ORIGINAL ARTICLE

Neuroprotective effect of nicotine against kainic acid excitotoxicity is associated with alpha-bungarotoxin insensitive receptors subtype of nAChRs

Vladimir Riljak, Jan Benes, Jaroslav Pokorný, Jaromir Myslivecek

Charles University, First Medical Faculty, Institute of Physiology, Prague, Czech Republic

Correspondence to: Vladimír Riljak, MD., PhD.
Institute of Physiology, First Faculty of Medicine
Charles University in Prague
Albertov 5, 128 00, Prague 2, Czech Republic.
tel: +420 224968443; fax: +420 224918816; e-mail: vladimir.riljak@lf1.cuni.cz

Submitted: 2011-11-04 Accepted: 2011-11-30 Published online: 2012-01-15

Key words: **nicotine; kainic acid; hippocampus; seizure; Fluoro-Jade B; α-bungarotoxin insensitive receptors**

Neuroendocrinol Lett 2011; **32**(6):816–820 **PMID:** 22286796 NEL320611A16

Abstract

OBJECTIVES: Our previous study showed that administration of nicotine is capable to protect the neurons of hippocampus against the kainic acid induced damage. Here we tested the hypotheses that multiple nicotine administration would prevent the effects of kainic acid on neuronal nicotinic receptor subtypes densities (α-bungarotoxin sensitive and α-bungarotoxin insentive) and on hippocampal cell degeneration.

METHODS: Radioligand binding study was used to detect the particular nAChR subtypes densities. Two histochemical methods (bis-benzimide staining and Fluoro-Jade B dye) were used to detect and evaluate neuronal degeneration.

RESULTS: Our study shows that: a) kainic acid single administration increased the number of α-bungarotoxin insentive nicotinic receptors, b) nicotine was able to prevent such changes, c) repeated nicotine administration is capable to attenuate the damage of CA1 and CA3 areas of the hippocampus. No effect on α-bungarotoxin sentive nicotinic receptors was observed. Our data therefore reveal the importance of α-bungarotoxin insentive nicotinic receptors in the response to kainite and the ability of nicotine to prevent such changes both in the cell degeneration and in number of receptors.

CONCLUSION: Nicotine administration influences α-bungarotoxin insensitive receptors and repeated administration is capable to protect against toxicity caused by kainic acid in hippocampal area.

INTRODUCTION

Nicotine, the principal substance in cigarette smoke, is highly addictive legal drug exerting variety of effects such as: influence of learning, memory, mood and regulation of stress and anxiety (Ferrea & Winterer 2009); perturbation of some physiological parameters i.e. heart rate or body temperature (Pelissier *et al.* 1998), increases cerebral blood flow (Uchida *et al.* 1997), cortex excitability (Riljak *et al.* 2010) or modulation of immune response (Ferrea & Winterer 2009). In recent years the evidence of beneficial effects of nicotine has arise and nicotine has become a candidate that could mediate neu-

roprotection of CNS. Variety of experimental designs confirmed that nicotine is effective against neuronal damage caused by different chemotoxins (Borlongan *et al.* 1995; Kaneko *et al.* 1997; Marin *et al.* 1994; Riljak *et al.* 2007), decrease the toxic action of β-amyloid and cognitive deficit in Alzheimer´s disease (Buckingham *et al.* 2009) and protects the nigrostriatal system in various model of Parkinsonism (Maggio *et al.* 1998). Majority of above mentioned nicotine properties is associated with activation of neuronal nicotinic acetylcholine receptor (nAChRs). nAChRs are ligand gated cationic pentameric channel distributed throughout the central nervous system. This receptor family consists of different subtypes of nAChRs and each subtype exhibit specific anatomical distribution, biophysical and physiological properties. nAChRs are formed from α and β subunits (α_2–α_{10}, β_2–β_4); different combination of these subunits is responsible for pharmacological heterogeneity of receptor family (Marin *et al.* 1994). In general, α-bungarotoxin sensitive receptors consist of seven α subunits $(\alpha7)_5$ and α-bungarotoxin insentive receptors have this composition of subunits: $(\alpha4)_2(\beta2)_3$, i.e. it consist of two α4 subunits and three β2 subunits. Kainic acid is rigid analogue of glutamate causing (when administered intraperitoneally or intracerebroventricularly) massive neuronal depolarization and this effect may finally leads to functional overload of neuronal circuits and cell dead (Ben-Ari & Cossart 2000; Sperk 1994). One of the most sensitive structures is hippocampus, because this part of CNS exhibits high density of kainite receptors (Huettner 2003), thereby administration of kainaite causes the massive degeneration of CA3 and CA1 field of hippocampus (Riljak *et al.* 2007; Sperk 1994), partially by direct toxic effect and partially by seizure generating epileptiform seizures predominantly in CA3 region of hippocampus (Ben-Ari & Cossart 2000). Such seizures are able to propagate to other limbic structures leading to pattern similar to those one observed in human temporal lobe epilepsy (Ben-Ari & Cossart 2000; Sperk 1994). Our previous study (Riljak *et al.* 2007) showed that administration of nicotine is capable to protect the neurons of hippocampus against the kainic acid induced damage and this result was in line with observations of others research groups (Borlongan *et al.* 1995; Kim *et al.* 2000). Here we tested the influence of multiple nicotine administration on neuronal nicotinic receptor subtypes densities (α-bungarotoxin sensitive and α-bungarotoxin insentive) and the effect of such pretreatment on hippocampal cell degeneration. To reach our goal we used the combination of receptor binding study together with methods of histochemistry.

