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Effect of Magnesium Pre-treatment on the Hippocampal NOS Activity during Long-lasting Intermittent Hypoxia

Jandová K., Langmeier M., Marešová D., Pokorný J., Trojan S.
Institute of Physiology of the First Faculty of Medicine, Charles University in Prague, Czech Republic.

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Mailing address: Kateřina Jandová, MD., Institute of Physiology of the First Faculty of Medicine, Charles University, Albertov 5, 128 00 Prague 2, Czech Republic, Phone: +420 224 968 443, e-mail: katerina.jandova@lf1.cuni.cz

Abstract: Influence of magnesium pre-treatment during repetitive hypoxia was studied in the hippocampus of rats by histochemical analysis (NADPH-diaphorase staining). NADPH-diaphorase occurs concurrently with NO-synthase that is responsible for NO synthesis. Rat pups were kept together with their mother for 8 hours a day in a hypobaric chamber at a simulated altitude of 7 000 m since the day of birth till the 17th day. The first group of animals was exposed to the repeated hypoxia; the second group under the same conditions was pre-treated by magnesium before the exposition to the hypoxia. Both groups were compared with intact control animals and intact animals treated with magnesium. The experimental and control animals were the transaortically perfused with 4% buffered neutral formaldehyde under thiopental anaesthesia at the age of 35 days. Brains were processed for NADPH-d staining. We estimated the density of NADPH-d positive neurons in CA1 and CA3 areas of the hippocampus and in the dentate gyrus. Intermittent hypoxia brings about higher numbers of NADPH-diaphorase positive neurons of CA1 and CA3 of the hippocampus and of the dorsal blade of dentate gyrus, in the comparison with either group of control animals. In the hilus and ventral blade of the dentate gyrus, on the contrary, the number of NADPH-d positive neurons was smaller. Magnesium pre-treatment during hypoxia decreased number of nitrergic neurons in all areas of the hippocampus except CA1 area, where the effect of magnesium was not significant. These results demonstrate that magnesium can probably have a neuroprotective effect.

Introduction

Perinatal hypoxic–ischemic brain damage is a major cause of acute mortality and chronic neurological morbidity in infants and children. Between 20–50% of asphyxiated newborns with hypoxic–ischemic encephalopathy die within the newborn period, and up to 25% of the survivors will exhibit permanent neuropsychological outputs (including mental retardation, cerebral palsy, epilepsy or learning disability) [1].

The brain is more susceptible to hypoxia than other organ, as it has high oxygen demand and only little energy is produced by anaerobic mechanisms [2, 3]. Hypoxia in experimental animals evokes series of electrophysiological [4, 5], behavioural [6] and morphological changes [7, 8, 9, 10].

Perinatal hypoxia leads to the alterations of energy metabolism, intracellular calcium level, release of excitatory amino acids and inhibition of protein biosynthesis. The formation of oxygen radicals during the reperfusion phase following an ischemic insult, induction of the NO system, and inflammatory reactions result from the neuronal damage [11].

One of possible mechanism of brain injury is the activation of some enzymes as nitric oxide synthases, resulting in nitric oxide (NO) production [12]. NO is produced from L-arginine (L-Arg) by nitric oxide synthase (NOS). There are at

least three different forms of this enzyme [13], the endothelial (eNOS) that is responsible for cardiovascular actions, the inducible (iNOS) found originally in macrophages and involved mainly in immunological processes and the neuronal (nNOS). Neuronal NOS is a constitutive enzyme, which is expressed only by a few percent of neurons. The production of NO is a calmodulin- dependent process; therefore, it must be preceded by the elevation of intracellular Ca^{2+}-concentration [14]. It has been observed that nNOS produces NO almost exclusively after activation of N-methyl-D-aspartate (NMDA) receptors [15].

Many studies have shown that nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) may correspond to NOS, and it is therefore suggested that neurons containing NADPH-d might be capable of producing NO [16].

Clinical and experimental studies describe effect of some ions on the function and structure of the brain in hypoxic/ischemic conditions and during the reperfusion period. Some publications describe vascular influence of magnesium-increase in cerebral blood flow by vasodilatation of cerebral arteries and decrease of blood pressure with no effect on heart rate [17]. The key role plays magnesium as an important cation necessary for the functioning of over 300 enzymes in many intracellular mechanisms [18]. Magnesium as a blocker of NMDA receptors and voltage-gated calcium channels [19] regulates calcium concentration within the cell and modulates posttraumatic neurochemical changes mediated by these elevated intracellular calcium levels.

