Changes in the Number of Nitrergic Neurons in Rats Hippocampus Following Nicotine Administration

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Abstract: Nicotine is a very widely used drug of abuse, which exerts a number of neurovegetative behavioural effects by interacting with the neuronal nicotinic acetylcholine receptor. Using histochemical analysis (NADPH-diaphorase and Fluoro-Jade B dye), the influence of intraperitoneal administration of nicotine on neurons of the hippocampus in 35-day-old male rats of the Wistar strain was studied. At the age of 37 days, the animals were transcardially perfused with 4% paraformaldehyde under deep thiopental anaesthesia. Cryostat sections were stained to identify NADPH-diaphorase positive neurons that were then quantified in CA1 and CA3 areas of the hippocampus, in the dorsal and ventral blade of the dentate gyrus and in the hilus of the dentate gyrus. In the same areas, using Fluoro-Jade B dye, signs of neurodegeneration were classified, using Fluoro-Jade B dye. Nicotine administration increased the number of NADPH-diaphorase positive neurons in the CA3 area of the hippocampus and in the hilus of the dentate gyrus with no effect in the remaining areas studied. Fluoro-Jade staining did not reveal any degenerating neurons in the hippocampus as an effect of nicotine administration.

Introduction
Nicotine is a very widely abused drug, which exerts a number of neurovegetative, behavioural effects by interacting with the neuronal nicotinic acetylcholine receptor. Experiments conducted in recent years point to an interesting fact: that (-)-nicotine has or could have positive effects on a variety of diseases, mainly on central nervous system disorders (currently especially Parkinson’s disease and Alzheimer’s disease) [1, 2]. Animal experimental models further show that nicotine could be used for treatment (even if only symptomatic) of a variety of other central nervous system (CNS) disorders, for example Huntington chorea, Parkinson’s disease, the consequences or prevention of hypoxia, schizophrenia, etc. [3, 4, 5, 6, 7, 8, 9, 10].

Nitric oxide (NO) is produced from L-arginine by NOS. Studies conducted in 90’s suggest that NO mediates changes in cerebral blood flow under certain physiological [11] and pathological conditions, such as kainic acid induced seizures [12] (kainic acid is one of the most common substance, which is widely used as a model of human temporal epilepsy with complex symptomatology). NO also acts as an interneuronal messenger which interferes with glutamate transmission [13] and is involved in glutamate neurotoxicity [14].

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+}. 

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concentration [15]. $\text{Ca}^{2+}$ influx is induced by activation of glutamate receptors, preferentially NMDA receptors [16].

Fluoro-Jade B, fluorochrome, could be used for detecting neuronal degeneration [17]. The hippocampus (particularly CA3 and CA1 areas) is especially sensitive to the neurotoxic effect of the many substances. The neural events in this brain structure have been studied intensively [18] due to its comparatively simple anatomy, its involvement in a variety of neurodegenerative conditions as well as due to its probable role in memory formation. The problem addressed in the present paper concerned the question whether and how can intraperitoneal application of nicotine influence individual brain structures of young rats.

**Material and methods**

Male rats of the Wistar strain of our own breed were used for the experiments. Total eight animals were used, four in each group. Two brains in each group were evaluated histochemically for NADPH-diaphorase staining and 2 brains were evaluated for combination of the staining Fluoro-Jade B and Hoechst in each group. In each brain 25–30 sections were examined or quantified. On the 35th day of age animals were given a single intraperitoneal injection of nicotine (1 mg/kg). Animals of the control group received normal saline in equal doses. When aged 37 days, 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, submerged for 1 hour into 20% sucrose for cryoprotection, and sliced in the frontal plane into 40 $\mu$m thick sections with a cryostat. To identify effects of experimental treatment, two different histochemical methods were used:

1) **NADPH-diaphorase staining**
2) Combination of the Fluoro-Jade B and bis-benzimide (Hoechst 33342)

Ad 1. For the NADPH-diaphorase 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 $\beta$-NADPH reductase (Sigma), 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 precleaned 0.5% gelatine-coated microscope slides (Menzel-Gläser), air-dried and coverslipped with microscope cover glasses (Menzel-Gläser) using D.P.X. neutral mounting medium (Aldrich) [8]. NADPH-d positive neurons (Figure 1) were then quantified in five regions of the hippocampal formation [19]:

1) In CA1 area of the hippocampus,
2) In CA3 area of the hippocampus,

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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

All hippocampal section within the AP planes 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 unpaired t-test and ANOVA were used (level of significance was set at p<0.001).

