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Nicotine reduces mortality of developing rats exposed to high-altitude hypoxia and partially suppresses the duration of cortical epileptic afterdischarges

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Abstract. Nicotine has been repeatedly reported as substance possessing neuroprotective properties. This study focused on the possible beneficial effects of nicotine against the high-altitude hypoxia (9000 m for one hour). 15 min prior to hypoxia exposition rats (12- and 35-day-old) were treated with nicotine. Next day electrodes have been implanted and the effects of nicotine and hypoxia (or both factors) on duration of afterdischarges (ADs) were tested. Administration of nicotine declined the hypoxia-induced mortality in 35-day-old animals. Nicotine pretreatment had no effect on ADs duration in 12-day-old pups, therefore brought about suppression of ADs in 35-day-old animals. Taken together, our data show that nicotine exhibits an anticonvulsant effect that is age-dependent. The mechanisms of nicotine neuroprotective properties include probably the influence of calcium homeostasis, increase synthesis of variety of growth factors, inhibition of the caspase cascades and antioxidant capability of nicotine.

Key words: Brain cortex — Afterdischarges — Hypoxia — Nicotine

Abbreviations: EcoG, electrocorticography; ADs, afterdischarges.

Introduction

Hypoxia and its consequences in central nervous system have been studied for many years because of its clinical significance (Vannucci and Vannucci 2005). Extent of oxygen deficiency (brain ischemia) could be classified as the focal and global one. The first mentioned term means the interruption of the blood supply to some region of the brain (hemorrhagic or ischemic stroke), the latter term describes interruption of oxygen delivery into the whole organism and brain is not excluded (cardiac arrest or decreased partial pressure of oxygen in ambient atmosphere, anemia, impairment of respiratory system) (Ginsberg and Busto 1989; Hartman et al. 2005; Cervantes et al. 2008). In the following text the term hypoxia is used for the global decrease of oxygen supply to the brain, being aware that it is certain simplification. Consequences of hypoxia depend on its intensity, repetition of hypoxic insult, length of hypoxia period and such consequences are age-related as well. Hypoxia affects the central nervous system homeostasis (causing massive neuronal depolarization) *via* excitotoxic damage and this damage could finally lead to cell death by overloading the neuronal circuits (Doble 1999; Kalincik and Maresova 2005). Therefore the mechanisms (or drugs) capable to protect or at least ameliorate the consequences of hypoxia are intensively searched. One of the most promising neuroprotective substances is nicotine. Our laboratory demonstrated previously, that nicotine is capable to decrease the neuronal damage induced by kainic acid (Riljak et al. 2007), to influence the rat seizures susceptibility (Riljak et al. 2010) and change the electrocorticography (ECoG) pattern and behaviour of experimental animals (Hralova et al. 2010). Series of neuroprotective nicotine´s effects have been reported: nicotine ameliorates the Parkinson´s (Singh et al. 2010) and Alzheimer´s related pathological changes (Shimohama and Kihara 2001) and enhances the learning and memory deficit during the process of aging (Carrasco et al. 2006). Therefore we decided to test effects of nicotine on global hypoxia. We used cortical epileptic afterdischarges (ADs) as an experimental model (Mares 2010; Maresova et al. 2010) and two distinct

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age groups were tested to reveal the possible developmental aspects. The younger group (12-day-old) represents morphologically and functionally immature central nervous system and in some aspects corresponds with the early postnatal stage of human brain, while the older one (35-day-old) reflects the adolescent stage (Pometlova et al. 2010).

Materials and Methods

Animals

Male Wistar rats (of our own breed) 12 and 35 days old were used in experiments. Animals were housed at a constant temperature (23 ± 1°C) and relative humidity (60%) with a fixed 12 h light/dark cycle and fed (or their mothers) with food and water *ad libitum*. On the testing day animals were transported into the experimental room, weighed, marked and randomly assigned into particular experimental groups. All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and in agreement with the guidelines of the Animal Protection Law of the Czech Republic.

