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Changes in the Number of Nitrergic Neurons Following Kainic Acid Administration and Repeated Long-term Hypoxia

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Summary

Using histochemical analysis (NADPH-diaphorase) we have been investigating the influence of intraperitoneal administration of kainic acid (KA), hypoxia and combination of both these factors on neurons of the hippocampus and on the primary auditory cortex (PAC) in male rats of the Wistar strain. Kainic acid was administered to 18-day-old animals, which were exposed to long-lasting repeated hypoxia from the 2nd till the 17th day of age in a hypobaric chamber (for 8 h a day). At the age of 22 or 90 days, the animals were transcardially perfused with 4 % paraformaldehyde under deep thiopental anesthesia. Cryostate sections were stained to identify NADPH-diaphorase positive neurons that were then quantified in the hippocampus, in the dentate gyrus and in the PAC. In 22-day-old animals both hypoxia and KA increased the number of NADPH-diaphorase positive neurons in the hilus, CA1, CA3 areas of the hippocampus and in the PAC. On the contrary, KA given to hypoxic animals lowered the number of NADPH-diaphorase positive neurons in the dentate gyrus. In 90-day-old animals, hypoxia and KA given to both normoxic and hypoxic animals lowered the number of NADPH-diaphorase positive neurons in some areas of the central nervous system.

Key words
Kainic Acid • Nitric Oxide • Hypoxia • Hippocampus • Primary auditory cortex

Introduction

Brain ischemia and reperfusion activate independently multiple crucial terminal pathways involving loss of membrane integrity, progressive proteolysis, and inability to check these processes. The changes during hypoxia lead to nitric oxide synthase (NOS) activation, resulting in nitric oxide production (White *et al.* 2000).

Nitric oxide (NO) is produced from L-arginine by NOS. There are at least three different forms of this enzyme, 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 one (nNOS). Although all forms can be found in the CNS, the specific actions on neurotransmission may be attributed primarily to NO produced by nNOS located in neurons.

Neuronal NOS is a constitutive enzyme, which is expressed only by a small percentage of neurons. The production of NO is a calmodulin-dependent process, which must be preceded by an elevation of intracellular Ca^{2+} concentration (Kiss 2000). Ca^{2+} influx is induced by activation of glutamate receptors, preferentially NMDA receptors (Prast and Philippu 2000).

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 to produce NO.

NO production can be stimulated not only by hypoxia. Kainic acid also increases NO production (Montecot *et al.* 1997), although by different mechanisms. Kainic acid (KA), [2-carboxy-4 (1-methyl-ethenyl)-3-pirrolidinacetic acid] is a rigid structural analogue of glutamic acid (Zagulska-Szymczak *et al.* 2000). The potent excitotoxic and epileptogenic effects of kainate were thought to be due to the existence of specific receptors for kainate. Such a hypothesis was supported by the demonstration of high affinity binding sites for [^{3}H]kainate in the rat brain (Bleakman and Lodge 1998). KA is an agonist of two kinds of ionotropic, non-NMDA glutamate receptors (AMPA and KA receptors) (Zagulska-Szymczak *et al.* 2000).

Because of the marked sensivity of limbic structures to the neurotoxic effects of KA, which are evidently due to the great density of binding sites for excitatory amino acids in these regions, KA-induced seizures are considered to be a satisfactory model of human partial seizures with a complex symptomatology (Velíšková *et al.* 1988, Koryntová *et al.* 1997, Kubová *et al.* 2001). As an excitotoxin of the mammalian central nervous system, KA induces neural cell death by apoptosis as well as by necrosis (Akhlaq *et al.* 2001).

KA is a very important tool for probing multiple brain genome responses, as it establishes a link between the transient seizure activity and long-term alterations in neural structures and functions in several brain regions. The hippocampus (particularly CA3 and CA1 areas) is especially sensitive to the excitatory and neurotoxic effect of KA and is the area of its primary action. The neural events in this brain structure have been studied intensively (Zagulska-Szymczak *et al.* 2000) due to its relatively simple anatomy, its involvement in a variety of neurodegenerative conditions as well as its probable role in memory formation.

The problem addressed in the present paper concerned the question whether and how can long-lasting repeated perinatal hypoxia and KA influence individual brain structures of young and adult rats.

