

Morphological Changes in the Hippocampus Following Nicotine and Kainic Acid Administration

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Summary

Using histochemical analysis (NADPH-diaphorase, Fluoro-Jade B dye and bis-benzimide 33342 Hoechst) we studied the influence of intraperitoneal administration of nicotine (NIC), kainic acid (KA) and combination of both these substances on hippocampal neurons and their changes. In experiments, 35-day-old male rats of the Wistar strain were used. Animals were pretreated with 1 mg/kg of nicotine 30 min prior to the kainic acid application (10 mg/kg). After two days, the animals were transcardially perfused with 4 % paraformaldehyde under deep thiopental anesthesia. Cryostat sections were stained to identify NADPH-diaphorase positive neurons that were then quantified in the CA1 and CA3 areas of the hippocampus, in the dorsal and ventral blades of the dentate gyrus and in the hilus of the dentate gyrus. Fluoro-Jade B positive cells were examined in the same areas in order to elucidate a possible neurodegeneration. In animals exposed only to nicotine the number of NADPH-diaphorase positive neurons in the CA3 area of the hippocampus and in the hilus of the dentate gyrus was higher than in controls. In contrast, KA administration lowered the number of NADPH-diaphorase positive cells in all studied hippocampal areas and in both blades of the dentate gyrus. Massive cell degeneration was observed in CA1 and CA3 areas of the hippocampus and in the hilus of the dentate gyrus after kainic acid administration. Animals exposed to kainic acid and pretreated with nicotine exhibited degeneration to a lesser extent and the number of NADPH-diaphorase positive cells was higher compared to rats, which were exposed to kainic acid only.

Key words

Kainic acid • Nitric oxide • Nicotine • Hippocampus • Neurodegeneration

Introduction

Nicotine, the main alkaloid found in tobacco, and its analogues exert a number of neurovegetative, behavioral and psychological effects on the central nervous system (CNS), which depend on the activation of specific cholinergic receptors (Benowitz 1986).

Cholinergic receptors can be divided into two types, muscarinic and nicotinic, based on the pharmacological action of various agonists and antagonists (James and Norberg 1995).

Nicotine has been reported to have a positive effect in patients with some neurodegenerative diseases (such as Alzheimer's and Parkinson's diseases) and

nicotine has been suggested for the treatment of these CNS disorders (Ryan *et al.* 2001, James and Norberg 1995). Progression of these diseases is related to oxidative stress, resulting from either a decrease in antioxidant levels or an increase in reactive oxygen species or reactive nitrogen species. The role of nicotine in neurodegenerative disorders is still incompletely understood, because there is evidence that nicotine has both antioxidant and prooxidant effects (Newman *et al.* 2002).

Kainic acid (KA), the analogue of excitatory amino acid L-glutamate, interacts with specific receptors in the central nervous system. Systemic injection of KA causes severe convulsions, seizure-induced brain damage and increased seizure susceptibility in the rat (Ben-Ari 1985, Sperk 1994). These sequel closely resemble those of human temporal lobe epilepsy. Therefore, KA-induced epilepsy has become a widely accepted animal model for this and some other neurological disorders (Ben-Ari 1985, Nadler 1980).

Although many brain areas are affected by KA administration, the hippocampus seems to be one of the most influenced areas (Ben Ari *et al.* 1979a,b, Gottesfeld and Jacobowitz 1979, Kohler *et al.* 1979, Nadler *et al.* 1980, Sperk *et al.* 1983). The neurodegeneration of cells in the CA3 area of the hippocampus brings about epileptiform activity of the CA1 pyramidal cells, which is characterized by enhanced NMDA-mediated excitatory phase with apparent loss of GABA-mediated postsynaptic inhibition (Turner and Wheal 1991). Systemic kainic acid administration leads to neuronal degeneration, characterized among others by cell loss, when kainic acid is administered once or repeatedly (Riljak *et al.* 2005). The cell loss is not specific for kainic acid administration only but also for other neurotoxic insults (e.g. alcohol) (Milotová *et al.* 2005).

