ISSN 1990-519X, Cell and Tissue Biology, 2021, Vol. 15, No. 5, pp. 482–492. © Pleiades Publishing, Ltd., 2021.
Russian Text © The Author(s), 2021, published in Tsitologiya, 2021, Vol. 63, No. 2, pp. 163–175.

Effect of Prenatal Hypoxia on Cytoarchitectonics and Ultrustructural Organisation of Brain Regions Related to Olfaction in Rats

N. L. Tumanova[a], D. S. Vasilev[a, *], N. M. Dubrovskaya[a, b], N. N. Nalivaeva[a], and I. A. Zhuravin[a, †]

[a] *Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, St. Petersburg, 194223 Russia*

[b] *St. Petersburg Pediatric Medical State University of the Ministry of Healthcare of the Russian Federation, St. Petersburg, 194100 Russia*

**e-mail: dvasilyev@bk.ru*

Received November 11, 2020; revised November 29, 2020; accepted November 30, 2020

Abstract—Using light and electron microscopy it was shown that on the 20th day after birth in rats subjected to prenatal hypoxia on day 14 of embryonic development (E14, 7% O_2, 3 h) in the central parts of the olfactory system—the hippocampus and entorhinal cortex, there were significant neurodegenerative changes and decreased numbers of neurons, while in the peripheral part of the olfactory system, namely olfactory bulbs, no changes were observed. Immunohistochemical analysis also revealed changes in the content and which distribution of a metallopeptidase, neprilysin (NEP), in the entorhinal cortex and hippocampus of rats subjected to prenatal hypoxia. These data allow us to conclude that the impairment of the olfactory function in young rats, caused by maternal hypoxia during pregnancy and which manifested itself as worsened performance in the food search task, is underlined by the pathological changes in the cells of the olfactory system as well as by the decreased content of NEP.

Keywords: entorhinal cortex, hippocampus, neurodegeneration, olfactory behavior, olfactory bulbs, ontogenesis, neprilysin, prenatal hypoxia

DOI: 10.1134/S1990519X21050114

INTRODUCTION

One of the most important areas of modern biomedicine is to elucidate the causes and to search for early diagnosis of neurodegenerative disease, which are often associated with disturbance of the olfactory analyzer. The deterioration of the sense of smell may indicate the development of neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's diseases (Djordjevic et al., 2008; Doty, 2012; Morozova et al., 2014; Murphy, 2019), Huntingon's disease (Barresi et al., 2012) and other forms of dementia (Carnemolla et al., 2020). Disruption of olfactory function is often accompanied by increased mortality in the elderly (Liu et al., 2019). Olfactory disorders manifest themselves earlier than cognitive or motor disorders and become more apparent and severe as the neurodegenerative process progresses.

In recent years, functional MRI imaging helps to visualize the olfactory structures of the brain, contributing significantly to understanding the causes of the development of olfactory disorders in neurodegenerative diseases (Wang et al., 2010). According to MRI data, the decrease in the volume of olfactory bulbs and tracts correlates with a decrease in cognitive function, analyzed by the Mini Mental State Assessment (MMSE) score (Thomann et al., 2009). In AD and Parkinson's disease, olfactory disorders can be associated with both the gray matter atrophy of the olfactory bulbs, primary olfactory cortex, hippocampus, thalamus and hypothalamus, and the increase in the number of inhibitory neurons in the olfactory system (Wang et al., 2010). On the other hand, bulbectomy in different types of rodents (mice, guinea pigs and rats) leads to behavioral, morphological, biochemical and immunological changes characteristic of the development of neurodegeneration characteristic to AD (for review see Gulyaeva et al., 2017).

In the studies from our laboratory it was shown that prenatal hypoxia on the 14th day of embryonic development of rats (E14, 7% O_2 for 3 hours) leads to impaired morpho-functional properties of the nervous tissue of the parietal cortex and hippocampus, decreased density of the dendritic spines and delayed neurogenesis, morphogenesis and formation of the plasticity of the nervous system (Vasilev et al., 2016; Tumanova et al., 2018). Prenatal hypoxia also leads to changes in the metabolism of amyloid-β peptide (Aβ) and an increase in the content of its precursor protein

Abbreviations: APP—β-amyloid precursor protein, AD—Alzheimer's disease, Aβ—β-amyloid peptide.

† Deceased.

(APP), as well as to a decrease in the activity of α-secretase and an increase in the activity of β-secretase, cleaving APP, which leads to a shift in the balance of APP catabolism towards the formation of Aβ (Nalivaeva et al., 2004). Similar changes are also observed in a brain ischemia model in adult rats (Nalivaeva et al., 2005). Along with these pathological changes in the cortex and hippocampus we have also observed a deficit of the amyloid-degrading enzymes neprilysin and endothelin-converting enzyme (Nalivaeva et al., 2004, 2012). In humans, a combination of these factors can lead to the accumulation of amyloid peptide in the nervous tissue, the death of nerve cells, development of neurodegeneration and dementia.

In addition to morphological and biochemical changes in brain tissue, prenatal hypoxia in rats also leads to impaired cognitive functions in postnatal ontogenesis, which are detected by testing animals in a two-level radial maze, novel object recognition and development of instrumental reflex paradigms (Dubrovskaya and Zhuravin, 2009; Zhuravin et al., 2010). Thus, prenatal hypoxia in rats can be considered as a zootropic model of the early stages of human neurodegenerative diseases. However, studies of the effects of prenatal hypoxia on the formation of the structures participating in the olfactory function of animals have not yet been conducted.

The aim of this study was to analyse the effects of prenatal hypoxia on the morpho-functional characteristics of brain regions associated with smell sensing as well on olfactory function in rats. For this we used methods of light and electron microscopy, immunohistochemistry as well as the analysis of the efficacy of food search by smell.

MATERIALS AND METHODS

Animals. The males from the offspring of female Wistar rats of the control and experimental (normobaric hypoxia) groups were used. All experiments were carried out in accordance with the protocol of use of laboratory animals approved by IEFB RAS, based on the directive of the European Communities Council Directive #86/609 for the Care of Laboratory Animals.

Model of prenatal hypoxia. Pregnant female rats were subjected to normobaric hypoxia on the 14th day of gestation (E14) in a special chamber (100 L in volume) equipped with the systems of thermoregulation, ventilation, CO_2 absorption and gas analysis. To create hypoxia the content of oxygen in the chamber was decreased linearly from 20.7 to 7% and maintained at this level for 3 h. Concentration of CO_2 in the chamber was not higher than 0.2% and the temperature was kept at 22°C. Not more than 10 rats were kept simultaneously in the chamber. Female rats from the control group were kept during the same period of time under normal oxygen content. On the 20th day of pregnancy (1 day before the offspring birth) each female was put in a separate cage. On the second day after birth only 8 pups were left in each brood. The day of birth was considered as P0.

