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Zaniewska, M., Alenina, N., Wydra, K., Fröhler, S., Kuśmider, M., McCreary, A. C., Chen, W., Bader, M. and Filip, M. (2015), Discovering the mechanisms underlying serotonin (5-HT)2A and 5-HT2C receptor regulation following nicotine withdrawal in rats. Journal of Neurochemistry, 134: 704–716. doi: 10.1111/jnc.13192

which has been published in final form in:

Journal of Neurochemistry
2015 Jul 26; 134(4): 704-716
doi: 10.1111/jnc.13192
Publisher: Wiley-Blackwell

Article Type: Original Article

Discovering the mechanisms underlying serotonin (5-HT)$_{2A}$ and 5-HT$_{2C}$ receptor regulation following nicotine withdrawal in rats

Magdalena Zaniewska[1,2*], Natalia Alenina[2], Karolina Wydra[1], Sebastian Fröhler[3], Maciej Kuśmider[4], Andrew C. McCreary[5], Wei Chen[3], Michael Bader[2], Małgorzata Filip[1]

[1]*Laboratory of Drug Addiction Pharmacology, Department of Pharmacology, Institute of Pharmacology, Polish Academy of Sciences, Smętna 12, 31-343 Kraków, Poland;*

[2]*Molecular Biology of Peptide Hormones, Department of Cardiovascular Research, Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Str. 10, 13125 Berlin, Germany;*

[3]*Laboratory for New Sequencing Technology, Berlin Institute for Medical Systems Biology, Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Str. 10, 13125 Berlin, Germany;*

[4]*Laboratory of Biochemical Pharmacology, Department of Pharmacology, Institute of Pharmacology, Polish Academy of Sciences, Smętna 12, PL31-343 Kraków, Poland;*

[5]*Brains-On-Line, PO Box 4030, 9701 EA, Groningen, The Netherlands*

***Corresponding author:** Magdalena Zaniewska, Laboratory of Drug Addiction Pharmacology, Department of Pharmacology, Institute of Pharmacology, Polish Academy of Sciences, Smętna 12, 31-343 Kraków, Poland; Tel: +48 12 6623268; Fax: +48 12 6374500; E-mail: zaniew@if-pan.krakow.pl

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/jnc.13192

Running title: Nicotine withdrawal and 5-$HT_{2A/2C}$ receptors

Keywords: 5-HT_2 receptor autoradiography; deep sequencing; nicotine withdrawal; rat; real time PCR

Abbreviations: 5-HT, serotonin; 5-HT_{2A} receptor, serotonin 2A receptor; 5-HT_{2C} receptor, serotonin 2C receptor; NS, non-specific binding; T – total binding

Discovering the mechanisms underlying serotonin (5-HT)$_{2A}$ and 5-HT_{2C} receptor regulation following nicotine withdrawal in rats

Abstract

We have previously demonstrated that nicotine withdrawal produces depression-like behavior and that serotonin (5-HT)$_{2A/2C}$ receptor ligands modulate that mood-like state. In the present study we aimed to identify the mechanisms (changes in radioligand binding, transcription or RNA-editing) related to such a behavioral outcome. Rats received vehicle or nicotine (0.4 mg/kg, sc) for 5 days in home cages. Brain 5-$HT_{2A/2C}$ receptors were analyzed on day 3 of nicotine withdrawal. Nicotine withdrawal increased [^{3}H]ketanserin binding to 5-HT_{2A} receptors in the ventral tegmental area and ventral dentate gyrus, yet decreased binding in the nucleus accumbens shell. Reduction of [^{3}H]mesulergine binding to 5-HT_{2C} receptors was seen in the ventral dentate gyrus. Profound decrease in the 5-HT_{2A} receptor transcript level was noted in the hippocampus and ventral tegmental area. Out of five 5-HT_{2C} receptor mRNA editing sites, deep sequencing

data showed a reduction in editing at the E site and a trend toward reduction at the C site in the hippocampus. In the ventral tegmental area, a reduction for the frequency of CD 5-HT_{2C} receptor transcript was seen. These results show that the reduction in the 5-HT_{2A} receptor transcript level may be an auto-regulatory response to the increased receptor density in the hippocampus and ventral tegmental area during nicotine withdrawal, while decreased 5-HT_{2C} receptor mRNA editing may explain the reduction of receptor labelling in the hippocampus.

Introduction

Nicotine is one of the major psychoactive constituents of tobacco smoke that has a strong abuse potential in humans and laboratory animals. The most common withdrawal symptoms in humans include nicotine craving, irritability, anxiety, difficulty of concentration, increased appetite and depressed mood (reviewed by Kenny and Markou, 2001; Markou, 2008). Prevention of the latter mood state has become an important issue in the management of nicotine-use or addiction.

Many pharmaco-behavioral studies have shown that withdrawal from chronic nicotine exposure produced depression-like behavior in mice (15-day voluntary drug intake or passive drug administration) and rats (experimenter-delivered drug for 5 days), as measured by exaggerated immobility time in the forced swim test (Mannucci et al., 2006; Ribeiro-Carvalho et al., 2011; Zaniewska et al., 2010). The above data also showed that such behavioral sequelae were either completely diminished by the 5-HT precursor 5-hydroxytryptophan, or were attenuated by 5-HT_{2A} receptor antagonists or 5-HT_{2C} receptor agonists (Mannuci et al., 2006; Zaniewska et al., 2010). During withdrawal from repeated nicotine exposure (delivered by the experimenter or through osmotic mini-pumps) profound changes in the 5-HT system (i.e., significant reductions in brain 5-HT content and/or neurotransmitter turnover) have been found

(Benwell and Balfour, 1979; Mannuci et al., 2006; Slotkin and Seidler, 2007; Yasuda et al., 2002) and were further linked to the functional supersensitivity of 5-HT_{2A} (Suemaru et al., 2001; Zaniewska et al., 2009) and 5-HT_{2C} receptors (Zaniewska et al., 2010).

5-HT_{2A} and 5-HT_{2C} receptor-mediated neurotransmission can be regulated at multiple levels, including transcription (alterations at the transcription level), post-transcriptional RNA processing (alternative splicing), post-translational modifications, trafficking and protein complex formation. Additionally, the expression of the 5-HT_{2C}, but not the 5-HT_{2A}, receptor gene can be post-transcriptionally regulated by RNA editing (Fig. 1) (Burns et al., 1997; Niswender et al., 1998). Importantly, the 5-HT_{2C} receptor is the only known G protein coupled receptor that was shown to undergo editing. In this process, adenosine deaminases acting on RNA (ADARs) convert one or more of five closely spaced adenosine residues (termed A, B, E, C and D editing sites) into inosines which are read as guanosines by the translational machinery. Changes in gene-encoded Ile, Asn and Ile (INI) at positions 156, 158 and 160 (in humans), located within the second intracellular loop of the receptor, may lead to the generation of up to 24 protein isoforms. These RNA-edited 5-HT_{2C} receptor isoforms have differential distribution patterns throughout the brain and, as evidenced by *in vitro* or *in vivo* studies, may have very different receptor function, including changes in constitutive activity, as well as altered agonist potency, ligand binding affinity and selectivity of G-protein coupling (Berg et al., 2001; Burns et al., 1997; Fitzgerald et al., 1999; Herrick-Davis et al., 1999; Niswender et al., 1999; Olaghere da Silva et al., 2010; Price et al., 2001; Wang et al., 2000). Furthermore, editing has been shown to affect alternative splicing (Martin et al., 2013) or drive receptor localization, *i.e.*, enhance cell surface expression of highly edited 5-HT_{2C} receptors (VGV isoform) by modulating intracellular trafficking efficacy (Marion et al., 2004).

Studies in humans have revealed a link between changes in 5-HT_{2C} receptor RNA editing and psychiatric disorders, including depression (Gurevich et al., 2002b; Iwamoto and Kato, 2003; Lyddon et al., 2013). Similarly, a modified profile of RNA-edited receptor isoforms was reported in rodents with depression-like phenotypes or reduced brain 5-HT levels which are regarded as core to depression (Bhansali et al., 2007; Gurevich et al., 2002a; Iwamoto et al., 2005). To date, the impact of nicotine withdrawal-induced depression-like behavior on 5-HT_{2C} receptor editing has not yet been studied.

