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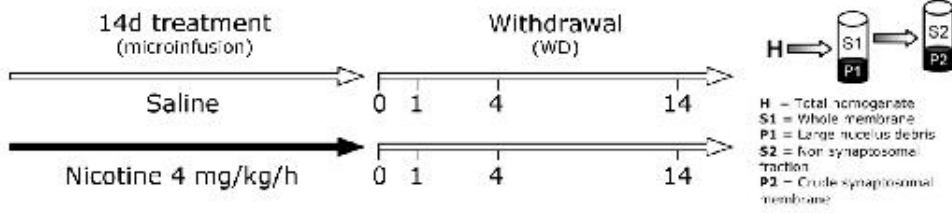
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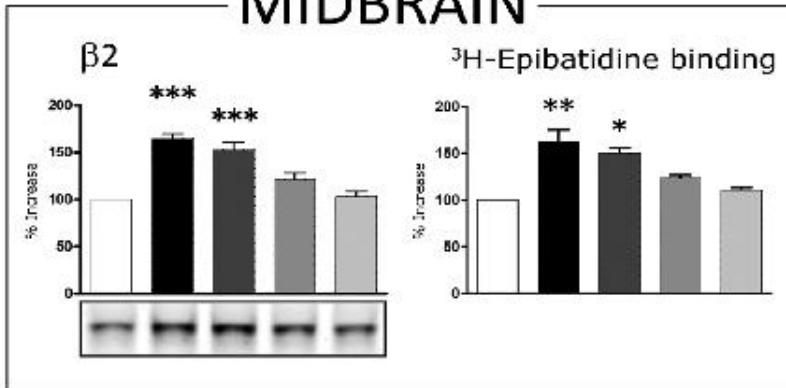
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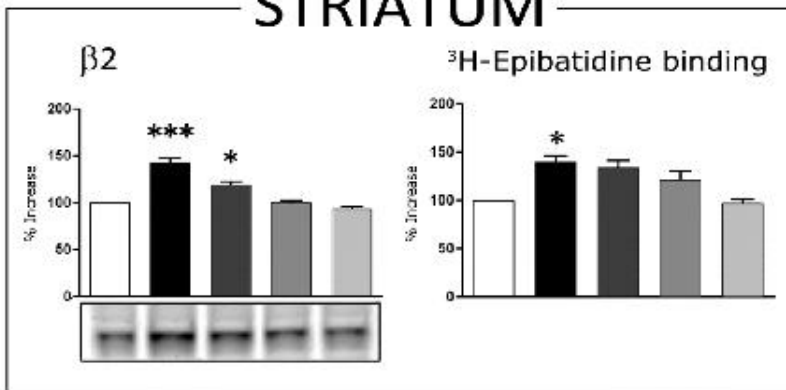
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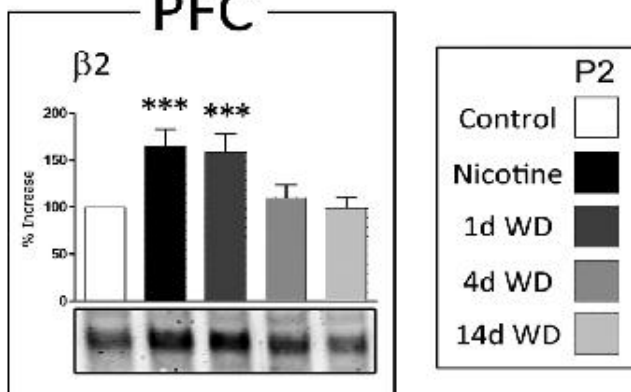
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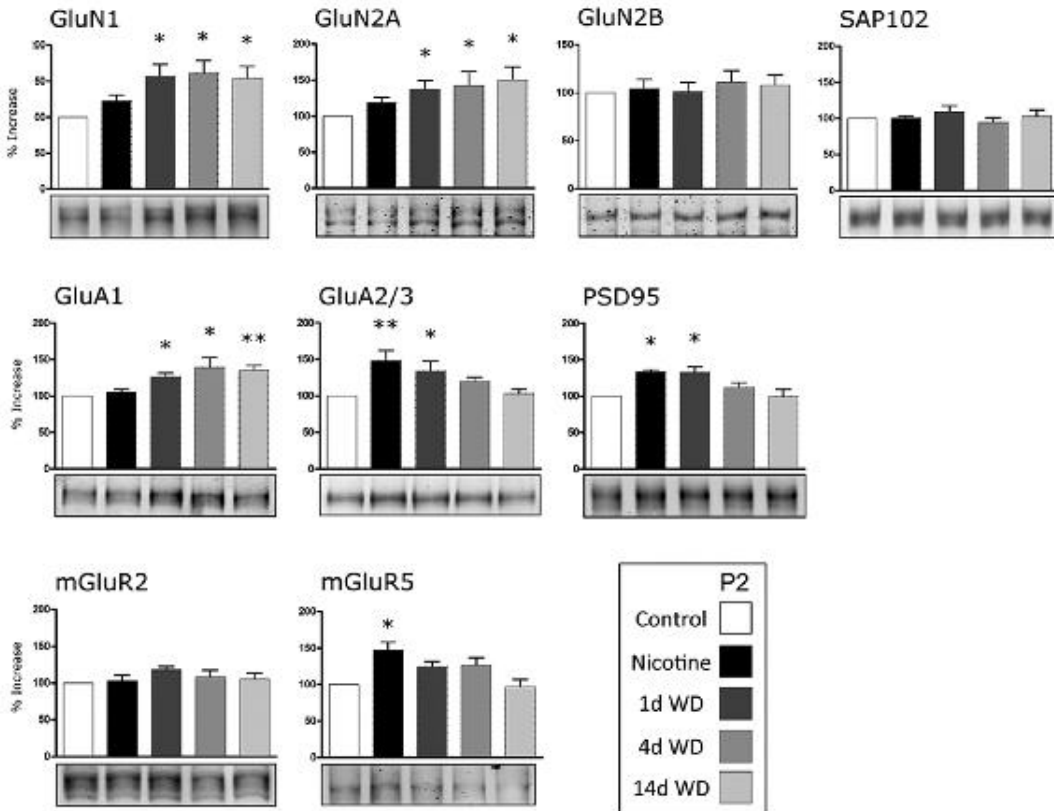
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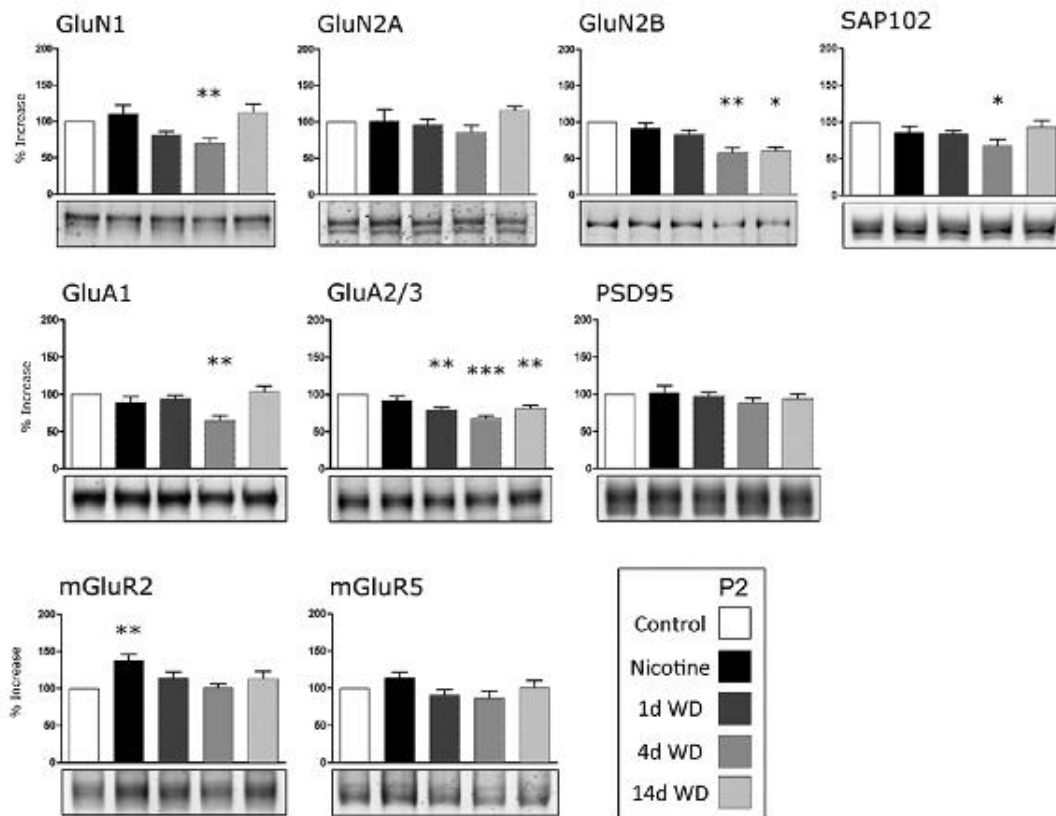
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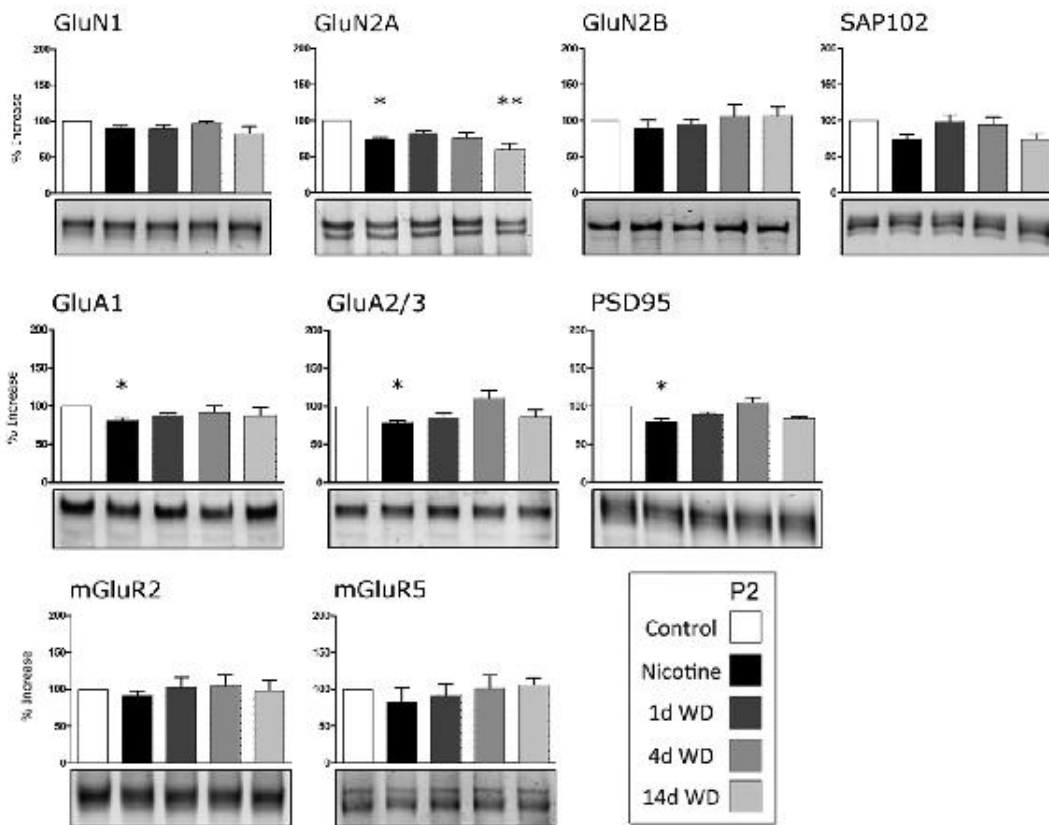
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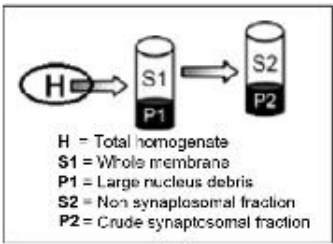
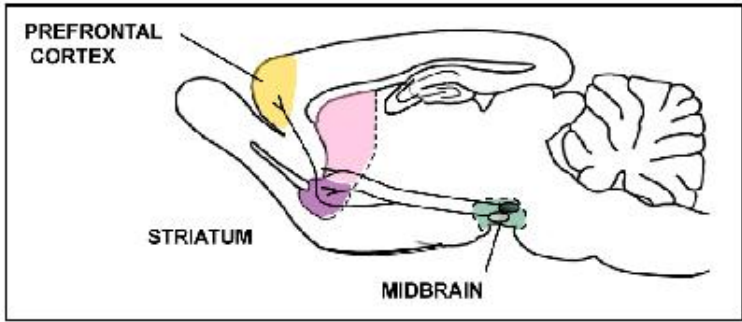
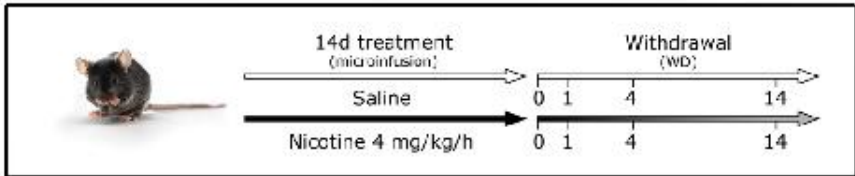
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PREFRONTAL CORTEX