MATERIALS AND METHODS

Subjects

Wistar rats of our own breed were housed at a constant temperature (23±1 °C) and relative humidity (60%) with a fixed 12 h light/dark cycle with lights on at 07:00 and all testing occurred between 09:00 and 17:00. Animals were fed with food and water *ad libitum* and there were housed two a cage. Animals enter the experiment at postnatal day 30 (PD30), experiment ended on PD35 (perfusion, decapitation). 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.

Drugs and administration

All substances were administered intraperitoneally (i.p.), nicotine and kainic acid were dissolved in physiological saline to reach desired concentration. Nicotine (or physiological saline) has been administered twice a day (09:30 and 14:00) for three days, fourth day of an experiment schedule animals were treated with kainic acid (or physiological saline), so finally four experimental groups have been created:

Nicotine group: animals treated with 0.25 mg/kg (0.25 mg of nicotine dissolved in 1ml of physiological saline, recalculated volume 0.001 ml of solution per 1 g of animal body weight) twice a day (cumulative dose of nicotine 1.5 mg/kg). Fourth day animals received single injection of physiological saline in equal volume.

Kainic acid group: animals were sham-treated with saline injection twice a day, fourth day rats were treated with single injection of kainic acid in dose 5 mg/kg (5 mg of kainic acid dissolved in 1 ml of physiological saline, recalculated volume 0.001 ml of solution per 1 g of animal body weight).

Kainic acid + nicotine group: animals treated with 0.25 mg/kg (0.25 mg of nicotine dissolved in 1 ml of physiological saline, recalculated volume 0.001 ml of solution per 1 g of animal body weight) twice a day (cumulative dose of nicotine 1.5 mg/kg). Fourth day animals received single injection of of kainic acid in dose 5 mg/kg (5 mg of kainic acid dissolved in 1 ml of physiological saline, recalculated volume 0.001 ml of solution per 1 g of animal body weight).

Control group: animals treated all four days with physiological saline. First three days twice a day (volumes equal to nicotine volume), fourth day single injection only (volume equals to kainic acid injection volume).

General experimental procedure

One day after last treatment (fifth day) animals were sacrificed by transcardial perfusion for histological part of study and by decapitation for radioligand binding study.

Histology

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 μm

thick sections with a cryostat at –20 °C. 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 and the ventral blade of the dentate gyrus.

Radioligand binding experiments

The brains were quickly removed from the skull after decapitation and hippocampal tissue has been separated. Both hippocampi were used as one tissue bolus (regardless the side) and frozen rapidly (–20°C).

Membrane preparation

The tissue was weighed and homogenized for two or three pulses of 20–30 s in a homogenizer (Ultra-Turrax® T25 basic IKA®-Werke 24,000 r.p.m.) in ice cold Tris-EDTA buffer (Tris-HCl 50 mmol/l, EDTA 2 mmol/l, pH adjusted to 7.4). The tubes were cooled on ice throughout.

Membranes were prepared as follows: the homogenate was centrifuged at 600 *g* for 10 minutes (Hettich Micro 22R), the supernatant was collected and the sediment was re-suspended in buffer and centrifuged again. The second supernatant was collected, mixed with first and centrifuged for 25 minutes at 31,990 *g*. The supernatant was discarded; the sediment was re-suspended in buffer and centrifuged as before (25 minutes, 31,990 *g*).