In the present study we analysed possible protective effect of magnesium on the density of NADPH- diaphorase positive neurons in the developing brain (in 35-day-old rats) exposed to chronic hypoxia.

This work extends our previous study of the hypoxia and magnesium effects on 25-day-old rats [20].

Materials and Methods

Experiments were performed on 35-day-old male rats (Wistar strain). There were 16 animals in all experimental groups, with 4 in each group. Following groups of rats were studied:

1. Control rats not exposed to hypoxia (C)
2. Control rats not exposed to hypoxia repeatedly injected with magnesium sulphate (300 mg/kg i.p.) (C+Mg)
3. Rats exposed to repeated hypobaric hypoxia (H)
4. Rats exposed to repeated hypobaric hypoxia as above and repeatedly injected with magnesium sulphate (300 mg/kg i.p.) before the hypoxia exposition (H+Mg)

Rats, together with their mother, were exposed to hypoxia for 8 hours a day (except day 6, 7, 13 and 14) in a hypobaric chamber at a simulated altitude of 7 000 m since the day of birth till the 17^{th} day.

Animals were studied at the age of 35 days, eighteen days after the end of exposition to hypobaric hypoxia. Rat pups were transaortically perfused under deep thiopental anaesthesia with 4% neutral paraformaldehyde. The brain was removed, postfixed for one hour in 4 % buffered paraformaldehyde and then submerged for 20 hours into 20 % sucrose for cryoprotection. The brain was sliced in the frontal plane into 40 μm thick sections with a cryostat. Brain slices were processed for NADPH-diaphorase staining.

CA1 and CA3 areas of the hippocampus, dorsal blade, ventral blade and hilus of the dentate gyrus (DB DG and VB DG) between the AP plane 2.5 mm and 4.0 mm posterior to bregma were subjected to quantification of nitrergic neurons under a light microscope Olympus Provis AX 70 (Colour figures 9, 10). For the statistical evaluation, the t-unpaired test and ANOVA were used (level of significance was set at $p < 0.05$ (*), $p < 0.01$(**), $p < 0.001$ (***)).

Results

The results show that the repeated hypobaric perinatal hypoxia enhanced the density of NADPH-d positive neurons compared to the control group which we consider as baseline, in CA1 by 16% ($p < 0.01$), in CA3 area of the hippocampus by 49% ($p < 0.001$) and in the dorsal blade of the dentate gyrus by 15% ($p < 0.001$). In the hilus and ventral blade of the dentate gyrus, on the contrary, the number of NADPH-d positive neurons was lower, in the hilus by 12% ($p < 0.05$) and in the ventral blade of the dentate gyrus by 54% ($p < 0.001$) (Figure 1).

Magnesium pre-treatment in control rats decreased number of nitrergic neurons in all areas of the hippocampus: in CA1 by 14% ($p < 0.05$), in CA3 by 39% ($p < 0.001$), in the hilus by 60% ($p < 0.001$), in the ventral blade by 58% ($p < 0.001$) and in the dorsal blade of the dentate gyrus by 18% ($p < 0.001$) (Figure 2).

The same results – magnesium pre-treatment reduces density of NADPH-d positive neurons elevated by hypoxia. Except the CA1 hippocampal area, where changes were not significant, lower density of NADPH-d positive neurons was observed in CA3 area of hippocampus (by 19%, $p < 0.001$), in the hilus (by 40%, $p < 0.001$), in the ventral blade (by 14%, $p < 0.05$) and in the dorsal blade of the dentate gyrus (by 20%, $p < 0.001$) (Figure 3).

Discussion

Many studies have shown that NADPH-diaphorase can correspond with the neuronal NOS, and it is therefore suggested that neurons containing NADPH-d are likely to be capable of producing NO [21].

NADPH-d reactivity has been detected in various regions of the nervous system of mammals including the rat. The coexistence of NADPH-d reactivity and neurotransmitter or neuropeptide reactivity has been demonstrated in certain populations of neurons [22].

