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Long-lasting Changes in the Density of Nitrergic Neurons Following Kainic Acid Administration and Chronic Hypoxia

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Summary

Using histochemical analysis (NADPH-diaphorase) we have investigated 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 hours a day). At the age of 1 year, 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 CA1 and CA3 areas of the hippocampus, in the dentate gyrus and in the PAC. Both, hypoxia and KA lowered the number of NADPH-diaphorase positive neurons in the hilus, dorsal and ventral blades of the dentate gyrus, CA1 and CA3 areas of the hippocampus. On the contrary, KA given to the hypoxic animals increased the number of NADPH-diaphorase positive neurons in the dorsal blade of the dentate gyrus and PAC.

Key words

Hypoxia • Kainic acid • Nitric oxide • Hippocampus • Primary auditory cortex

Introduction

During brain ischemia and reperfusion multiple independently-fatal terminal pathways are activated involving loss of membrane integrity, progressive proteolysis, and inability to control these processes. The changes started by 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. Three different forms of this enzyme are known, the endothelial (eNOS) 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. These neurons are considered to be interneurons. The production of NO is a calmodulin-dependent process, therefore, it must be preceded by 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).

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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.

Changes in NO production were found not only after the influence of hypoxia. Kainic acid (KA) is also responsible for the difference in the number of NADPH-d positive neurons (Montecot *et al.* 1997). Kainic acid, [2-carboxy-4 (1-methyl-ethenyl)-3-pirrolidinacetic acid] is a 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 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.* 1987, 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 of 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 it is the area of its primary action (Ben-Ari *et al.* 1981, Zagulska-Szymczak *et al.* 2000). The neural events in the named 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.

In the model of perinatal hypoxia, animals were exposed to long-lasting repeated hypobaric hypoxia from the 2nd till the 17th day of age. It has been shown that in 22-day-old animals hypoxia and kainate given to normoxic animals resulted in an increase of the number of NADPH-d positive neurons in the hilus, CA1, CA3 areas of the hippocampus and in the primary auditory cortex. Kainate in combination with hypoxia lowered the number of NADPH-d positive neurons in the hilus, dorsal and ventral blades of the dentate gyrus (Langmeier *et al.* 2002, Benešová *et al.* 2004). In 3-month-old adult rats both, hypoxia and kainate given to normoxic, as well as hypoxic animals, lowered the number of NADPH-d positive neurons in some areas of the central nervous system, preferentially in CA1 and CA3 areas of the hippocampus (Benešová *et al.* 2003, 2004).

The problem addressed in this paper concerned the question whether and how can the changes in the number of NADPH-d positive neurons started by long-lasting repeated perinatal hypoxia and KA influence individual brain structures of old rats. Our intention was to asses the life-long dynamics of this effect. We were interested in the fact whether the changes of the density of nitrergic neurons caused by perinatal hypoxia and kainate tend to persist, become more profound or if we can expect any reparation during a longer period of time. We have therefore chosen 1-year-old rats as a representative group of old animals.

Methods

Male rats of the Wistar strain of our own breed were used for the experiments. There were 40 animals in all the experimental groups, with 10 in each group. We histochemically evaluated 8 brains in all the groups, 2 in each. In each brain 25-30 sections were quantified. Young animals from the 2nd till the 17th day of age 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 hours a day. On the 18th day of age, i.e. one day after the final day of the exposition to hypoxia, 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.* 1987).

Animal groups used in the experiment:

1) animals exposed to hypoxia from the 2nd till the 17th day of age,
2) animals exposed to hypoxia from the 2nd till the 17th day of age, followed by KA i.p. injection on the 18th day of age,
3) animals not exposed to hypoxia, and
4) animals not exposed to hypoxia, followed by KA i.p. injection on the 18th day of age.