Because of very limited amount of tissue available, the affinity constants (K_D) were determined on diaphragm muscle and whole brain preparation and were similar as published previously for a given radioligand, tissue and species (Sihver *et al.* 1998; Yu *et al.* 2007). Homogenates were incubated in a single saturating concentration of the radioligand (2,000 pmol/l [^{3}H]bungarotoxin for bungarotoxin-sensitive nicotine receptors, 1,000 pmol/l [^{3}H]cytisine for bungarotoxin-insensitive nicotine receptors) and the B_{max} values were computed from $B_{max}=B\times([L] + K_L) / [L]$, where B=bound radioligand [fmol/mg of protein], L=radioligand concentration [fmol/l], and $K_L=K_d$ [fmol/l] of the radioligand. All tissue samples were processed in triplicates.

Non-specific binding was determined in the presence of 100 µmol/l tubocurarine for bungarotoxin-sensitive receptors and 100 µmol/l nicotine for bungarotoxin-insensitive receptors. Incubation conditions were 120 min at 37 °C for bungarotoxin-sensitive nicotine receptors and 90 min at 4 °C for bungarotoxin-insensitive receptors.

The incubations were terminated by rapid filtration through Whatman GF/B glass fibre filters pre-soaked with 0.5% polyethylenimine in a Brandel cell harvester (Brandel Inc., Gaithersburg, USA). Radioactivity retained on the filters was measured by scintillation counting in Bray's solution after desiccation overnight.

Statistical analysis

Results are presented as mean±S.E.M. and each group represents an average of 5–17 animals. Statistical differences among groups were determined by one-way analysis of variance (ANOVA), and for multiple comparisons an adjusted t-test modified by SNK (Student-Newman-Keuls) correction was used. Values of $p<0.05$ were considered to be significant.

RESULTS

Systemic kainic acid administration

Histological analysis, using Fluoro-Jade B dye, revealed neuronal degeneration in CA1 and CA3 area of the hippocampus in experimental group treated with kainic acid only. Bis-benzimide staining (Hoechst 33342) confirm the presence of apoptotic bodies and condensed nuclei reflecting apoptosis in pyramidal layer of the hippocampal formation. Both blade of dentate gyrus were relatively spared.

Radioligand binding study showed that the densities of α-bungarotoxin sensitive receptors remained unaffected, when compared with any other group. Mutual comparison confirmed significant difference between kainic acid group and experimental group treated with kainic acid and nicotine, when densities of α-bungarotoxin insensitive receptors were analyzed.

Systemic nicotine administration

No Fluoro-Jade B positive cells have been detected in any region of the hippocampus in animals treated by nicotine only. Bis-benzimide staining didn´t visualized

any signs of neither apoptosis nor necrosis. Radioligand binding study showed that treatment with nicotine caused the decline in α-bungarotoxin insensitive receptor densities, when mutualy compared with control group, changes in densities of α-bungarotoxin sensitive receptors weren't detect.

Systemic nicotine and kainic acid administration

Radioligand binding study revealed, that administration of nicotine, kainic acid, or both of these substances doesn't change the densities of α-bungarotoxin sensitive nicotinic receptors. Completely different situation was observed in the part of the binding study, when α-bungarotoxin insensitive receptor densities were determined. Administration of kainic acid (and saline as a pretreatment) brings about higher the receptor density when compared with control group. Administration of nicotine caused the decrease in receptor densities, when compared with control group. Pretreatment of animals with nicotine prevented the effects of kainic acid treatment and returned the values of bungarotixine insetive receptors to the control level.

The neuronal degeneration was present in smaller extend, Fluoro-Jade B positive cells were less numerous and this density decline was predominantly present in CA1 area CA3 area and in the hillus of dentate gyrus.

DISCUSSION

The main aim of our study was to test the effect of repeated nicotine administration on kainic acid induced hippocampal neuronal circuit's degeneration. Kainic acid binds to glutamate non-NMDA receptors and causes massive depolarization of neurons followed by distinct seizure pattern (status epilepticus). Prolonged seizures may finally lead to neurodegeneration and cell death. As confirmed by the methods of immunohistochemistry repeated nicotine administration is capable to attenuate the damage of CA1 and CA3 areas of the hippocampus. These finding are supports by our previous data as well (Riljak *et al.* 2007). In this experiment we decided to administer nicotine in more (and low-concentrated) consecutive doses. This experimental pattern decreases great majority of nicotine side-effects (mortality, breathing pathology), while the protective effect of nicotine is still pronounced. Radiologand binding study revealed following importontant data: administration neither kainic acid nor nicotine brings any change in α-bungarotoxin sensitive receptors. The receptor-densities changes were detect only in the group of α-bungarotoxin insensitive receptors. There is an evidence, that activation of nAChRs is protective against various neurotoxic stimuli (Maggio *et al.* 1998; Marin *et al.* 1994; Riljak *et al.* 2007). Hippocampal nAChRs (present in hippocampal interneurons and pyramidal cells mainly) modulate variety of functions and contribute to synaptic plasticity significantly. From our observations it seems to be very probable, that simple nicotine administration decreases the density of α-bungarotoxin insensitive receptors probably via receptor down-regulation. It has been shown repeatedly that chronic nicotine administration increases the number of neuronal nicotinic receptors (but this does not mean increased level of functions mediated by these receptors), while acute treatment was able to desensitize or decrease the number of nicotinic receptors (for review see Kellar *et al.* 1999). Moreover, not all nicotinic receptor subtypes are affected in the same way by chronic exposure to nicotine (i.e., there are differences between bungarotoxin sensitive and insensitive receptors – see also (Kellar *et al.* 1999)). These data are in