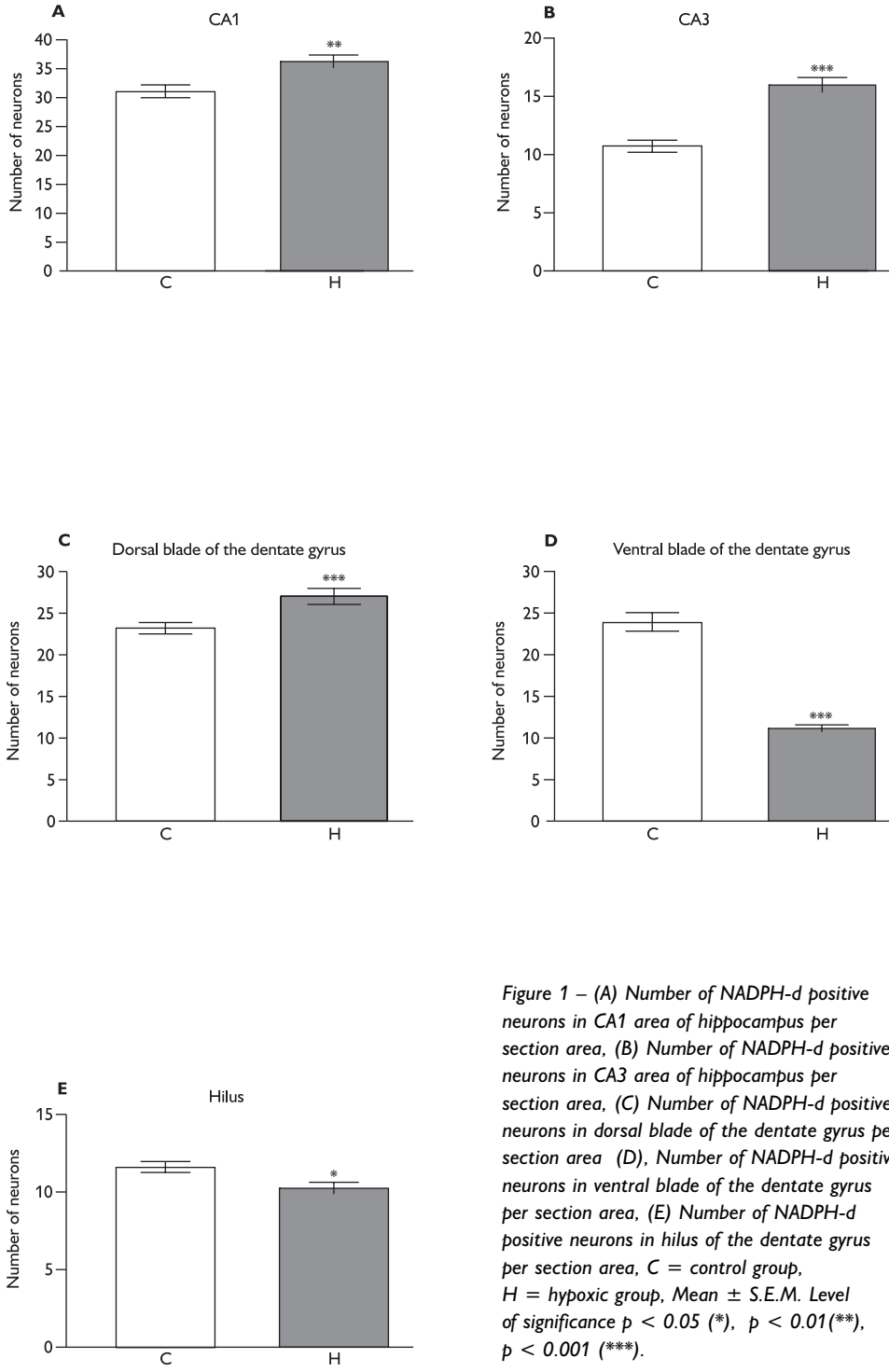

Figure 1 – (A) Number of NADPH-d positive neurons in CA1 area of hippocampus per section area, (B) Number of NADPH-d positive neurons in CA3 area of hippocampus per section area, (C) Number of NADPH-d positive neurons in dorsal blade of the dentate gyrus per section area (D), Number of NADPH-d positive neurons in ventral blade of the dentate gyrus per section area, (E) Number of NADPH-d positive neurons in hilus of the dentate gyrus per section area, C = control group, H = hypoxic group, Mean ± S.E.M. Level of significance $p < 0.05$ (*), $p < 0.01$(**), $p < 0.001$ (***).

