ORIGINAL ARTICLE

Nicotine effects on rat seizures susceptibility and hippocampal neuronal degeneration

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Abstract

OBJECTIVES: Nicotine is a widely used drug of abuse exerting number of effects on the central nervous system. The study was aimed at the effects of nicotine in various doses on the excitability of cerebral cortex and – by using the methods of histochemistry – to identify possible signs of neuronal degeneration after nicotine administration.

METHODS: Cortical afterdischarges were elicited by repeated stimulation of the right sensorimotor cortex. The duration of evoked ADs was monitored in animals treated with nicotine (0.75 or 1.0 mg/kg) and in animals treated with saline. Methods of histochemistry (Fluoro-Jade B and bis-benzimide) were used to detect possible neuronal degeneration in hippocampus or in cerebral cortex.

RESULTS: No Fluoro-Jade B positive cells were found in hippocampi of control animals, or animals treated with nicotine. ADs length was significantly influenced by administration of nicotine.

CONCLUSION: Nicotine in 0.75 or 1.0 mg/kg dose leads to the decrease in ADs duration, influences the seizure susceptibility, and doesn't cause any detectable neuronal damage.

Abbreviations:

CNS	- Central Nervous System
ADs	- Cortical afterdischarges
ECoG	- Electrocorticography

INTRODUCTION

Nicotine is a widely used drug of abuse, exerting number of effects on central nervous system (CNS), like the modulation of neurovegetative functions, influence of learning, memory and generally the cognitive functions (Ferrera & Winterer 2009). Administration of nicotine leads to its interaction with specific cholinergic receptors and these nicotinic receptors have been proposed to mediate the very interesting characteristic of nicotine – the neuroprotective potential (Buckingham *et al.* 2009; Shimohama 2009). The latter effect has recently attracted interest of many research groups mainly because of the perspective positive effect in patients with neurodegenerative diseases (such as Alzheimer's and Parkinson's diseases) or due to the expected beneficial effect on cognitive functions during the process of ageing (Carrasco *et al.* 2006). On the other hand nicotine, when reaching central nervous system (CNS) in significant dose, can strongly influence the internal microenviroment of vital CNS regions like cerebral cortex or hippocampus, causing the functional overload and inducing the oxidative stress (Newmann *et al.* 2002). All these mechanisms can cause higher sei-

zure susceptibility in the cortex and a cell death. From this point of view nicotine acts as a positive as well as a negative agent, while the effect is limited mainly by the dose of this substance and the way of administration. In this study we focused on the effect of a single dose of intraperitoneally applied nicotine in two important brain regions – cerebral cortex and hippocampus.

MATERIALS AND METHODS

Animals

All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in agreement with the guidelines of the Animal Protection Law of the Czech Republic.

Electrophysiological experiments were performed with freely moving 35-day-old Wistar rats of our own breed. They were housed at a constant temperature (23 ± 1 °C) and relative humidity (60%) with a fixed 12 h light/dark cycle and fed with food and water *ad libitum*. There were at least 8 animals in each group for electrophysiological analysis and 4 animals in the histological study.

Animals were randomly assigned into the following subgroups:

- Control group – animals received a single injection of the normal saline solution
- Animals received a single injection of 0.75 mg/kg nicotine
- Animals received a single injection of 1 mg/kg nicotine

Experimental substances and procedures

All drugs were applied intraperitoneally. Nicotine (Sigma) was dissolved in normal saline solution and injected in the dose 0.75mg or 1mg per kilogram of animal body weight, recalculated volume: 0.02ml of the solution per 10g of animal body weight. Saline (control group) was applied in the equal volumes.