All authors declare that do not have conflict of interest , with no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted.



Nicotinic and Glutamate receptors analysis

Supplementary data

Figure 1 **Specificity of anti- β 2 antibodies**

The specificity of the anti- β 2 Abs was tested by immunoprecipitation experiments as previously described (Gotti et al, 2008) and by Western blotting in areas of wild type (WT), heterozygote (Het) and knockout (KO) mice

The specificity of the Abs were tested by incubating blots of WT, Het and KO β 2 mice with antibodies directed against the β 2 subunit. 10 μ g of 2% Triton cortex extracts from the different genotypes were separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 9% acrylamide and then electrophoretically transferred to nitrocellulose membranes .

As shown in Fig. 1, the anti- β 2 Ab recognized a band of 52 kDa (corresponding to the expected size of the β 2 subunit) in the extracts taken from the WT and Het mice but not in the extract prepared from KO mice

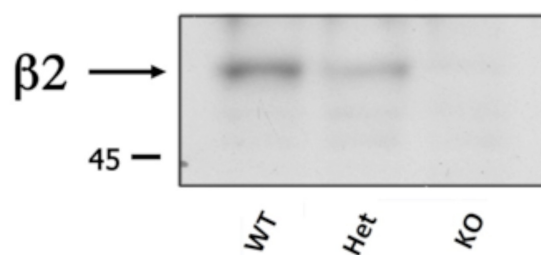
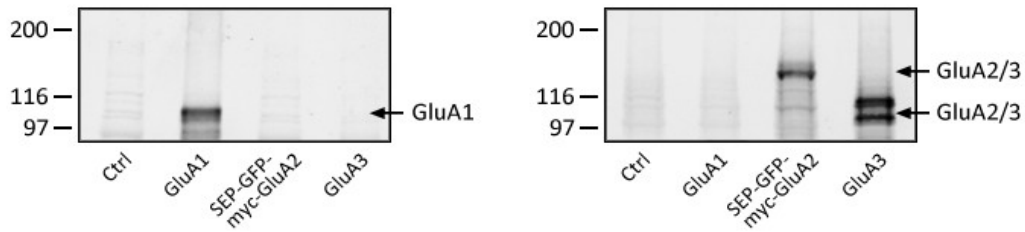


Figure 2 **Specificity of the Abs directed against the GluA1, GluA2 and GluA3 AMPAR subunits**

HEK 293 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 1% L-Glutamine, 100 units/ml penicillin G, and 100 μ g/streptomycin in a humidified atmosphere containing 10% CO₂. The cDNAs encoding GluA1, GluA3 and the SEP-GFPmycGLuA2 construct were transfected into the HEK 293 cells at 30% confluency. The cell transfections were carried out in 100 mm Petri dishes using 30 μ L of JetPEI™ (Polypus, France) (1 mg/ml, pH 7.2) and 10 μ g of cDNAs. After 48 h transfection, the cells were collected, washed with PBS by centrifugation, and used for Western blotting analysis