Hypoxia procedure

A 15 min rest after the injection (see below) of any substance (nicotine, vehicle or no treatment) was followed by exposure to hypobaric hypoxia (6.4 kPa), i.e. to the simulated altitude of 9000 m, which was reached in 2 min (30 kPa/min) and lasted 60 min. After returning to ambient air pressure (approximately 101 kPa) with the speed of 30 kPa/min, animals were left to recover at resting conditions for 24 hours, and then the electrophysiology procedure was conducted.

Drugs and experimental groups

Nicotine (Sigma) or its vehicle were applied intraperitoneally (nicotine was dissolved in saline), in the dose 1 mg/kg. The solutions were freshly prepared for each experiment. The recalculated volume *per* body mass was the same for both substances (nicotine or vehicle) (1 ml of solution/kg of body mass).

Experimental animals, 12-day-old and 35-day-old, were assigned into the following groups: animals exposed to hypoxia ($n = 14$, $n = 24$, respectively), animals treated with vehicle and exposed to hypoxia ($n = 8$, $n = 19$, respectively), animals treated with nicotine and exposed to hypoxia ($n = 10$, $n = 18$, respectively).

Electrophysiology – ADs duration 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 (placed into nasal bone). Recording and other experimental manipulations were carried out after the recovery of righting and suckling reflexes (i.e. approximately 15 min after the surgery), then the cortical ADs were elicited by stimulation of the right sensorimotor cortex. We used constant current 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 recorded.

Statistics

Electrophysiological data were subjected to nonparametric tests. To compare the differences in ADs length in six subsequent stimulations in particular groups Friedman test followed by Wilcoxon signed rank test were used. To compare the differences between the hypoxia exposed rats and nicotine pretreated rats (exposed to hypoxia as well) within a given session, Kruskal-Wallis test and the Mann-Whitney test were used. The error bars in graphs indicate the standard error of the mean.

Results

Nicotine, saline and hypoxia effects on rat's mortality

All12-day-old animals exposed to hypoxia survived regardless of the treatment.

There were 83% mortality in 35-day-old animals of the control group (hypoxia with no treatment), 31% mortality in the group treated with saline (and exposed to hypoxia) and 0% in the group treated with nicotine (and exposed to hypoxia).

Age-related differences in cortex excitability

Analysis of the ADs length and mutual comparison of ADs length after the 1st stimulation (used as a baseline) with the following five stimulations confirmed the prolongation,

typically observed in such immature animals like the 12-day-old rats. All subsequent stimuli (2^{nd}–6^{th}) significantly prolonged the response of cortical neuronal populations (Fig. 1). Repeated stimulation in older experimental animals (35-day-old) revealed the significant shortening of ADs after the 3^{rd}, 4^{th} and 5^{th} cortical stimulation. ADs length after the very last stimulation - the sixth one was statistically of the same length as the first one (Fig. 1). The duration of analyzed ADs was significantly larger in 12-day-old group compared to the older experimental group (Fig. 2). The types of ECoG graphoelements analysis revealed the spike and waves predomination in 35-day-old animals, while analysis the younger group confirmed that the typical picture of observed ADs was the train of fast sharp waves. The spikes of low amplitude were recorded only rarely.

Age-related effects of nicotine pretreatment and hypoxia on cortex excitability

Administration of nicotine (as a hypoxia pretreatment) had no effect on the length of cortical ADs in the younger experimental group exposed to hypoxia (Fig. 3A). All subsequent stimuli compared with the very first AD (baseline) were of same duration. Nicotine in our experimental paradigm was not able to bring any increase or decrease of the cortical excitability.