Methods

Male Wistar rats of our own breed were used for the experiments. There were 16 animals in the experimental groups. From the 2nd till the 17th day of age the young animals were, together with their mother, exposed to long-lasting repeated hypoxia in a hypobaric chamber at a simulated altitude of 7000 m, always for 8 h a day. The 18th day of age, i.e. one day after the final day of the exposition to hypoxia, the animals were given a single intraperitoneal injection of KA (2.5 mg/kg). This sublethal dose is responsible for partial seizures with complex symptomatology (Velíšková *et al.* 1988).

Eight animal groups used in the experiment:

– young animals exposed to hypoxia
– young animals exposed to hypoxia, followed by KA i.p. injection the 18th day of age
– young animals not exposed to hypoxia
– young animals not exposed to hypoxia, followed by KA i.p. injection the 18th day of age
– adult animals, exposed to hypoxia from the 2nd till the 17th day of age
– adult animals, exposed to hypoxia from the 2nd till the 17th day of age, followed by KA i.p. injection the 18th day of age
– adult animals, not exposed to hypoxia
– adult animals, not exposed to hypoxia, followed by KA i.p. injection the 18th day of age

Animals aged 22 or 90 days were perfused under deep thiopental anestesia with 4 % paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. The brain was removed, postfixed for one hour in 4 % buffered paraformaldehyde and then submerged for 1 h into 20 % sucrose for cryoprotection. The brain was sliced in the frontal plane into 40 μm thick sectiones with a cryostate. The free-floating sections were placed in 0.1 M phosphate buffer and incubated in 0.1 M phosphate buffer containing 0.5 mg/ml β-NADPH (Sigma) reductase, 0.2 mg/ml Nitro blue tetrazolium (NBT, Sigma) and 0.3 % Triton for 4 h at 37 °C in thermostat. Following the reaction, the sections were rinsed in 0.1 M phosphate buffer and kept at 8 °C for 16 h. The histochemically reacted sections were mounted on pre-cleaned 0.5 % gelatin-coated Microscope Slides (Menzel-Gläser), air dried, coverslipped with Microscope Cover Glasses (Menzel-Gläser) using D.P.X. Neutral Mounting Medium (Aldrich) (Wang *et al.* 2001). NADPH-d positive neurons were then quantified in five regions of the hippocampal formation (Fig.1): i) in CA1 area of the hippocampus, ii) in CA3 area of the hippocampus, iii) in the hilus of the dentate gyrus, iv) in the dorsal blade of the dentate gyrus, v) in the ventral blade of the dentate gyrus, vi) in the primary auditory cortex [Te 1 - temporal area 1 (primary auditory cortex) (Schober 1986)]. In each animal, 25-30 sections were quantified.