Nitric oxide (NO) is produced from L-arginine by nitric oxide synthase (NOS). There are at least three different forms of that enzyme, the endothelial (eNOS) which is responsible for cardiovascular actions, the inducible one (iNOS) found originally in macrophages and involved mainly in immunological processes and a neuronal form (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. 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 receptor (Prast and Philippu 2001).

Neuronal nitric oxide plays an important role in brain function. Studies conducted in the nineties suggested that NO mediates changes in cerebral blood flow not only under certain physiological (Iadecola 1993) but also under pathological conditions, such as KA-induced seizures (Rigaud-Monnet *et al.* 1994, 1995). There are only few studies which examined the possible modulation of KA-induced syndrome by nicotine. They indicate that nicotine pretreatment attenuates KA-induced neurodegeneration and that this alkaloid lowers the behavioral changes, which typically accompany KA application (Semba *et al.* 1996, Shytle *et al.* 1995). For example, Borlongan *et al.* (1995) reported that nicotine pretreatment blocks KA-induced wet dog shakes (WDS). The animals receiving nicotine before KA application displayed only mild limbic convulsions with sporadic incidents of head and jaw clonus. In contrast, animals receiving saline before KA administration displayed prominent convulsions accompanied by excessive salivation, severe convulsions and hyperkinesia in addition to consistent WDS (Borlongan *et al.* 1995).

Three main problems are concerned in this paper. First, whether and how can changes in the number of NADPH-diaphorase positive neurons induced by nicotine application and KA application influence brain structure of young rats. Second, if nicotine pre-treatment can modulate expression of KA-induced structural changes. Third, if nicotine has some degenerative potential in the CNS.

Methods

Male 35-day-old Wistar rats from our own breeding colony were used for the experiments. They were housed at a constant temperature (23 °C) and relative humidity (60 %) with a fixed 12 h light/dark cycle and fed with food and water *ad libitum*. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies. There were 24 animals in all the experimental groups, six animals in each group:

- 1) 35-day-old animals exposed to nicotine (1 mg/kg) i.p.
- 2) 35-day-old animals exposed to kainic acid (10 mg/kg) i.p.