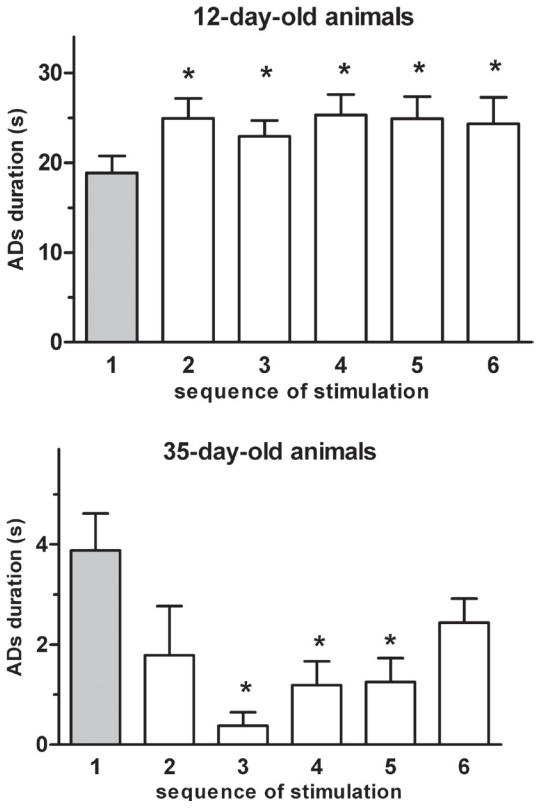

Figure 1. Duration of ADs in control (untreated) 12-day-old animals (upper graph) and 35-day-old animals (bottom graph). Grey column: baseline – duration of the first afterdischarges (ADs). Results were calculated as mean ± SEM; * $p < 0.05$ in comparison with the ADs after the first stimulation.

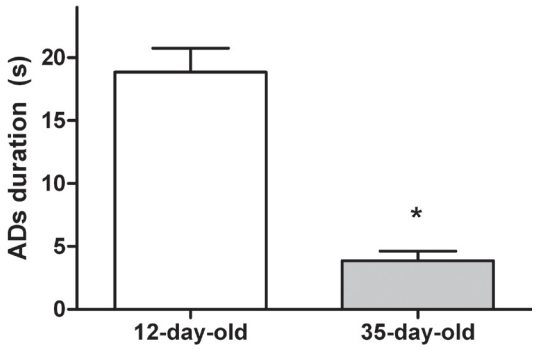

Figure 2. Duration of the 1^{st} ADs in control (untreated) 12-day-old and 35-day-old animals. Results were calculated as mean ± SEM; * $p < 0.01$.

Nicotine administration brought following changes in the older experimental group: the 3^{rd}, 4^{th}, 5^{th} and 6^{th} ADs compared to the baseline ADs (after the first stimulation) were significantly shorter. Repeated stimulation lead to the decrease in ADs duration and this effect did not disappear even after the 5^{th} stimulation. Comparison of the group exposed to hypoxia and treated with vehicle with the group exposed to hypoxia and treated with nicotine confirmed significantly shorter ADs in the group treated with nicotine – the 4^{th} and 5^{th} ADs were shorter (Fig. 3B).

Discussion

The main aim of our study was to test the effect of nicotine treatment on hypoxia-induced changes in two different age groups. It is well known, that ECoG pattern and ADs length changes with the age (Mares et al. 1982). ECoG pattern of ADs in immature 12-day-old animals was represented mainly by the train of sharp rhythmic waves, while predomination of spike-and-wave rhythm in 35-day-old animals was observed. ADs were much longer in younger experimental animals (Fig. 2), reflecting the insufficiency of cortical inhibitory mechanisms at this developmental stage. Immaturity of seizure arresting mechanism is caused (among others mechanisms) by the development of inhibitory mediator systems in the brain (Mares and Kubova 2008). The older animals are capable to arrest the seizures in our experimental arrangement of subsequent stimulations – comparing the baseline (duration of very first ADs) with the following ones revealed the typical "U-shape" curve, which means graphical representation of the postictal depression phenomenon