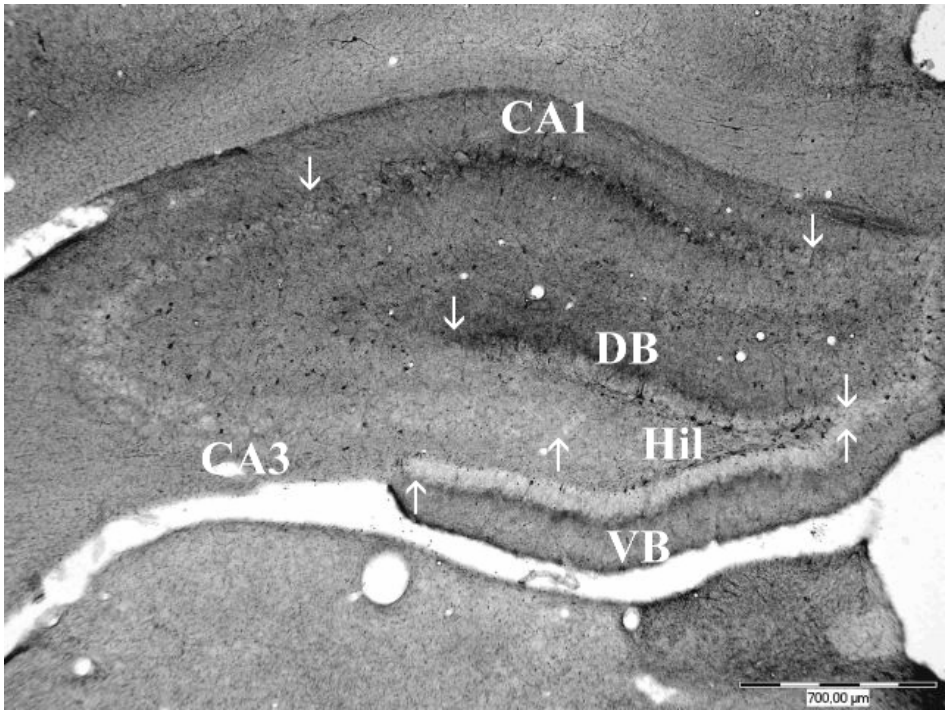

Fig. 1. Frontal section of hippocampus (AP 2.5) of experimental animal (NADPH-d staining). Bar = 700 µm. (CA1 = CA1 area of hippocampus, CA3 = CA3 area of hippocampus, Hil = hilus of the dentate gyrus, DB = dorsal blade of the dentate gyrus, VB = ventral blade of the dentate gyrus.)

The effect of KA was studied in two distinct hippocampal regions: in the dentate gyrus and cornu amonis (CA) subfields. The granule cells comprise the most prominent neuronal layer of the dentate gyrus. The polymorphic layer of the dentate gyrus (also referred to as the hilus) is another area frequently mentioned in reference to changes following KA insult. The CA subfields, or the hippocampus proper, consists of CA1 and CA3 regions (Lorente De Nó 1934).

The material was examined and NADPH-d positive neurons quantified under a light microscope Olympus Provis AX 70.

For the statistical evaluation, the t-test and ANOVA were used (level of significance $p<0.05$).

Results

22-day-old animals

In young animals, the repeated hypobaric hypoxia increased the number of NADPH-d positive neurons in the hilus (Fig. 2C), CA1 (Fig. 2A), CA3 (Fig. 2B) areas and in the primary auditory cortex (Fig. 2F) as compared to the control group. In the dorsal (Fig. 2D) and ventral (Fig. 2E) blades of the dentate gyrus the difference from the controls was not significant. KA given to the normoxic animals increased the number of NADPH-d positive neurons in the hilus (Fig. 2C), CA1 (Fig. 2A), CA3 (Fig. 2B) areas of the hippocampus and also in the primary auditory cortex (Fig. 2F), but no changes occurred in the dorsal (Fig. 2D) and ventral (Fig. 2E) blades of the dentate gyrus. On the contrary, KA given to the hypoxic animals lowered the number of NADPH-d positive neurons in the hilus (Fig. 2C), dorsal (Fig. 2D) and ventral (Fig. 2E) blades of the dentate gyrus, and increased the number of NADPH-d positive neurons in the primary auditory cortex (Fig. 2F). However, no changes were found in CA1 (Fig. 2A) and CA3 (Fig. 2B) areas of the hippocampus (Langmeier *et al.* 2002).

90-day-old animals

In the adult animals, the long-lasting intermittent perinatal hypoxia lowered the number of NADPH-d positive neurons in CA1 (Fig. 3A) and CA3 (Fig. 3B) areas of the hippocampus as compared to the control group. In the hilus (Fig. 3C), dorsal (Fig. 3D) and ventral (Fig. 3E) blades of the dentate gyrus and in the primary auditory cortex (Fig. 3F) the difference from controls was not significant. KA given to the normoxic animals also lowered the number of NADPH-d positive neurons in CA1 (Fig. 3A) and CA3 (Fig. 3B) areas and in the primary auditory cortex (Fig. 3F) and did not cause any

changes in the hilus (Fig. 3C) and dorsal blade (Fig. 3D) of the dentate gyrus. KA given to the hypoxic animals also lowered the number of NADPH-d positive neurons in CA1 (Fig. 3A) and CA3 (Fig. 3B) areas. The difference from controls was not significant in the hilus (Fig. 3C) and ventral blade (Fig. 3E) of the dentate gyrus, in the dorsal blade of the dentate gyrus (Fig. 3D) and in the primary auditory cortex (Fig. 3F) (Benešová *et al.* 2003).