Light microscopy. Light microscopy analysis was performed in 20-day old (P20) rat pups of the control ($n = 10$) and experimental ($n = 9$) groups. Brain tissue was fixed by trans-cordial perfusion with neutral 10% formalin in a phosphate buffer (PBS, 4°C, pH 7.4). Frozen coronal plane 20 μm thick brain slices were prepared using a Leica CM 1510S cryostat (Leica Microsystems, Germany). For analysis the slices of the olfactory bulb (5.2–7.0 mm from bregma) (Paxinos and Watson, 2005), the hippocampus and entorhinal cortex (4.5–5.5 mm from bregma) were selected, and Nissl stained. The condition of the nervous tissues was evaluated using an ImagerA microscope (Zeiss, Germany). Quantitative comparison of the cells in the CA1 area of the hippocampus and of the entorhinal cortex was performed in 20 μm thick slices from which the first one was selected at random with the distance between further analysed slices being 40 μm. Slice images were processed using Videotest: Master Morphology 4.2 computer program (VideoTest, Russia). From 6 slices containing the dorsal hippocampus or entorhinal cortex we calculated average total cell number and number of neurons (on the area of 10000 μm^2) for each animal from the control ($n = 8$) and experimental ($n = 8$) groups.

Electron microscopy. For analysis the brains of 20-day old rat pups (P20) (in the control group $n = 5$, in hypoxia group $n = 4$) was fixed by trans-cordial perfusion with a mixture of 1% glutaraldehyde and 1% formaldehyde in 0.1 M PBS (pH 7.4). The blocks containing the olfactory bulbs, entorhinal cortex or hippocampus were additionally treated with 1% OsO_4, contrasted with uranyl acetate, dehydrated and embedded in araldite glue according to a standard protocol (Tumanova et al., 2018). Ultra-thin 50 nm sections of analysed brain areas were prepared using LKB-III (LKB, Sweden) and analyzed using an FEI Tecnai V2 transmission electron microscope (FEI, United States).

Immunohistochemistry. For the analysis the sections of the olfactory (5.2–7.0 mm form bregma, Fig. 1a) (Paxinos and Watson, 2005), entorhinal cortex and CA1 area of the hippocampus (4.5–5.5 mm from bregma, Figs. 2a, 3a) from the animals of the control and experimental groups ($n = 8$ in each age group) were used. The brains were fixed in 10% solution of formalin in 0.1 M phosphate buffer (pH 7.4) and then sectioned using a cryostat Leica CM 1510S (Leica Microsystems, Germany). To reduce autofluorescence, sections were incubated in 0.1 M glycerine (Sigma, Germany) in 0.1 M phosphate buffer (pH 7.4) under visual control until maximal reduction of fluorescence in the wavelength range of 490–550 nm. Nonspecific binding of antibodies was prevented by

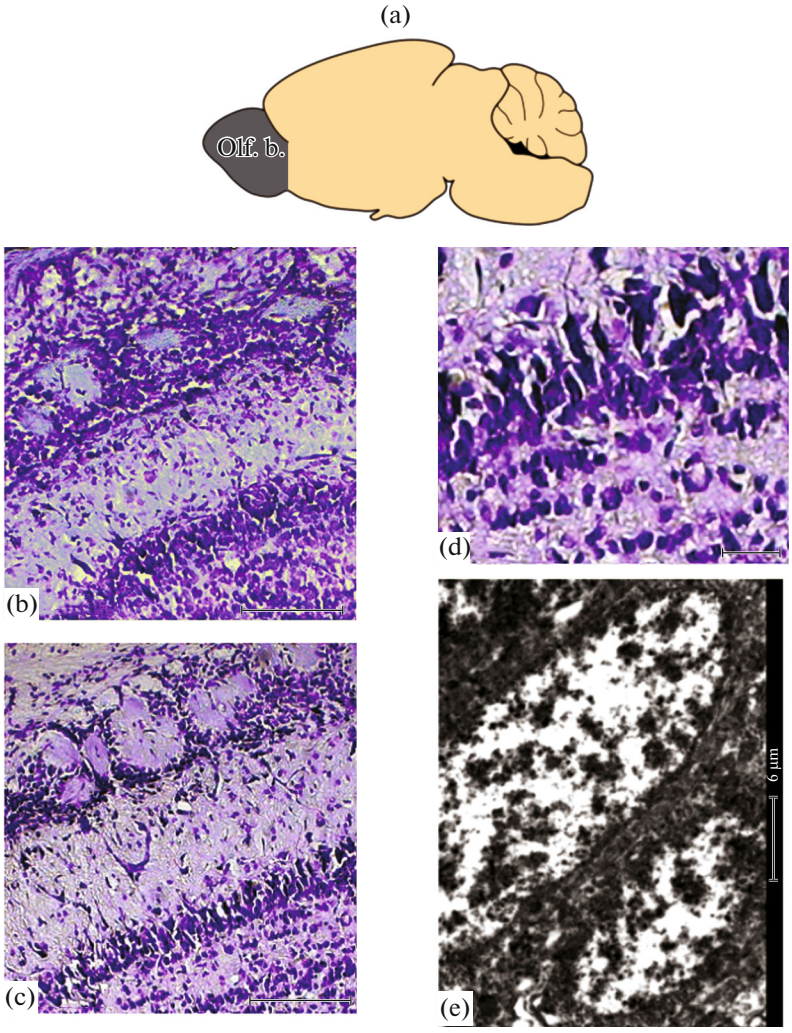

Fig. 1. Olfactory bulbs of 20-days old rat pups subjected to prenatal hypoxia. (a) Schematic representation of the analysed area of the olfactory bulb (Olf. b.)—(adapted from Paxinos and Watson, 2005). (b–d) Representative microphotographs of the olfactory bulb tissue of rat pups from the control (b) and experimental (c, d) groups; Nissl staining. (d) The layer of mitral cells in the olfactory bulb of rat pups subjected to prenatal hypoxia; Nissl staining. Scale: 200 (b, c) and 20 (d) μm. (e) An electron microscopy image of the olfactory bulb of a hypoxic animal; shown are the mitral cells with a narrow rim of cytoplasm.

1 h incubation of sections in 2% bovine serum albumin (Sigma, Germany) in 0.1 M phosphate buffer (pH 7.4) to which 0.01% Triton-X100 was added to allow permeability of cellular membranes. For immunohistochemical analysis of neprilysin distribution rabbit polyclonal antibody Anti-CD10 (EPR5904, ab126593; Abcam, UK, dilution 1 : 100) was used. Visualisation of binding was performed using FITC-conjugated secondary antibody against rabbit IgG (ab96902, Abcam; UK, dilution 1 : 500). For a negative control of non-specific binding in the sections of analysed brain areas of each animal the immunochemical reaction was performed in the absence of the primary antibody. Control rat liver and kidney tissues with characteristic high content of neprilysin were used as a positive control. Immunofluorescence was registered using a Leica DMR microscope with a Leica TCS SL confocal scanner (Leica Microsystems, Germany). FITC fluorochrome excitation was achieved using a He/Ar-laser beam with 488 nm wavelength. FITC fluorescence was recorded in the wavelength of 496–537 nm. The intensity of FITC fluorescence in the area of nervous tissues of 10000 μm^2 was evaluated using Video Test Master-Morphology software (Video Test, Saint-Petersburg, Russia). For each animal, the average value of fluorescence for 6 sections has been calculated and normalised by detracting the average value obtained for the negative controls (immunochemical reaction without the primary antibody). The averaged normalised values for FITC signal for the animals of the control (n = 8) and experimental groups (n = 8) were compared.