Taken together, the behavioral and biochemical data prompted us to verify the hypothesis that exposure to nicotine followed by withdrawal induces changes in the binding pattern and gene expression of 5-HT_{2A} and 5-HT_{2C} receptors. We therefore administered nicotine for five days and on day 3 of drug withdrawal animals were sacrificed and changes in brain 5-HT_{2A} and 5-HT_{2C} receptors were analyzed. We have previously demonstrated that 5-day nicotine injections induced behavioral sensitization and conditioned locomotor activity in rats and such a dosing schedule has been shown to produce changes in the 5-HT_{2A} and 5-HT_{2C} receptor labeling in the brain (Zaniewska et al., 2015). The same treatment was accompanied by increased BOLD response in the brain regions related to drug addiction, including the hippocampus, nucleus accumbens, prefrontal cortex or ventral tegmentum (Li et al., 2008) or induced dopamine release in the nucleus accumbens in rats (Rahman et al., 2007) similarly as tobacco smoking in humans (Cosgrove et al., 2014). Thus, this animal model serves as a relevant initial screening method not only for evaluating the potential therapeutic efficacy of novel drugs, but also for evaluating the cellular and molecular changes following exposure to nicotine. The early withdrawal period was chosen based on previous data, demonstrating maximal depression-like effects (an increase in

immobility time in the forced swim test) on this day of drug cessation and efficacy of 5-$HT_{2A/2C}$ receptor ligands in attenuating this mood-like state (Zaniewska et al., 2010). Here we enrich the previously published behavioral data (Zaniewska et al., 2010) from day 3 of nicotine withdrawal by showing other parameters: swimming and climbing. Receptor autoradiography was analyzed in several brain areas (Fig. 2A) in which 5-HT_{2A} or 5-HT_{2C} receptors are known to be present (López-Giménez et al., 2002) and/or that are essential in the process of nicotine withdrawal (Malin and Goyarzu, 2009). The transcript level of 5-HT_{2A} receptors was analyzed only in these brain structures (the hippocampus and ventral tegmental area) in which the robust (> 50%) changes in radioligand binding were reported. Analogous brain areas were chosen to assess 5-HT_{2C} receptor mRNA level and editing. Both these brain regions are implicated in nicotine dependence and depression-like behavior in rodents (Cohen et al., 2015; Drevets et al., 2008; Malin and Goyarzu, 2009). In addition, in the hippocampus we noted significant changes in radioligand binding to 5-HT_{2C} receptors. Transcript level was analyzed by real time PCR. Deep sequencing technology was used to gain information about the 5-HT_{2C} receptor mRNA editing.

Material and Methods

Animals

Male Wistar rats (Charles River, Germany) weighing 220-250 g (9 weeks old) at the beginning of the experiment were used. The animals were housed 4-7 per cage (58 cm x 37 cm x 20 cm) in a colony room maintained at 21 ± 1ºC and 40-50% humidity under a 12-h light-dark cycle (lights on at 6:00). Rodent chow and water were available *ad libitum*. All the experiments were conducted during the light phase of the light-dark cycle (8:00-14:00), in accordance with *The*

National Institutes of Health Guide for the Care and Use of Laboratory Animals and the approval of the Bioethics Commission (at the Institute of Pharmacology Polish Academy of Sciences) and compliant with the Polish Law (August 21, 1997). All the efforts were made to minimize animal suffering and to reduce the number of rats used.

Repeated nicotine administration

Drugs

(-)-Nicotine bitartrate (Sigma-Aldrich, USA) was dissolved in 0.9% saline. The pH was adjusted to 7.0 using 20% NaOH. Drug was injected subcutaneously in a volume of 1 ml/kg. The dose of nicotine (expressed as that of the free base) and pretreatment time were optimized in our laboratory, as described previously (Zaniewska et al., 2010).

Measurement of depression-like behavior in animals withdrawn from nicotine

Rats were given nicotine (0.4 mg/kg) or vehicle (1 ml/kg) for 5 days in home cages. The forced swim test was performed on day 3 of nicotine withdrawal.

Forced swim test

On the first day of forced swim test, rats withdrawn from nicotine were placed individually in a cylinder (50-cm high and 23 cm in diameter) filled with 30 cm of water (25 ± 1°C) for 15 min (the pre-test). They were then removed, dried with towels, and placed in a warmer enclosure for 15 min before they were returned to their home cages. The cylinders were emptied and cleaned between rats. 24 h after the pre-test, the rats were retested for 5 min under identical conditions. Two blinded observers scored each retest session. The following parameters were measured manually: the immobility, swimming and climbing. A rat was rated to be immobile if it was

making only movements necessary to keep its head above water; swimming was recorded if a rat was actively making swimming movement that caused it to move within the center of cylinder and swim below the surface of water (diving); climbing behavior was recorded if a rat was making forceful thrashing movements with its forelimbs against the walls of cylinder.

Withdrawal from chronic nicotine treatment

Animals were given nicotine (0.4 mg/kg) or vehicle (1 ml/kg) for 5 days in home cages. Following 3 days of nicotine withdrawal, animals were killed by decapitation and their brain 5-$HT_{2A/2C}$ receptors were analyzed by receptor autoradiography, real time PCR or deep sequencing.

Quantitative receptor autoradiography

Immediately after decapitation, the brains were dissected, frozen on dry-ice and stored at -70°C until use. Frozen brains were cut on a cryostat (Leica Cm 1850, Germany) at -22 ± 1°C. 12-µm sections were then mounted on a single gelatin-coated slide (5 coronal sections per each slide; Fig. 2A) and kept at -20°C until assayed. Autoradiographic analyses were performed according to the method of Syvälahti et al. (2006) with certain modifications (Zaniewska et al., 2015). Briefly, the 5-HT_{2A} receptor antagonist [ethylene-^{3}H]ketanserin hydrochloride (Perkin Elmer, USA; 1 nM; s.a.: 67.0 Ci/mmol) and the 5-HT_{2C} receptor inverse agonist [N^6-methyl-^{3}H]mesulergine (Amersham Biosciences; 4.2 nM; s.a.: 67.0 Ci/mmol) were used as radioligands. The slides were first pre-incubated in Buffer A (170 mM Tris-HCl; Sigma-Aldrich; pH 7.5). Next, the incubation was performed in Buffer A containing the appropriate radioligand and the blocking substance I (prazosin hydrochloride (Tocris Bioscience, UK; 1 µM; to block [^{3}H]ketanserin binding to α_1-adrenoceptors) or spiperone hydrochloride (Sigma-Aldrich; 100

nM; to block [^{3}H]mesulergine binding to 5-HT$_{2A}$ receptors)) (total binding: T). The non-specific binding (NS) was quantified by incubating separate sections in Buffer A with the appropriate radioligand, blocking substance I, and blocking substance II (M100,907 (Abbott Healthcare Products B.V., The Netherlands; 5 μM; 5-HT$_{2A}$ receptor autoradiography) or methysergide maleate (Tocris Bioscience; 5 μM; 5-HT$_{2C}$ receptor autoradiography)). After the incubation, all slides were washed in Buffer A and then in distilled water.

Radiolabeled dried tissue sections were exposed to tritium-sensitive screens (The Bass IP-TR, 20 cm x 25 cm, Fujifilm, Japan) in photosensitive cassettes (The Bass IP Cassettes, 20 cm x 25 cm, Fujifilm) together with the autoradiographic [^{3}H]microscale (Amersham Biosciences). Images were read by Image Reader v.1.1 (The Bass-5000 IP, Fujifilm). Quantitative analysis of autoradiograms was performed using Multi Gauge 3.0 software (Fujifilm). Optical densities of grey values on the film (relative optical density) were converted into bound radioactivity using the standard polynomial regression curve as a standard. Optical densities, which were the differences between the optical density of T and NS binding, were expressed as nCi/mg tissue relative to the specific activity of the appropriate radioligand. Changes in receptor labeling are expressed in fmol of bound radioligand per mg of tissue (± SEM) and are also presented as the mean percentage of changes with relation to the control value (100%).

Brain structures were identified by a comparison of autoradiographic images with appropriate figures from the rat brain atlas of Paxinos and Watson (1998). Measurement of the optical densities of 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors was performed on both sides of the frontal cortex, prefrontal (cingulate, infralimbic and prelimbic) cortex, claustrum, dorsal striatum, nucleus accumbens, hippocampus (ventral dentate gyrus) and ventral tegmental area, and additionally – in the case of 5-HT$_{2C}$ receptors – the lateral septum, thalamus, amygdala,

substantia nigra and choroid plexus (Fig. 2B, C). Only the data from these brain structures in which significant changes in 5-HT_{2A} and 5-HT_{2C} receptor labeling were noted following withdrawal from chronic nicotine treatment are shown in the paper (Fig. 2A).

Sample preparation for transcript analyses

The transcript level of 5-HT_{2A} and 5-HT_{2C} receptors and editing status of 5-HT_{2C} receptor mRNA were analyzed in the hippocampus and ventral tegmental area. Immediately after the end of behavioral procedure (the same nicotine dosing schedule as for autoradiography analysis), animals were killed by decapitation and the brains were rapidly dissected and chilled in ice-cold saline. The brain was positioned ventral side up and a coronal cut was made at the level of the mammillary nuclei (its posterior end; approximately 5.6 mm posterior to bregma). The ventral tegmental area was dissected out by making a ca. 1.5-mm punch (around 0.5 to 2.0 mm dorsal to the ventral edge of the anterior brain portion). Next, the hippocampus (including dentate gyrus, CA3, CA2, CA1 and subiculum) was dissected out. Tissues were frozen on dry ice and stored at -80°C until use. Total RNA was isolated from the above brain areas using TRIzol Reagent (Invitrogen, Germany). The RNA quality and concentration of all samples were assessed by NanoDrop^{TM} 1000 spectrophotometer (Peqlab, Erlangen, Germany). Due to different RNA concentrations in the hippocampus and ventral tegmental area, either 1.0 or 0.2 µg, respectively, of isolated RNA was treated with DNAse I (Roche Life Science, Indianapolis, USA) and reverse transcribed using random hexamer primers and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV; Invitrogen).