The animals were kept in standard breeding

conditions, during feeding there were 10 rat pups together with one mother. On average, 60 % of the rats survived for one year. In the control group the survival was 90 %, in the hypoxic group 50 %, in the kainate group 60 % and in the kainate and hypoxic group 40 %. The animals died due to hypoxic or toxic alterations. In the kainate group, behavioral changes like automatisms were observed. Animals aged 1 year were perfused under deep thiopental anesthesia 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. Each section was taken and evaluated. 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 and coverslipped with microscope cover glasses (Menzel-Gläser) using D.P.X. neutral mounting medium (Aldrich) (Wang *et al.* 2001). NADPH-d positive neurons (Fig. 1) were then quantified in five regions of the hippocampal formation and in the primary auditory cortex:

1) in CA1 area of the hippocampus,
2) in CA3 area of the hippocampus,
3) in the hilus of the dentate gyrus,
4) in the dorsal blade of the dentate gyrus,
5) in the ventral blade of the dentate gyrus, and
6) in the primary auditory cortex [Te 1 - temporal area 1 (primary auditory cortex) (Schober 1986).

Hippocampus and neocortex between the AP plane 2.5 mm and 4.0 mm posterior to the bregma were subjected to quantification of NADPH-d positive neurons under a light microscope Olympus Provis AX 70.

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

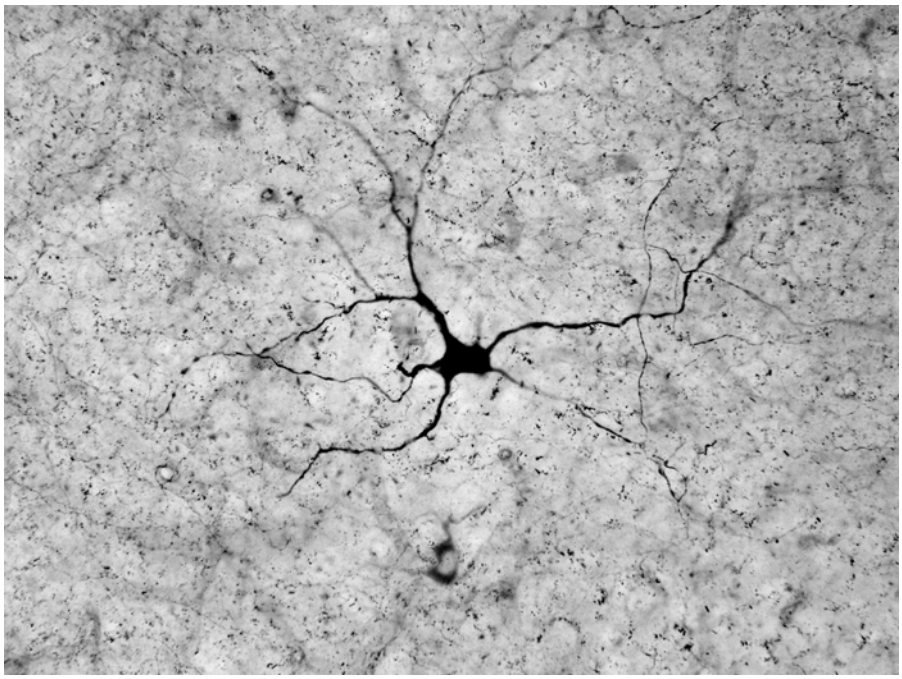

Fig. 1. NADPH-d positive interneuron in the second cortical layer in the primary auditory cortex. NADPH-d staining. Direct magnification 400x.