Analysis of animal behavior. Experiments were performed in 30 day old rat pups of the control group

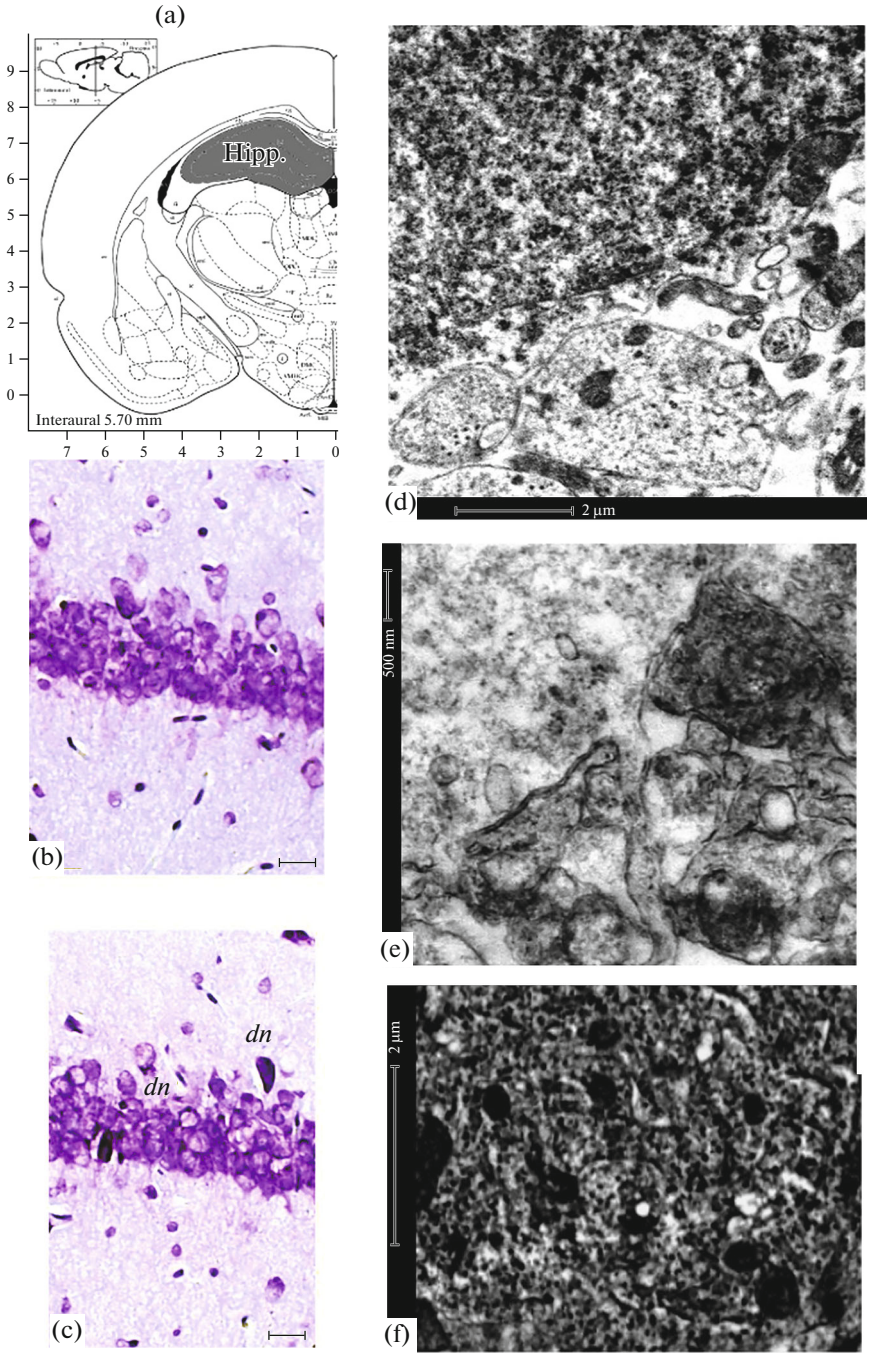

Fig. 2. Neurodegenerative changes in the CA1 area of the hippocampus of 20-day old rat pups after prenatal hypoxia. (a) Schematic representation of the analysed area of the hippocampus (Hipp.)—(adapted from: Paxinos and Watson, 2005). (b, c) Microphotographs of the CA1 area of the hippocampus of rat pups from the control (b) and experimental (c) groups; *dn*—a degenerating neuron; Nissl staining, scale—20 μm. (d–f) Electron microscopy images of the CA1 area of the hippocampus of rat pups from the control (d) and experimental (e–f) groups: a normal neuron (d), a degenerating neuron with the lysis of organelles in the cytoplasm (e), a degenerating neuron with hyperchromic cytoplasm and lysosomes (f).

(intact control, $n = 15$) and the group subjected to prenatal hypoxia (experimental group, "hypoxia," $n = 9$). In the "food search" paradigm we have used our modification of the test described in (Sun et al., 2016). Animal testing was performed in a cage with non-transparent walls with the floor dimensions of 100 × 100 cm and a height of 30 cm. The floor of the chamber had 16 holes of 2 cm diameter. During the experiments scented food pellets (pieces of an oatmeal cookie of diameter 0.5 cm) were placed at random in

two holes 0.5 cm below the floor under the layer of sawdust. In each testing the position of pellets was changed. Before testing the animals had two days of food deprivation. Testing was performed daily for 6 days. During 15 min of the test the number of pellets found in each trial was scored as 0, 1 or 2. In addition, during the testing cycle, the total number of sniffs of all the holes in the floor of the experimental chamber was recorded. After finding the second piece of the biscuits, the testing stopped.

Statistical analysis of the data. The data obtained were processed using the SigmaStat 3.0 software package using the two-tailed *t*-test and a non-parametric Mann–Whitney *U* test. The differences were considered significant at $p < 0.05$.

RESULTS

Morphology of Brain Cortical Areas

(1) Olfactory bulbs. The analysis of the Nissl-stained sections of the olfactory bulbs (the peripheral olfactory analyzer) revealed no significant differences in the structure of the nerve tissue of the olfactory bulbs of the control and hypoxic rats (Figs. 1b, 1c). Figure 1d shows a layer of the mitral cells formed by several concentrically located rows of neurons in the olfactory bulb of the animal subjected to prenatal hypoxia. In the electron microscopy images the mitral cells had a large size and a large nucleus (Fig. 1e) and did not differ from the cells of this layer in the control animals. The electron microscopy examination of the fibers and neurons of the olfactory bulbs also found no differences between the control and hypoxic animals.

(2) Hippocampus. Using the light optometric Nissl method structural changes of the neurons in the CA1 area of the hippocampus have been detected in rat pups subjected to prenatal hypoxia compared to age-matched controls. The average number of neurons in the pyramidal layer of the CA1 area was $15.4 \pm 5.8\%$ lower than in controls ($U = 8.1$, $p = 0.03$). In some neurons the swelling of cell bodies and their processes as well as the appearance of numerous vacuoles and lysis of organelles in cytoplasm (chromatolysis) were observed, while other neurons were characterised by the shrivelling of cell bodies and their processes, as well as by the compaction of the cytoplasm (hyperchromatosis, Figs. 2b, 2c). On electron microscopy images of brain sections from these animals the neurons with changed structure were seen alongside normal cells (Fig. 2d). Chromatolysis of neurons in the hippocampus of rats subjected to hypoxia was less common than hyperchromatosis. Figure 2e shows a neuron degenerated via chromatolysis with a swollen body and lysed organelles in the cytoplasm, from whose body the axon with a varicose thickening departs, and next to it there is a modified axosomatic contact with a dark axonal terminal belonging to a hyperchromatic neuron. In the cytoplasm of such neurons (Fig. 2f) it was difficult to distinguish organelles (ER and mitochondria), but the lysosomes were often seen. In the neuropil of the hippocampus, dark shrivelled dendritic processes and terminals of hyperchromic cells with agglutinated synaptic vesicles were observed.