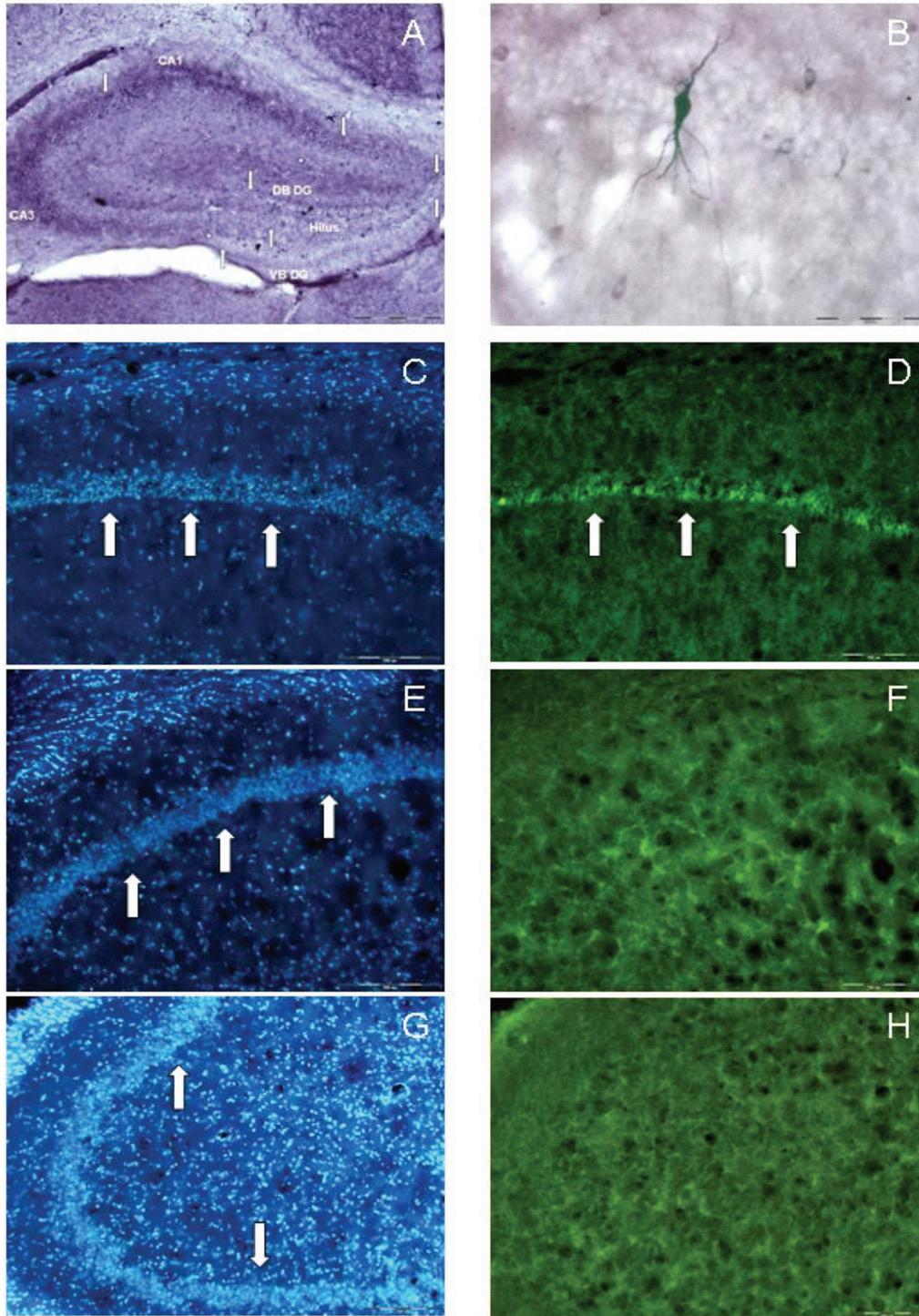


Fig. 1. Histological analyses of the hippocampus. **A.** Hippocampus (the arrows show the borders of investigated areas): CA1 and CA3 areas of the hippocampus, hilus of the dentate gyrus, DB DG - dorsal blade of the dentate gyrus, VB DG - ventral blades of the dentate gyrus. NADPH-diaphorase staining. Direct magnification 40x. **B.** NADPH-diaphorase positive cell in CA1 area of the hippocampus. Direct magnification 200x. **C.** CA1 area of the hippocampus (arrows). Bis-benzimide, Hoechst 33342 staining. Kainic acid-treated rat (10 mg/kg). Direct magnification 100x. **D.** CA1 area of the hippocampus (arrows show degenerating cells). Fluoro-Jade B staining. Kainic acid-treated rat (10 mg/kg). Direct magnification 100x. **E.** CA1 area of the hippocampus (arrows). Bis-benzimide, Hoechst 33342 staining. Nicotine treated rat (1 mg/kg) 30 min prior kainic acid administration (10 mg/kg). Direct magnification 100x. **F.** CA1 area of the hippocampus. Fluoro-Jade B staining. Nicotine-treated rat (1 mg/kg) 30 min prior kainic acid administration (10 mg/kg). Direct magnification 100x. **G.** CA1 and CA3 areas of the hippocampus (arrows). Bis-benzimide, Hoechst 33342 staining. Nicotine-treated rat (1 mg/kg) 30 min prior kainic acid administration (10 mg/kg). Direct magnification 100x. **H.** CA1 and CA3 areas of the hippocampus. Fluoro-Jade B staining. Nicotine-treated rat (1 mg/kg) 30 min prior kainic acid administration (10 mg/kg). Direct magnification 100x.