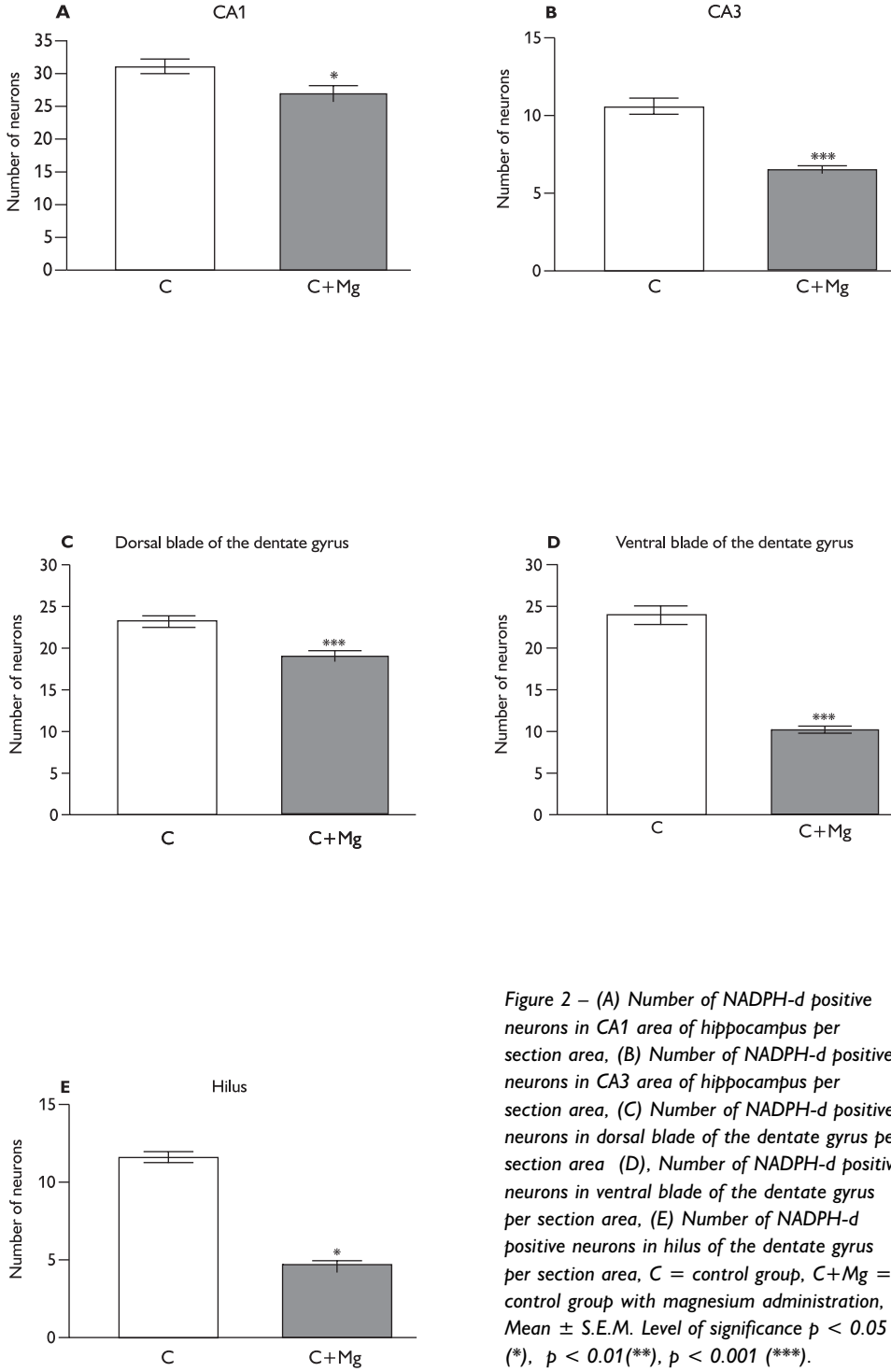

Figure 2 – (A) Number of NADPH-d positive neurons in CA1 area of hippocampus per section area, (B) Number of NADPH-d positive neurons in CA3 area of hippocampus per section area, (C) Number of NADPH-d positive neurons in dorsal blade of the dentate gyrus per section area (D), Number of NADPH-d positive neurons in ventral blade of the dentate gyrus per section area, (E) Number of NADPH-d positive neurons in hilus of the dentate gyrus per section area, C = control group, C+Mg = control group with magnesium administration, Mean ± S.E.M. Level of significance $p < 0.05$ (*), $p < 0.01$(**), $p < 0.001$ (***).