Electrophysiology – ADs analysis

For ADs analysis six silver electrodes were implanted through the cranium under deep anaesthesia: two stimulation electrodes (right sensorimotor cortex), three registration electrodes (left sensorimotor cortex, left and right visual cortex) and reference electrode (prefrontal cortex). Recording and other experimental manipulations were carried out after the recovery of rightening reflexes (i.e. approximately 15 min after the surgery), then the nicotine or saline were administered and after 15 minutes the cortical afterdischarges were elicited by stimulation of the right sensorimotor cortex. We used constant current (CC) stimulation (bipolar pulses – pulse period 1 ms; duration of stimulation 15 s; frequency 8 Hz; intensity 3–5 mA, which is sufficient for ADs eliciting). The basic stimulation intensity level was set at 3 mA. In case of no response, another stimulation of 4 mA was used 5 min after the first stimulation. The process was similarly repeated with 5 mA stimulation. Finally, if no epileptic graphoelements appeared after the 5 mA stimulation, the animal was excluded from the experiment. If a distinct response (epileptic graphoelements) was recorded, the stimulation was repeated five times at one-minute intervals (timed from the end of each seizure to the beginning of the next stimulation). The duration of evoked ADs and the shape of evoked graphoelements were monitored. The paired, unpaired t-test and ANOVA in GraphPadPrism were used for evaluation of the results.

Histological analysis

For the histological part of the study animals were exposed to nicotine (0.75 or 1 mg/kg) or to normal saline solution. After 24 hours animals were perfused under the 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 µm thick sections with a cryostat. Then two different histochemical methods were used:

A. Fluoro-Jade B (Histo-Chem Inc.) is an anionic fluorescein derivative useful for the histological staining of neurons undergoing degeneration (Schmued & Hopkins 2000)
B. Hoechst 33342 (bis-benzimide) (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. 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), coverslipped using D.P.X. neutral mounting medium and allowed to dry.

The tissue was examined using an epifluorescent microscope OLYMPUS AX 70 PROVIS with blue (450–490 nm) excitation light. Following regions in the hippocampal area were analysed for possible signs of degeneration or apoptosis: CA1 area of the hippocampus, CA3 area of the hippocampus, the hilus of the dentate gyrus, the dorsal blade of the dentate gyrus, the ventral blade of the dentate gyrus, and the sensorimotor cerebral cortex.

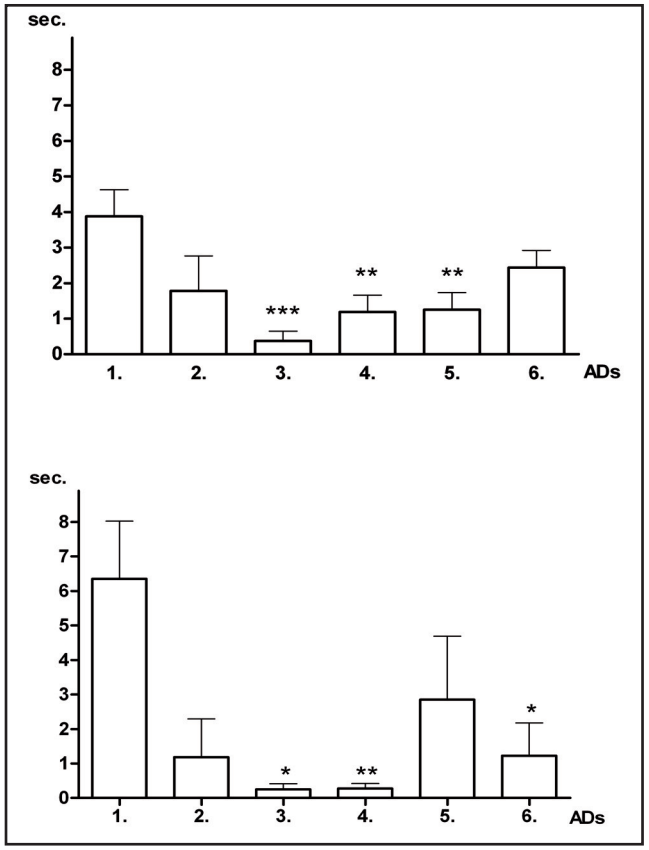

Fig.1. Duration of ADs in 35-day-old rats. Upper graph – animal treated with dissolvent only, lower graph animal treated with 1.0 mg of nicotine. 1–6 sequence of stimulation. Y axis represent the duration of ADs (seconds), * indicates results significant at $p<0.05$, ** indicates results significant at $p<0.01$, *** indicates results significant at $p<0.001$, all in comparison with ADs after the 1st stimulation. Upper graph displays the so called "U-shape", the postictal inhibition (see text).