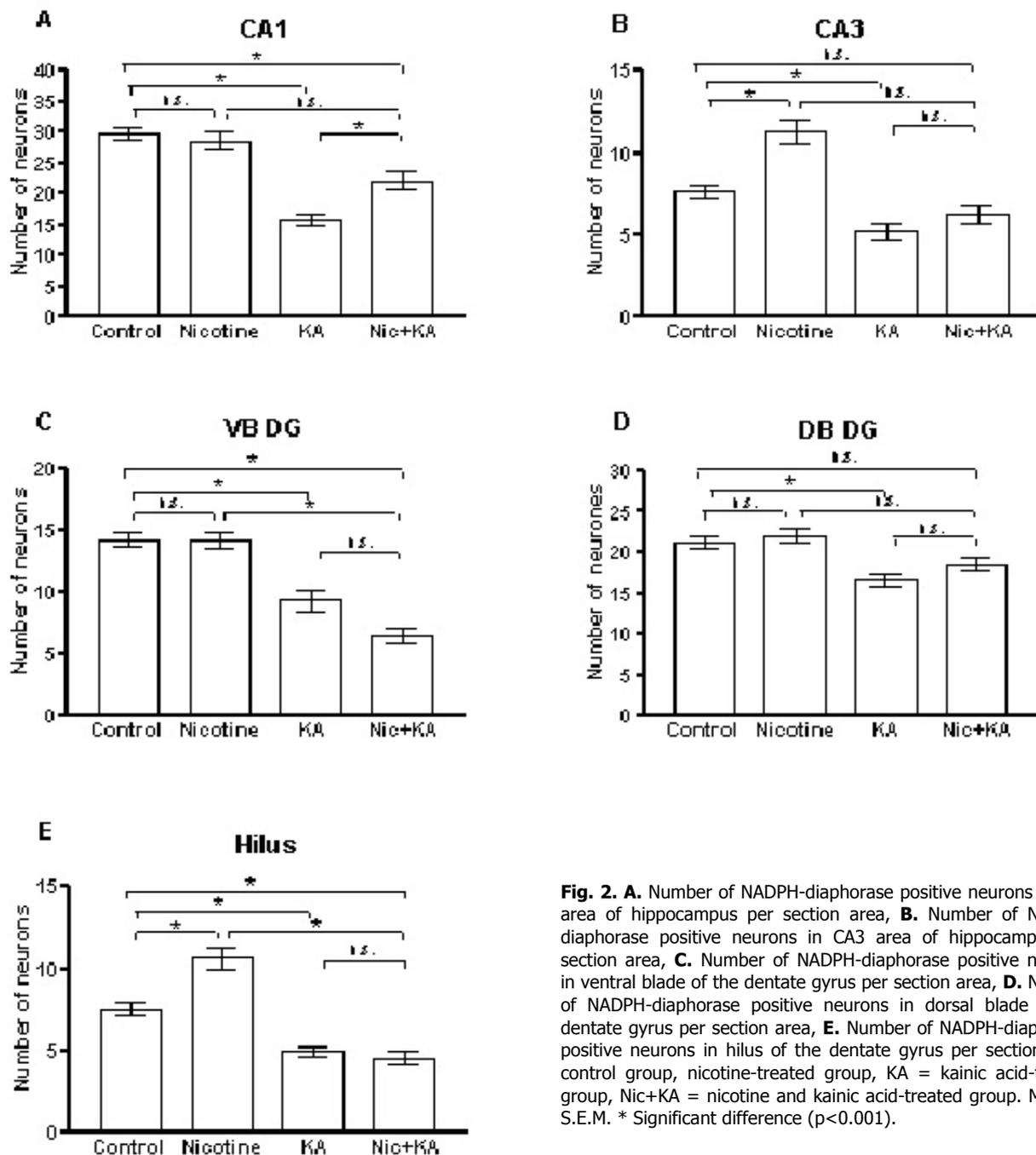


Fig. 2. **A.** Number of NADPH-diaphorase positive neurons in CA1 area of hippocampus per section area, **B.** Number of NADPH-diaphorase positive neurons in CA3 area of hippocampus per section area, **C.** Number of NADPH-diaphorase positive neurons in ventral blade of the dentate gyrus per section area, **D.** Number of NADPH-diaphorase positive neurons in dorsal blade of the dentate gyrus per section area, **E.** Number of NADPH-diaphorase positive neurons in hilus of the dentate gyrus per section area; control group, nicotine-treated group, KA = kainic acid-treated group, Nic+KA = nicotine and kainic acid-treated group. Mean \pm S.E.M. * Significant difference ($p < 0.001$).