(3) Entorhinal cortex. In the entorhinal cortex of rat pups subjected to prenatal hypoxia the number of pathological changes in the cells via chromatolysis was higher than via hyperchromatolysis. This was observed both at the light and electron microscopy levels, especially in the layers II and III (Figs. 3b, 3c). In experimental animals along with normal cells (Fig. 3d), neurons with changed structures were observed. The electron microscopy images show signs of neurodegenerative changes of the chromatolysis type such as the swelling of cell bodies and their processes, appearance of large vacuoles, and lysis of organelles in the cytoplasm (Fig. 3e). In rare cases single ER cisternae with ribosomes could be seen. In the neurons degenerating via hyperchromatolysis there was shrivelling of cell bodies and their processes; the cytoplasm became more electron dense, the volume of the nucleus decreased and around it a rim of dark cytoplasm with detectable organelles (ER and mitochondria) could be seen (Fig. 3f). In the cytoplasm of such neurons there was an increased number of lysosomes indicating the destructive processes. In the entorhinal cortex (as well as in the hippocampus) of rat pups subjected to prenatal hypoxia a large number of glial cells with processes located around degenerating neurons was detected compared to the age matched controls. The total amount of cells was decreased by $19.4 \pm 4.8\%$ ($U = 5.2$, $p = 0.012$) while the average number of neurons was lower by $21.7 \pm 3.5\%$ ($U = 2.7$, $p = 0.007$) compared to controls.

Thus, morphological analysis of two such important brain areas of central origin which are related to olfactory function—the entorhinal cortex and hippocampus—in rats subjected to prenatal hypoxia revealed pathological changes in neurons during the early stage of postnatal development.

Neprilysin distribution in nervous tissue. The immunohistochemical analysis of neprilysin distribution in the brain areas was performed compared to the negative controls in the absence of the primary antibody against neprilysin (Figs. 4a–4i), when practically no autofluorescence and nonspecific labelling of brain tissues with the secondary antibody was observed. In the liver and kidney tissues used as positive controls a significant fluorescence was observed in the range of wavelength of FITC emission testifying to the viability of the antibodies used (Figs. 4j, 4k).

According to the data of immunohistochemical analysis of neprilysin distribution in the brain tissue, this protein is localised both in the neuropil and in the vicinity of cell bodies (Figs. 4b, 4e, 4h). Due to a rather wide distribution of neprilysin, the quantitative analy-

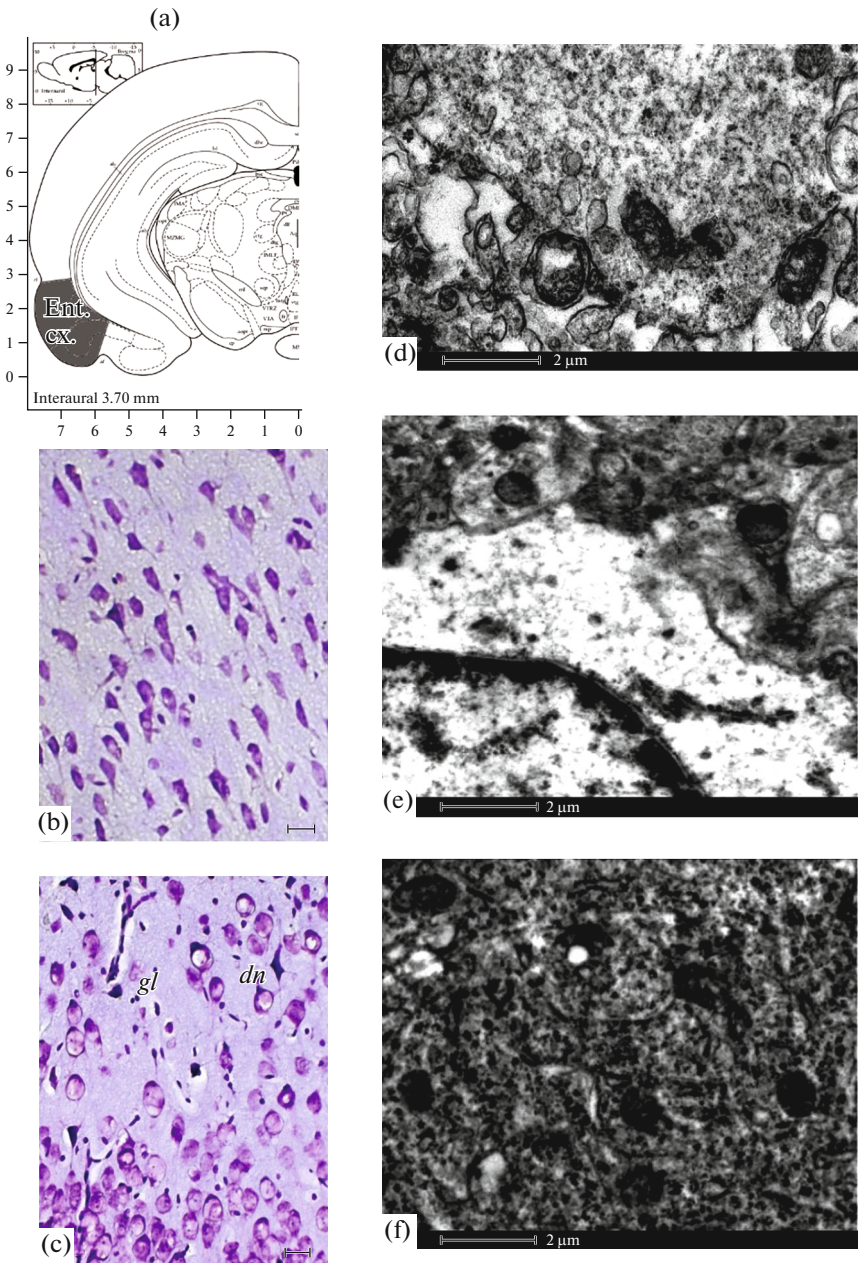

Fig. 3. Neurodegenerative changes in the entorhinal cortex of 20-day old rat pups subjected to prenatal hypoxia. (a) Schematic representation of the analysed area of the entorhinal cortex (Ent. cx.) (adapted from: Paxinos and Watson, 2005). (b–c) Microphotographs of the areas of the entorhinal cortex of rat pups from the control (b) and experimental (c) groups; *gl*—glial cells, *dn*—a degenerating neuron, Nissl staining, scale—20 μm. (d–f) Electron microscopy images of the entorhinal cortex of rat pups form the control (d) and experimental (e–f) group: a normal neuron (d), a degenerating neuron with the lysis of organelles in the cytoplasm (d) and a degenerating neuron with hyperchromic cytoplasm (e).