(shortening of the 3rd, 4th and 5th ADs). All these data agree with the thesis of rhythmic phenomena development of thalamocortical origin (Mares et al. 1982). Our interest was focused mainly on nicotine influence on short-term hypoxia consequences. It is well known, that hypoxia-ischemia is one of the most common factors leading to epileptogenesis in newborns and hypoxia is even capable to decrease seizure threshold throughout adulthood (Jensen et al. 1992, 1998). One of the early-onset mechanisms accompanying the oxygen deficit is the neuronal depolarization (Balestrino 1995). Oxygen deprivation could lead to excitotoxicity induced by so-called glutamate loop (oversecretion of glutamate) and by excessive intracellular calcium level. Such mechanisms could enhance the metabolic block of oxidative phosphorylation, decrease the intracellular pH and increase lactic acid concentration which finally leads to inadequate energy supply of neuronal cells (Doble 1999). All mentioned factors contribute to the break-down of ion distribution on neuronal membrane. Nicotine attracts the interest of researches mainly because of its possible neuroprotective properties. One of the pilot works on this field conducted by Borlongan and coworkers described the suppressing effect of nicotine on wet dog shakes caused by administration of kainic acid in rats (Borlongan et al. 1995). Other authors confirmed the nicotine as powerful drug that is able to ameliorate consequences of processes associated with aging and neu-