Ad 2. After cryostat sectioning free-floating sections were placed in 0.1 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 minutes, in 70% ethanol solution for 1 minute, in distilled water for 1 minute, in 0.01% potassium permanganate (KMnO₄) for 15 minutes with gentle shaking. Slides were washed in distilled water three times. Staining proceeded in dim place by immersing slides into 0.001% Fluoro-Jade B solution for 30 minutes with occasional gentle shaking. After that slides were rinsed in the distilled water three times for 1 minute. Slides were then immersed in 0.01% Hoechst staining solution for 10 minutes 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-d evaluation (each section was taken and evaluated). The tissue was examined under the epifluorescence illumination with blue (450–490 nm) excitation light.

Results
The results show that nicotine brings about higher numbers of NADPH-diaphorase positive neurons in CA3 area of the hippocampus (Figure 1) and hilus of the dentate gyrus (Figure 1) in the comparison with either group of control animals. Furthermore, it did not change the number of NADPH-diaphorase positive neurons in CA1 area of the hippocampus (Figure 1), in the ventral blade of the dentate gyrus (Figure 1) and in the dorsal blade of the dentate gyrus (Figure 1).

Brain tissue from nicotine exposed rats contained no Fluoro-Jade B positive neurons. (Colour figures 11, 12) illustrate a typical section (animal, which received 1mg/kg nicotine) with Fluoro-Jade B/Hoechst combination labelling at low magnifications. The Hoechst staining did not detect any cells with fine granulated nucleus; the neuronal cells were classified as intact in all areas of hippocampus. (Colour figures 13, 14) illustrate the typical section from the brain of control animal (normal saline solution) with Fluoro-Jade B/Hoechst combination labelling at low magnifications.

Discussion
Our findings show that nicotine administration brings about higher numbers of NADPH-d positive neurons in 2 examined areas of the hippocampus in 35-day-old
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Figure 1 – Number of NADPH-d positive neurons in CA1 area of hippocampus per section area, Number of NADPH-d positive neurons in CA3 area of hippocampus per section area, Number of NADPH-d positive neurons in dorsal blade of the dentate gyrus per section area, Number of NADPH-d positive neurons in ventral blade of the dentate gyrus per section area, Number of NADPH-d positive neurons in hilus of the dentate gyrus per section area, Mean ± S.E.M. Level of significance p < 0.001 (*)
animals. 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 [19].

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 [19]. 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 [20]. Thus, the relatively simple NADPH-d histochemical technique allows us to characterize the nitrergic systems in the central nervous system. NADPH-d positive neurons are probably interneurons.

Our findings also suggest that nicotine might induce higher expression of nNOS, which could contribute to the neuronal integration as responding to the different pathophysiological demands. Some experiments have shown that the heavily positive NADPH-d neurons are relatively resistant to neurotoxic insults [12]. The observed changes and their mutual comparison lead us to the conclusion that the dose 1mg/kg (albeit this dose is rather high) of nicotine administered intraperitoneally did not induce neurodegeneration in any area of the hippocampus in 35-day-old animals, when Fluoro-Jade B dye was used for evaluation.

We can speculate, that nicotine may acts as an neurotoxic agent, which induced NADPH-d positively in hilus of the dentate gyrus an CA3 area of the hippocampus (this areas are unresisting to many toxic insults), but it did not induce any neuronal degeneration. Nicotine can operate as an agent which (by NADPH-d induced positivity) may protect against some neurotoxic drugs e.g. kainic acid (KA) and many others [25]. KA-induced seizures are widely used as a model for human temporal lobe epilepsy [21]. If our theory is true, than nicotine pre-treatment could be a protective factor against KA effect. In recent literature only few studies exist, which have discussed this hypothesis. For example, in the experiments of Shytle et al. [22] the incidence of KA-induced wet dog shakes (a paroxysmal shaking of the head, neck and trunk) was lower in rats pre-treated with nicotine 15 minutes before kainic acid administration, than in those pre-treated only with normal saline. That behavioural study also demonstrates nicotine protective potential in vivo. On the other hand extensive literature suggests that nicotine can similarly protect against other different toxic insults in in vitro systems [8], including its effect against MPTP-induced toxicity in nigral neurons [9].

Nicotine might also act also as an antioxidant [23]. Discussions proceed that nicotine can increase expression of some neurotrophic factors, which are crucial for neuronal maintenance, survival and regeneration [24].

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The precise mechanism, by which nicotine increases the number of NADPH-diphorase positive neurons and the implications for brain plasticity, eventually for its protective potential remain to be elucidated. To clarify these questions more studies are necessary.

References

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