sis of the average values of immunochemical reaction against this protein was performed in the analysed area of the nervous tissue which contained both cells and neuropil. In rat pups subjected to prenatal hypoxia (Figs. 4c, 4f, 4i), the intensity of immunochemical reaction against neprilysin in the cortical areas of the brain was lower than in control animals by 33.4% in the entorhinal cortex (t = 3.81; p = 0.002) and by 17.4% in the CA1 area of the hippocampus (t = 2.81; p = 0.014, Fig. 4l). In the tissue of the olfactory bulbs there was no difference in the average intensity of immunostaining between control rats and rats subjected to prenatal hypoxia (t = 0.66; p = 0.052). However, in hypoxic rats, we observed changes in neprilysin topography with its main localisation in the central part of the olfactory bulb. The data obtained demonstrate that prenatal hypoxia results in a decrease

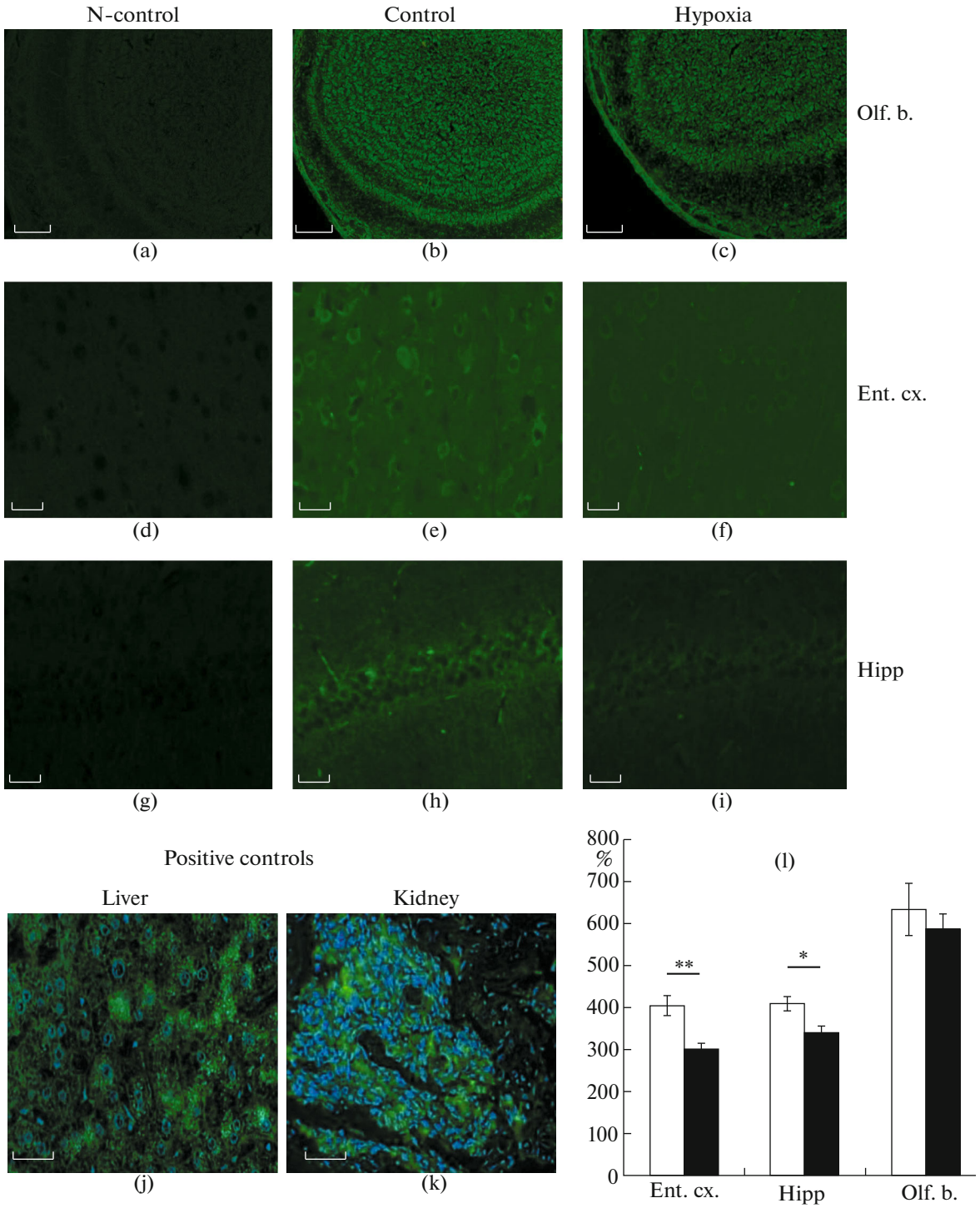

Fig. 4 Effect of prenatal hypoxia on distribution of NEP (FITC-positive green color signal) in the tissue of the olfactory bulb (Olf. b.), entorhinal cortex (Ent. cx.) and CA1 area of the hippocampus (Hipp) of 20-days old rat pups (b, c, e, f, h, i). (a, d, g) Negative (N) control of immunohistochemical staining of brain tissue in the absence of the primary antibody against neprilysin (no immunochemical reaction). (j–k) Examples of positive control of the immunochemical reaction; the tissues of liver (j) and kidney (k) characterised by a high content of neprilysin from a control animal were used (green color—FITC); nuclei were stained by a nonspecific dye DAPI (blue color). (l) Results of densitometric analysis of the fluorescence of FITC-positive structures in the tissue of the entorhinal cortex (Ent. cx.), CA1 area of the hippocampus (Hipp) and olfactory bulb (Olf. b.) 20-day old rat pups with normal development (control, while bars, $n = 8$) and rat pups subjected to prenatal hypoxia (black bars, $n = 8$); data presented as the mean and the standard error of the mean in % of the averaged values of FITC fluorescence in the tissue of the negative control (N-control); the differences between the control and "hypoxia" groups are significant at * $p < 0.05$ and ** $p < 0.01$ (t-test). Scale: 100 (a–c) and 30 (d–k) μm.

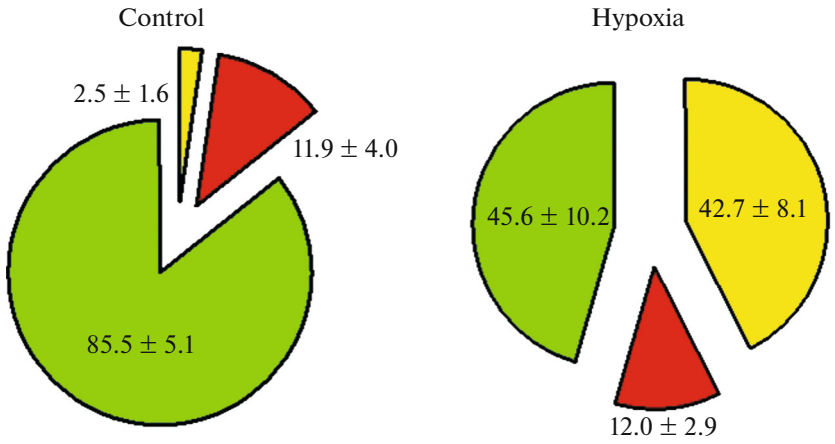

Fig. 5. Schematic representation of comparative efficiency of food search between control rat pups and pups subjected to prenatal hypoxia. Different colors indicate groups of rat pups that found 2 (green), 1 (red) and no (yellow) food pellets, numbers (the mean and the standard error of the mean) indicate corresponding values (%). Differences between the corresponding parts of the figures in the control ($n = 15$) and "hypoxia" ($n = 9$) groups are statistically significant at $p < 0.01$.

of neprilysin levels in the entorhinal cortex and hippocampus of developing rat pups.