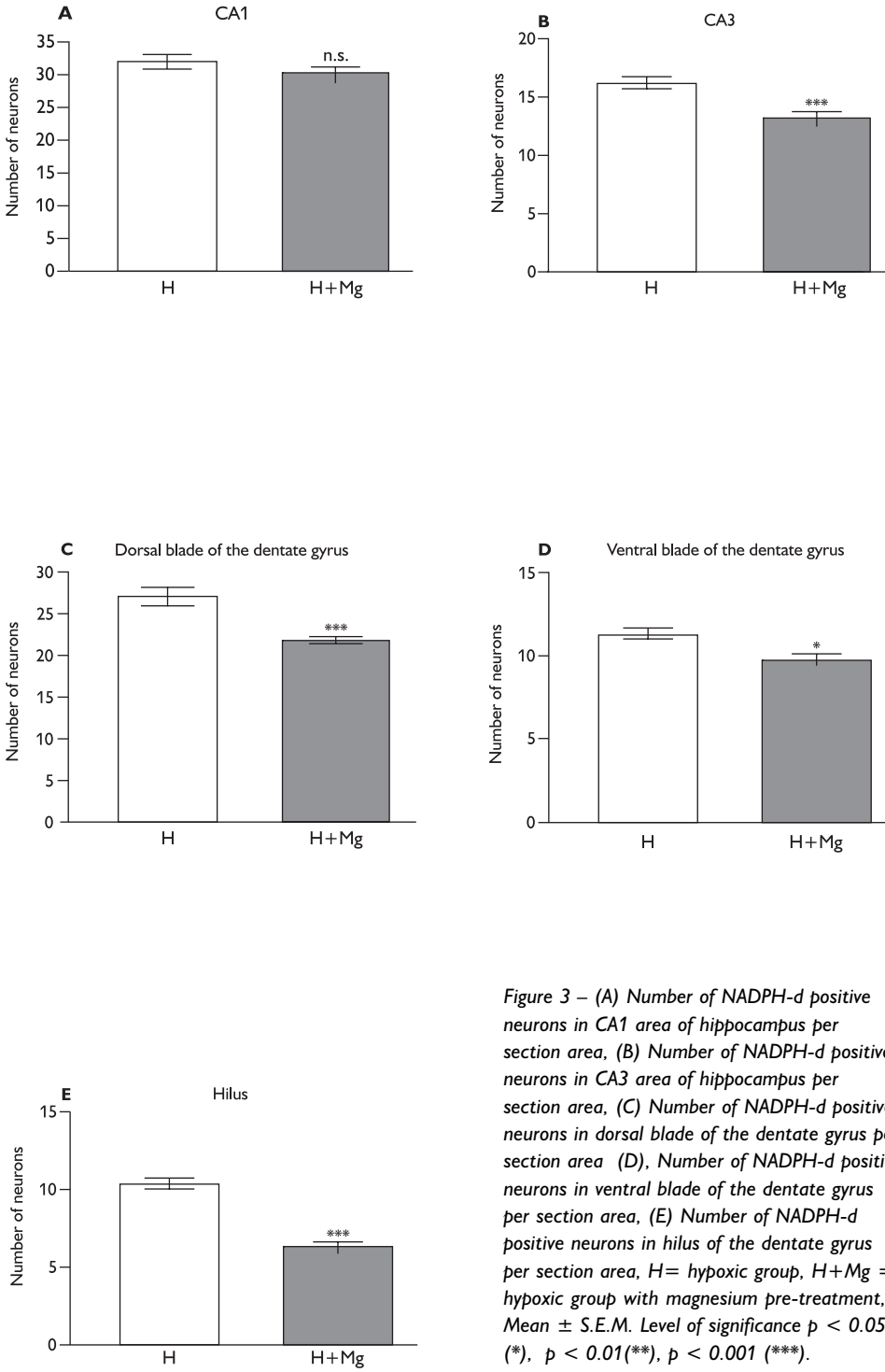

Figure 3 – (A) Number of NADPH-d positive neurons in CA1 area of hippocampus per section area, (B) Number of NADPH-d positive neurons in CA3 area of hippocampus per section area, (C) Number of NADPH-d positive neurons in dorsal blade of the dentate gyrus per section area (D), Number of NADPH-d positive neurons in ventral blade of the dentate gyrus per section area, (E) Number of NADPH-d positive neurons in hilus of the dentate gyrus per section area, H= hypoxic group, H+Mg = hypoxic group with magnesium pre-treatment, Mean ± S.E.M. Level of significance $p < 0.05$ (*), $p < 0.01$(**), $p < 0.001$ (***).

Our findings show that long-lasting intermittent perinatal hypoxia increased the number of NADPH-d positive neurons in CA1, CA3 areas of the hippocampus and in dorsal blade of the dentate gyrus.

After the pre-treatment with magnesium, density of nitrergic neurons in hilus and ventral blade of the dentate gyrus was lower then only after the hypoxia. Changes of density of NADPH-diaphorase positive neurons in the hilus of the dentate gyrus occurred immediately after the last exposure to hypoxia [23]. This could support the theory that hypoxia causes a rapid loss of high-energy phosphates, generalized depolarization, progressive proteolysis, and loss of membrane integrity, due to damage to membrane lipids by lipolysis. The post-ischemic suppression of protein synthesis, which could explain the loss of neurons, was reported in numerous studies [24].

Magnesium pre-treatment of rats exposed to intermittent hypobaric hypoxia evoked decline of nitrergic neurons density in all studied areas. Possible protective effect of the magnesium administration can be hypothetically explained by an encroachment on gene expression of nitric oxide synthase. NMDA receptor and voltage-gated calcium channels blockade by magnesium lead to reduction in intracellular calcium concentration which can suppress synthesis of calcium dependent nNOS and subsequent lower synthesis of NO [25].