RESULTS

Electrophysiology-ADs analysis

Electrophysiology analysis of brain excitability in the control group (saline) revealed that in comparison with the 1st ADs, the 3rd, 4th, and 5th seizures were significantly shorter – the manifestation of postictal depression. The 2nd and 6th seizures remained unchanged (see Fig. 1).

In the experimental group which received 0.75 mg/kg of nicotine, all subsequent ADs (2nd–6th) were significantly shorter then the first one. ADs after the 2nd, 3rd, 4th, 5th and 6th stimulation were significantly shorter also in comparison with the control group.

Animals of the group that received 1.0 mg/kg of nicotine had the 3rd, 4th and 6th ADs shorter, then those after the first stimulation. The comparison with the control group revealed that ADs duration was significantly shorter after the 3rd, 4th and 5th stimulation (see Fig. 2).

Histochemistry

Detection of possible neuronal degeneration didn't confirm any neurotoxic effect of nicotine. No Fluoro-Jade B positive cells were found in hippocampi of control animals, or in animals treated with nicotine. Hoechst staining didn't confirm any apoptotic changes regardless of dose of nicotine and the same results were observed in animals treated with normal saline solution. The same results were found in the cerebral cortex. No signs of degeneration, or apoptosis were identified.

DISCUSSION

The aim of our study was to analyze effects of a single dose of nicotine on the rat brain excitability and on the neuronal degeneration in cerebral cortex and hippocampus. The arrangement of our experiment allowed observing the effect of drugs (in this case nicotine) on two important central nervous system regions – the cortex and the hippocampus. Hippocampus is highly suitable for such study design as it is a region with anatomically well organized structure which has a very long postnatal development. Such characteristic allows studying hippocampus by various methods of histology and correlate results in different age groups (Milotová *et al.* 2008, Malinowska-Kolodziej *et al.* 2009, Mourek *et al.* 2009). Testing the rat brain excitability using of ECoG and analysis of ADs length allows effectively reveal the level of susceptibility and excitability of the neuronal circuits and register changes of the brain homeostasis (Kalinčík & Marešová 2005, Marešová *et al.* 2010). Seizure susceptibility is maintained by the equilibrium/disequilibrium of excitatory and inhibitory mechanisms. Administration of any active substance allows detecting changes in excitability by prolongation or shortening of ADs (or negative effects on ADs duration). For the present experiment 35-day-old rats were used, because they have the inhibitory systems already developed and are able to prevent the prolongation of ADs after the repeated stimulation (Marešová *et al.* 2010). In the typical animal of this age group the ADs duration restores to the duration of the first one after the 4th stimulation. The graphical representation of such relation has so called "U-shape" (Figure 1). This distinctive electrophysiological pattern is modified when 1mg of nicotine is administered. The ADs after the last (6th) stimulation is still significantly shorter compared to that after the first stimulation. Figure 2 clearly shows that the pattern of excitability in control animals differs significantly from those treated with 0.75 or 1 mg of nicotine. Nicotine tends to shorten the duration of ADs and this change can be observed even after the 2nd and following stimulations. Analysis of ADs duration showed that both doses of nicotine, used in our experiment were probably very similar in their effect on cortical excitability and ADs duration. Nicotine administered in high dose is capable to produce tonic-clonic convulsion mediated centrally and the administration of nicotinic cholinergic receptor antagonists is able to block the effect of nicotine. Some literature sources documented, that nicotine adminis-