Real Time PCR

mRNA expression analysis was performed using Real Time PCR with SYBR Green I detection kit (Qiagen, Hilden, Germany) with the following primers: 5-HT_{2A} receptor (NM_017254.1): HT2A5 – 5'-AACGGTCCATCCACAGAG-3'; HT2A3 – 5'-AACAGGAAGAACACGATGC-3' (109-bp amplicon size; Shi et al., 2008); 5-HT_{2C} receptor (NM_012765): HT2C5 – 5'-ATCATGGCAGTAAGCATGG-3'; HT2C3 – 5'-ATCTTCATGATGGCCTTAGTCCG-3' (302-bp amplicon size; Dracheva et al., 2009); β-actin (NM_031144): β-Act fw – 5'-TACAATGAGCTGCGTGTG-3'; β-Act rev – 5'-CACAGCCTGGATGGCTAC-3'; 147-bp amplicon size). All the reactions were run in triplicates using iCycler iQ5 (BIO-RAD, USA). A negative control lacking cDNA was included for each primer pair. The $2^{-\Delta\Delta CT}$ method was applied to analyze the relative changes in 5-HT_{2A} and 5-HT_{2C} receptor gene expression (Livak and Schmittgen, 2001), normalized to the mRNA level of housekeeping gene, β-actin. Results are shown as $2^{-\Delta\Delta C_T}$ means of the tested gene.

Deep sequencing

Quantification of 5-HT_{2C} receptor mRNA variants was done using deep sequencing. The edited region of 5-HT_{2C} receptor was amplified by PCR using Taq DNA Polymerase (Invitrogen) with the following primers: forward primer HT5C51 – 5'-XXXXXXTTTTCAACTGCGTCCATCAT GCACCT-3', reverse primer HT2C3 – 5'-ATCTTCATGATGGCCTTAGTCCG-3'. For each of 16 conditions (2 treatments: vehicle/nicotine, 4 animals per each treatment group, 2 brain areas: the hippocampus/ventral tegmental area) a different six nucleotide barcode (XXXXXX, e.g., ACTGAC, CTGACA, TGACAC) at 5' end of the forward primer was used to differentiate

between conditions. PCR products (122 bp in length) were purified by centrifugation using Wizard SV gel and PCR Clean Up System (Promega, Madison, USA). The quality and concentration of PCR amplicons were assessed by NanoDrop^{TM} spectrophotometer. 200 ng of each barcoded PCR product were pooled and a standard Illumina TruSeq DNA sequencing library was created for the barcoded pool of the 16 PCR amplicons of the targeted 5-HT_{2C} receptor editing region. The library was sequenced for 2x101 nt paired-end on an Illumina HiSeq 2000. Due to the short amplicon length, overlaps between forward and reverse read of a fragment were resolved using ea-utils (Aronesty, 2011) by merging forward and reverse read for each of the ~180 million fragments into combined reads of average length 122nt. Merged, demultiplexed reads were mapped onto a 5-HT_{2C} receptor transcript reference (5'-TTTTCAACTGCGTCCATC ATGCACC T C T G CG CCATATCGCTGGACCGGTATGTAGCAATACGTAATCCTATTG AGCATAGCCGGTTCAATTCGCGGACTAAGGCCATCATGAAGAT-3') using Burrows-Wheeler Alignment (BWA) without seeding, allowing for up to 4 mismatches. Mappings were converted into the Sequence Alignment/Map (SAM) format using SAMtools (Li and Durbin, 2009; Li et al., 2009). A custom perl script was used to obtain amplicon counts and to assess position specific editing for each of the amplicons.

The following editing parameters were calculated: the level of site-specific editing of the 5-HT_{2C} receptor mRNA (%), the frequency of observed 5-HT_{2C} receptor mRNA variants (%) and the frequency of predicted 5-HT_{2C} receptor protein isoform (%).

Statistical Analysis

Data are expressed as means (± SEM). Comparisons between means representing changes from control values were made using Student's *t*-test. All comparisons were made with an experiment

wise type I error rate (α) set at $p < 0.05$.

Results

Nicotine withdrawal increases immobility time and reduces swimming behavior in rats withdrawn from nicotine

On day 3 of withdrawal from repeated (for 5 days) nicotine treatment a statistically significant increase in immobility time ($p < 0.001$) and decrease in swimming behavior ($p < 0.05$) was observed (Fig. 3).

Nicotine withdrawal alters 5-HT_{2A} and 5-HT_{2C} receptor binding in various brain structures

We first analyzed the effect of nicotine withdrawal on radioligand binding to 5-HT_{2A} and 5-HT_{2C} receptors in brains of rats repeatedly (for 5 days) treated with vehicle or nicotine.

[^{3}H]ketanserin binding to 5-HT_{2A} receptors in animals withdrawn from repeated nicotine administration

In control animals receiving vehicle repeatedly, [^{3}H]ketanserin binding to 5-HT_{2A} receptors in different areas of the rat brain varied from 3.7 to 57 fmol/mg tissue, with the maximum radioligand binding seen in the prefrontal cortex (Tab. 1).

On day 3 of nicotine withdrawal, an increase in [^{3}H]ketanserin binding to 5-HT_{2A} receptors was seen in the ventral tegmental area (81%) and ventral dentate gyrus (49%), and a decrease in the labelling of 5-HT_{2A} receptors appeared in the nucleus accumbens shell (39%)

(Tab. 1).

[^{3}H]mesulergine binding to 5-HT$_{2C}$ receptors in animals withdrawn from repeated nicotine administration

In animals receiving vehicle, [^{3}H]mesulergine binding to 5-HT$_{2C}$ receptors in different areas of the rat brain varied from 7.5 to 234 fmol/mg tissue, with the maximum radioligand binding seen in the choroid plexus (Tab. 2).

On day 3 of nicotine withdrawal, a lower (33%) level of 5-HT$_{2C}$ receptor labelling was observed in the ventral dentate gyrus, but higher (by 17%) levels of binding were seen in the choroid plexus (Tab. 2).

Nicotine withdrawal changes the expression levels of 5-HT$_{2A}$ receptors, but not 5-HT$_{2C}$ receptors

We next evaluated whether the nicotine withdrawal changes the expression levels of 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors in the hippocampus and ventral tegmental area.

The expression level of 5-HT$_{2A}$ receptor gene measured by real time PCR on day 3 of withdrawal from repeated nicotine administration significantly decreased in the whole hippocampus (62%) and ventral tegmental area (57%) (Fig. 4A, B).

No change in the level of mRNA encoding for 5-HT$_{2C}$ receptors was observed in the whole hippocampus or ventral tegmental area in rats withdrawn from chronic nicotine administration (Fig. 5A, B).

Nicotine withdrawal affects 5-HT_{2C} receptor mRNA editing

We assessed whether the editing of 5-HT_{2C} receptor mRNA in the hippocampus and ventral tegmental area is altered by nicotine withdrawal.

Deep sequencing analysis of 5-HT_{2C} receptor mRNA editing in rats treated repeatedly with vehicle revealed that in the whole hippocampus and ventral tegmental area the highest frequency (12-21%) was detected for ABD, AB, ABE and ABCD receptor mRNA variants (Tabs. 3, 4). Out of the 24 predicted protein isoforms, the most abundant in either of the above brain structures were VNV, VNI, VDI and VSV 5-HT_{2C} receptor isoforms (Tabs. 3, 4). Editing was significantly lower at the A (8%; $p = 0.028$) and B (9%; $p = 0.036$) site in the hippocampus compared with the ventral tegmental area (Fig. 6A, B).

Repeated treatment with nicotine followed by a three-day withdrawal period produced a statistically significant decrease (30%; $p = 0.043$) in the editing level at the E site in the hippocampus; a trend toward reduction (5%; $p = 0.063$) was also noted at the C editing site (Fig. 6A). At the same time, the frequency of EC, CD and ECD 5-HT_{2C} receptor mRNA variants (corresponding to IGI, ISV and IGV protein isoform, respectively) was decreased (by 19, 32 and 42%, respectively) in nicotine withdrawn animals as compared to control group, but this did not reach statistical significance ($p = 0.14$, 0.38 and 0.25, respectively) (Tab. 3).

In the ventral tegmental area of nicotine withdrawn animals, the frequency of CD mRNA variant, which corresponds to ISV receptor protein isoform, significantly decreased (28%; $p = 0.0082$) (Tab. 4). The site-specific editing of 5-HT_{2C} receptor mRNA in the ventral tegmental area was not altered by the drug treatment (Fig. 6B).

