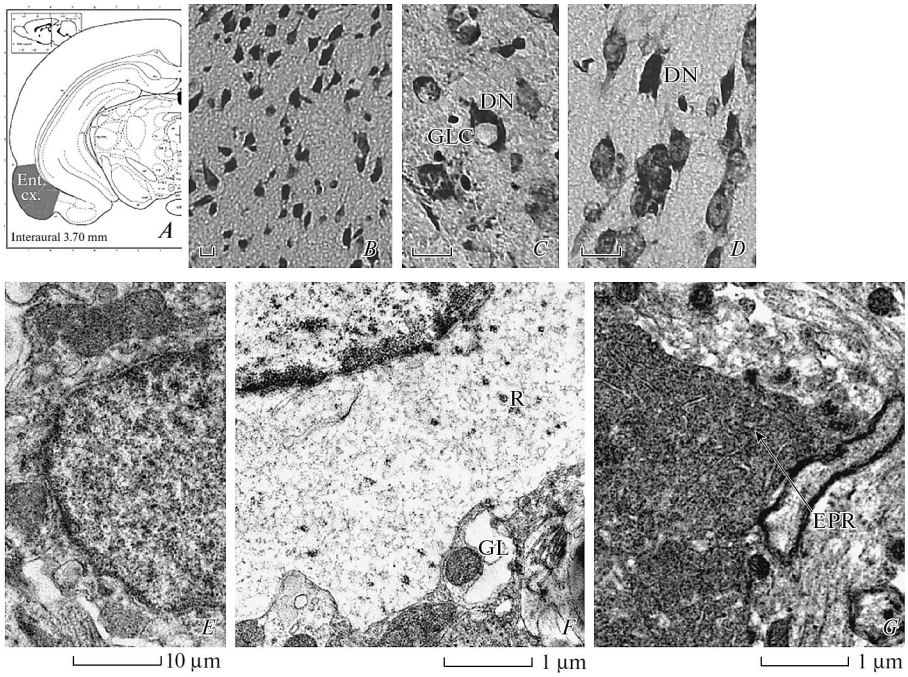

Fig. 3. Neurodegenerative changes in the entorhinal cortex of 20-day-old rat pups after prenatal hypoxia. *A*) Diagram showing the positions of the areas of the entorhinal cortex analyzed (with modifications from Paxinos and Watson, 2007). *B–D*) Photomicrographs of tissues in layers II–III of the entorhinal cortex of rats from the control group (*B*) and the experimental groups (*C*, *D*). Nissl stain. Scale bar – 20 μm. DN – Degenerating neurons; GLC – glial cells. *E–G*) Electron micrographs of entorhinal cortex tissue from rats of the control (*E*) and experimental (*F*, *G*) groups. Scale bars: *E* – 10 μm; *F*, *G* – 1 μm. *E*) A normal neuron; *F*) a degenerating neuron with cytoplasmic organoid lysis; *G*) a degenerating neuron with hyperchromic cytoplasm. GL – glial cell process; R – single ribosomes; EPR – endoplasmic reticulum.

the parietal cortex can occur only in rats subjected to prenatal hypoxia on E14, and not E18 [6, 7]. Studies of the parietal cortex also showed differences between the control and hypoxia groups in the numbers of pyramidal neurons only in the first month of postnatal ontogeny, but not in adult animals. The present study demonstrated similarity in the nature of the actions of prenatal hypoxia on the cell composition and state of neurons in the parietal and entorhinal cortex during postnatal ontogeny in rats. It should be noted that there were differences between the study areas of the cerebral cortex in the pattern of changes in cell composition. On P20–30 the parietal cortex of rat pups exposed to hypoxia on E14 showed changes in the number of nonpyramidal neurons [7], while this was not seen in the entorhinal cortex. The selective action of prenatal hypoxia on the population of excitatory cerebral cortex neurons could lead to impairments to the balance of excitatory and inhibitory processes during further development [18]. Studies of the excitability of neurons in different parts of the cortex and hippocampus are therefore relevant in the light of the results obtained here.

It is interesting to assess the question of possible impairments to cortex-hippocampus interactions as the cause of structural changes and long-term potentiation in the hippocampus of rat pups subjected to prenatal hypoxia. We have previously described structural changes in the hippocampus of rat pups from the offspring of females subjected to hypoxia on day 14 of pregnancy [8]. These changes included moderate levels of neuron death in the pyramidal layer of field CA1 and decreased numbers of mushroom-type dendritic spines (containing the cytoskeletal marker protein synaptopodin [19]). The decrease in the number of labile spines is evidence of changes in neural network plasticity [20]; later studies showed that such decreases were accompanied by impairment to the process of long-term potentiation and cognitive dysfunction [9]. Analogous changes to axospinous contacts have been described by other authors in conditions of impaired afferentation of the hippocampus from the cerebral cortex [10]. This suggests that may be derangement of the interaction between the entorhinal cortex and hippocampus in animals with prenatal pathology. The results obtained here provide evidence supporting this suggestion, as they show impaired formation of entorhinal cortex projection neurons involved in afferentation of the hippocampus. Naturally, this suggestion cannot be regarded as the only possible cause of the effects of hypoxia on E14 on hippocampal structure and function. Thus, the literature contains data indicating that hypoxia on E17 can impair neuroblast migration in the hippocampus [21], though the effect of this impaired cell migration on the formation of neural networks during postnatal ontogeny remains unstudied. It should be noted that a direct effect of prenatal hypoxia on the formation of the hippocampus may be explained by structural changes in hypoxia on E18, but not in hypoxia

on E14. In the case of hypoxia on E14, the hypothesis that the cortex-hippocampus interaction is impaired comes to be of some interest and may be a subject for future experimental studies.

Thus, the results obtained here provide evidence that prenatal hypoxia affects nervous tissue formation in the entorhinal cortex in rats.

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