

## LACK OF CHANGES IN CORTICAL [<sup>3</sup>H]-MUSCIMOL BINDING IN RATS SENSITIZED TO NICOTINE-INDUCED ENHANCEMENT OF DOPAMINE METABOLISM

*Halina Sienkiewicz-Jarosz<sup>1</sup>, Agnieszka I. Członkowska<sup>2</sup>,  
Małgorzata Lehner<sup>3</sup>, Piotr Maciejak<sup>3</sup>, Marek Siemiątkowski<sup>4</sup>,  
Janusz Szynkler<sup>2</sup>, Aleksandra Wisłowska<sup>2</sup>, Małgorzata Zienowicz<sup>2</sup>,  
Andrzej Bidziński<sup>3</sup>, Wojciech Kostowski<sup>4</sup>, Adam Płaźnik<sup>2,3,#</sup>*

<sup>1</sup>Department of Neurology, <sup>3</sup>Department of Neurochemistry, <sup>4</sup>Department of Pharmacology and Physiology of the Nervous System, Institute of Psychiatry and Neurology, Sobieskiego 9, PL 02-957 Warszawa;

<sup>2</sup>Department of Experimental and Clinical Pharmacology, Medical University, Krakowskie Przedmieście 26/28, PL 00-927 Warszawa, Poland

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It was proposed that chronic nicotine treatment may induce adaptive changes in GABA<sub>A</sub> receptors, thus leading to the attenuation of a GABAergic inhibition of dopaminergic neurons. This putative mechanism might underlie the sensitization to nicotine-induced increase in locomotor activity and dopamine metabolism; i.e. phenomena highly significant to the dependence-producing effects of this psychostimulant. To test this hypothesis, in the present study we have analyzed the influence of acute and repeated treatment of rats with nicotine on the binding of a highly selective and competitive GABA<sub>A</sub> receptor agonist, [<sup>3</sup>H]-muscimol. The binding was investigated by autoradiography in different brain cortical structures. It was found that nicotine given at the dose stimulating locomotor activity (0.6 mg/kg, *sc*), markedly increased striatal HVA concentration in the group of animals chronically pretreated (for 6 days) with this psychostimulant. Neither acute nor repeated nicotine administration changed in a significant way the [<sup>3</sup>H]-muscimol binding to brain cortical structures. Thus, the hypothesis about the role of adaptive changes in GABA<sub>A</sub> receptors in the enhancement of the biochemical and behavioral effects of nicotine was not confirmed.

**Key words:** *nicotine, dopamine, [<sup>3</sup>H]muscimol, autoradiography, microdialysis, behavior, rat*

## INTRODUCTION

It is well recognized that repeated pretreatment with nicotine significantly enhances the locomotor and biochemical effects of subsequent acute administration of this psychostimulant in animals. This phenomenon may be highly significant to the dependence-producing effects of nicotine. Accordingly, it was shown that locomotion stimulating effect of nicotine was substantially enhanced after pretreatment of animals with 12 daily injections of nicotine (0.5 mg/kg, *sc*) [12]. Nicotine pretreatment elevated also dopamine release in the prefrontal cortex, whereas it did not affect this response in the nucleus accumbens [12]. These results were interpreted as demonstrating that the behavioral sensitization after chronic nicotine treatment is accompanied by an enhanced dopamine release specifically within the cortical brain structures. However, subsequent experiments showed that repeated nicotine may enhance dopamine metabolism also in the other brain structures, e.g. the striatum [11].

Numerous reports indicate that dopamine release in different brain structures, including cortex, substantia nigra, hypothalamus, striatum and nucleus accumbens, is under tonic GABAergic inhibition mediated by GABA<sub>A</sub> receptors [2, 3, 5, 15, 16, 18]. Moreover, several recent biochemical and electrophysiological studies have demonstrated that nicotinic agonists stimulate the release of GABA from different brain structures, *via* stimulation of AChRs containing  $\alpha 4\beta 2$  subunits [1, 4, 7–10, 14]. Neuronal nicotinic receptor activation stimulated GABA release from CA1 neurons of the rat hippocampal slices [1], and in the cultured cortical neurons [9]. Therefore, it is conceivable, that an enhanced dopamine release after repeated nicotine treatment may be secondary to some adaptive changes occurring in GABA<sub>A</sub> receptors, due to their persistent stimulation by GABA, leading to the attenuation of a GABAergic inhibition of dopaminergic neurons. This might be an intrinsic mechanism of development of sensitization to the nicotine-induced enhancement of locomotor activity and dopamine release.