References

1. VANNUCCI S. J., HAGBERG H.: Hypoxia-ischemia in the immature brain. *J. Exp. Biol.* 207: 149–3154, 2004.
2. TROJAN S.: Adaptation of the central nervous system to oxygen deficiency during ontogenesis. *Acta Univ. Carol. Med. Monogr.* 85: 5–180, 1978.
3. MISHRA O. P., DELIVORIA-PAPADOPOULOS M.: Cellular mechanisms of hypoxic injury in the developing brain. *Brain Res. Bull.* 48: 233–238, 1999.
4. MAREŠOVÁ D., VALKOUNOVÁ I., JANDOVÁ K., BORTELOVÁ J., TROJAN S.: Excitability changes of cortical neurons during the postnatal period in rats exposed to prenatal hypobaric hypoxia. *Physiol. Res.* 50: 215–219, 2001.
5. MAREŠOVÁ D.: Changes of evoked epileptic seizures after the short term hypobaric hypoxia in the young rats. *Prague Med. Rep.* 105: 381–390, 2004.
6. ŠIMONOVÁ Z., ŠTĚRBOVÁ K., BROŽEK G., KOMÁREK V., SYKOVÁ E.: Postnatal hypobaric hypoxia in rats impairs water maze learning and the morphology of neurones and macroglia in cortex and hippocampus. *Behav. Brain Res.* 141: 195–205, 2003.
7. LANGMEIER M., POKORNY J., MARES J., MARES P., TROJAN S.: Effect of prolonged hypobaric hypoxia during postnatal development on myelination of the corpus callosum in rats. *J. Hirnforsch.* 28: 385–395, 1987.
8. LANGMEIER M., POKORNÝ J., MAREŠ J., TROJAN S.: Changes of the neuronal structure produced by prolonged hypobaric hypoxia in infant rats. *Biomed. Biochim. Acta.* 48: 204–207, 1989.
9. WILLIAMS P. A., DOU P., DUDEK F. E.: Epilepsy and synaptic reorganization in a perinatal rat model of hypoxia-ischemia. *Epilepsia* 45: 1210–1218, 2004.
10. YANG S. N., HUANG C. B., YANG C. H., LAI M. C., CHEN W. F., WANG C. L., WU C. L., HUANG L. T.: Impaired SynGAP expression and long-term spatial learning and memory in hippocampal