Animal behavior. The results of animal testing in the paradigm of food search for a fixed time showed that on average in the intact rat pups ($n = 15$) the efficiency of search was 1.83 ± 0.06 points while in the age matched experimental group subjected to prenatal hypoxia ($n = 9$) this parameter was lower by 43% ($t = 4.1$, $p < 0.01$) and comprised 1.04 ± 0.18 points. More detailed analysis of the studied behavior has revealed that rat pups subjected to prenatal hypoxia more often had an unsuccessful result with zero scoring ($U = 21$, $p < 0.01$) and they rarely could find both pieces of cookies ($t = 3.5$, $p < 0.01$) (Fig. 5).

The number of sniffs of the holes in the floor of the experimental chamber during food search was not different both in intact rat pups and pups subjected to prenatal hypoxia and comprised 12.7 ± 0.86 and 14.9 ± 1.20, respectively.

DISCUSSION

The formation of the olfactory system begins in the embryonic period, which is important for the survival of newborns and the development of sensory systems of the brain in the early postnatal period (Franks and Isaacson, 2005), and its disorders lead to significant rearrangements in the development of neuronal networks involved in the olfactory behavior of animals (Pardo et al., 2018). Our research has shown that prenatal hypoxia has a significant effect on the formation of molecular-cell characteristics of the olfactory system and its functioning in rats in the first month of postnatal development.

It is known that the olfactory analyzer consists of several parts. Its peripheral part is represented by receptor cells located in the olfactory epithelium of the nasal mucosa and the olfactory nerve formed by their axons. The fibers of the olfactory nerve end in the olfactory bulb, where sensory information is transmitted to the secondary neurons. From the olfactory bulb, the axons of nerve cells go into the olfactory cortex, which is divided into 5 main areas (the anterior olfactory nucleus, the pyriform cortex, the olfactory tubercle, the cortico-medial group of the amygdala, part of the entorhinal cortex), each of which has connections with the central and limbic structures. Projections of neurons of the olfactory bulbs have a very wide representation in the cortical structures (Ghosh et al., 2011; Sosulski et al., 2011), and their activation leads to stimulation of cells in different parts of the brain (Arzi and Sobel, 2011). On the other hand, numerous centrifugal fibers from the brain come to the olfactory bulb. Via these fibers, the limbic centers modulate the activity of the olfactory bulb and thus one smell can have different meanings depending on the animal's condition and behavioral context.

Our morphological study of the olfactory bulbs of rats subjected to prenatal hypoxia has not revealed any significant structural changes in neurons and fibers of the olfactory tract, as well as in the cells of the mitral layer whose axons form the projections from the olfactory bulbs to the central parts of the brain. This may be due to the fact that the bulk of neurons of the olfactory bulbs are formed in rats after the 15th–16th day of embryonic development (Bailey et al., 1999), while in our work the experimental pathological impact on pregnant females was carried out on the 14th day of pregnancy. However, our morphological study of two brain regions associated with olfactory function (the entorhinal cortex and hippocampus) of rats subjected to prenatal hypoxia showed pathological changes in neurons already at an early stage of brain development (P20). The changes observed characterize such patho-

logical cell conditions as chromatolysis, hyperchromatosis and lysosome accumulation. In addition, in both brain structures we observed a decrease in the number of neurons and activation of glial cells and their processes surrounding degenerating neurons. Moreover, in the entorhinal cortex these changes were more pronounced than in the hippocampus.

As we have shown earlier, prenatal hypoxia on E14 disrupts the formation and migration of neuroblasts to the lower layers of the entorhinal cortex, which leads to a decreased number of neurons in them (Vasilyev et al., 2020). The death of the projection neurons of the entorhinal cortex also disrupts the afferents of the neurons of the hippocampus and, as a result, leads to their degeneration. In turn, the disruption of the connections between the entorhinal cortex and the hippocampus with other cortical centers and the olfactory bulbs can change the character of animal reaction to olfactory stimuli.

The method used by us to test the olfactory behavior of rats is based on the search for food by smell and can reflect both the motivation and the state of the animal olfactory system (Bianchi et al., 2014). In our studies, the effectiveness of food search in animals that had prenatal hypoxia was much lower than that of intact peers, and their search results were more likely to be zero and less than 100 percent. However, the number of sniffing of the holes in the floor of the experimental chamber in the process of searching for food which reflect motivation, did not differ in intact animals and animals subjected to prenatal hypoxia. This observation allows us to conclude that prenatal hypoxia violates the olfactory function in animals, and not the motivation of food search. Previously, the scientific literature has reported on the effects of adverse prenatal impacts on more complex forms of olfactory behavior related to associative learning and memory (Tiul'kova, 2010; Akers et al., 2011). The data obtained in this study demonstrate the effects of prenatal hypoxia on execution of food search by smell already at the early stages of animal development, which is undoubtedly due to the morphological changes revealed by us in the entorhinal cortex and hippocampus.

The olfactory bulbs are the only brain structure in which NEP is synthesized in significant amounts before birth and in the early days of rat life (Dutriez et al., 1992), indicating its important role in the functioning of the olfactory system. The literature actively discusses data on the participation of NEP in the regulation of the peptidergic system of the brain (Nalivaeva et al., 2020). It is known that neprilysin cleaves somatostatin—the main mediator of peptidergic transmission in the olfactory bulbs (Nocera et al., 2019) and hippocampus (Barnes et al., 1995). Moreover, neprilysin is the major amyloid-degrading enzyme, and the deficiency of its content and activity plays an important role in the pathogenesis of AD (for review see: Nalivaeva and Turner, 2019). Moreover, the entorhinal cortex and hippocampus suffer first from the accumulation of β-amyloid peptide during the development of AD (Reilly et al., 2003).

Disruption of somatostatin metabolism in olfactory bulbs also underlies smell disturbance in AD (Saiz-Sanchez et al., 2010). Our results of immunochemical staining of neprilysin in the olfactory structures are consistent with the results of other authors who performed similar staining in the tissues of the cerebral cortex and hippocampus (Pacheco-Quinto et al., 2016) with predominant presynaptic localization (Fukami et al., 2002; Iwata et al., 2004) and in the bodies of parvalbumin-positive neurons (Pacheco-Quinto et al., 2016).

As we have shown before, prenatal hypoxia leads to a decrease in the expression and activity of NEP in the cerebral cortex and hippocampus of the rat brain (Nalivaeva et al., 2012). Our results on the differences in the nature of the distribution and content of neprilysin in the olfactory bulbs, entorhinal cortex and hippocampus of rats subjected to prenatal hypoxia indicate a functional link between the amount of this peptidase, changes in the state of the nervous tissue and the olfactory behavior of animals. The decrease in neprilysin content in the entorhinal cortex and hippocampus of the rat brain after prenatal hypoxia may be a prerequisite to the imbalance of Aβ towards its accumulation, cell death and neurodegeneration. In humans, such changes can lead to the development of a sporadic form of AD and, associated with it, disruption of olfaction.

Thus, it can be concluded that the disturbance of olfactory function in young rats, resulting from prenatal hypoxic insult on the 14th day of embryonic development, may be associated with the pathological changes in neurons of the entorhinal cortex and hippocampus and with the decreased content in them of the neuropeptidase neprilysin. In this case, the neurodegenerative changes in the entorhinal cortex of animals subjected to prenatal hypoxia are manifested more strongly than in the hippocampus, which may indicate a more pronounced effect of hypoxia on its formation.