- 3) 35-day-old animals exposed to nicotine (1 mg/kg), followed in 30 min by KA i.p. injection (10 mg/kg)
- 4) control 35-day-old animals exposed to normal saline in equal doses

Four brains of animals in each group were stained by NADPH-diaphorase method and 2 brains in each group were stained by combination of Fluoro-Jade B and Hoechst staining. In each brain 25-30 sections were examined and quantified. At the age of 35 days, animals received single intraperitoneal injection of nicotine, kainic acid or both substances. Animals of the control

group received normal saline in equal volumes. Two days later, all animals were perfused under deep thiopental anaesthesia with 4 % paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. Brains were removed, postfixed for one hour in 4 % buffered paraformaldehyde and then submerged for 1 h into 20 % sucrose for cryoprotection. Each brain was sliced in the frontal plane into 40 μ m thick sections with a cryostat. Then two different histochemical methods were used:

NADPH-diphorase staining

For the NADPH-diphorase examination 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), 0.2 mg/ml Nitro blue tetrazolium (NBT, Sigma) and 0.3 % Triton (Sigma) for 4 h at 37 °C in thermostat. Following the reaction, sections were rinsed in 0.1 M phosphate buffer and kept at 8 °C for 16 h. The histochemically reacted sections were mounted on precleaned 0.5 % gelatine-coated microscope slides (Menzel-Gläser), air-dried and cover slipped with microscope cover glasses (Menzel-Gläser) using D.P.X. neutral mounting medium (Aldrich) (Wang *et al.* 2001). NADPH-diphorase positive neurons were then quantified in five regions of the hippocampal formation (each section was evaluated) (Fig. 1):

- 1) CA1 area of the hippocampus,
- 2) CA3 area of the hippocampus,
- 3) the hilus of the dentate gyrus,
- 4) the dorsal blade of the dentate gyrus,
- 5) the ventral blade of the dentate gyrus

Hippocampus between the AP plane 2.5 mm and 4.0 mm posterior to the bregma was subjected to quantification of NADPH-diphorase positive neurons under the light microscope Olympus Provis AX 70 with epifluorescence illumination. For the statistical evaluation, ANOVA and the unpaired t-test (GraphPadPrism) were used (level of significance was set at $p < 0.001$).

Combination of Fluoro-Jade B and bis-benzimide 33342 Hoechst

Fluoro-Jade B (FJB, Histo-Chem Inc.) is an anionic fluorescein derivative useful for the histological staining of neurons undergoing degeneration (Schmued and Hopkins 2000). Hoechst 33342 (Sigma) staining was used as an apoptotic marker, which detects apoptotic nuclei with condensed and/or fragmented DNA.

After cryostat sectioning, free-floating slices were placed in 0.1 M phosphate buffer. Tissue sections were then mounted onto gelatinized slides and allowed to dry at room temperature. Slides were then placed in staining racks (one slide/slot for even staining) and immersed in 100 % ethanol solution for 3 min, in 70 % ethanol solution for 1 min, in distilled water for 1 min, in 0.01 % potassium permanganate (KMnO_4) (Sigma) for 15 min with gentle shaking. Slides were washed in distilled water three times. Staining proceeded in a dim place by immersing slides into 0.001 % Fluoro-Jade B