To test this hypothesis, in the present study we have analyzed the influence of an acute and repeated treatment of rats with nicotine on the binding of a highly selective and competitive GABA<sub>A</sub> receptor agonist, [<sup>3</sup>H]-muscimol, to the cortical and hippocampal brain structures. Special attention was

paid to the cortical structures, to corroborate and extend the findings reported by Nissel et al. [12] (see above). The effect of nicotine was verified in a behavioral (open field test), and biochemical (*in vivo* microdialysis of striatal HVA concentration, as an indicator of dopamine metabolism), control experiments. It is noteworthy that sensitization of animals to the nicotine-induced locomotor stimulation was recently shown by us after repeated administration of this psychostimulant (0.6 mg/kg, *sc*), using exactly the same experimental procedure [17].

## MATERIALS and METHODS

### Animals

The experiments were carried out on adult male Wistar rats weighing 300–350 g. All animals were acclimatized to their cages for 5 days before surgery. They were housed with water and food *ad libitum* under a 12 h light-dark cycle, and at a controlled temperature (20°C). All experiments were conducted between 10 a.m. and 4 p.m.. The experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609 EEC). All experimental procedures using animal subjects were approved by the Committee for Animal Care and Use at the Institute of Psychiatry and Neurology.

### Nicotine administration

Nicotine-di-tartrate (RBI, Natic, MA, USA) was dissolved in saline and administered *sc* in a volume of 1 ml/kg. Nicotine solutions were adjusted to pH = 7.0–7.2 with diluted NaOH. The animals received a single (0.1 or 0.6 mg/kg, *sc*) or 6 repeated, once-a-day, injections of the drug (0.6 mg/kg, *sc*). The control group received the appropriate volume of saline.

### Open field test

The open field test was performed in a sound-proof chamber under dim light and continuous white noise (65 dB) without previous habituation. The open field apparatus consisted of two round arenas (80 cm in diameter) with 30 cm high walls. During 20 min observation, locomotor activity, the number of central entries and the time spent in the central sector of the open field (50 cm in diameter) were recorded and analyzed with the PC-based

Videomot System (TSE, Bad Homburg, Germany). The parameter of thigmotaxis was calculated as a ratio of the number of entries into central part of testing arena, to the rat locomotor activity and multiplied by 1000, and was designed as an index of emotional behavior [17]. The higher value of the score the lower thigmotaxis and the more pronounced anxiolytic-like effect. Nicotine (0.1 and 0.6 mg/kg, *sc*) was administered acutely 5 min before open field test.

### Microdialysis

After acclimatization, the rats were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and a guide cannula was stereotaxically implanted into the right striatum, according to the coordinates given in the atlas of Paxinos and Watson [13] (0.6 mm anteriorly to the bregma, 3.5 mm laterally to the sagittal suture, 5.5 mm below the dura). The guide cannula was fixed to the skull with jewelry screws and dental acrylic cement. Seven days later the rats were subjected to the microdialysis study.

Two days prior to the dialysis experiment, the rats were transported from their home cages to the room where dialysis experiments took place and were habituated to the experimental setting. On the day of the study, a concentric microdialysis probe (CMA/11, 6 kDa cut-off, O. D. 0.24 mm, CMA/Microdialysis, Sweden) was inserted into the guide cannula. The probe was connected to a microinfusion pump by tubing, and Ringer's solution (in mM: NaCl 147; KCl 4.0; CaCl<sub>2</sub> 2.4) was perfused at a constant rate of 2 µl/min. Following 2 h of perfusion for stabilization, four consecutive samples were collected to measure basal levels of monoamines before nicotine or saline administration. Subsequently, a challenge injection of nicotine (0.6 mg/kg, *sc*) or saline was given. Perfusate samples were collected at 30-min intervals for the next 240 min into 1 ml vials and immediately loaded directly into injector. The extracellular concentrations of HVA (homovanillic acid) were determined by a fully automated high performance chromatography system (HPLC) with electrochemical detection [18].

For histological verification of cannula and probe placement, the brains were removed and stored in 5% formaldehyde solution. The frozen tissue was sectioned into the slices to establish the place of perfusion.