Discussion

Previously we have shown that administration of nicotine for 5 days produces a depression-like phenotype reflected by increased immobility time in the forced swim test (from day 1 to 10) following drug withdrawal (Zaniewska et al., 2010). Although the depression-like state seems to be transient and disappears on day 30 of drug withdrawal, a reduction in time spent on swimming, a 5-HT-related parameter (Slattery et al., 2005), was reported on withdrawal day 3 (the present study) and persisted until day 30 (Zaniewska et al., unpublished observations). Our detailed pharmacological analyses performed on day 3 of nicotine withdrawal – when we reported the maximal depression-like behavior – revealed that pharmacological blockade of 5-HT_{2A} receptors and stimulation of 5-HT_{2C} receptors significantly attenuated the depression-like state (Zaniewska et al., 2010). Here, for the first time, we report a dramatic increase in radioligand 5-HT_{2A} receptor labelling in the ventral tegmental area and ventral dentate gyrus of the hippocampus and a less prominent reduction in radioligand binding in the nucleus accumbens shell in rats on day 3 of nicotine withdrawal. In opposition to 5-HT_{2A} receptor binding, we demonstrate a decrease in 5-HT_{2C} receptor binding in the ventral part of the dentate gyrus. In addition, we provide the mechanistic characteristics of the above 5-$HT_{2A/2C}$ receptor regulation.

The increases in 5-HT_{2A} receptor labelling in the ventral tegmental area and hippocampus during nicotine withdrawal could be potentially explained by a regional decrease in 5-HT levels. Indeed, our present behavioral findings showing reduced swimming – a behavior sensitive to changes in 5-HT neurotransmission (Slattery et al., 2005) – in rats during nicotine cessation partially support the above hypothesis. Moreover, a reduction in the content of 5-HT and/or its metabolite 5-HIAA has been found in rat hippocampus or mouse whole brain following short term (for 4 or 7 days) once daily nicotine (0.4 or 0.5 mg/kg) administration and its 24-h

withdrawal (Benwell and Balfour, 1979; Yasuda et al., 2002). Other studies showed a decrease in the diencephalic 5-HT content and increased immobility time in the forced swim test in mice following withdrawal (days 15-60; earlier withdrawal days have not been tested) from nicotine (2 mg/kg; injected four times daily for 15 days), which were normalized after administration of the selective 5-HT reuptake inhibitor paroxetine (Mannucci et al., 2006), suggesting that reduced 5-HT activity contributes to alterations in the forced swim test of nicotine withdrawn animals. Also, the enhanced electrophysiological responsiveness of dorsal raphe 5-HT neurons to 5-HT_{1A} receptor agonist was reported on days 3 and 4 of withdrawal from nicotine (a 12-day exposure to 6 mg/kg/day) delivered through osmotic mini-pumps in rats (Rasmussen and Czachura, 1997), suggesting decreased presynaptic 5-HT-feedback inhibition during drug cessation. Although the above findings were obtained using different dosing schedules/models and were assessed on different withdrawal days, the recurring observation is the existence of the deficit in 5-HT function during nicotine withdrawal. In addition, the functional supersensitivity of 5-HT_{2A} receptors (evidenced by the enhanced behavioral response to 5-HT_{2A} receptor agonist) was reported in rodents following 24-h or 5-day cessation of chronic (for 5 or 7 days) nicotine administration (Suemaru et al., 2001; Yasuda et al, 2002; Zaniewska et al., 2009).

On the basis of our 5-HT_{2A} receptor autoradiography studies using receptor antagonist [^{3}H]ketanserin, which binds to high- and low-affinity state receptors with similar affinity (Lohse et al., 1984), we may assume that we provide information on the total density of 5-HT_{2A} receptors and therefore, speculate that the augmentation in 5-HT_{2A} receptor binding may reflect an increase in receptor density.

As a first step in defining the molecular mechanism underlying the effect of nicotine

withdrawal on 5-HT_{2A} receptor binding we analyzed the transcript level of the 5-HT_{2A} receptor and noted a significant decrease in 5-HT_{2A} receptor mRNA level in both studied brain structures. So far, published data on the 5-HT_{2A} receptor expression level during nicotine withdrawal showed no change either in the receptor density or the transcript level of 5-HT_{2A} receptors in the mouse midbrain, the region containing the ventral tegmental area (Hayslett and Tizabi, 2005). Species differences (mice *vs.* rats), experimental design (twice *vs.* once daily nicotine injections) or the fact that the latter research group examined the transcript level in the early (16-18h) phase of drug withdrawal (*vs.* 72h withdrawal in our study) may account for such discrepancies.

Dissociation between 5-HT_{2A} receptor synthesis (mRNA expression) and protein abundance (binding assay/autoradiography) – as a part of a presumed compensatory response – was noted earlier by some authors after chronic administration of several drugs, including 5-HT_{2A} receptor agonists (5-HT or DOI) or nicotine (Ferry et al., 1994; Hayslett and Tizabi, 2005; Shi et al., 2008). Indeed, *in vitro* studies (Ferry et al., 1994) have shown that brief, but not long-term, exposure to 5-HT evoked a transient increase in the level and stability of receptor mRNA and a concurrent decrease in receptor density.

Taken together, one of the possible mechanisms associated with 5-HT_{2A} receptor regulation following nicotine withdrawal could be regionally-specific (the hippocampus and, possibly, ventral tegmental area) reductions in 5-HT levels which further lead to 5-HT_{2A} receptor up-regulation, thereby enhancing sensitivity of neurons to the attenuated 5-HT tone. Moreover, it can be speculated that the reduction in the 5-HT_{2A} receptor synthesis in the ventral tegmental area and hippocampus may have occurred as a counter-regulatory mechanism to increased

abundance of receptor protein in these brain areas. Certainly, identification of other regulatory mechanisms (e.g., post-transcriptional or post-translational receptor modifications), by which nicotine withdrawal leads to increased labelling of 5-HT_{2A} receptors is a challenge for future research.

Besides increased ketanserin binding to 5-HT_{2A} receptors in the ventral tegmental area and ventral hippocampus, we also reported almost a 40% decrease in radioligand binding in the nucleus accumbens shell, a brain region implicated in the depressive phenotypes observed during drug withdrawal (Shirayama and Chaki, 2006). Whether the decline in 5-HT_{2A} receptor binding sites in the nucleus accumbens, where 5-HT_{2A} receptors are located on dopaminergic terminals, is due to the utilization of the whole pool of 5-HT_{2A} receptor mRNA for the synthesis of the receptor in the ventral tegmental area, in which 5-HT_{2A} receptors are present on the cell bodies of dopaminergic neurons, or due to the trafficking/internalization processes and subsequent degradation of 5-HT_{2A} receptors would remain to be established in further experiments.

In the present work, we also report dramatic decreases in [^{3}H]mesulergine binding to 5-HT_{2C} receptors in the ventral dentate gyrus of the hippocampus during nicotine withdrawal. Earlier studies in rats showed reductions in 5-HT_{2C} receptor binding in the ventral hippocampus (CA1), which were suggested to be linked with anhedonia (Leventopoulos et al., 2009). Importantly, mesulergine is an inverse agonist at 5-HT_{2C} receptors and therefore is predicted to preferentially interact with the inactive (low-affinity state) form of the receptor rather than the active form (Barker et al., 1994; Berg et al., 1999), suggesting that when used as a radioligand it 'labels' only the population of inactive receptors. Thus, the decreases in, presumably, low-affinity 5-HT_{2C} receptor binding sites may be either due to the general decrease in the number of

receptors (receptor down-regulation) or conformational changes (availability of binding sites) in the receptor. While receptor down-regulation seems less probable for two reasons: 1) decreased 5-HT level in the hippocampus (Benwell and Balfour, 1979) and/or 2) enhanced functional sensitivity of 5-HT_{2C} receptors (Zaniewska et al., 2010) during nicotine withdrawal, one possible explanation could be the conversion of 5-HT_{2C} receptors from low to high-affinity state (due to transcriptional, post-transcriptional and/or translational modifications) as a compensatory adaptation for the decreased availability of 5-HT.

To identify mechanisms responsible for the alteration in radioligand binding in the hippocampus we first performed real time PCR analysis, but no significant changes in the transcript level of 5-HT_{2C} receptors were seen, suggesting the existence of another mechanism explaining the changes in receptor binding. Using a deep sequencing method, we discovered decrease in the editing at the E site and a tendency for a reduction at the C site in the hippocampus. We also found pronounced, but non-significant, reduction in the expression of the ECD 5-HT_{2C} receptor mRNA variant. It might be speculated that the expression of the predicted IGV receptor isoform (encoded by ECD) was also reduced, however, caution must be taken when estimating the receptor isoform expression directly from mRNA levels (e.g., 5-HT_{2A} receptors, see above).

To our knowledge, this study provides the first evaluation of 5-HT_{2C} receptor RNA editing patterns in rats with a putative depression-like phenotype following nicotine withdrawal. In agreement with our findings, clinical data show the reduction in editing efficiency across all editing sites and frequency of all transcripts edited at E and C in the prefrontal cortex of

depressed patients; this effect paralleled increases in highly functioning INI and AD receptor isoforms (Lyddon et al., 2013). Other clinical studies showed only a slight non-significant decrease in the frequency of E, C and D site editing (Zhu et al., 2012), no change in editing (Niswender et al., 2001) or even an increase in editing efficiency of site D (Iwamoto and Kato, 2003) in the prefrontal cortex of patients with major depression. Preclinical observations show that the learned helplessness rat model of depression was associated with increased editing of site E in the cortex (Iwamoto et al., 2005). Although not completely consistent, the recurring finding in these studies is altered editing at E and/or C site in depression-related behaviors. It would certainly be of interest to perform such studies in nicotine-withdrawn individuals demonstrating affective-like symptoms.