solution for 30 min with occasional gentle shaking (Schmued and Hopkins 2000). Thereafter the slides were rinsed in the distilled water three times for 1 min. Slides were then immersed in 0.01 % Hoechst staining solution for 10 min and dehydrated (in ethanol series), cover-slipped using D.P.X. neutral mounting medium and allowed to dry. Fluoro-Jade B positive neurons were studied in the same hippocampal regions as those used for NADPH-diphorase evaluation (each section was evaluated). The tissue was examined using an epifluorescent microscope with blue (450-490 nm) excitation light. For the semiquantitative analysis, Fluoro-Jade B positive cells were counted by two independent workers in all slices. Each area of the hippocampus in the slice was evaluated by the scale: (0) no FJB positive cells in field of view, (+) sporadically present FJB cells in field of view, (++) groups of FJB positive cells in field of view (+++) massive alteration, the study area is completely undergoing degeneration.

Results

The results clearly show that the nicotine administration increased the number of NADPH-diphorase positive neurons in the hilus of the dentate gyrus and CA3 area of the hippocampus, compared to the control group (Fig. 2B, E). In other areas the difference from controls was not significant (Fig. 2A, C, D). KA given to the animals decreased the number of NADPH-diphorase positive neurons in all examined areas of the hippocampus (Fig. 2). The combination of both of these substances decreased the number of NADPH-diphorase positive neurons in the CA1 area of the hippocampus, compared to the control group, but the number of NADPH-diphorase positive cells was significantly higher when compared with only KA-exposed rats (Fig. 2A). Fluoro-Jade B staining revealed the massive neurodegeneration after KA application (Fig. 1C, D). The most affected regions were CA3 and CA1 areas of the hippocampus and hilus of the dentate gyrus. Both blades of the dentate gyrus remained intact. This confirmed the previous finding of other authors (French *et al.* 1982, de Montigny and Tardif 1981). Semiquantitative analysis showed that nicotine pretreatment before KA administration lowered the number of Fluoro-Jade B positive cells compared to rats, which were pretreated by KA only (Fig. 1E, F, G, H). This finding was especially prominent in CA1 and CA3 area of the hippocampus and in the hilus of the dentate gyrus (Table 1). Nicotine

Table 1. Semiquantitative analyze of Fluoro-Jade B positive cells in experimental groups: control rats received equal dose of normal saline, kainic acid-treated rats (10 mg/kg), nicotine-treated rats (1 mg/kg), and rats pretreated with nicotine (1 mg/kg) 30 min prior to kainic acid administration (10 mg/kg).

Area	Control n = 108	Kainic acid n = 109	Nicotine n = 112	Nicotine + kainic acid n = 112
CA1	29.80 ± 0.97	15.73 ± 0.90	28.59 ± 1.43	22.12 ± 1.40
CA3	7.62 ± 0.36	5.20 ± 0.46	11.26 ± 0.74	6.22 ± 0.53
Hilus	7.50 ± 0.37	4.97 ± 0.31	10.62 ± 0.63	4.556 ± 0.41
Ventral blade of dentate gyrus	14.27 ± 0.56	9.36 ± 0.88	14.27 ± 0.67	6.50 ± 0.67
Dorsal blade of dentate gyrus	21.33 ± 0.74	16.58 ± 0.68	22.03 ± 0.90	18.56 ± 0.83

Data are means ± S.E.M. Each area of the hippocampus in the slice was evaluated by the scale: (0) no FJB positive cells in field of view, (+) sporadically present FJB cells in field of view, (++) groups of FJB positive cells in field of view (+++) massive alteration, the study area is completely undergoing degeneration.

administration produced no degeneration in any area of the hippocampus, albeit this dose (1 mg/kg) was rather high. Morphological analysis, using Hoechst 33342, confirmed that KA administration caused neuronal morphological changes (condensed nuclei, apoptotic bodies), reflecting apoptosis in the pyramidal layer of the hippocampal formation. No morphological signs of apoptosis (evaluated by Hoechst 33342) were found in animals receiving nicotine before KA administration.