### Autoradiography

A detailed description of the method for receptor autoradiography has been published earlier [6]. The dose of the nicotine (0.6 mg/kg, *sc*) was previously established as effective in behavioral test. Rats were divided into 3 groups: SCH – control rats repeatedly treated with saline, SNA – animals treated repeatedly with saline and given an acute injection of nicotine on the last sixth day, NCH – rats treated repeatedly with nicotine, and given the same drug on the last sixth day. Ten minutes after the last injections, the brains were rapidly removed, frozen in isopentane (–30 to –40°C) and stored at –70°C. The coronal (12 µm) sections were cut on microtome at –20°C, thaw-mounted onto gelatinized glass slides and stored at –20°C until used (1 to 2 days). Forty two and sixty slices from each structure, of control and experimental animals, respectively, were taken for examination. Frozen sections were brought to room temperature 30 min prior to assay. Slides were preincubated in 50 mM Tris-citrate buffer (pH 7.1) for 20 min at 4°C to remove endogenous competitors. Then they were incubated for 40 min at 4°C in the same Tris-citrate buffer supplemented with 10 nM [<sup>3</sup>H]-muscimol (19.1 Ci/mmol, Amersham). Non-specific binding was estimated in the presence of 0.2 mM GABA. The tissues were then rinsed in the cold buffer for 1 min and rapidly in-out dipped in distilled water. The slides were dried under a cold stream of air, placed in X-ray cassettes and exposed to tritium-sensitive film ([<sup>3</sup>H]-Hyperfilm, Amersham) at 4°C together with standards ([<sup>3</sup>H]-microscale, Amersham). After 6-week exposure, the films were developed using Kodak LX-24 film developer, washed in water, and then placed in Kodak fixer. The autoradiograms were analyzed with the image analysis system (Analytical Imaging Station, Imaging Research Inc., St. Catharines, Canada). Optical densities were converted into nCi/mg of tissue equivalent using the standard curve. Non-specific binding of [<sup>3</sup>H]-muscimol was negligible.

### Statistical analysis

The open field data are shown as means ± SEM. They were analyzed by one-way ANOVA, followed by Newman-Keuls post hoc test. Microdialysis data were calculated as percent changes in relation to baseline levels according to the following scheme: the average of the four samples preceding a challenge injection of nicotine or saline

was defined as 100% and used as a baseline for the following 12 samples. Two-way ANOVA for repeated measures was conducted, with the used drug considered as between-subject measure and time course as within-subject measure. The ANOVA was followed by Newman-Keuls post-hoc test, when appropriate. The autoradiographic data are shown as mean  $\pm$  SEM, and were analyzed using one-way ANOVA; *p* values of less than 0.05 were considered to be significant.

## RESULTS

Nicotine dose-dependently increased rat locomotor activity in the open field test ( $F_{2,21} = 9.74$ ,  $p = 0.001$ ) (Tab. 1). Post-hoc analysis revealed the significant effect of the drug at the dose of 0.1 mg/kg ( $p < 0.05$ ) and 0.6 mg/kg ( $p < 0.01$ ). Nicotine increased the number of central entries ( $F_{2,21} = 3.85$ ,  $p = 0.037$ ), time spent in the central sector ( $F_{2,21} = 5.26$ ,  $p = 0.01$ ), and it decreased the thigmotaxis ( $F_{2,21} = 3.27$ ,  $p = 0.05$ ). These effects reached the level of significance after the higher dose of nicotine (0.6 mg/kg) (central entries,  $p < 0.05$ ; time spent in the central sector,  $p < 0.01$ ; and the anti-thigmotactic effect,  $p < 0.05$ ).

Nicotine administered acutely (0.6 mg/kg, *sc*) did not produce significant changes in the concentration of HVA (treatment,  $F_{1,6} = 2.58$ ,  $p = 0.16$ ; time,  $F_{11,66} = 1.44$ ,  $p = 0.17$ ; interaction,  $F_{11,66} = 0.96$ ,  $p = 0.48$ ) (Fig. 1). After repeated treatment of rats with nicotine, there appeared a significant effect of a nicotine challenge on the HVA concentration in the rat striatum (treatment,  $F_{1,6} = 17.6$ ,  $p = 0.005$ ; time,  $F_{11,66} = 1.53$ ,  $p = 0.14$ ; interaction,  $F_{11,66} = 2.29$ ,  $p = 0.02$ ) (Fig. 1). Post-hoc analysis revealed that nicotine significantly increased striatal HVA level ( $p < 0.01$ ) at the 390th minute of