Importantly, editing at the E and C sites has been shown to be crucial for attenuating the receptor efficiency to stimulate G protein. The IGV 5-HT_{2C} receptor variant, similarly to other isoforms containing glycine (VGI and VGV) at position 158 (i.e., edited at both E and C site) in humans, has been demonstrated to exhibit the most prominent reduction in agonist potency for G-protein coupling stimulation compared with the non-edited INI 5-HT_{2C} receptor isoform (Wang et al., 2000). In addition, the fully edited VGV receptor isoform has been shown to have the lowest ligand independent (constitutive) receptor activity, while the partially edited VSV or non-edited INI receptor isoforms exhibited the intermediate or the highest levels of constitutive activity, respectively (Marion et al., 2004; Nisewender et al., 2001). At the behavioral level, mice solely expressing the VGV isoform of the 5-HT_{2C} receptor exhibited an antidepressant-like phenotype (evidenced as decreased immobility time in the forced swim test), while the absence of editing in INI mice induced a depression-like profile (Mombereau et al., 2010). Therefore, it

can be speculated that the decrease in editing in the hippocampus in the present study could contribute to the depression-like phenotype on day 3 of nicotine withdrawal.

A logical reason for reduced editing during nicotine withdrawal may be the perturbations in tonic levels of 5-HT in the hippocampus, as they decreased editing at E and C sites and enhanced the expression of receptor isoforms exhibiting higher sensitivity to 5-HT (*i.e.,* those edited at sites A, B and D) in the mouse forebrain neocortex (Gurevich et al., 2002a). Reduced 5-HT tone, through reduced 5-HT_{2C} receptor mRNA editing, may decrease the synthesis of low-affinity state receptors, and therefore lead to the attenuated binding of [^{3}H]mesulergine – a ligand preferring low-affinity state receptors. This argument is further supported by the finding that mice solely expressing VGV 5-HT_{2C} receptor isoform – occurring predominantly in the low affinity receptor state – exhibited enhanced [^{3}H]mesulergine binding (Martin et al., 2013). Therefore, in opposite direction, we could expect that decrease in editing at E and C sites (i.e., decrease in the expression of Gly-containing receptor isoforms) during nicotine withdrawal leads to the reduction in radioligand binding to these – more active – receptors. At the behavioral level, this could be reflected by enhanced functional response of these receptors to their agonist (Zaniewska et al., 2010).

There is some concern, however, regarding the physiological role of changes in editing at sites E and C corresponding to receptor transcripts representing < 2% of all receptor variants. Since these rare isoforms may be expressed on specific types of neurons or can be linked with specific environmental stimuli, their importance cannot be discounted. Further studies are necessary to target these discrete populations of receptors.

It must be mentioned that there is inconsistency in the brain regions used for autoradiographic and transcript analyses (ventral dentate gyrus *vs.* whole hippocampus). Certainly, the use of the same subfields of the hippocampus would provide more consistency between two analyses. However, since the expression of 5-HT_{2C} receptors in the dorsal hippocampus is sparse (*cf.* Holmes et al., 1995), it seems doubtful that subtle changes noted in editing of 5-HT_{2C} receptor RNA are due to the fact that for this analysis we used the whole hippocampus instead of the ventral dentate gyrus.

In the present work we also assessed the 5-HT_{2C} receptor editing in the ventral tegmental area, a brain area in which we did not observe significant changes in [^{3}H]mesulergine binding to 5-HT_{2C} receptors or transcript level of the receptor during nicotine withdrawal. Here, we noted significantly lower (28%) levels of the mRNA variant CD encoding for the ISV protein isoform. As shown previously, the ISV receptor isoform did not affect G-protein coupling *in vitro* (Wang et al., 2000). Thus, decreased expression of the ISV receptor isoform in the ventral tegmental area during nicotine withdrawal may not have been sufficient to affect the binding of mesulergine to 5-HT_{2C} receptors in this brain area.

In summary, we propose that nicotine withdrawal evokes an increase in the number of 5-HT_{2A} receptors in ventral tegmental area and hippocampus and attenuates editing of 5-HT_{2C} receptor mRNA in the hippocampus which results in a shift of the RNA editing profile towards a population of less edited (and thus more active) receptors.

The present treatment approach suffers from certain limitations. In fact, nicotine was experimenter-delivered, in contrast to the voluntary drug intake models that better mirror the addictive behavior in humans. In addition, the duration of exposure of rats to nicotine was very

short. Whether the changes in 5-HT_{2A} receptor density and editing pattern of 5-HT_{2C} receptor reported in the present study could be "bio-markers" of a depression-like state during nicotine withdrawal is a challenge for future research using more complex behavioral models (e.g., nicotine self-administration) and performing extend analysis on different time-points of nicotine withdrawal.

Acknowledgements:

This research was supported by the grant no. N N401 2798 33 from the Ministry of Science and Higher Education (Warszawa) and statutory funds from the Department of Pharmacology, Institute of Pharmacology Polish Academy of Sciences (Kraków) and Department of Cardiovascular Research, Max-Delbrück-Center for Molecular Medicine (Berlin). This paper originated from the financial support from the DAAD program to Magdalena Zaniewska.

Portions of these data were presented at the 24th European College of Neuropsychopharmacology Congress (Paris, 2011).

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Tables

Table 1. [^{3}H]ketanserin binding to 5-HT$_{2A}$ receptors in rats withdrawn from repeated nicotine administration

STRUCTURE	VEH (fmol/mg tissue)	NIC (fmol/mg tissue (% of VEH))
prelimbic cortex	53.43±4.56	59.54±2.05 (111)
infralimbic cortex	56.53±4.58	54.84±2.36 (97)
cingulate cortex	39.51±3.52	39.48±1.74 (100)
dorsal striatum	8.68±1.55	6.77±1.13 (78)
nucleus accumbens		
- core	23.34±2.68	16.50±1.72 (71)
- shell	26.14±1.36	15.87±2.14 (61)**
ventral dentate gyrus	18.36±1.14	27.30±2.22 (149)*
ventral tegmental area	3.73±0.45	6.74±0.94 (181)*

Animals were treated with vehicle (VEH) or nicotine (NIC; 0.4 mg/kg) for five days and three days later they were killed by decapitation. Data are expressed as means (± SEM) of data from 4-6 rats/group. *A priori* comparisons (Student's *t*-test): *$p < 0.05$, **$p < 0.01$ *vs.* VEH group.

data showed a reduction in editing at the E site and a trend toward reduction at the C site in the hippocampus. In the ventral tegmental area, a reduction for the frequency of CD 5-HT_{2C} receptor transcript was seen. These results show that the reduction in the 5-HT_{2A} receptor transcript level may be an auto-regulatory response to the increased receptor density in the hippocampus and ventral tegmental area during nicotine withdrawal, while decreased 5-HT_{2C} receptor mRNA editing may explain the reduction of receptor labelling in the hippocampus.

Introduction

Nicotine is one of the major psychoactive constituents of tobacco smoke that has a strong abuse potential in humans and laboratory animals. The most common withdrawal symptoms in humans include nicotine craving, irritability, anxiety, difficulty of concentration, increased appetite and depressed mood (reviewed by Kenny and Markou, 2001; Markou, 2008). Prevention of the latter mood state has become an important issue in the management of nicotine-use or addiction.

Many pharmaco-behavioral studies have shown that withdrawal from chronic nicotine exposure produced depression-like behavior in mice (15-day voluntary drug intake or passive drug administration) and rats (experimenter-delivered drug for 5 days), as measured by exaggerated immobility time in the forced swim test (Mannucci et al., 2006; Ribeiro-Carvalho et al., 2011; Zaniewska et al., 2010). The above data also showed that such behavioral sequelae were either completely diminished by the 5-HT precursor 5-hydroxytryptophan, or were attenuated by 5-HT_{2A} receptor antagonists or 5-HT_{2C} receptor agonists (Mannuci et al., 2006; Zaniewska et al., 2010). During withdrawal from repeated nicotine exposure (delivered by the experimenter or through osmotic mini-pumps) profound changes in the 5-HT system (i.e., significant reductions in brain 5-HT content and/or neurotransmitter turnover) have been found

ACD ABCD	0.68±0.10 12.42±0.62	0.69±0.02 12.56±0.26	VSV	13.10±0.55	13.25±0.27
AECD ABECD	0.20±0.03 0.37±0.01	0.20±0.03 0.39±0.02	VGV	0.56±0.04	0.60±0.04
B	0.38±0.04	0.42±0.04	MNI		
BE	0.0059±0.0015	0.013±0.008	MDI		
BC	0.049±0.007	0.062±0.016	MSI		
BD	0.33±0.02	0.34±0.02	MNV		
BEC	0.0008±0.0002	0.0025±0.0011	MGI		
BED	0.009±0.005	0.004±0.001	MDV		
BCD	0.066±0.018	0.083±0.009	MSV		
BECD	0.00078±0.0003	0.00036±0.00018	MGV		
E	0.21±0.02	0.19±0.02	IDI		
EC	0.088±0.007	0.071±0.007	IGI		
ED	0.28±0.09	0.23±0.02	IDV		
ECD	0.067±0.022	0.039±0.003	IGV		
C	0.92±0.04	0.91±0.05	ISI		
CD	1.41±0.47	0.96±0.10	ISV		
D	3.11±0.86	2.83±0.09	INV		

Animals were treated with vehicle (VEH) or nicotine (NIC; 0.4 mg/kg) for five days and three days later they were killed by decapitation. RNAs from the hippocampus were isolated, reverse transcribed and amplified by PCR and deep sequenced. 5-HT_{2C} receptor mRNA variants (e.g., AB, ABE, ABC) and the corresponding amino acid isoforms (e.g., INI, VNI) are indicated. Data are expressed as frequency means (± SEM) of data from 4 rats/group.