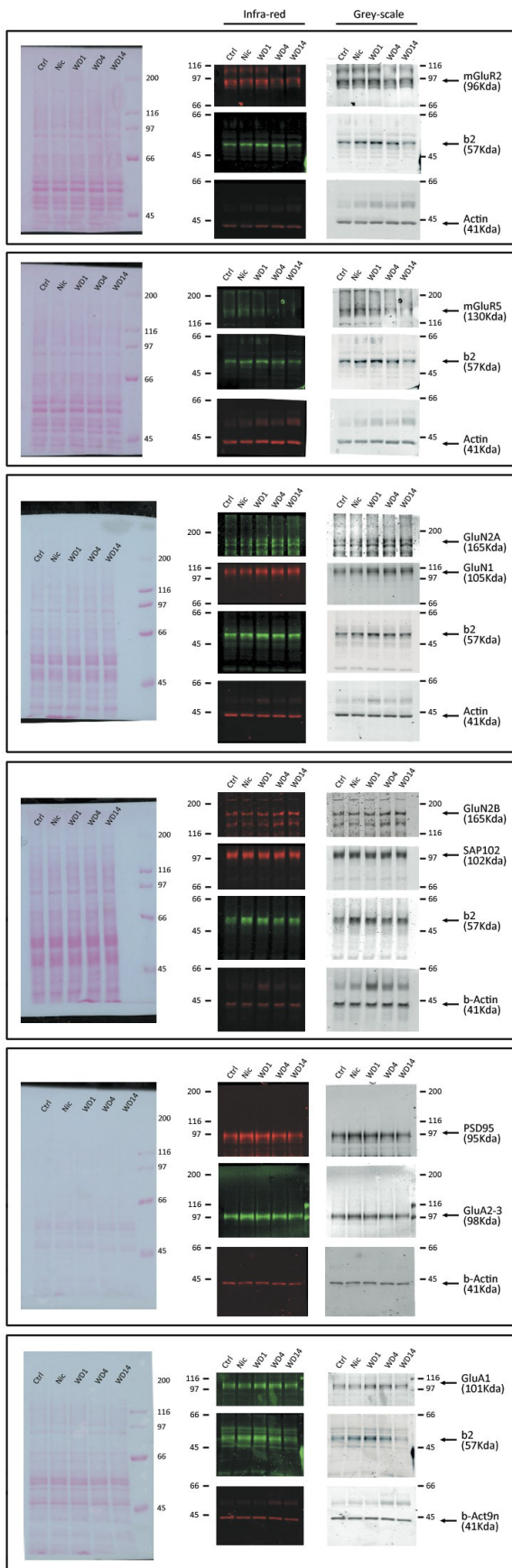
5-10 μ g of the transfected cells were separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7,5% acrylamide and then electrophoretically transferred to nitrocellulose membranes. The blots were incubated with 1 μ g of the anti GluA1 Abs (Left) or the anti-GluA2-A3 Abs. As shown in Fig2 anti GluA1 Abs do not recognise neither the GluA2 nor the GluA3 proteins. Vice versa anti Glu2/3 Abs do not recognise GluA1 subunit and recognised both SEP-GFPmycGLuA2 and GluA3 subunits.

Figure 3: Original Western blots from which figures have been derived

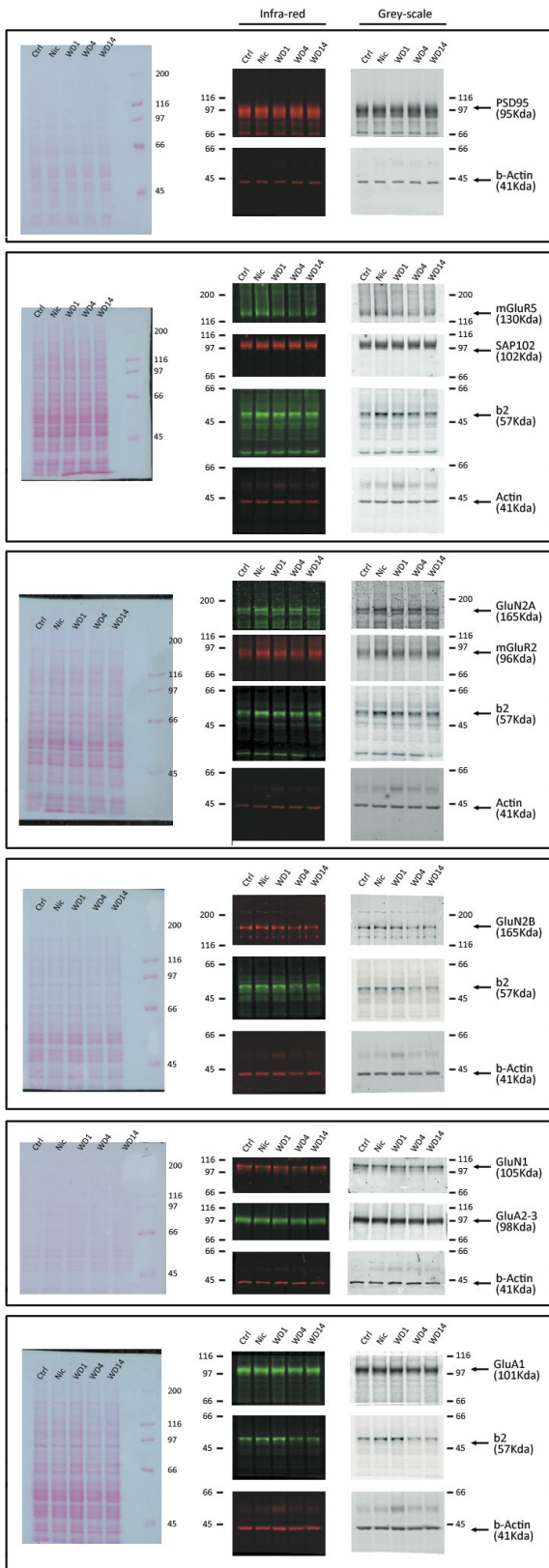
In the figures below, western blots from the prefrontal cortex, striatum and midbrain incubated with the indicated antibodies are shown. In each figure on the left is shown the ponceau of the western blot, in the middle the labelling of the appropriate secondary antibody (anti-rabbit Ly-Cor IRDye800RD; anti-mouse Ly-Cor IRDye680RD), on the right the grey scale of the secondary antibody labelling. Each western has been cut in two, three or four pieces and incubated with the indicated primary polyclonal or monoclonal antibodies (middle) and then revealed with the Lycor secondary antibodies. On the left of each panel there is the grey scale of the western shown in the middle.

The IR signal was measured using an Odyssey CLx - Infrared Imaging System. The signal intensity of the Western blot bands was quantified using iStudio software.

MIDBRAIN



STRIATUM



Chronic nicotine and withdrawal affect glutamatergic but not nicotinic receptor expression in the mesocorticolimbic pathway in a region-specific manner.

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Highlights

- Chronic nicotine up-regulates heteromeric β 2-containing nicotinic receptors in the mesocorticolimbic pathway.
- These receptors are rapidly removed from mesocorticolimbic synapses upon nicotine cessation.
- Glutamate receptor expression in this pathway is regulated in a region-specific manner during nicotine exposure and withdrawal.

ABSTRACT

Tobacco addiction is a complex form of dependence process that leads high relapse rates in people seeking to stop smoking. Nicotine elicits its primary effects on neuronal nicotinic cholinergic receptors (nAChRs), alters brain reward systems, and induces long-term changes during chronic nicotine use and withdrawal.

We analysed the effects of chronic nicotine treatment and withdrawal on the mesocorticolimbic pathway (a brain reward circuit in which addictive drugs induce widespread adaptations) by analysing the expression of nAChRs in the midbrain, striatum and prefrontal cortex (PFC) of mice receiving intravenous infusions of nicotine (4 mg/kg/h) or saline (control) for 14 days and mice sacrificed two hours, and one, four and 14 days after treatment withdrawal.

We biochemically fractionated whole tissue homogenates in order to obtain crude synaptosomal membranes. Western blotting analyses of these membrane fractions, ligand binding and immunoprecipitation studies, showed that chronic nicotine up-regulates heteromeric $\beta 2^*$ nAChRs in all three mesocorticolimbic areas, and that these receptors are rapidly removed from synapses upon the cessation of nicotine treatment. The extent of nicotine-induced nAChR up-regulation, and the time course of its reversal were comparable in all three areas.