ACKNOWLEDGMENTS

The authors express their deep gratitude to O.S. Aleekseeva (IEPhB RAS) for providing technical assistance with modelling prenatal hypoxia in rats, as well as to the Center for the Collective Use of Scientific Equipment for Physiological, Biochemical and Molecular Biological Research (CCU) of IEPhB RAS.

FUNDING

Supported by Russian Foundation for Basic Research (project no. 19-015-00232) and (partially) by the State Assignment for the Sechenov Institute of Evolutionary

Physiology and Biochemistry RAS no. AAAA-A18-118012290373-7.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare no conflict of interests.

Statement on the welfare of animals. The work with the animals was carried out in accordance with the approved protocol of use of laboratory animals by Sechenov the Institute of Evolutionary Physiology and Biochemistry RAS, based on the directive of the European Community Council for the Humane Treatment of Experimental Animals (European Communities Board Directive #86/609 for the Care Laboratory of Animals).

AUTHOR CONTRIBUTIONS

TNL: morphological research, writing of the article; VDS: morphological and immunohistochemical studies, statistical data processing; DNM: behavioral experiments and statistical data processing, NNN: data analysis, writing and editing of the article; ZIA: model of prenatal hypoxia, general management of the project and analysis of the data. The text and graphic images of the article have been approved by all co-authors.

REFERENCES

Akers, K.G., Kushner, S.A., Leslie, A.T., Clarke, L., van der Kooy, D., Lerch, J.P., and Frankland, P.W., Fetal alcohol exposure leads to abnormal olfactory bulb development and impaired odor discrimination in adult mice, *Mol. Brain*, 2011, vol. 4, p. 29. http://www.molecular-brain.com/content/4/1/29.

Arzi, A. and Sobel, N., Olfactory perception as a compass for olfactory neural maps, *Trends Cogn. Sci.*, 2011, vol. 15, p. 537.
https://doi.org/10/1016/j.tics.2011.09.007

Bailey, M.S., Puche, A.C., and Shipley, M.T., Development of the olfactory bulb: evidence for glia–neuron interactions in glomerular formation, *J. Comp. Neurol.*, 1999, vol. 415, no. 4, p. 423–448.
https://doi.org/10.1002/(SICI)1096-9861(19991227)415:4<423::AID-CNE2>3.0.CO;2-G

Barnes, K., Doherty, S., and Turner, A.J., Endopeptidase-24.11 is the integral membrane peptidase initiating degradation of somatostatin in the hippocampus, *J. Neurochem.*, 1995, vol. 64, p. 1826.
https://doi.org/10/1046/j.1471-4159.1995.64041826.x

Barresi, M., Ciurleo, R., Giacoppo, S., Foti Cuzzola, V., Celi, D, Bramanti, P., and Marino, S., Evaluation of olfactory dysfunction in neurodegenerative diseases, *J. Neurol. Sci.*, 2012, vol. 323, p. 16.
https://doi.org/10/1016/j.jns.2012.08.028

Bianchi, P., Bettini, S., Guidi, S., Ciani, E., Trazzi, S., Stagni, F., Ragazzi, E., Franceschini, V., and Bartesaghi, R., Age-related impairment of olfactory bulb neurogenesis in the Ts65Dn mouse model of Down syndrome, *Exp. Neurol.*, 2014, vol. 251, p. 1.
https://doi.org/10.1016/j.expneurol.2013.10.018

Carnemolla, S.E., Hsieh, J.W., Sipione, R., Landis, B.N., Kumfor, F., Piguet, O., and Manuel, A.L., Olfactory dysfunction in frontotemporal dementia and psychiatric disorders: a systematic review, *Neurosci. Biobehav. Rev.*, 2020, vol. 118, p. 588.
https://doi.org/10.1016/j.neubiorev.2020.08.002

Djordjevic, J., Jones-Gotman, M., De Sousa, K., and Chertkow, H., Olfaction in patients with mild cognitive impairment and Alzheimer's disease, *Neurobiol. Aging*, 2008, vol. 29, p. 693.
https://doi.org/10.1016/j.neurobiolaging.2006.11.014

Doty, R.L., Olfactory dysfunction in Parkinson disease, *Nat. Rev. Neurol.*, 2012, vol. 8, p. 329.
https://doi.org/10.1038/nrneurol.2012.80

Dubrovskaya, N.M. and Zhuravin, I.A., Ontogenetic characteristics of behavior in rats subjected to hypoxia on day 14 or day 18 of embryogenesis, *Neurosci. Behav. Physiol.*, 2010, vol. 40, p. 231.
https://doi.org/10.1007/s11055-009-9235-2

Dutriez, I., Salès, N., Fournié-Zaluski, M.C., and Roques, B.P., Pre- and post-natal ontogeny of neutral endopeptidase 24-11 ("enkephalinase") studied by in vitro autoradiography in the rat, *Experientia*, 1992, vol. 48, p. 290.
https://doi.org/10.1007/BF01930479

Franks, K.M. and Isaacson, J.S., Synapse-specific downregulation of NMDA receptors by early experience: a critical period for plasticity of sensory input to olfactory cortex, *Neuron*, 2005, vol. 47, p. 101.
https://doi.org/10.1016/j.neuron.2005.05.024

Fukami, S., Watanabe, K., Iwata, N., Haraoka Jo, Bao Lu, Gerard, N.P., Gerard, C., Fraser, P., Westaway, D., St. George-Hyslop, P., and Saido, T.C., Aβ-degrading endopeptidase, neprilysin, in mouse brain: synaptic and axonal localization inversely correlating with Aβ pathology, *Neurosci. Res.*, 2002, vol. 43, p. 39.
https://doi.org/10.1016/S0168-0102(02)00015-9

Ghosh, S., Larson, S.D., Hefzi, H., Marnoy, Z., Cutforth, T., Dokka, K., and Baldwin, K.K., Sensory maps in the olfactory cortex defined by long-range viral tracing of single neurons, *Nature*, 2011, vol. 472, p. 217.
https://doi.org/10.1038/nature09945

Gulyaeva, N.V., Bobkova, N.V., Kolosova, N.G., Samokhin, A.N., Stepanichev, M.Yu., and Stefanova, N.A., Molecular and cellular mechanisms of sporadic Alzheimer's disease: Studies on rodent models in vivo, *Biochemistry* (Moscow), 2017, vol. 82, p. 1088.
https://doi.org/10.1134/S0006297917100029

Iwata, N., Mizukami, H., Shirotani, K., Takaki, Y., Muramatsu, S., Lu, B., Gerard, N.P., Gerard, C., Ozawa, K., and Saido, T.C., Presynaptic localization of neprilysin contributes to efficient clearance of amyloid-β peptide in mouse brain, *J. Neurosci.*, 2004, vol. 24, p. 991.
https://doi.org/10.1523/JNEUROSCI.4792-03.2004

Liu, B., Luo, Z., Pinto, J.M., Shiroma, E.J., Tranah, G.H., Wirdefeldt, K., Fang, F., Harris, T.B., and Chen, H., Relationship between poor olfaction and mortality among community-dwelling older adults: a cohort study, *Ann. Intern. Med.*, 2019, vol. 170, p. 673.
https://doi.org/10.7326/M18-0775

Morozova, S.V., Savvateeva, D.M., and Petrova, E.I., Olfactory disorders in patients with neurodegenerative diseases, *Nevrol. Zh.*, 2014, no. 1, p. 4.