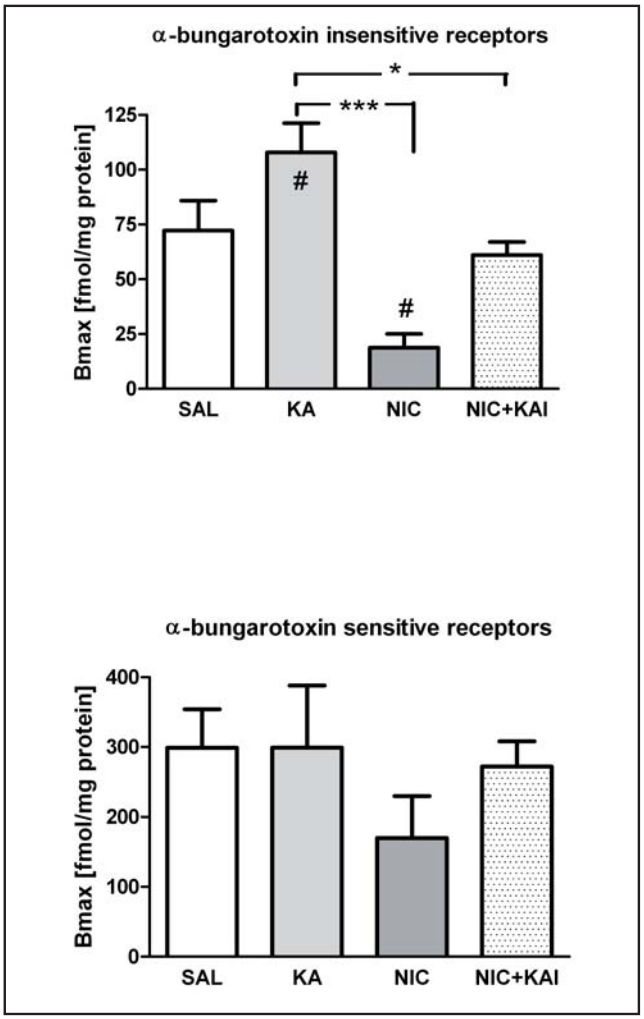

Fig. 1. Changes of receptor binding in hippocampus. Appropriate type of receptor is marked in the heading. Abscissa: Particular experimental group. Ordinate: receptor binding expressed as B_{max} [fmol/mg prot.]. SAL – animals treated with normal saline solution only, KA – animals treated with saline and kainic acid, NIC animals treated with saline and nicotine, NIC+KA – animals treated with nicotine and kainic acid. * indicate differences significant at $p<0.05$ between the particular columns, *** indicate differences significant at $p<0.001$ between the particular columns. # indicates results significant at $p<0.05$ between the saline treated group (control, white column) and kainic acid (nicotine respectively) treated groups. Error bars were calculated as ± SEM.

concordance with our result about different effects of nicotine treatment on α-bungarotoxin sensitive and insensitive receptors.

The data about the effects of kainate on nicotinic receptors are new. There is only one report that describes the effects of nicotine on kainate induced seizures (Shin *et al.* 2007). These authors reported that the effects of nicotine was mediated via α7 (bungarotoxin sensitive) nicotinic receptors rather than α4β2 (bungarotoxin sensitive) nicotinic receptors. But they did not evaluate the binding to these receptors, what can be as discussed by Keller and coworkers (Kellar *et al.* 1999) different from the function.

We can therefore conclude that nicotine has preventive effect on kainite induced cell damage and that this effect is caused by bungarotoxine insentive nicotinic receptors.

ACKNOWLEDGEMENT

This work was supported by grants: GACR 305/09/P136, MSM 00216 208 16, and GACR 309/09/0406.