Results

The results show that the repeated hypobaric perinatal hypoxia lowered the number of NADPH-d positive neurons (Fig. 1) in all the investigated areas of the hippocampus, compared to the control group which we consider as baseline (Fig. 2A-2E). In the primary auditory cortex, on the contrary, the number of NADPH-d

positive neurons was increased (Fig. 2F). KA given to the normoxic animals also lowered the number of NADPH-d positive neurons in CA1 (Fig. 2A) and CA3 (Fig. 2B) areas of the hippocampus, in the hilus (Fig. 2C), in the dorsal (Fig. 2D) and ventral (Fig. 2E) blades of the dentate gyrus, but did not cause any changes in the primary auditory cortex (Fig. 2F). KA given to the hypoxic animals increased the number of NADPH-d positive neurons in the dorsal blade (Fig. 2D) of the dentate gyrus and in the primary auditory cortex (Fig. 2F). Furthermore, it did not change in CA1 (Fig. 2A), CA3 (Fig. 2B) areas of the hippocampus, in the hilus (Fig. 2C) and ventral blade (Fig. 2E) of the dentate gyrus.

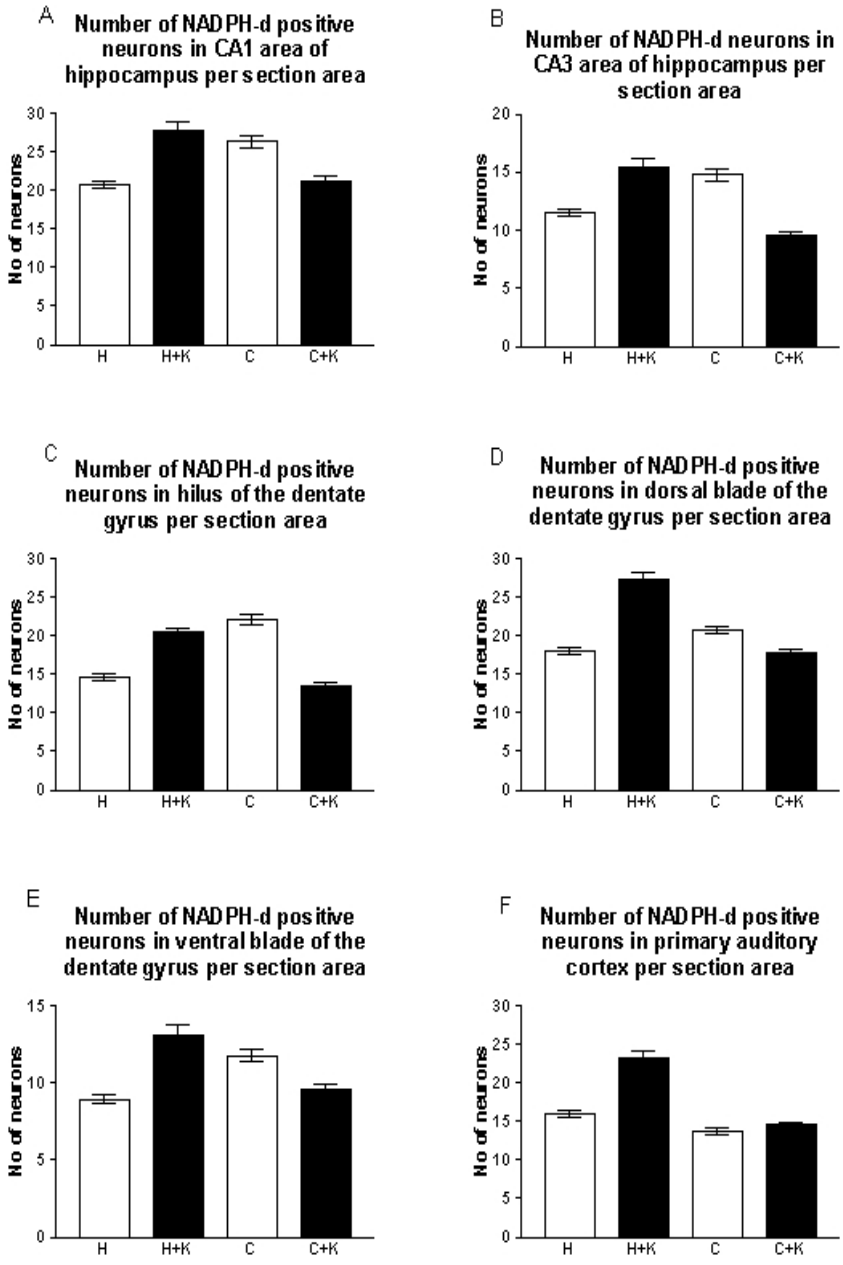

Fig. 2. (**A**) Number of NADPH-d positive neurons in CA1 area of hippocampus per section area, (**B**) Number of NADPH-d positive neurons in CA3 area of hippocampus per section area, (**C**) Number of NADPH-d positive neurons in hilus of the dentate gyrus per section area, (**D**) Number of NADPH-d 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. Mean ± S.E.M.

Discussion

Our findings show that long-lasting intermittent perinatal hypoxia lowered the number of NADPH-d positive neurons in all examined areas of the hippocampus in adult animals, which had been influenced by hypoxia during their perinatal life. It seems to be the same effect as we observed in CA1 and CA3 areas of the hippocampus in 90-day-old rats (Benešová *et al.* 2003). 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 (White *et al.* 2000). It is evident that translation in the post-ischemic brain is not regionally homogenous; the cortex, hippocampal CA1 and hilus, and caudate show severe and prolonged suppression of protein synthesis, whereas the hippocampal dentate gyrus and brainstem structures are less affected. In several of these studies the CA1 zone, which is the most susceptible to neuronal death following ischemia, never recovered protein synthesis, indicating that a prolonged deficit in post-ischemic protein synthesis correlates with selective vulnerability (White *et al.* 2000).

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 are likely to be capable of producing NO (Montecot *et al.* 1997). 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 (Wang *et al.* 2001). The method used to demonstrate nitrergic elements in the brain is based on 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 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 NADPH-d activity and NOS immunoreactivity in the nervous system 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 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).