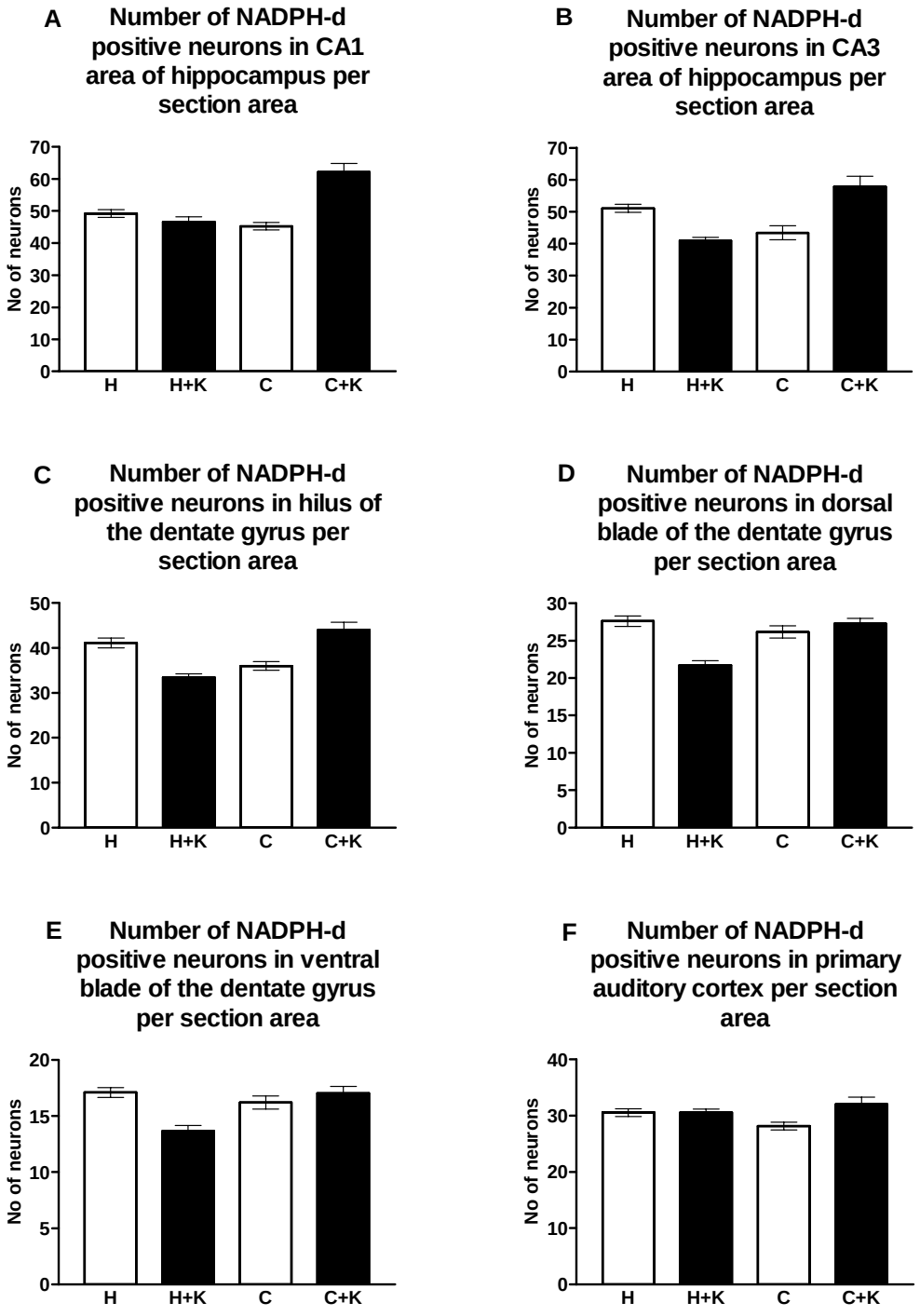

Fig. 2. 22-day-old animals. (**A**) Number of NADPH-d positive neurons in CA1 area of hippocampus per section area, (**B**) Number of NADPH-d positive neurons in CA3 area of hippocampus per section area, (**C**) Number of NADPH-d positive neurons in hilus of the dentate gyrus per section area, (**D**) Number of NADPH-d positive neurons in dorsal blade of the dentate gyrus per section area, (**E**) Number of NADPH-d positive neurons in ventral blade of the dentate gyrus per section area, (**F**) Number of NADPH-d positive neurons in primary auditory cortex per section area. H = hypoxic group, H + K = hypoxic and kainate group, C = control group, C + K = control and kainate group. Means ± S.E.M.