We also analysed the expression of glutamate receptor subunits (GluRs) and scaffold proteins, and found that it was altered in an area-specific manner during nicotine exposure and withdrawal.

As the functional properties of GluRs are determined by their subunit composition, the observed changes in subunit expression may indicate alterations in the excitability of mesocorticolimbic circuitry, and this may underlie the long-term biochemical and behavioural effects of nicotine dependence

Kew words: Chronic nicotine; nicotine withdrawal; nicotinic acetylcholine receptors; metabotropic glutamate receptors; NMDA and AMPA glutamate receptors

1. INTRODUCTION

Tobacco smoking is responsible for about five million premature deaths a year throughout the world directly and indirectly (as a result of exposure to passively inhaled tobacco smoke). Nicotine is generally regarded as the primary psychoactive component of tobacco that promotes the tobacco-smoking habit (addiction), and reduces stress and anxiety [1]. Nicotine alters a number of neuronal circuits by acting on neuronal nicotinic acetylcholine receptors (nAChRs), a heterogeneous family of ligand-gated cation channels that is widely distributed in the brain [2]. It has been repeatedly demonstrated that tobacco use in humans and chronic nicotine treatment in rodents induce an increase in the expression of nAChRs, (primarily the $\alpha 4\beta 2^*$ -nAChR subtype), that varies in different brain regions [3-5]). The reinforcing and motivational properties of nicotine are at least partially mediated by alterations in the activity of dopamine (DA) neurons in the mesocorticolimbic pathway. These neurons originate in the ventral tegmental area (VTA) and project to two principal targets: the nucleus accumbens (NAc) and the prefrontal cortex (PFC). Their activity in the VTA is regulated by complex interactions involving acetylcholine, glutamate, GABA, and DA transmissions (for review see [6]). By acting on the $\alpha 7$ nAChRs localised in presynaptic PFC-VTA glutamatergic terminals, nicotine stimulates glutamate release, and this facilitates the burst firing of VTA DA neurons; at the same time, it increases the release of GABA by acting on $\alpha 4\beta 2$ receptors located on inhibitory GABAergic afferents or interneurons, which modulates the bursting activity of DA neurons [7]. Moreover, by binding to the nAChR subtypes expressed by DA neuron cell bodies or terminals, nicotine increases DA release in the NAc. The increase in glutamatergic transmission and the direct activation of DA neurons mediated by nAChRs are mainly responsible for the reinforcing effect of nicotine, whereas the reduction in GABA transmission alters the perception of its aversive effects [8].

Glutamate binds to metabotropic (mGluRs) a class of slowly-acting G protein coupled receptors [10,11] and ionotropic (iGluRs), glutamate receptors (GluRs) [12]. The iGluRs are comprised mainly of α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (AMPA receptors) and N-methyl-D-aspartate (NMDA) receptors (NMDARs) that are primarily located on post-synaptic somatodendritic membranes and concentrated in dendritic spines, although some

may have a presynaptic localization [12]. AMPARs and NMDARs are both heterotetramers: AMPARs consist of various combinations of GluA1–GluA4 subunits and NMDARs have two obligatory GluN1 subunits and two modulatory GluN2 (GluN2A–D) and/or GluN3 (GluN3A–B) subunits.

The reinforcing effect of nicotine is partially mediated by the indirect activation of iGluRs in the VTA. This has been shown by the fact that injecting competitive [13, 14] or non-competitive [15] NMDAR antagonists in the VTA decreases nicotine self-administration (SA) and inhibits the nicotine-induced increase in DA in the NAc. Moreover, blocking the post-synaptic mGluR5 [16, 17] or activating presynaptic mGluR2/3 [18] also decreases nicotine-induced neurochemical and behavioural effects.

However, much less is known about the changes induced by nicotine withdrawal. It is thought that alterations in DA signalling contribute to withdrawal symptoms after chronic nicotine exposure. The withdrawal of nicotine induces a hypofunctional DA state in animal models of nicotine SA [19] or passive nicotine administration [19, 20] that decreases brain reward function [21] and low DA levels may induce drug seeking in order to reverse the negative effects of nicotine withdrawal.

The mGluR2s and mGluR3s in the mesocorticolimbic pathway, which negatively modulate excitatory glutamate transmission are presynaptically expressed, whereas mGluR5s have a post-synaptic location outside of the postsynaptic density (reviewed in [6]) and AMPARs and NMDARs are also mainly located postsynaptically. Non-DA neurons in the VTA especially express GluN1/GluN2A NMDARs, whereas DA neurons predominantly express the GluN1/GluN2B/GluN2D subtype, GluA2* AMPARs (GluA1/GluA2 or GluA2/GluA3), or GluA1 homotetramers. The AMPARs in the striatum which mainly contain the GluA1 and GluA2 subunits, are densely expressed in medium spiny neurons and may be located synaptically and extrasynaptically, whereas the NMDARs mainly express GluN1/GluN2A, GluN1/GluN2B and GluN1/GluN2A/GluN2B subunits. The AMPARs and NMDARs in the PFC are expressed in the cell bodies and presynaptic compartments of pyramidal neurons.

Given the critical importance of the mesocorticolimbic circuit in governing motivated and addictive behaviour, it is important to examine the changes in GluRs induced by chronic nicotine

exposure and its withdrawal at different times because different changes in excitatory synaptic transmission have been found during the early and late phases of cocaine withdrawal [22, 23]. Moreover, different drugs of abuse induce different neuronal adaptations, mainly because of their different mechanisms of action [24].

The findings of a number of electrophysiological studies suggest that nicotine functionally up-regulates iGluRs [25, 26], but have not fully explained the effects of nicotine on different GluR subtypes in specific brain regions. In addition, very little is known concerning the effects of nicotine withdrawal on GluRs.

The aim of this study of non-contingent, continuous nicotine administration was to examine the changes in nAChR and GluR expression in the three principal areas of the mesocorticolimbic pathway (the midbrain, the striatum and the PFC) after chronic nicotine exposure and during nicotine withdrawal. The analysis was made after fractionating the tissues in order to identify the receptors belonging to specific cellular subdomains.

2. EXPERIMENTAL PROCEDURES

2.1 Antibody production and characterization

For the detection of nAChR subunits we used affinity-purified, subunit-specific, polyclonal antibodies (Abs), produced in rabbit against peptides derived from the intracytoplasmic loop (CYT) of nAChR subunits [27]. For the GluR subunits we produced Abs directed against the COOH peptide (EGYNVYGIESVKI) of the GluA2/3 subunit and of the N-terminal peptide (RTSDSRDHTRVDWKR) of the the GluA1 subunit. The specificity of the affinity-purified Abs was tested by western blotting studies using β 2 WT and β 2 KO mouse cortex extracts cDNAs (supplementary Fig. 1) or using cells transfected and non-transfected with GluA1, GluA2 and GluA3 cDNAs (supplementary Fig. 2).

The anti-GluN2B (clone N59/20), anti-PSD95 (clone K28/43) and anti-SAP102 (clone N19/2) Abs were from Antibodies incorporated (Davis, CA, USA); anti-GluN1 (clone 54.1) was from BD Pharmingen (Franklin Lakes, NJ, USA); anti-GluN2A (clone A3-2D10) was from Life technologies (Waltham, MA, USA); anti-mGluR5 was from Millipore (Billerica, MA, USA); anti-mGluR2 (clone mG2Na-s) was from Abcam (Cambridge, UK) and anti-actin (clone AC-40) was from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Animals and Materials

The study involved the use of 4-6 month old male, pathogen-free, C57BL/6 wild-type (WT) mice. All efforts were made to minimise animal suffering and to use the minimum number of animals necessary to reach statistically relevant results.

All of the animal experiments were reviewed and approved by the Institutional Animal Care and Utilization Committee at the University of Colorado, Boulder and were conducted in accordance with the [European Community Council Directive \(2010/63/UE\)](#):

All of mice were bred at the Institute for Behavioral Genetics, and were housed at 22°C with lights on from 7 a.m. to 7 p.m. and allowed free access to food and water.