Perinatal i.p. KA administration brought about reduced number of NADPH-d positive neurons in all examined regions of the hippocampus in adult rats which was also observed in CA1, CA3 areas of the hippocampus and in the primary auditory cortex in 90-day-old rats. The effect of KA had been 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). This was confirmed by our results. 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 evident from our results. The number of NADPH-d positive neurons in dorsal and ventral blades of the dentate gyrus was decreased after perinatal KA administration but these changes were not so evident as in CA1 and CA3 regions of the hippocampus. 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 possibly toxic alterations of these regions, depending on different sensitivity of the discussed regions to these possible factors. NADPH-d positive neurons are probably interneurons. The reason of their reduced number can also be a result of changes in gene expression. The color intensity of the neurons is also not the same in all studied regions, since it seems to be higher in the CA3 area than in CA1 region. This could be explained by a higher density of interneurons in this area.

On the contrary, the long-term repeated hypoxia and perinatal i.p. administration of KA brought about an increased number of NADPH-d positive neurons in some examined regions of the central nervous system, i.e. in

dorsal blade of the dentate gyrus and in the primary auditory cortex of adult male rats. The different response of the dentate gyrus can be supported by results of other authors. Domoic acid, which is structurally related to kainic acid, also causes neuronal excitation. Lesions in the hippocampus, caused by this agent were limited to the neurons in the CA1 and CA3 subfields of the hippocampus and the hilus of the dentate gyrus whereas granular neurons of the dentate gyrus were spared (Ananth *et al.* 2003). The different response of primary auditory cortex could be explained by lack of kainate receptors in this area. Our findings also suggest that hypoxia might induce higher expression of nNOS, which could contribute to the neuronal integration as responding to the different physiological demands under hypoxic stress. Factors such as hypoxia that enhance the releasing of glutamate from presynaptic afferent terminals would likely activate NO production *via* the NMDA-mediated mechanisms. In fact, NOS gene expression activated by hypoxia in central and peripheral neurons had recently been observed (Chang *et al.* 2003).

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