Table 4. Frequency for the detected 5-HT_{2C} receptor mRNA variants and predicted amino acid isoforms in the ventral tegmental area of rats withdrawn from repeated nicotine administration

mRNA variant	%		amino acid isoform	%	
	VEH	NIC		VEH	NIC
None	4.20±0.23	9.26±5.07	INI		
A	6.01±0.14	5.78±0.19	VNI	19.95±0.76	20.23±1.29
AB	13.95±0.63	14.44±1.16			
AE	0.12±0.02	0.15±0.01	VDI	15.55±0.35	13.25±1.46
ABE	15.43±0.36	13.09±1.45			
AC	1.17±0.05	1.12±0.08	VSI	10.94±0.11	10.93±0.71
ABC	9.78±0.08	9.81±0.75			
AD	1.92±0.11	1.92±0.10	VNV	23.36±0.16	22.48±0.74
ABD	21.44±0.05	20.56±0.65			
AEC	0.27±0.03	0.30±0.08	VGI	0.76±0.04	0.80±0.05
ABEC	0.50±0.06	0.50±0.05			
AED	0.084±0.003	0.087±0.049	VDV	1.35±0.10	1.08±0.19
ABED	1.27±0.09	1.00±0.22			
ACD	0.55±0.05	0.61±0.03	VSV	15.31±0.67	13.66±0.63
ABCD	14.76±0.70	13.05±0.62			
AECD	0.12±0.03	0.09±0.01	VGV	0.55±0.07	0.59±0.07
ABECD	0.43±0.04	0.51±0.06			
B	0.37±0.05	0.36±0.06	MNI		
BE	0.017±0.015	0.011±0.005	MDI		
BC	0.12±0.02	0.12±0.03	MSI		
BD	0.51±0.04	0.47±0.04	MNV		
BEC	0.00047±0.00037	0.00055±0.00033	MGI		
BED	0.0092±0.0066	0.010±0.009	MDV		
BCD	0.19±0.03	0.13±0.03	MSV		
BECD	0.0004±0.0002	0.00046±0.00018	MGV		
E	0.18±0.04	0.22±0.04	IDI		
EC	0.077±0.024	0.090±0.023	IGI		
ED	0.18±0.03	0.16±0.01	IDV		
ECD	0.022±0.005	0.027±0.010	IGV		
C	1.10±0.14	1.09±0.06	ISI		
CD	0.83±0.06	0.60±0.01**	ISV		
D	1.86±0.12	1.90±0.16	INV		

Animals were treated with vehicle (VEH) or nicotine (NIC; 0.4 mg/kg) for five days and three days later they were killed by decapitation. RNAs from the ventral tegmental area were isolated, reverse transcribed and amplified by PCR and deep sequenced. 5-HT_{2C} receptor mRNA variants (e.g., AB, ABE, ABC) and the corresponding amino acid isoforms (e.g., INI, VNI) are indicated.

Data are expressed as frequency means (± SEM) of data from 4 rats/group. Student's *t*-test: $^{**}p < 0.01$ *vs.* VEH group.

Figure legends

Fig. 1. Editing of 5-HT_{2C} receptor mRNA. 5-HT_{2C} receptor RNA undergoes adenosine-to-inosine (recognized as guanosines by ribosomes) RNA editing (named A, B, E, C and D) resulting in the generation of different mRNA variants. This further yields multiple amino acid sequences within the second intracellular loop of the receptor, *i.e.,* editing at A or A/B sites generates valine (Val; V) at this site, editing at B site converts isoleucine (Ile; I) into methionine (Met; M). Editing at the E site generates aspartate (Asp; D), editing at C site generates serine (Ser; S), while editing at both E and C generates glycine (Gly; G). Editing at D site converts I into V. INI – the non-edited receptor isoform, VGV – fully edited receptor variant (Burns et al., 1997).

Fig. 2. Representative autoradiograms of 5-HT_{2A} and 5-HT_{2C} receptors in the brain of rats receiving repeated vehicle treatment. The grey outlines in the left panel **(A)** show the brain areas in which optical densities were quantified. Central and right panels show representative autoradiograms of brain [^{3}H]ketanserin-labeled 5-HT_{2A} receptors **(B)** or [^{3}H]mesulergine-labeled 5-HT_{2C} receptors **(C)** of rats treated repeatedly with vehicle. A – prelimbic cortex, B – infralimbic cortex, C – cingulate cortex, D – dorsal striatum (caudate putamen), E – nucleus accumbens (core), F – nucleus accumbens (shell), G – choroid plexus, H – ventral dentate gyrus, I – substantia nigra (compact part), J – substantia nigra (reticular part), K – ventral tegmental

area.

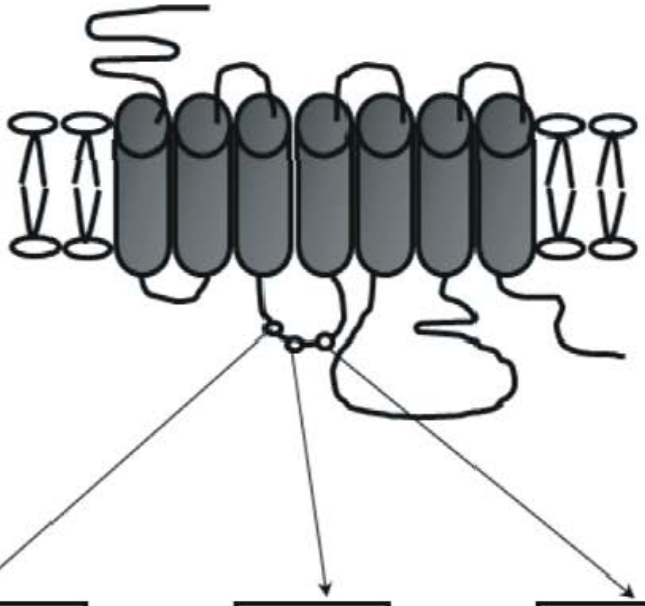
Fig. 3. Depression-like state in rats withdrawn from nicotine (0.4 mg/kg)-repeated administration measured in the forced swim test. The following parameters were measured: immobility, swimming and climbing in rats on day 3 of withdrawal from a 5-day nicotine or vehicle treatment. The results are shown as mean time (±SEM) of data from 7 rats/group. Student's *t*-test: $*p < 0.05$, $***p < 0.001$ *vs.* VEHICLE group.

Fig. 4. The transcript level of 5-HT_{2A} receptor in the rat brain. Animals were treated with vehicle or nicotine (0.4 mg/kg) for five days and three days later they were killed by decapitation. 5-HT_{2A} receptor transcript level was analyzed in the whole hippocampus (A) and ventral tegmental area (B) by real time quantitative PCR. Data were normalized to β-actin and represent means (± SEM) of data from 4 rats/group. Student's *t*-test: $**p < 0.01$ *vs.* VEHICLE group.