The (\pm)-[³H]-epibatidine ligand (Epi, specific activity: 66 Ci/mmol) was purchased from Perkin Elmer (Waltham, MA, USA); nonradioactive Epi, nicotine and α -bungarotoxin (α -Bgtx) were purchased from Tocris Bioscience (Bristol, UK). Liquid nicotine was purchased from

Sigma/Aldrich (St. Louis, MO) and redistilled periodically. All other reagents (PMSF, proteases inhibitors, chemicals) were also from Sigma-Aldrich.

2.3 Nicotine treatment

Nicotine was delivered through the cannula of silastic tubing inserted in the right jugular vein of each mouse as described by [28]. After surgery each mouse was transferred to an individual infusion chamber and its cannula was connected to a 1 mL syringe mounted on an infusion pump (Harvard Apparatus, Holliston, MA, USA). Sterile saline was continuously infused at a rate of 35 μ L/h for two days before nicotine treatment was begun. The mice were divided into two treatment groups: one received 4.0 mg/kg/h of nicotine (free base) and the other saline (control group). The nicotine dose was chosen because it induces strong nAChR up-regulation as observed in previous chronic nicotine treatment studies ([28], Moretti et al., unpublished data) with approximately 210 \pm 40ng/ml nicotine in the serum and corresponding to plasma concentrations of 1.3 \pm 0.2 μ M (28), that is higher than that present in the blood of smokers where the peak nicotine level can reach 0.6 μ M but usually it is 0.3 μ M (reviewed in [29]). After 14 days of treatment, nicotine administration was stopped. The animals in the nicotine group were divided into four subgroups and were subsequently sacrificed after, respectively, two hours (time 0), 1, 4, and 14 days (see upper part Fig. 1). The control animals were also divided into four groups and sacrificed at the same times.

2.4 Tissue preparation

Mice were euthanized by means of cervical dislocation, their brain was rapidly removed from the skull, and the areas of interest were dissected, placed in 1.5 ml Eppendorf tubes and quickly frozen on dry ice before being stored at -80°C.

2.4.1 Crude synaptosomal preparation

The midbrain, striatum, and PFC tissues were separately washed copiously in 0.32 M sucrose/10 mM TRIS buffer and homogenised in defined volumes of the same sucrose/Tris buffer using an Ultraturrax homogeniser and glass-Teflon tissue grinder. The homogenates (H) were centrifuged at 1,000 g for five minutes, and supernatant S1 was further centrifuged at

12,000 g for 20 min. The resulting crude synaptosomal preparations (P2) were suspended in isotonic buffer (Tris-HCl 50 mM, NaCl 120 mM, KCl 5 mM, MgCl₂ 1 mM, CaCl₂ 2.5 mM, pH 7.5). The protein concentrations were measured using the Pierce™ BCA Protein Assay Kit.

2.5 Immunoblotting and densitometric quantification of Western blot bands

The GluR and nAChR subunits were analysed by means of Western blotting as previously described [27,30]. In brief, depending on the brain area and the target subunit, 2.5, 5, 7.5, 10 or 20 μ g of the crude synaptosomal (P2) fractions obtained from the midbrain, striatum or PFC of the saline or nicotine-treated mice were diluted 1:1 (v/v) with Laemmli buffer, separated by means of SDS-polyacrylamide gel electrophoresis using 7.5% acrylamide, and electrophoretically transferred to nitrocellulose membranes with 0.45 mm pores (Schleicher and Schull II, Dassel, Germany). The same amount of P2 protein was always loaded on the same gel and the loading was further verified by actin staining.

The blots were blocked overnight in 4% non-fat milk in Tris-buffered saline, washed in a buffer containing 4% non-fat milk and 0.3% Tween20 in Tris-buffered saline, and incubated for 2 h with the primary antibody at the following concentrations or dilutions (GluA1, GluA2/3, and β 2: 1–2.5 mg/ml; GluN2A 1:500 GluN1 1:1500; GluN2B 1:600; mGluR5 1:1000; mGluR2 1:2000; PSD95 1:4000; SAP102 1:1000; actin 1:3000-12000 depending on the proteins loaded). They were then incubated for 1h with the appropriate secondary antibody (anti-rabbit Ly-Cor IRDye800RD; anti-mouse Ly-Cor IRDye680RD). After another series of washes, the membranes were dried overnight in the dark at RT. The IR signal was measured using an Odyssey CLx - Infrared Imaging System. The signal intensity of the Western blot bands was quantified using iStudio software. The optical density ratio was calculated by taking the optical density of the control saline as 100%. The data are expressed as mean values \pm S.E.M. of 10-15 separate experiments using each antibody.

2.6 ³H-Epipatidine binding studies

After incubating the P2 fractions obtained from the different brain areas of the different groups with 1 μ M α -Bungarotoxin for two hours, in order to block binding of ³H-Epi to α 7-nAChR sites, the binding experiments were carried out by incubating aliquots of the membrane with 1

nM ³H-Epi for two hours at RT. Non-specific binding (average of 5–10% of total binding) was determined in parallel samples containing 250 nM unlabelled Epi. At the end of the incubation, the samples were filtered on a millipore apparatus using a glass GFC filter soaked in 0.5% polyethylenimine, washed with 15 ml of buffer solution (sodium phosphate, 10 mM, pH 7.4, and 50 mM NaCl), and counted in a Tri-Carb 2100TR β-counter (Perkin Elmer)

2.7 Statistical analysis

The data from the binding and Western blotting studies were expressed as mean values ± SEM and analysed by means of one-way ANOVA followed by a Bonferroni *post hoc* test (parametric data) or by Kruskal–Wallis test followed by Dunn's *post hoc* test (non-parametric data). The accepted level of significance was $p < 0.05$. All of the statistical analyses were made using Prism software, version 5 (GraphPad).

3. RESULTS

3.1 Nicotine treatment

C57Bl/6 mice were implanted with a jugular catheter and, four days later, started receiving intravenous infusions of nicotine (4 mg/kg/h) or saline (control) for 14 days. Separate groups of mice were sacrificed two hours, and one, four or 14 days after the last infusion (see Figure 1). The three main areas of the mesocorticolimbic circuit (midbrain, striatum and PFC) were dissected and frozen. In order to define the distinct subcellular compartments in which the receptors are localised, whole tissue homogenates were fractionated as described in Methods in order to obtain the S1 fraction containing all of the cellular membranes, and then the P2 fraction containing the crude synaptosomal membranes. The S2 fraction contained mainly soluble proteins and some internal membranes, but no plasma membranes (data not shown).

3.2 Effects of nicotine exposure and withdrawal on nAChRs

We analysed nAChR expression in the mesocorticolimbic pathway under control conditions and after nicotine exposure (evaluated two hours after the cessation of chronic treatment) by means of ³H-Epi binding to the membrane-bound nAChRs in the P2 fraction of nicotine-treated animals. Moreover, as our previous binding and immunoprecipitation studies [31,32] have shown that more than 90% of the high affinity heteromeric receptors that bind ³H-Epi in the mesocorticolimbic pathway contain the β 2 subunit, we quantified the effect of nicotine on the expression of heteromeric receptors in the P2 fraction using anti- β 2 subunit-specific Abs. Western blot and binding studies showed that the P2 crude synaptosomal fraction of the three regions of the chronically nicotine-treated mice had higher levels of β 2*-nAChRs than the control mice (midbrain: Western blot: +64.4±5.3%, binding: +62±13.0%; striatum: Western blot: +43±3.6%, binding: +40±5.8%, and PFC: Western blot: +65±17.1%) (see Fig. 1). We did not assay binding in the PFC because of the limited amount of material.

It has been previously reported that exposure to nicotine increases the striatal expression of the α 4 β 2*-nAChR subtype, but there are conflicting data concerning its effects on α 6 β 2*-nAChR expression [4,33,34]. We used subunit-specific Abs to immunoprecipitate the ³H-

Epi-labelled receptors present in the 2% Triton X-100 extract obtained from the striatal P2 fraction, and found that there was a significant increase in $\alpha 4$ (control 65.6 ± 11 vs nicotine 103.9 ± 6.5 fmol/mg of protein, $n=4$; paired t test $p=0.046$) and $\beta 2$ subunits (control 67.1 ± 9.1 vs nicotine 107.5 ± 9.2 fmol/mg of protein, $n=4$; paired t test $p=0.0013$), and a significant decrease in the expression of $\alpha 6\beta 2^*$ -nAChRs in the nicotine-treated animals (control 16.4 ± 0.4 vs nicotine 13.5 ± 0.3 fmol/mg of protein, $n=4$; paired t test $p=0.029$), thus indicating that the nicotine-induced increase in $\beta 2^*$ -nAChRs is only due to the increase in the $\alpha 4\beta 2^*$ -nAChR subtype.