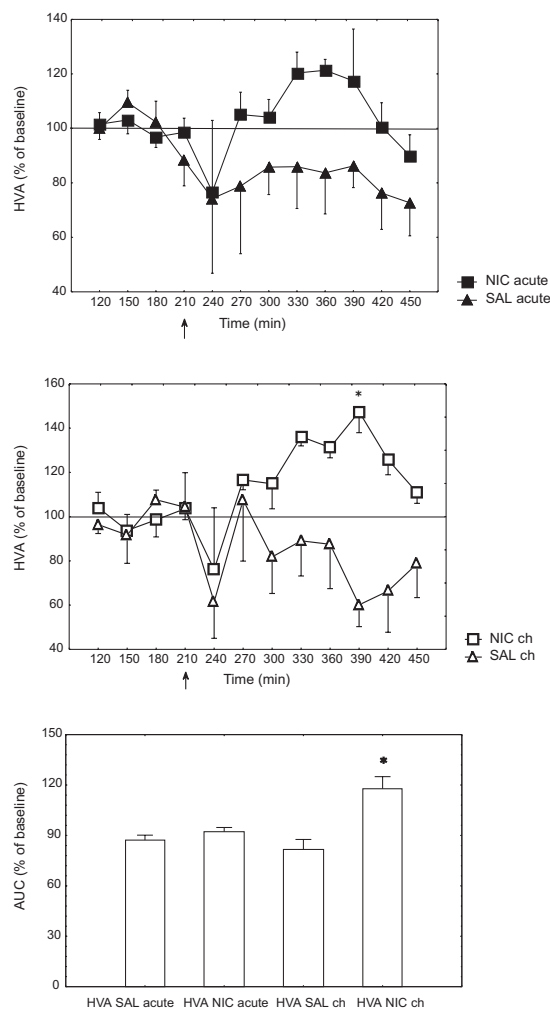


Fig. 1. Changes in extracellular concentrations of HVA (homovanillic acid) in the rat striatum after a single (acute) or repeated (ch) six daily injections of nicotine (NIC, 0.6 mg/kg) or saline (SAL). Nicotine or saline were injected *sc* at 210 min (the arrow), after four basal microdialysate samples were collected. The figure shows time-course and the cumulative effect (HVA, over a period of 240–450 min after drug injection), expressed as an area under the curve (AUC). Data are expressed as percent changes in relation to baseline levels. Results are shown as means  $\pm$  SEM. \*  $p < 0.01$  differs from appropriate control group ( $n = 4$  rats per group)

Table 1. The influence of acute nicotine administration on motor activity, number of entries into the central part of the open field, time spent in the central sector and the anti-thigmotactic effect (calculated as a ratio of the number of entries into the central part of the open field to the rat locomotor activity, and multiplied by 1000). Nicotine (0.1 and 0.6 mg/kg, *sc*) was administered acutely 5 min before open field test. The anti-thigmotactic ratio was calculated for each rat separately and then the mean value for each experimental group was computed. The data are shown as means  $\pm$  SEM. N – number of rats. \*  $p < 0.05$ , \*\*  $p < 0.01$  vs. control group

Group	N	Motor activity	Entries into central sector	Time spent in central sector	Anti-thigmotactic effect
Control	8	4508 $\pm$ 240	1.7 $\pm$ 0.7	3.5 $\pm$ 1.1	3.51 $\pm$ 1.42
Nicotine 0.1 mg/kg	8	6128 $\pm$ 603*	6.5 $\pm$ 2.5	11.8 $\pm$ 4.8	9.36 $\pm$ 3.22
Nicotine 0.6 mg/kg	8	7530 $\pm$ 531**	10.2 $\pm$ 2.7*	36.3 $\pm$ 11.8**	12.92 $\pm$ 2.88*