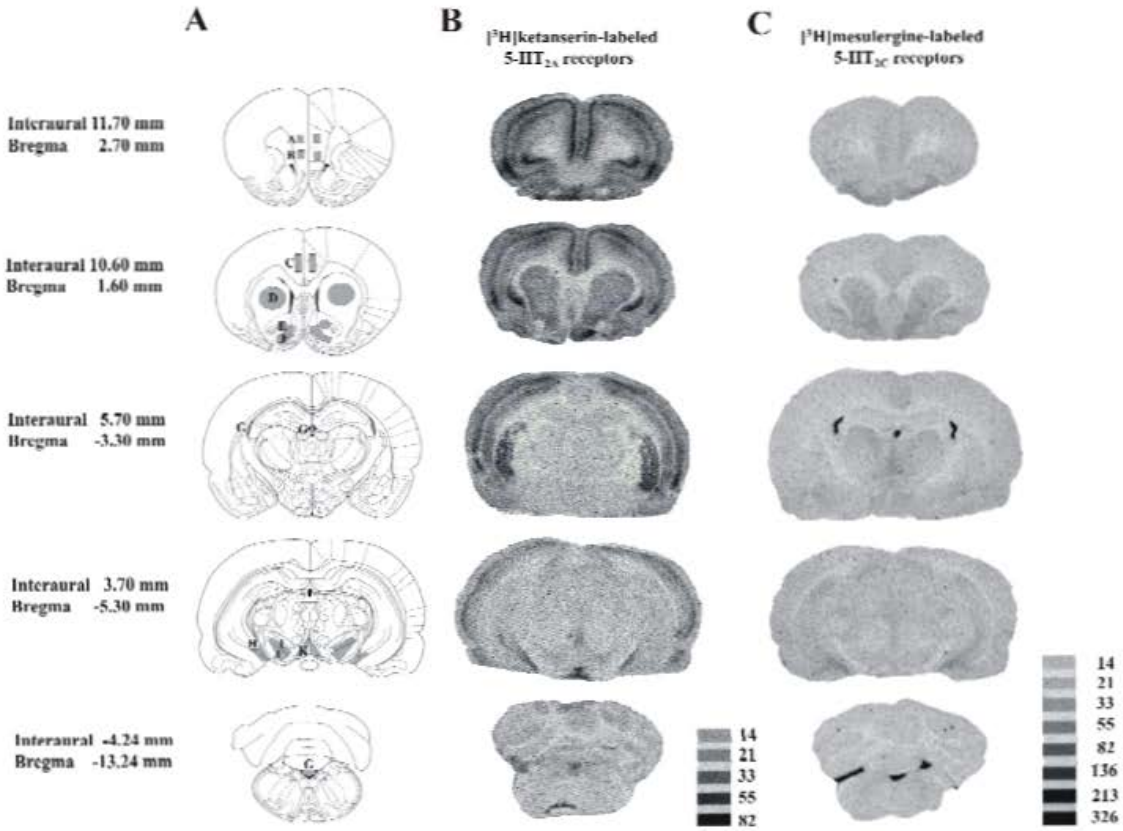
Fig. 5. The transcript level of 5-HT_{2C} receptor in the rat brain. Animals were treated with vehicle or nicotine (0.4 mg/kg) for five days and three days later they were killed by decapitation. 5-HT_{2C} receptor transcript level was analyzed in the whole hippocampus (A) and ventral tegmental area (B) by real time quantitative PCR. Data were normalized to β-actin and represent means (± SEM) of data from 4 rats/group.

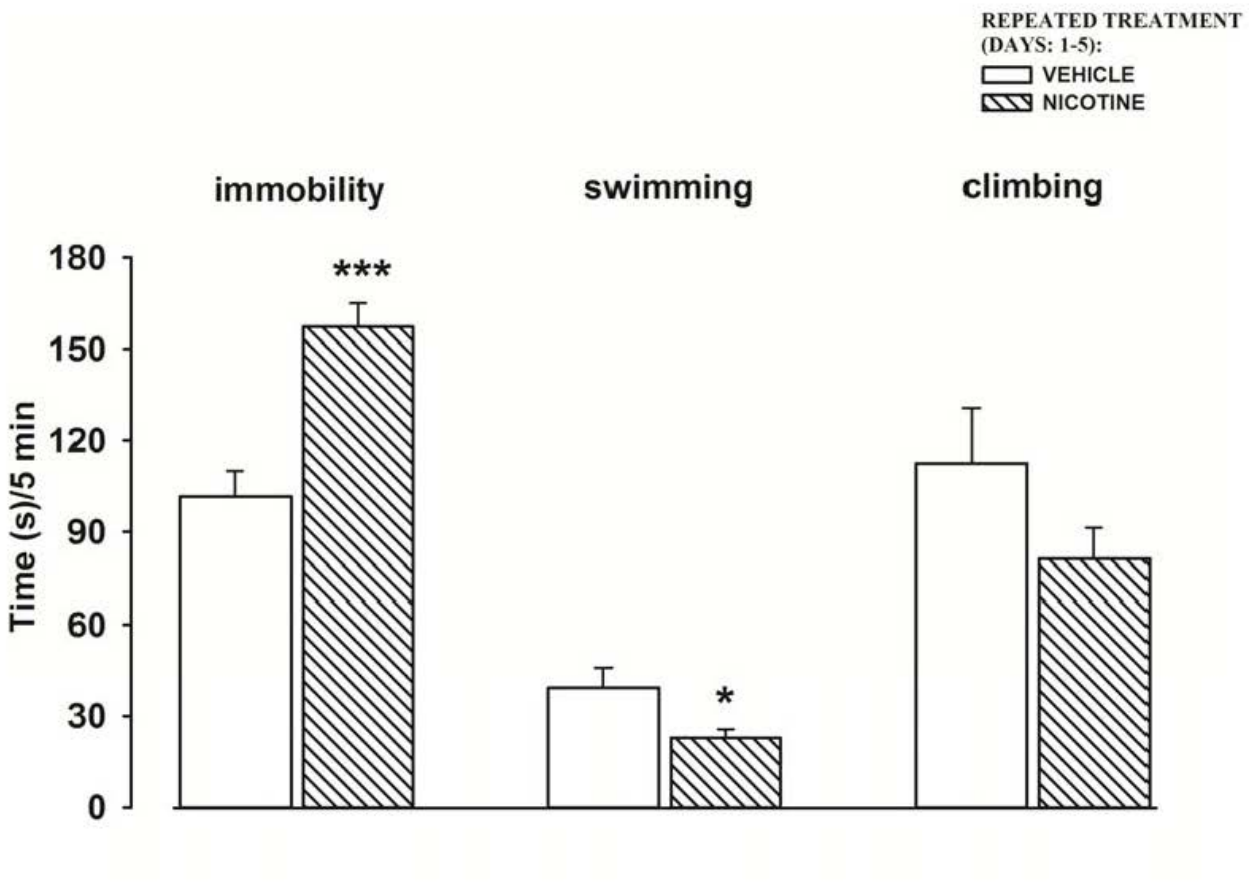
Fig. 6. mRNA editing level at the five editing sites (A, B, E, C and D) of the 5-HT_{2C} receptor gene in the rat brain. Animals were treated with vehicle or nicotine (0.4 mg/kg) for five days and three days later they were killed by decapitation. 5-HT_{2C} receptor mRNA editing status was analyzed in the whole hippocampus (A) and ventral tegmental area (B) by deep sequencing technology. Data are expressed as site-specific editing of the 5-HT_{2C} receptor mRNA (± SEM) of data from 4 rats/group. Student's *t*-test: $*p < 0.05$ *vs.* VEHICLE group.