The binding studies of the striatum and midbrain, and the Western blots of all three brain areas showed that $\beta 2^*$ -nAChR expression gradually decreased during nicotine withdrawal, and had returned to control levels by withdrawal day (WD) 14. The P2 data indicate that nAChR expression was still significantly increased in the midbrain (Western blot: $53 \pm 7.2\%$, binding: $50 \pm 6\%$), striatum (Western blot: $19.9 \pm 2.5\%$; binding: $50 \pm 6\%$) and PFC (Western blot: $58.1 \pm 20\%$) on WD1, but had decreased to control levels by WD4.

These findings confirm that chronic nicotine up-regulates heteromeric $\beta 2^*$ nAChRs in the three principal mesocorticolimbic areas, and that receptors rapidly return to control levels upon nicotine cessation. The extent and temporal dynamics of nicotine-induced nAChR up-regulation are comparable in all three areas, thus supporting the idea that they are due to a common mechanism(s).

3.3 Mesocorticolimbic GluR expression after nicotine treatment and withdrawal

Previous studies have shown that addictive drugs such as cocaine induce long-lasting changes in the brain (reviewed in [35]), including widespread adaptations of glutamatergic synapses in the mesocorticolimbic pathway [35]. We therefore analysed the expression of iGluRs and mGluRs, and scaffold proteins specific for the different GluRs in the P2 fractions of the midbrain, striatum and PFC at different times after chronic nicotine treatment.

All of the immunoblots (Fig. [s](#) 2, 3 and 4) showed that the GluN1 subunit was visualised at a molecular weight (Mr) of 110kDa, GluN2A and GluN2B at an Mr of 160-180 kDa, GluA1 and GluA2/3 at an Mr of 110 kDa, mGluR2 at an Mr of 100 kDa, and mGluR5 at an Mr of 130 kDa.

The figures show representative blots of the different conditions and the quantitative data obtained from 10-15 Western blotting experiments.

3.3.1 Midbrain.

Chronic nicotine treatment did not affect the expression of midbrain GluN1, GluN2A or GluN2B subunits. However, starting from WD1, the levels of GluN1 and GluN2A increased during withdrawal. The increase in GluN1 was $+56.6\pm 17\%$ at WD1, $+61.1\pm 17.8\%$ at WD4 and $+54.1\pm 16.8\%$ at WD14, and the increase was statistically significant at all three time points ($n=10$; one way ANOVA, $p<0.05$). The increase in GluN2A was $+37.1\pm 12.5\%$ at WD1, $+42\pm 20\%$ at WD4, $+49.9\pm 18.6$ at WD14 and once again was statistically significant at all three time points ($n=10$; one way ANOVA, $p<0.05$).

The changes in calcium-permeable GluA1-containing AMPARs and the calcium-impermeable GluA2/3-containing AMPARs went in opposite directions during nicotine treatment and withdrawal (Fig. 2). Nicotine treatment selectively increased the expression of GluA2/3 ($+48\pm 13\%$, $p<0.01$), which was still high on WD1 ($+34\pm 13\%$, $p<0.05$), but had decreased by WD4 ($19\pm 5.2\%$, ns) and had returned to control levels by WD14. The increased levels of GluA2/3 after nicotine treatment and on WD1 were paralleled by increases in the levels of synaptic scaffold protein PSD95, which were $+32.8\pm 3\%$ after nicotine treatment, $+32.2\pm 7.7\%$ on WD1 (both $p<0.05$), $+11.8\pm 6.6\%$, on WD4, (ns), and returned to control levels by WD14. The expression of GluA1 was not affected by chronic nicotine treatment, but was increased during withdrawal: $+25.7\pm 5.5\%$, ($p<0.05$) on WD1, $+38.9\pm 13\%$ ($p<0.05$) on WD4, and 34.7 ± 7 ($p<0.01$) on WD14.

Finally, nicotine treatment specifically up-regulated mGluR5 ($47.0\pm 10.9\%$, $p<0.05$), but this effect was no longer statistically significant on WD1 ($23.8\pm 7.7\%$, ns) or WD4 ($25.6\pm 11.3\%$, ns), and the levels returned to those of controls by WD14. There was no change in mGluR2 after nicotine exposure or during withdrawal (Fig. 2). These findings show that the midbrain GluR subtypes that are up-regulated during chronic nicotine treatment are different from those that are up-regulated during withdrawal.

3.3.2 Striatum

Western blot analysis of the crude total striatal synaptosomal fractions (P2) showed that nicotine exposure did not change the level of expression of GluN1 ($109.6 \pm 12.1\%$, ns) or GluN2B ($91.4 \pm 7.8\%$, ns) subunits. However, there was a significant decrease in GluN1 ($-30.4 \pm 7\%$, $p < 0.01$) and GluN2B ($-43 \pm 7\%$, $p < 0.01$) on WD4. GluN2B down-regulation was long-lasting as it was still present on WD14 ($-40 \pm 5.5\%$, $p < 0.05$), whereas the expression of the GluN1 subunit had returned to control level by WD14. The level of GluN1 at P14 is similar to that of control and this is probably due to the fact that although not significant, there is an increase in GluN2A-subunits (which are associated with the GluN1 subunits in NMDARs), and this may compensate for the decrease of GluN1 subunits associated with GluN2B. The reduction in NMDAR expression in the P2 fraction on WD4 was associated with a significant reduction in the scaffold protein SAP102 ($-32.7 \pm 8.7\%$, $p < 0.05$), thus suggesting that the remodelling of striatal synapses may occur by WD4 (Fig. 3).

In comparison with the saline-treated samples, the levels of the AMPAR subunits in the P2 fractions were unchanged after nicotine treatment, but significantly decreased during withdrawal. The level of GluA2/3 was down-regulated on WD1 ($-21 \pm 3.5\%$, $p < 0.01$), and remained down-regulated throughout the withdrawal period (WD4 $-32 \pm 3.7\%$, $p < 0.001$; WD14 $-19 \pm 4.5\%$, $p < 0.01$). The level of GluA1 was also unaffected by nicotine treatment and on WD1, but decreased on WD4 ($-35.5 \pm 6.6\%$, $p < 0.01$) and had returned to control level by WD14.

Nicotine did not affect the expression of mGluR5, but strongly up-regulated mGluR2 ($+38.1 \pm 8.1\%$, $p < 0.01$). However, the up-regulation decreased on WD1 and had returned to control level by WD4 after which remained unchanged.

These data clearly indicate that nicotine treatment does not affect the expression of striatal iGluRs, but the withdrawal of nicotine treatment leads to a marked decrease in synaptic iGluR expression. In contrast, the expression of mGluR2 was increased by nicotine treatment. It is also interesting to note that almost all of the iGluRs and SAP102 were decreased on WD4 (Fig. 3).

3.3.3 Prefrontal cortex

Chronic nicotine treatment did not affect the expression of GluN2B and GluN1 in PFC, but reduced the expression of GluN2A ($-26 \pm 3\%$, $p < 0.05$). The decrease in GluN2A was biphasic it

was non-significant on WD1 ($-17\pm 3.1\%$) and WD4 (24.1 ± 7.2 , ns), but significant on WD14 ($-40\pm 8.6\%$, $p<0.01$) (Fig. 4).

Exposure to nicotine decreased the expression of GluA1 ($-19.1\pm 4\%$, $p<0.05$) and GluA2/3 ($-22\pm 3.8\%$, $p<0.05$) (Fig. 4). The differences were no longer significant on WD1 ($-12\pm 3.1\%$, ns) or WD4 (-14.8 ± 5.5 , ns) and remained almost constant until WD14. The decrease in AMPA subunits after nicotine treatment was paralleled by a significant decrease in scaffold protein PSD95 (-20.4 ± 4.1 , $p<0.05$). These data suggest that chronic nicotine reduces PFC glutamatergic synaptic transmission, mainly via mechanisms that remove iGluRs from the synapses.