Discussion

Systemic administration of KA in rats readily produces various motor signs including convulsive seizures (Ben-Ari 1985) and massive neuronal damage can be observed in the hippocampal formation (Sperk 1994, Riljak *et al.* 2005). We have confirmed that intraperitoneal administration of KA brought about death of neurons in the CA1 and CA3 areas of the hippocampus and in the hilus of the dentate gyrus. Mechanisms contributing to KA-induced seizures and brain damage are still not completely clear. The main causes are so-called axon sparing lesions, lesions mediated through activation of excitatory pathways, non-selective seizure-related brain damage, changes in the blood-brain barrier and possibly some other (Sperk 1994). One cause of the high sensitivity of CA1 and CA3 areas of the hippocampus can be the high concentration of KA receptors upon the neurons of this structure (Sawada *et al.* 1988, Yoshihara *et al.* 2003).

When the character of neuronal injury is concerned, two major forms of morphologically distinct

cell death have been observed under various neuropathologic conditions necrosis and apoptosis (Johnson and Deckwerth 1993, Nicotera and Lipton 1999). It is often difficult to decide whether the cell died by the necrotic or apoptotic process (Columbano 1995). The mechanism of neuronal death in the hippocampus, demonstrated in our experiments, is most likely apoptotic. But the necrotic process in the hippocampus cannot be excluded without any other histological method.

When the rats were pretreated by nicotine, neuronal degeneration was attenuated. This observation was confirmed by Fluoro-Jade B staining. Hoechst 33342 staining allowed to identify the cells with condensed, fragmented nuclei, which both have been proposed to be signs of apoptosis. To decide if the neurons are dying by apoptotic process must be confirmed by other methods.

It has been proposed that nicotine in the CNS has antioxidant properties. This aspect was extensively reviewed (Newman *et al.* 2002). Nicotine might also act as an antioxidant and/or it can inhibit complex I of the electron transport chain, with a consequent reduction in the levels of reactive oxygen species (Cormier *et al.* 2003, Newman *et al.* 2002, Obata *et al.* 2002, Soto-Otero *et al.* 2002). Increased nicotine-induced expression of neurotrophic factors crucial for neuronal maintenance, survival and regeneration has also been discussed (Matarredona *et al.* 2001, Roceri *et al.* 2001).

In our experiments, kainic acid administration decreased the number of NADPH-diaphorase positive neurons. It can be the result of kainic acid-induced neurodegeneration in the hippocampal area, or it is a

consequence of kainic acid application that decreases the number of all neurons and the NADPH-diaphorase positive neurons are not excluded. It has been proposed that some other insults (for example perinatal long-lasting hypobaric hypoxia) result in a decreased number of NADPH-diaphorase positive neurons (Benešová *et al.* 2005, Marešová *et al.* 2005, Jandová *et al.* 2006). Considering, that the dose of kainic acid used in our experiment reached the convulsive limit, the cell loss was not surprising. Some authors, who studied the effects of subconvulsive doses of the kainic acid, did not find any structural changes in the hippocampus (Koryntová *et al.* 1997). Our prediction must be confirmed in future studies, which will quantify the whole neuronal population.

In this and our previous experiments the nicotine administration was associated with higher numbers of NADPH-diaphorase positive neurons in CA3 area of the hippocampus and hilus of the dentate gyrus in comparison with either group of control animals (Riljak *et al.* 2006). NADPH-diaphorase positive neurons were repeatedly reported to be relatively resistant to the injury

caused by status epilepticus (Koh *et al.* 1986, Lerner-Natoli *et al.* 1994), but the findings related to vulnerability or survival of this neuronal population after status epilepticus are still controversial. If such speculations are true, increased number of the NADPH-diaphorase positive cells following nicotine application may act as a protective factor against excitotoxin-induced degeneration. On the other hand, it has been demonstrated that NO (free radical synthesized by NO-synthase which is colocalized with NADPH-diaphorase) and superoxide radicals combine to produce peroxynitrite that spontaneously decomposes forming hydroxyl radicals and nitrogen dioxide, which can produce cell damage (Ischiropoulos *et al.* 1992). The role of NO in the CNS is still not fully understood and many questions remain to be elucidated.

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