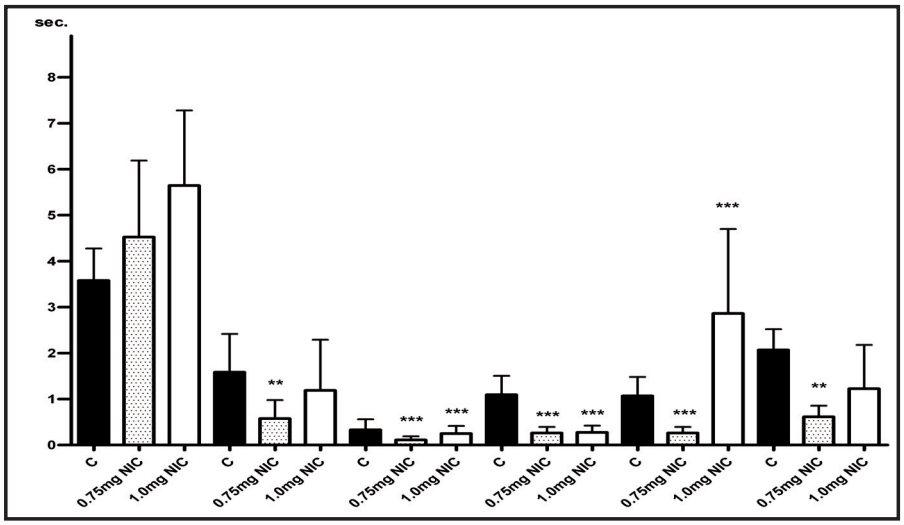

Fig. 2. Afterdischarges duration in 35-day-old rats treated with dissolvent (C), lower dose of nicotine (0.75 mg NIC) and higher dose of nicotine (1.0mg NIC). The "graph-triplets" represent a single stimulation. All six stimulations are depicted at the figure. * indicates results significant at $p<0.05$, ** indicates results significant at $p<0.01$, *** indicates results significant at $p<0.001$, all in comparison of ADs duration between the control group and groups treated with nicotine.

tered systemically in sub-convulsive doses (app 4–6 mg/kg) is able to decrease seizure threshold in experiments with electroconvulsion. Nicotine has been even referred to decrease the anticonvulsive effect of some seizure-preventing drugs (Czuczwar *et al.* 2003). Our experiment showed that nicotine in much lower doses (0.75 or 1.0 mg/kg) is able to decrease the duration of ADs in the experimental arrangement with repeated electrical stimulation. Our result signalize, that the dose could be the crucial factor for the possible pro-convulsive or non-convulsive effect of nicotine. Nicotine has been repeatedly reported as a drug with neuroprotective properties (Borlongan *et al.* 1995, Riljak *et al.* 2007). The mechanism by which nicotine is able to prevent the damage of particular parts of CNS is still unclear. There is evidence suggesting that nicotine could have antioxidant properties in the central nervous system (Newman *et al.* 2002). The antioxidant properties of nicotine may be intracellular via activation of the nicotinic receptors or extracellular by acting as a radical scavenger. More electrophysiological experiments are needed to confirm the hypothesis, that nicotine administered in low dose could decrease the animal seizure susceptibility and probably change the seizure threshold. We speculate that nicotine exhibits a broad spectrum of properties strongly dependent on the dose and this spectrum includes neuroprotective effect as well as neurotoxic one. The histological part of our study confirmed that nicotine in doses used in present experiment didn't cause neuronal degeneration in the hippocampus or in the cortex. The Fluoro-Jade B staining is very effective for identification the possible neuronal damage and nicotine administered in dose 0.75 or 1.0 mg/kg didn't lead to any Fluoro-Jade B positivity. We may conclude that nicotine in mentioned doses leads to the decrease in ADs duration, influence the seizure susceptibility of treated animal and doesn't cause any structural neuronal damage.

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