4. DISCUSSION

The mesocorticolimbic system is a critical pathway for nicotine dependence and, as in the case of other forms of drug addiction, glutamatergic transmission is a major regulator of addictive behaviour. This is the first comprehensive examination of the effects of chronic nicotine exposure and withdrawal on the expression of nAChRs and GluRs in the mesocorticolimbic system, a brain reward circuit in which addictive drugs induce widespread adaptations.

We found that non-contingent, chronic nicotine treatment has different effects on nAChR and GluR expression in this pathway. Nicotine up-regulated $\beta 2^*$ -nAChRs in all three areas, and its withdrawal led to rapid decrease in the levels of these receptors, whereas its effects on GluRs were region and subtype specific.

Western blotting and binding studies indicated that nicotine-induced nAChR up-regulation occurs during chronic nicotine treatment, but disappears within four days of nicotine withdrawal. Previous studies [3-5, 36] have shown that nicotine up-regulates $\beta 2^*$ -nAChRs by increasing the $\alpha 4\beta 2^*$ -nAChR subtype, and significantly decreasing the striatal $\alpha 6\beta 2^*$ -nAChRs. Furthermore *in vitro* studies have shown that nicotine favours the maturation and assembly of the $\alpha 4\beta 2^*$ -nAChR subtype in the endoplasmic reticulum by acting as a protein chaperone, although other mechanisms such as decreased $\alpha 4\beta 2$ degradation and/or turnover (reviewed in [37]) may also play a role.

Studies of rodent models of nicotine withdrawal syndrome have shown that affective signs during withdrawal are mediated solely by central nAChR populations, particularly $\beta 2^*$ -nAChRs [38, 39], as shown by the fact that the withdrawal syndrome can be precipitated by the $\beta 2^*$ -nAChR selective antagonist dihydro- β -erythroidine.

The signs of withdrawal after chronic nicotine exposure are partially mediated by a hypofunctional DA state, that is reflected in decreased brain reward function [20]. The observed transient up-regulation of $\alpha 4\beta 2^*$ -nAChR subtype in the mesocorticolimbic system may contribute to the withdrawal syndrome observed after the cessation of chronic nicotine treatment as Nashmi et al. [36] found a specific nicotine-induced up-regulation of the $\alpha 4^*$ -nAChRs located on the GABA inputs of midbrain DA neurons. It is possible that these nAChRs, which are no longer

desensitised by nicotine during withdrawal, may over-inhibit DA neurons and also lead to some withdrawal symptoms [20].

Unlike the similar up-regulation of nAChR expression after nicotine exposure and fast return to control levels following nicotine withdrawal in the three studied brain regions, the changes in GluRs were highly heterogeneous in time and location. These findings show that passive chronic nicotine administration may alter glutamatergic transmission within the mesocorticolimbic system by selectively modulating GluR subunit expression. The major difference probably reflects the fact that nicotine has a direct effect on the processes of nAChR biosynthesis by favouring the formation of high affinity $\alpha 4\beta 2$ receptors with $(\alpha 4)_2(\beta 2)_3$ stoichiometry, whereas the changes in GluRs are due to circuit-specific functional adaptations of glutamatergic transmission to nicotine exposure.

The effects of nicotine treatment and withdrawal on midbrain GluR expression are larger, more diverse, and longer lasting than the changes in the striatum or prefrontal cortex. In particular, chronic nicotine treatment increased the expression of GluA2/3 AMPARs and PSD95 in the P2 fractions of the midbrain but had no effect on GluA1 AMPARs or NMDAR subunit expression. This suggests that AMPA-mediated glutamatergic transmission was potentiated in some synapses of the midbrain. This is in line with the findings of previous electrophysiological studies showing that nicotine increases the AMPA/NMDA ratio in dopaminergic VTA neurons, although these studies could not provide any insights into the effects of nicotine on the actual expression of GluRs in the VTA [25,26].

Another important finding was the increased level of mGluR5, a receptor subtype that interacts with NMDARs [40] and is important in mediating the reinforcing and motivational effects of nicotine in the VTA and NAc [17].

The increase in iGluR expression in mesocorticolimbic regions is specific to the midbrain, because we did not find any change in any of the iGluR subunits in the striatum, and GluA1, GluA2/3 and GluN2A actually decreased in the PFC. As chronic nicotine exposure promotes increased evoked glutamate release in the PFC [41], we speculate that the nicotine-induced down-regulation of iGluRs in the PFC and increase in the inhibitory autoreceptor mGluR2 in the striatum may be homeostatic mechanisms that occur in response to the hyperstimulation of the glutamatergic synapses in these regions.

Other authors have studied the effect of nicotine on the expression of GluRs in mesolimbic areas. Like Kenny *et al.* [14], we found a decrease in GluN2A and GluR2/3 in the PFC, but Wang *et al.*[42] found that GluN1, GluN2A and GluN2B increased. However like Wang *et al.* [42] we did not find any significant change in AMPAR and NMDAR subunits in the striatum, but there was an increase in GluR2/3 in the midbrain.

One major difference between our results and those described by Wang *et al.* [42] and Kenny *et al.*[14] is that their GluR subunit expression data come from tissue homogenates, whereas we used a crude synaptosomal fraction that presumably only contains mature GluRs. The altered GluR subunit expression levels that they observed may at least partially, reflect alterations in the levels of subunits that have not yet been incorporated into mature plasma membrane receptors. This possibility is also suggested by the fact that they did not find a parallel increase in the GluN1 subunit required for the assembly of functional NMDARs. Moreover, a number of methodological differences may account for these apparent discrepancies: we used mice, whereas they used two different strains of rats; our mice received nicotine by constant infusion instead of nicotine SA (although with two very different access schedules). Finally, we harvested brain tissues two hours after the discontinuation of nicotine, whereas Kenny *et al.* [14] did so approximately 23 hours after the final nicotine SA (similar to our WD1) and Wang *et al.* [42] approximately 30 min after the last nicotine infusion (similar to our WD0).

One limitation of our analysis is that the crude synaptosomal fraction we analysed contains both pre- and post-synaptic components with their various receptors and channels. It is known that iGluRs and mGluRs are present at pre- and post-synaptic sites, and that exposure to nicotine can affect the function and localisation of presynaptic AMPARs and NMDARs in NAc synaptosomes [43, 44]. Therefore, our findings cannot establish whether the changes in GluR expression in P2 took place at pre- or post-synaptic level.

We found that nicotine withdrawal leads to a return to basal levels of Ca²⁺-impermeable GluA2/3 in P2 fractions from the midbrain, and this is paralleled by an increase to above-basal levels in Ca²⁺-permeable GluA1 AMPARs and in NMDARs containing the GluN1 and GluN2A subunits. These changes persisted throughout the 14 days of withdrawal.

A decrease to below basal levels in both synaptic GluA1- and GluA2/3-containing AMPARs and GluN1- and GluN2B-containing NMDARs was detected in the striatum following withdrawal (in some cases lasting up to 14 days after the cessation of nicotine treatment), whereas there was no significant change in PFC GluRs during withdrawal. Though the functional relevance of these changes needs to be assessed by means of studies targeting specific glutamatergic synapses, it is clear that complex and long-lasting alterations take place in GluRs and therefore glutamate transmission in the mesocorticolimbic system during nicotine withdrawal. The progressive development and persistence of some of these changes is in line with the evidence of very long-lasting behavioural alterations during abstinence from tobacco smoke / nicotine exposure [45, 46].

In summary, nicotine exposure mainly affects GluR proteins and scaffold proteins in the midbrain, but nicotine withdrawal elicits area-specific changes also in the striatum and PFC. It is worth noting that many of the alterations in GluRs develop slowly after nicotine withdrawal, peaking at WD14. Alterations in glutamatergic transmission after the cessation of chronic nicotine treatment [47] may be persistent and contribute to the long-lasting alterations in brain neurochemistry and behaviour observed after chronic nicotine exposure.