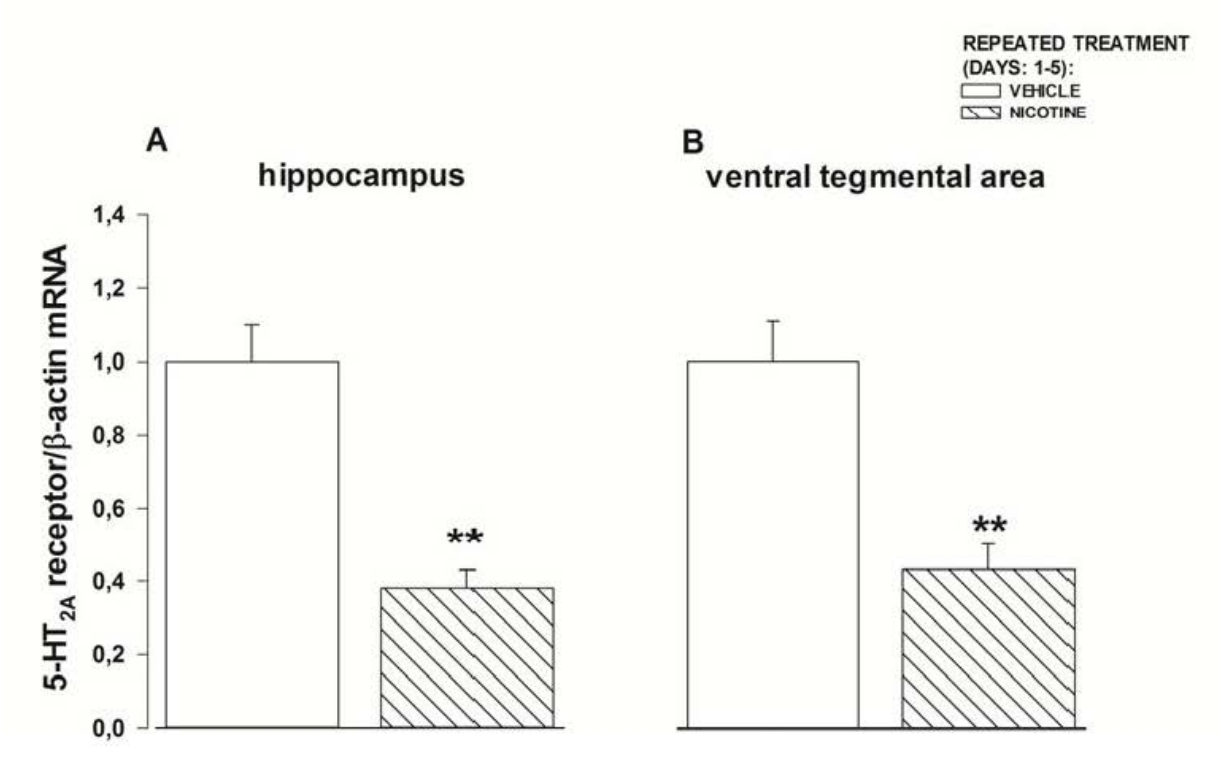
5-HT$_{2C}$ RECEPTOR mRNA EDITING

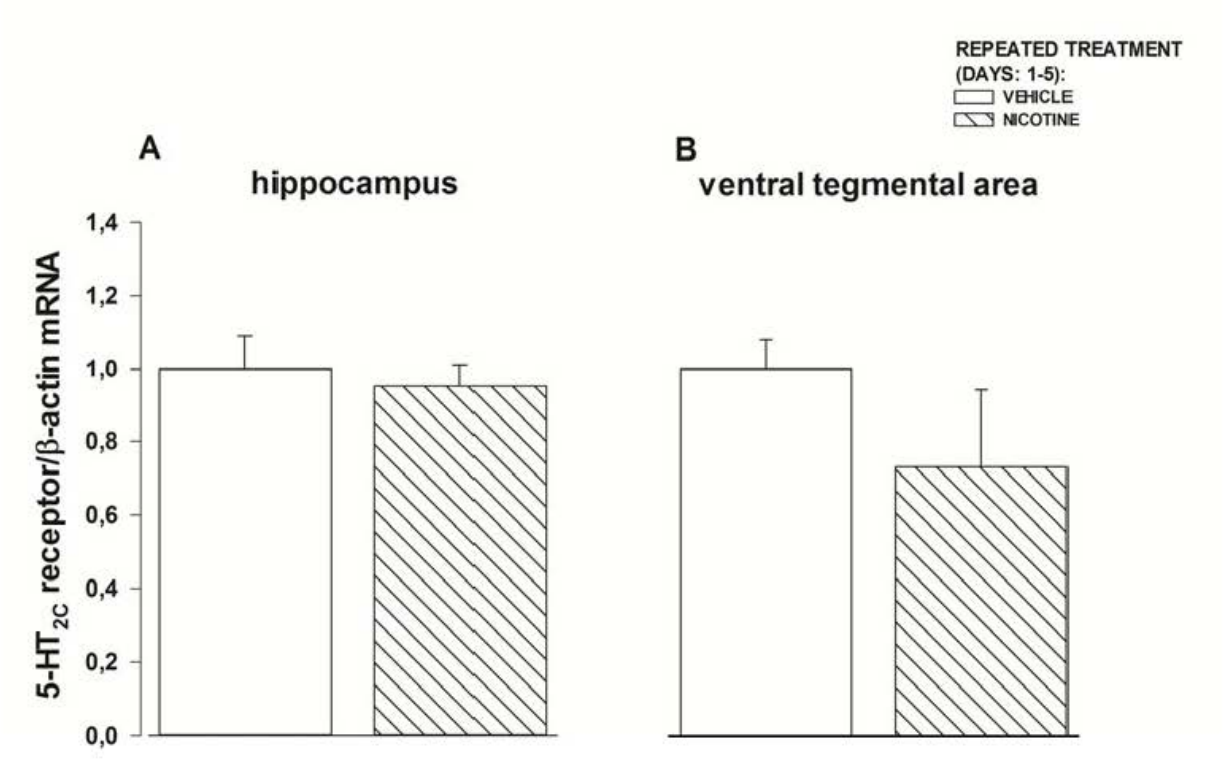


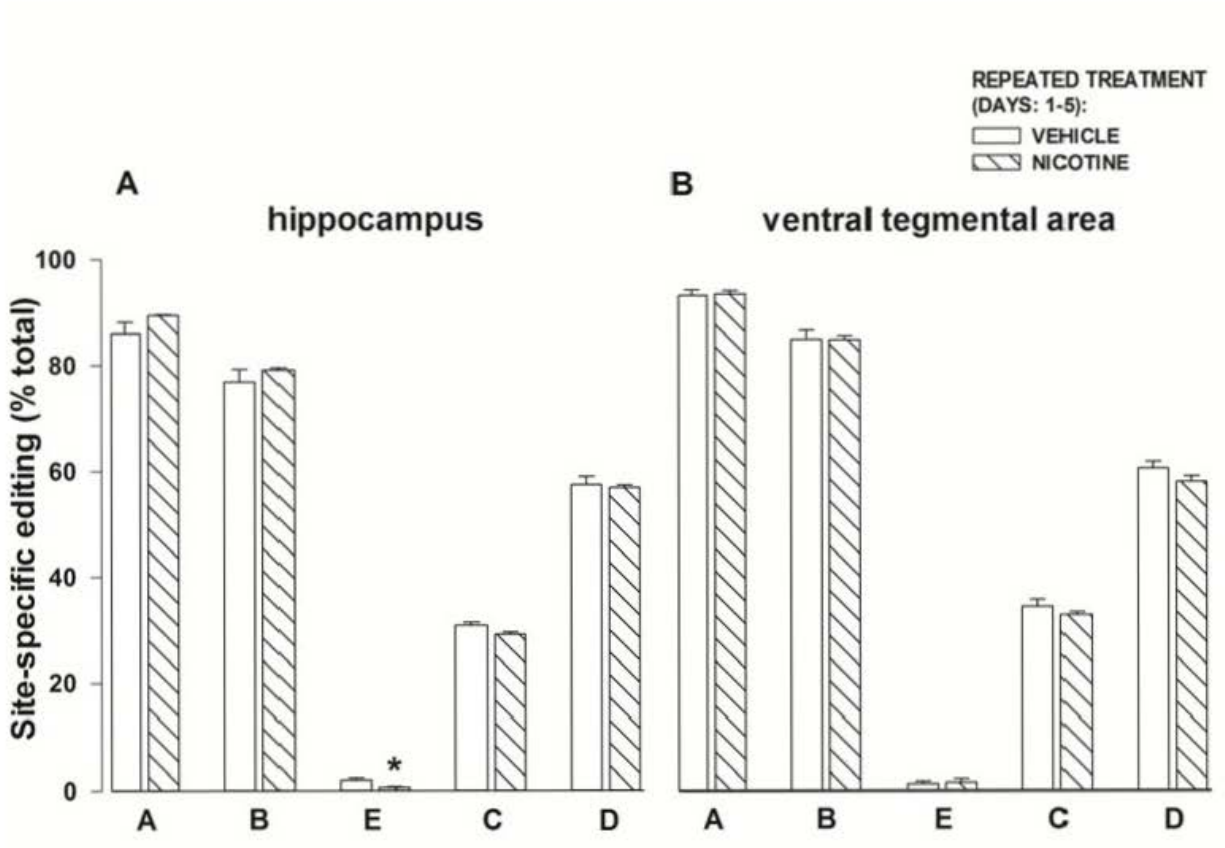
amino acid position	156	157	158	159	160	
mRNA editing sites	A B		EC		D	
non-edited mRNA amino acid	AUA Ile (I)	CGU Arg (R)	AAU Asn (N)	CCU Pro (P)	AUU Ile (I)	INI
partially edited mRNA amino acid	**G**UA Val (V)	CGU Arg (R)	AAU Asn (N)	CGU Arg (R)	**G**UU Val (V)	VNV
	AU**G** Met (M)	CGU Arg (R)	**G**AU Asp (D)	CGU Arg (R)	AUU Ile (I)	MDI
fully edited mRNA amino acid	**G**UA Val (V)	CGU Arg (R)	A**G**U Ser (S)	CGU Arg (R)	**G**UU Val (V)	VSV
	GU**G** Val (V)	CGU Arg (R)	**GG**U Gly (G)	CGU Arg (R)	**G**UU Val (V)	VGV


A
B
C
[3H]ketanserin-labeled
5-HT2A receptors
[3H]mesulergine-labeled
5-HT2C receptors
Interaural 11.70 mm
Bregma 2.70 mm
Interaural 10.60 mm
Bregma 1.60 mm
Interaural 5.70 mm
Bregma -3.30 mm
Interaural 3.70 mm
Bregma -5.30 mm
Interaural -4.24 mm
Bregma -13.24 mm
14
21
33
55
82
14
21
33
55
82
136
213
326


REPEATED TREATMENT
(DAYS: 1-5):
VEHICLE
NICOTINE
immobility
swimming
climbing
Time (s)/5 min
180
150
120
90
60
30
0
***
*


REPEATED TREATMENT
(DAYS: 1-5):
VEHICLE
NICOTINE
A
hippocampus
B
ventral tegmental area
5-HT2A receptor/β-actin mRNA
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
**
**


REPEATED TREATMENT
(DAYS: 1-5):
VEHICLE
NICOTINE
A
hippocampus
B
ventral tegmental area
5-HT2C receptor/β-actin mRNA
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0


REPEATED TREATMENT
(DAYS: 1-5):
VEHICLE
NICOTINE
A
hippocampus
B
ventral tegmental area
Site-specific editing (% total)
100
80
60
40
20
0
*
A
B
E
C
D
A
B
E
C
D