REFERENCES

1 Ben-Ari Y, Cossart R (2000). Kainate, a double agent that generates seizures: two decades of progress. Trends Neurosci. **23**: 580–587.
2 Borlongan CV, Shytle RD, Ross SD, Shimizu T, Freeman TB, Cahill DW, Sanberg PR (1995). (–)-nicotine protects against systemic kainic acid-induced excitotoxic effects. Exp Neurol. **136**: 261–265.
3 Buckingham SD, Jones AK, Brown LA, Sattelle DB (2009). Nicotinic acetylcholine receptor signalling: roles in Alzheimer's disease and amyloid neuroprotection. Pharmacol Rev. 61: 39–61.
4 Ferrea S, Winterer G (2009). Neuroprotective and neurotoxic effects of nicotine. Pharmacopsych. 42: 255–265.
5 Huettner JE (2003). Kainate receptors and synaptic transmission. Prog Neurobiol. **70**: 387–407.
6 Kaneko S, Maeda T, Kume T, Kochiyama H, Akaike A, Shimohama S, Kimura J (1997). Nicotine protects cultured cortical neurons against glutamate-induced cytotoxicity via alpha7-neuronal receptors and neuronal CNS receptors. Brain Res. **765**: 135–140.
7 Kellar KJ, Dávila-García MI, Xiao Y (1999). Pharmacology of neuronal nicotinic acetylcholine recceptors: effects of acute and chronic nicotine. Nicotine Tob Res. **Suppl 1**: 117–20; discussion S139–40.
8 Kim HC, Jhoo WK, Ko KH, Kim WK, Bing G, Kwon MS, Shin EJ, Suh JH, Lee YG, Lee DW (2000). Prolonged exposure to cigarette smoke blocks the neurotoxicity induced by kainic acid in rats. Life Sci. **66**: 317–326.
9 Maggio R, Riva M, Vaglini F, Fornai F, Molteni R, Armogida M, Racagni G, Corsini GU (1998). Nicotine prevents experimental parkinsonism in rodents and induces striatal increase of neurotrophic factors. J Neurochem. **71**: 2439–2446.
10 Marin P, Maus M, Desagher S, Glowinski J, Prémont J (1994). Nicotine protects cultured striatal neurones against N-methyl-D-aspartate receptor-mediated neurotoxicity. Neuroreport. **5**: 1977–1980.
11 Nai Q, McIntosh JM, Margiotta JF (2003). Relating neuronal nicotinic acetylcholine receptor subtypes defined by subunit composition and channel function. Mol Pharmacol. **63**: 311–324.
12 Pelissier AL, Gantenbein M, Bruguerolle B (1998). Chronopharmacological effects of nicotine repeated administration on heart rate, body temperature and locomotor activity circadian rhythms in rats. Life Sci. 63: 2189–2197.
13 Riljak V, Maresova D, Pokorny J (2010). Nicotine effects on rat seizures susceptibility and hippocampal neuronal degeneration. Neuro Endocrinol Lett. **31**: 792–5.
14 Riljak V, Milotová M, Jandová K, Pokorný J, Langmeier M (2007). Morphological changes in the hippocampus following nicotine and kainic acid administration. Physiol Res. **56**: 641–649.
15 Schmued LC, Hopkins KJ (2000). Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. Brain Res. **874**:123–130.
16 Shin E-J, Chae JS, Jung M-E, Bing G, Ko KH, Kim W-K, Wie MB, Cheon M-A, Nah S-Y, Kim H-C (2007). Repeated intracerebroventricular infusion of nicotine prevents kainate-induced neurotoxicity by activating the α7 nicotinic acetylcholine receptor, Epilepsy Res. **73**: 292–298.
17 Sihver W, Gillberg PG, Nordberg A (1998). Laminar distribution of nicotinic receptor subtypes in human cerebral cortex as determined by [3H](-)nicotine, [3H]cytisine and [3H]epibatidine in vitro autoradiography. Neuroscience **85**:1121–1133.
18 Sperk G (1994). Kainic acid seizures in the rat. Prog Neurobiol. **42**: 1–32.
19 Uchida S, Kagitani F, Nakayama H, Sato A (1997). Effect of stimulation of nicotinic cholinergic receptors on cortical cerebral blood flow and changes in the effect during aging in anesthetized rats. Neurosci Lett. 228: 203–206.
20 Yu WF, Guan ZZ, Nordberg A (2007). Postnatal upregulation of alpha4 and alpha3 nicotinic receptor subunits in the brain of alpha7 nicotinic receptor-deficient mice. Neuroscience **146**: 1618–1628.