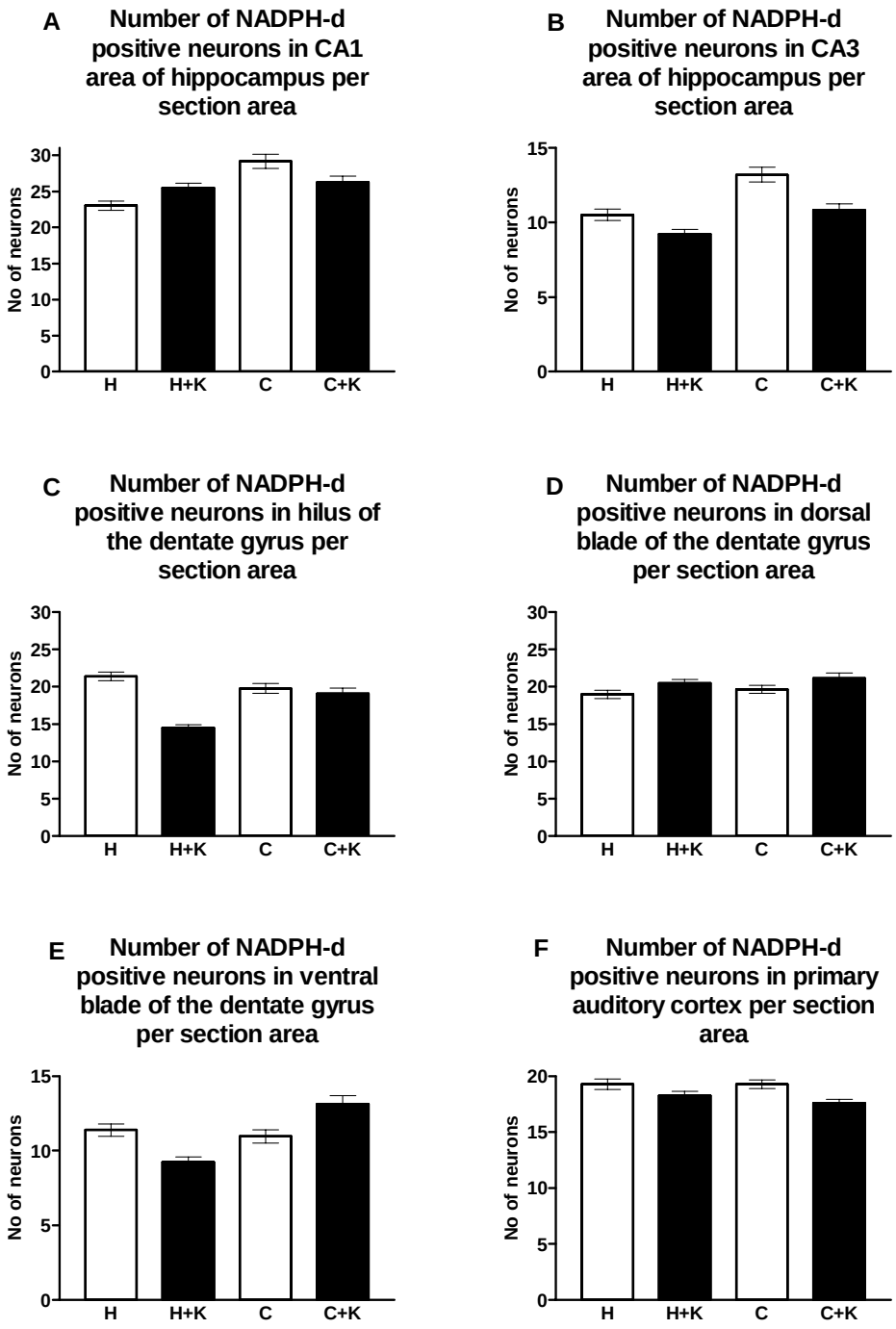

Fig. 3. 90-day-old animals. (**A**) Number of NADPH-d positive neurons in CA1 area of hippocampus per section area, (**B**) Number of NADPH-d positive neurons in CA3 area of hippocampus per section area, (**C**) Number of NADPH-d positive neurons in hilus of the dentate gyrus per section area, (**D**) Number of NADPH-d positive neurons in dorsal blade of the dentate gyrus per section area, (**E**) Number of NADPH-d positive neurons in ventral blade of the dentate gyrus per section area, (**F**) Number of NADPH-d positive neurons in primary auditory cortex per section area. H = hypoxic group, H + K = hypoxic and kainate group, C = control group, C + K = control and kainate group. Means ± S.E.M.