CA1 area from rats previously exposed to perinatal hypoxia-induced insults: beneficial effects of A68930. *Neurosci. Lett.* 371: 73–78, 2004.
11. BERGER R., GARNIER Y.: Pathophysiology of perinatal brain damage. *Brain Res. Rev.* 30: 107–134, 1999.
12. TURKYILMAZ C., TURKYILMAZ Z., ATALAY Y., SOYLEMEZOGLU F., CELASUN B.: Magnesium pre-treatment reduces neuronal apoptosis in newborn rats in hypoxia-ischemia. *Brain Res.* 955: 133–137, 2002.
13. GRIFFITH O. W., STUEHR D. J.: Nitric oxide synthases: properties and catalytic mechanism. *Annu. Rev. Physiol.* 57: 707–736, 1995.
14. KISS J. P.: Role of nitric oxide in the regulation of monoaminergic neurotransmission. *Brain Res. Bull.* 52: 459–466, 2000.
15. GARTHWAITE J., BOULTON C. L.: Nitric oxide signaling in the central nervous system. *Annu. Rev. Physiol.* 57: 683–706, 1995.
16. FLESCH I. E., HESS J. H., KAUFMANN S. H.: NADPH diaphorase staining suggests a transient and localized contribution of nitric oxide to host defence against an intracellular pathogen in situ. *Int. Immunol.* 6: 1751–1757, 1994.
17. KEMP P. A., GARDINER S. M., MARCH J. E., RUBIN P. C., BENNETT T.: Assessment of the effects of endothelin-1 and magnesium sulphate on regional blood flows in conscious rats, by the coloured microsphere reference technique. *Br. J. Pharmacol.* 126: 621–626, 1999.
18. VINK R., CERNAK I.: Regulation of intracellular free magnesium in central nervous system injury. *Front. Biosci.* 5: 656–665, 2000.
19. ISERI L. T., FRENCH J. H.: Magnesium: nature's physiologic calcium blocker. *Am. Heart J.* 108: 188–193, 1984.
20. MAREŠOVÁ D., JANDOVÁ K., BORTELOVÁ J., TROJAN S., TRNKOVÁ B.: Functional and morphological changes of the brain in rats exposed to intermittent hypobaric hypoxia after the repetitive magnesium administration. *Prague Med. Rep.* 106: 61–69, 2005.
21. MONTECOT C., BORREDON J., SEYLAZ J., PINARD E.: Nitric oxide of neuronal origin is involved in cerebral blood flow increase during seizures induced by kainate. *J. Cereb. Blood Flow. Metab.* 17: 94–99, 1997.
22. WANG T. J., LUE J. H., SHIEH J. Y., WEN C. Y.: The distribution and characterization of NADPH-d/NOS-IR neurons in the rat cuneate nucleus. *Brain Res.* 910: 38–48, 2001.
23. BENEŠOVÁ P., LANGMEIER M., BETKA J., TROJAN S.: Changes in the number of nitrergic neurons following kainic acid administration and repeated long-term hypoxia. *Physiol. Res.* 53: 343–349, 2004.
24. WHITE B. C., SULLIVAN J. M., DEGRACIA D. J., O'NEIL B. J., NEUMAR R. W., GROSSMAN L. I., RAFOLS J. A., KRAUSE G. S.: Brain ischemia and reperfusion: molecular mechanisms of neuronal injury. *J. Neurol. Sci.* 179: 1–33, 2000.
25. MANTELAS A., STAMATAKIS A., KAZANIS I., PHILIPPIDIS H., STYLIANOPOULOU F.: Control of neuronal nitric oxide synthase and brain-derived neurotrophic factor levels by GABA-A receptors in the developing rat cortex. *Brain Res. Dev. Brain Res.* 145: 185–195, 2003.