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6. FIGURE LEGENDS

Figure 1:

Upper part. Scheme of the treatment

A cannula was implanted in the jugular vein of adult C5BL/6J mice as described in Materials and Methods. After they had recovered from the anesthesia the mice received saline infusions for two days., Mice were then infused with nicotine (4mg/kg/h) or saline for two weeks. After 14 days of treatment, nicotine administration was stopped, and the animals in the nicotine group were divided into four subgroups that were subsequently sacrificed after respectively two hours (time 0 or nicotine), 1, 4, and 14 days. The control animals were also divided into four groups and sacrificed at the same times. Midbrain, striatum and PFC were dissected at each time point and biochemically fractionated. The total homogenates (H) of each area were centrifuged in order to separate whole membrane (S1) from the large nuclear debris (P1), after which S1 was further centrifuged in order to separate the non-synaptosomal fraction (S2) from the crude synaptosomal membrane (P2).

Lower part. Analysis of the high affinity nAChR content in the mesolimbic pathway.

The P2 fractions of the midbrain, striatum and PFC obtained as described in the upper part of the figure were used for Western blot and binding studies (only in midbrain and striatum). Each panel shows Western blot with the anti- $\beta 2$ antibody in P2 fractions of the indicated areas, and the binding of ^3H -epibatidine to the P2 fractions of midbrain and striatum is shown on the right of the respective panel. The western blot analysis is expressed as a ratio between the different nicotine conditions (time 0, 1, 4 and 14 day nicotine withdrawal) and control saline values.

The binding values are also expressed as ratio between the different nicotine conditions and control saline values.

For binding studies and western blotting analysis mean values \pm SEM of 4-5 experiments are reported. The binding and the Western blotting data were statistically analysed using one-way ANOVA followed by Bonferroni test (binding) or Dunn's test (Western blot) (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, respectively), significantly different from saline).

Figure 2: Analysis of midbrain GluR subunits and scaffold proteins after nicotine exposure or withdrawal

Midbrain expression of GluR subunits and scaffold proteins in the crude synaptosomal fractions P2 after nicotine exposure or withdrawal. Membrane proteins were separated on 7.5 % acrylamide SDS gels, electrotransferred to nitrocellulose and probed with antibodies as described in the Methods. The amounts of P2 membranes loaded for the antibody analysis were: 2.5 μ g (GluA2/3; PSD95); 5 μ g (GluN1; GluA1); 10 μ g (SAP102, GluN2A); 20 μ g (GluN2B, mGluR2, mGluR5, β 2). Identical amounts of P2 membrane proteins were always loaded on the same gel and the loading was further verified by actin staining. The western blot analysis is expressed as a ratio between the different nicotine conditions (time 0, 1, 4 and 14 day nicotine withdrawal) and control saline values. The graphs show the mean values \pm SEM obtained by analysis of five different experiments performed in duplicate or triplicate; underneath each graph is a representative blot of the corresponding time conditions. The Western blotting data were statistically analysed using one-way ANOVA followed by Dunn's test (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, respectively), significantly different from saline.

Figure 3: Analysis of striatal GluR subunits and scaffold proteins after nicotine exposure and withdrawal

The procedure and antibody analysis is the same as that described in Figure 2 apart from the amounts of loaded membranes, which were: 2.5 μ g (GluA2/3, scaffold proteins

PSD 95); 5 µg (GluN1, GluA1, mGluR2); 7,5 µg (GluN2A, SAP102); 10 µg (GluN2B, mGluR5 and β2). The western blot analysis is expressed as a ratio between the different nicotine conditions (time 0, 1, 4 and 14 day nicotine withdrawal) and control saline values. The Western blotting data were statistically analysed using one-way ANOVA followed by Dunn's test (*P< 0.05, ** P< 0.01 and ***P< 0.001, respectively), significantly different from saline

Figure 4: Analysis of prefrontal cortex GluR subunits and scaffold proteins after nicotine exposure and withdrawal

The procedure and antibody analysis is the same as that described in Figure 2 apart from the amounts of loaded membranes which were: 2.5 µg (GluA2/3, scaffold proteins PSD 95); 5 µg (GluN1, GluA1, mGluR2); 7,5 µg (GluN2A, SAP102); 10 µg (GluN2B, mGluR5 and β2). The western blot analysis is expressed as a ratio between the different nicotine conditions (time 0, 1, 4 and 14 day nicotine withdrawal) and control saline values. The Western blotting data were statistically analysed using one-way ANOVA followed by Dunn's test, (*P< 0.05, ** P< 0.01 and ***P< 0.001, respectively), significantly different from saline.



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REPLIES TO REVIEWERS

Reviewer 1

1. We have updated the reference number of the European Community Council Directive (2010/63/UE).
2. We have added the review of Matta et al. (2007) to the references.
3. We have corrected the spelling and typing errors.

Reviewer 2

- 1) We have corrected the spelling mistakes and the improper use/ deletion of punctuation throughout the manuscript
- 2) The band of the $\beta 2$ subunit was always identified because lanes containing cortical tissues from WT and KO $\beta 2$ mice (the tissues shown in Supplementary Figure 1) were always run in parallel. The quantification that we report only refers to the appropriate $\beta 2$ band. The $\beta 2$ protein is glycosylated and can sometimes vary in width.
- 3) We apologise for the mistake, which we have now corrected.
- 4) We uploaded the figures correctly, as they were seen by the other reviewers.

Reviewer 3

We agree that this study is descriptive, but believe that this is an effective way of providing basic information for further mechanistic studies at cellular and circuit level.

A number of functional and behavioural studies have previously suggested that nicotine can affect the function of glutamate receptors, but they did not provide

insights into the effects of nicotine on the expression of all the major glutamate



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receptor subunits in crucial nicotine target regions. Our study was not intended to address the question of what the changes mean biologically, but to provide a basis for future more mechanistic studies.

To the best of our knowledge, this is the first systematic study of the effects of chronic nicotine exposure on the expression of nAChRs and GluRs in the midbrain, striatum and prefrontal cortex. The findings clearly show:

- 1) the effect of chronic nicotine treatment on the expression of nAChRs and GluRs;
- 2) the effect of nicotine withdrawal on nAChRs and GluRs at different times after nicotine cessation;
- 3) region-specific heterogeneity in the response of the different classes of GluRs to different treatments.



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Milan

Prof. Emilio Clementi,
Editor-in-Chief,
Pharmacological Research

Milan, 8 November, 2015

Dear Sir,

Ref: YPHRS_2015_61. Chronic nicotine and withdrawal affect glutamatergic but not nicotinic receptor expression in the mesocorticolimbic pathway in a region-specific manner

Please find attached a revised version of the above cited manuscript, and our point-by-point replies to your reviewers' comments. The changes in the text are highlighted in red.

We hope that you will find the new version of the paper suitable for publication in *Pharmacological Research*, and look forward to hearing from you in due course.

Yours faithfully

Dr. Cecilia Gotti



NEUROSCIENCE INSTITUTE

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REPLIES TO REVIEWERS

Reviewer 1

1. We have updated the reference number of the European Community Council Directive (2010/63/UE).
2. We have added the review of Matta et al. (2007) to the references.
3. We have corrected the spelling and typing errors.

Reviewer 2

- 1) We have corrected the spelling mistakes and the improper use/ deletion of punctuation throughout the manuscript
- 2) The band of the $\beta 2$ subunit was always identified because lanes containing cortical tissues from WT and KO $\beta 2$ mice (the tissues shown in Supplementary Figure 1) were always run in parallel. The quantification that we report only refers to the appropriate $\beta 2$ band. The $\beta 2$ protein is glycosylated and can sometimes vary in width.
- 3) We apologise for the mistake, which we have now corrected.
- 4) We uploaded the figures correctly, as they were seen by the other reviewers.

Reviewer 3

We agree that this study is descriptive, but believe that this is an effective way of providing basic information for further mechanistic studies at cellular and circuit level.

A number of functional and behavioural studies have previously suggested that nicotine can affect the function of glutamate receptors, but they did not provide



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insights into the effects of nicotine on the expression of all the major glutamate receptor subunits in crucial nicotine target regions. Our study was not intended to address the question of what the changes mean biologically, but to provide a basis for future more mechanistic studies.

To the best of our knowledge, this is the first systematic study of the effects of chronic nicotine exposure on the expression of nAChRs and GluRs in the midbrain, striatum and prefrontal cortex. The findings clearly show:

- 1) the effect of chronic nicotine treatment on the expression of nAChRs and GluRs;
- 2) the effect of nicotine withdrawal on nAChRs and GluRs at different times after nicotine cessation;
- 3) region-specific heterogeneity in the response of the different classes of GluRs to different treatments.