Discussion

It has been described that hypoxia, as well as other processes including rapid loss of high-energy phosphates, generalized depolarization, progressive proteolysis, and loss of membrane integrity, stimulates nitric oxide (NO) production because of its probable influence on nitric oxide synthase (NOS) gene expression. The post-ischemic reperfusion involves protein synthesis inhibition at the level of translation

initiation, NO synthesis and radical-mediated peroxidation of polyunsaturated fatty acids, causing damage to membrane lipids (White *et al.* 2000). This could explain the increased number of NADPH-d positive neurons in some areas of the hippocampus and in the primary auditory cortex of animals exposed to hypoxia.

Many studies have shown that nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) may correspond to the neuronal NOS, and it is therefore suggested that neurons containing NADPH-d might be capable of producing NO (Montecot *et al.* 1997). NADPH-d reactivity has been detected in various regions of the nervous system of the mammals including the rat. The coexistence of NADPH-d reactivity and neurotransmitter or neuropeptide reactivity has been demonstrated in certain populations of neurons (Wang *et al.* 2001). The method used to demonstrate nitrergic elements in the brain is based on the histochemical reaction for NADPH-d. The most important and attractive reason for the interest of neuroanatomists in this technique arose when NADPH-d was identified as a marker of the neuronal NOS. Thus, the relatively simple NADPH-d histochemical technique was widely used to identify NO producing elements in the brain of representatives of all vertebrate classes. With the development of antibodies against NOS, many studies have used immunohistochemistry to characterize the nitrergic systems in the central nervous system. It has been repeatedly proved that in the nervous system, NADPH-d activity and NOS immunoreactivity is widely colocalized in the same sets of neurons. The exact match between the staining obtained with both technical approaches has been discussed in connection with several regions such as the olfactory receptors and their projections, cells in the cerebral cortex and in specific neuronal populations of the spinal cord (Moreno *et al.* 2002).

KA possibly causes NO synthesis enhancement by its binding on KA receptors (subpopulation of non-NMDA receptors), which might have caused the increased number of NADPH-d positive neurons in the hilus, CA1 and CA3 areas of the hippocampus and in the primary auditory cortex, following the KA administration. The effect of KA was studied in two distinct hippocampal regions: in the dentate gyrus and cornu amonis (CA) subfields. The granule cells comprise the most prominent neuronal layer of the dentate gyrus. The polymorphic layer of the dentate gyrus (also referred to as the hilus) is another area frequently mentioned in reference to changes following KA insult. The CA subfields, or the hippocampus proper, consists of CA1 and CA3 regions. The principal cell type of these regions is the pyramidal cell (Lorente de Nó 1934). CA3 pyramidal neurons are among the most responsive neurons to kainate in the brain (Franck 1984, Ben-Ari and Cossart 2000), and kainate seems to be a potent excitant of these neurons. This was confirmed by our results.

Our results indicate that hypoxia also lowers the density of KA receptors that may indirectly contribute to low NO production after the KA application. This may lead to the reduced damage of some neuronal subpopulations caused by long-term intermitent hypoxia (Jones *et al.* 1998).

The long-term repeated hypoxia and perinatal i.p. administration of KA brought about reduced number of NADPH-d positive neurons in some examined regions of central nervous system of the adult male rats. As an excitotoxin for the mammalian central nervous system, KA induces neural cell death by apoptosis as well as by necrosis (Akhlaq *et al.* 2001). It is well established that prolonged seizure activity can lead to irreversible brain damage, by both necrotic and apoptotic types of cell death, which has been recently reported as a consequence of seizures (Langmeier *et al.* 2003). Intraperitoneal administration of KA results in the death of neurons in the CA1 and CA3 areas. On the contrary, the dentate gyrus is consistently spared from KA-caused neurodegeneration. This is also seen in our results. The cellular processes caused by KA administration include excessive neuronal firing resulting in seizures, neuronal cell loss, neuronal plasticity and glial reactivation (Zagulska-Szymczak *et al.* 2000).

Our results showing a reduced number of neurons could be explained by hypoxic or eventually toxic alterations of these regions, depending on different sensitivity of the discussed regions to such factors.

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