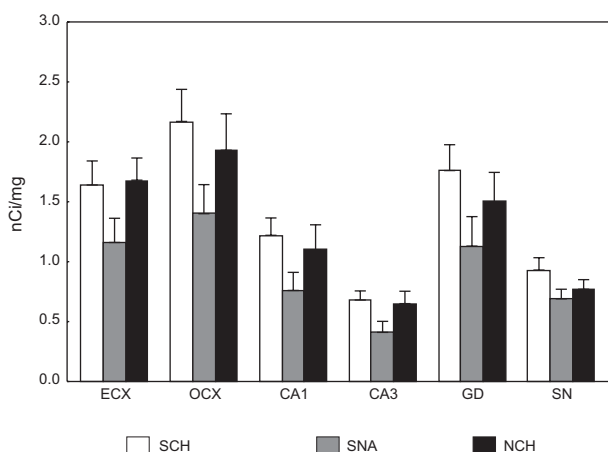


Fig. 2. Binding of [<sup>3</sup>H]-muscimol to the GABA<sub>A</sub> receptors in different brain structures after pretreatment of rats with nicotine. The data are shown as means ± SEM in nCi/mg. The number of rats in each experimental group varied from 7 to 9. ECX – entorhinal cortex, OCX – occipital cortex, CA1, CA3 – regions of the hippocampal formation, GD – dentate gyrus, SN – substantia nigra; SCH – control rats treated repeatedly with saline, SNA – animals treated repeatedly with saline and given an acute injection of nicotine (0.6 mg/kg) on the last sixth day, NCH – rats treated repeatedly with nicotine (0.6 mg/kg), and given the same drug (0.6 mg/kg, *sc*) on the last 6th day

microdialysis, in comparison with the rats treated repeatedly with saline and given an acute injection of a solvent. Accordingly, ANOVA showed a significant effect of experimental conditions on the total concentrations of HVA (AUCs) ( $F_{1,12} = 4.33$ ,  $p = 0.02$ ), with the strongest influence of a nicotine challenge in the group of the drug-pretreated animals ( $p = 0.02$ , vs. saline injected animals). The time-course and the cumulative effect for HVA presented as AUC ( $p < 0.05$ ) (lower part of the figure) are shown in Figure 1.

It was also shown that neither acute nor chronic treatment of animals with nicotine significantly changed [<sup>3</sup>H]-muscimol binding to the CA1 ( $F_{2,21} = 2.18$ ,  $p = 0.14$ ), the CA3 ( $F_{2,20} = 2.61$ ,  $p = 0.1$ ) regions of the hippocampus, the dentate gyrus ( $F_{2,21} = 1.88$ ,  $p = 0.18$ ), the occipital cortex ( $F_{2,21} = 2.21$ ,  $p = 0.13$ ), the entorhinal cortex ( $F_{2,20} = 2.00$ ,  $p = 0.16$ ), and the substantia nigra of the rat brain ( $F_{2,18} = 1.6$ ,  $p = 0.23$ ) (Fig. 2).

## DISCUSSION

The main finding of the present study is that repeated pretreatment of animals with nicotine did not change the specific binding of [<sup>3</sup>H]-muscimol

to the GABA<sub>A</sub> receptors in the hippocampus and cortex. There was only a tendency to decrease [<sup>3</sup>H]-muscimol binding after a single injection of the psychostimulant, corresponding with a short-term decrease in striatal HVA concentrations. This phenomenon occurred exactly at the point of time when the animals were sacrificed after nicotine administration for autoradiographic study.

Thus, the hypothesis about the role of adaptive changes in cortical GABA<sub>A</sub> receptors in the potentiation of the central effects of nicotine, was not confirmed. These negative findings were obtained in the brain structures considered important for the dependence-producing effects of nicotine, and where nicotine had been demonstrated to potently induce a release of GABA and dopamine (see Introduction). Nicotine was administered at the dose (0.6 mg/kg, *sc*) which significantly stimulated animal behavior. Moreover, repeated administration of nicotine at the same dose significantly stimulated striatal dopamine metabolism. Accordingly, the concentration of a main metabolite of dopamine, homovanillic acid, was significantly increased after injection of a challenge dose of nicotine only in the group of animals pretreated repeatedly with this psychostimulant. This observation indicates an enhancement of the effect of nicotine on dopamine metabolism. Recently, it was found by us using exactly the same experimental procedure, that repeatedly administered nicotine (0.6 mg/kg, *sc*) also significantly enhanced the drug-induced locomotor stimulation [17]. These findings show that the dose and the schedule of nicotine administration were sufficient to augment the effects of the psychostimulant.

Summing up, the role of cortical GABA<sub>A</sub> receptors in the enhancement of nicotine-induced central effects is not supported by our results. It is possible that other brain structures play a key role in the interaction between the GABAergic and dopaminergic systems, contributing to the central effects of nicotine.

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