Murphy, C., Olfactory and other sensory impairments in Alzheimer disease, *Nat. Rev. Neurol.*, 2019, vol. 15, p. 11. https://doi.org/10.1038/s41582-018-0097-5

Nalivaeva, N.N. and Turner, A.J., Targeting amyloid clearance in Alzheimer's disease as a therapeutic strategy, *Br. J. Pharmacol.*, 2019, vol. 176, p. 3447. https://doi.org/10.1111/bph.14593

Nalivaeva, N.N., Fisk, L., Kochkina, E.G., Plesneva, S.A., Zhuravin, I.A., Babusikova, E., Dobrota, D., and Turner, A.J., Effect of hypoxia/ischemia and hypoxic preconditioning/reperfusion on expression of some amyloid-degrading enzymes, *Ann. N.Y. Acad. Sci.*, 2004, vol. 1035, p. 21. https://doi.org/10.1196/annals.1332.002

Nalivaeva, N.N., Babusikova, E., Dobrota, D., and Turner, A.J., Effect of ischaemia and reperfusion on the content and degradation of amyloid precursor protein in the hippocampus of rats, *Neurochem. J.*, 2005, vol. 22, p. 207.

Nalivaeva, N.N., Belyaev, N.D., Lewis, D.I., Pickles, A.R., Makova, N.Z., Bagrova, D.I., Dubrovskaya, N.M., Plesneva, S.A., Zhuravin, I.A., and Turner, A.J., Effect of sodium valproate administration on brain neprilysin expression and memory in rats, *J. Mol. Neurosci.*, 2012, vol. 46, p. 569. https://doi.org/10.1007/s12031-011-9644-x

Nalivaeva, N.N., Vasilev, D.S., Dubrovskaya, N.M., Turner, A.J., and Zhuravin, I.A., Role of neprilysin in synaptic plasticity and memory, *Ross. Fiziol. Zh. im. I.M. Sechenova*, 2020, vol. 106, no. 10, p. 1191. https://doi.org/10.31857/S0869813920100076

Nocera, S., Simon, A., Fiquet, O., Chen, Y., Gascuel, J., Datiche, F., Schneider, N., Epelbaum, J., and Viollet, C., Somatostatin serves a modulatory role in the mouse olfactory bulb: neuroanatomical and behavioral evidence, *Front. Behav. Neurosci.*, 2019, vol. 13, p. 61. https://doi.org/10.3389/fnbeh.2019.00061

Pacheco-Quinto, J., Eckman, Ch.B., and Eckman, E.A., Major amyloid-β degrading enzymes endothelin-converting enzyme-2 and neprilysin are expressed by distinct populations of GABAergic interneurons in hippocampus and neocortex, *Neurobiol. Aging*, 2016, vol. 48, p. 83. https://doi.org/10.1016/j.neurobiolaging.2016.08.011

Pardo, G.V.E., Lucion, A.B., and Calcagnotto, M.E., Postnatal development of inhibitory synaptic transmission in the anterior piriform cortex, *Int. J. Dev. Neurosci.*, 2018, vol. 71, p. 1. https://doi.org/10.1016/j.ijdevneu.2018.07.008

Paxinos, G. and Watson, C., *The Rat Brain in Stereotaxic Coordinates*, Amsterdam: Elsevier AP, 2005.

Reilly, J.F., Games, D., Rydel, R.E., Freedman, S., Schenk, D., Young, W.G., Morrison, J.H., and Bloom, F.E., Amyloid deposition in the hippocampus and entorhinal cortex: quantitative analysis of a transgenic mouse model, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, vol. 100, p. 4837. https://doi.org/10.1073/pnas.0330745100

Saiz-Sanchez, D., Ubeda-Bañon, I., de la Rosa-Prieto, C., Argandoña-Palacios, L., Garcia-Muñozguren, S., Insausti, R., and Martinez-Marcos, A., Somatostatin, tau, and β-amyloid within the anterior olfactory nucleus in Alzheimer disease, *Exp. Neurol.*, 2010, vol. 223, p. 347. https://doi.org/10.1016/j.expneurol.2009.06.010

Sosulski, D.L., Lissitsyna Bloom, M., Cutforth, T., Axel, R., and Datta, S.R., Distinct representations of olfactory information in different cortical centres, *Nature*, 2011, vol. 472, p. 213. https://doi.org/10.1038/nature09868

Sun, S., Li, T., Davies, H., Li, W., Yang, J., Li, S., and Ling, S., Altered morphologies and functions of the olfactory bulb and hippocampus induced by miR-30c, *Front. Neurosci.*, 2016, vol. 10, p. 207. https://doi.org/10.3389/fnins.2016.00207

Thomann, P., Dos Santos, V., Toro, P., Schönknecht, P., Essig, M., and Schröder, J., Reduced olfactory bulb and tract volume in early Alzheimer's disease—a MRI study, *Neurobiol. Aging*, 2009, vol. 30, p. 838. https://doi.org/10.1016/j.neurobiolaging.2007.08.001

Tiul'kova, E.I., Vataeva, L.A., Samoilov, M.O., and Otellin, V.A., Mechanisms of formation of brain reactions to hypobaric hypoxia in different periods of prenatal development of rats, *Zh. Akusher. Zhensk. Bol.*, 2010, vol. 59, p. 99.

Tumanova, N.L., Vasiliev, D.S., Dubrovskaya, N.M., and Zhuravin, I.A., Changes in ultrastructure of the sensorimotor cortex accompanied by the motor behavior dysfunctions in the early ontogenesis of rats subjected to the prenatal hypoxia, *Tsitologiia*, 2018, vol. 60, p. 390. https://doi.org/10.31116/tsitol.2018.05.09

Vasilev, D.S., Dubrovskaya, N.M., Tumanova, N.L., and Zhuravin, I.A., Prenatal hypoxia in different periods of embryogenesis differentially affects cell migration, neuronal plasticity, and rat behavior in postnatal ontogenesis, *Front. Neurosci.*, 2016, vol. 10, p. 126. https://doi.org/10.3389/fnins.2016.00126

Vasilev, D.S., Tumanova, N.L., and Kalinina, D.S., Prenatal hypoxia disturbs the formation of pyramidal neurons in the entorhinal cortex of the rat brain, *Russ. J. Physiol.*, 2020, vol. 106, p. 127. https://doi.org/10.31857/S086981392010012X

Wang, J., Eslinger, P.J., Doty, R.L., Zimmerman, E.K., Grunfeld, R., Sun, X., Meadowcroft, M.D., Connor, J.R., Price, J.L., Smith, M.B., and Yang, Q.X., Olfactory deficit detected by fMRI in early Alzheimer's disease, *Brain Res.*, 2010, vol. 1357, p. 184. https://doi.org/10.1016/j.brainres.2010.08.018

Zhuravin, I.A., Vasilev, D.S., Dubrovskaya, N.M., Bagrova, D.I., Kochkina, E.G., Plesneva, S.A., Tumanova, N.L., and Nalivaeva, N.N., Cognitive impairments in ontogenesis of mammals after disturbed prenatal development, *Psikhiatriia*, 2010, vol. 4, no. 46, p. 36.