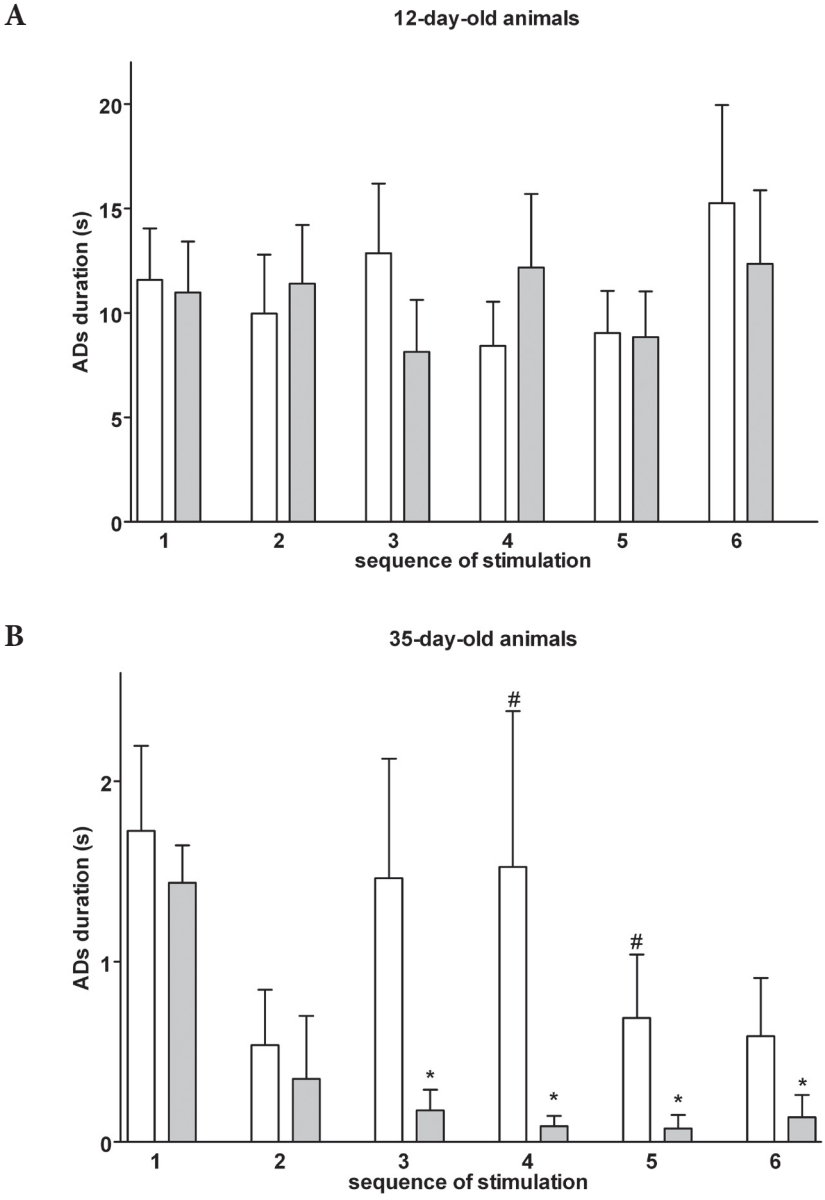

Figure 3. Duration of ADs in 12-day-old (A) and 35-day-old (B) rats. White columns – animals exposed to hypoxia and treated with vehicle. Grey columns – animals exposed to hypoxia and treated with 1.0 mg/kg of nicotine (i.p.). * indicate differences significant at $p < 0.05$ between the particular repetition of stimulation and the duration of the very first ADs (baseline) of the same animals; # indicates results significant at $p < 0.05$ between the hypoxia group (white column) and hypoxia and nicotine treated animals (grey column) within the particular session (stimulation). Results were calculated as mean ± SEM.

rodegeneration (Huang et al. 2009; Shimohama 2009). The mechanism of nicotine's protective effect to neuronal cells is not fully understood and is probably multifactorial – nicotine could influence the calcium homeostasis, increase synthesis of variety of growth factors, inhibit the caspase cascades or act as an antioxidant agent (Newman et al. 2002; Ferrea and Winterer 2009). We found that nicotine is able to rescue the older experimental animals from the death caused by hypoxia challenge. All mechanism mentioned above could be involved in the observed nicotine effect. The nicotine effects on ADs duration were observed only in the group of older animals. ADs in experimental pattern of repeated electrical stimulation were markedly shortened in this group, while the 12-day-old animals seem to remain unaffected by nicotine treatment. Observed differences could be related to the neuronal nicotinic acetylcholine receptors development. These receptors are formed from α and β subunits (α2–α10, β2–β4) and different combination of these subunits is responsible for different physiological and pharmacological properties of particular channel (Nai et al. 2003). The subunit composition differs during development and this could explain different effect of nicotine treatment at various ontogenetic stages of rats´ central nervous system (Cimino et al. 1995). It is very probable, that the dose is the crucial factor influencing the final effect of nicotine. It has been reported, that nicotine in dose 4–6 mg/kg is capable to decrease the seizure threshold in experiments with electroconvulsion, and nicotine is even able to decrease the anticonvulsant activity of some seizure-preventing drugs (Czuczwar et al. 2003). Our previous experiment tested the effect of 0.75 and 1 mg/kg of nicotine on naive animals (35-day-old) with the conclusion, that nicotine decreased the length of ADs in the experimental arrangement with repeated electrical stimulation (Riljak et al. 2010). This principle has been confirmed by our present work. Decrease of cortical excitability is probably limited to low nicotine dosage. Such dose (1 mg/kg and even less) shortens the ADs duration by stabilizing the neuronal micro-enviroment (calcium homeostasis, reactive oxygen species breakdown) and this finally leads to milder consequences of short-term hypoxia as confirmed by survival of the older experimental group. Surprising was the effect of vehicle (normal saline) on animals' survival rate. Injection of saline in our experiment decreased substantially the mortality of rats exposed to hypoxia. Some authors speculate, that the handling and intraperitoneal injection of saline represent a stress that can affect excitability of the neuronal circuits in the brain (Edwards et al. 2002). Such data could be only hardly compared with our experimental design, because animal were treated with nicotine or saline by using same injection procedure. To our knowledge, there is no clear explanation of observed effect of saline. To conclude, our study demonstrate that nicotine administration declines the hypoxia-induced mortality in 35-day-old animals and that nicotine is capable to suppress the ADs duration in this age group. While the nicotine administration neither prolongs nor suppresses ADs length in 12-day-old animals it seems to be very probably, that nicotine effects on hypoxia induced changes of brain cortex excitability is obviously age-dependent.

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