

**A comparative study of the effects of the intravenous self-administration or subcutaneous minipump infusion of nicotine on the expression of brain neuronal nicotinic receptor subtypes**

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Abbreviations: Ab, polyclonal antibody;  $\alpha$ Bgtx,  $\alpha$ Bungarotoxin;  $\alpha$ CntxMII,  $\alpha$ Conotoxin MII;  
Epi, epibatidine; MP, minipump; nAChR, nicotinic acetylcholine receptor; PMSF,  
phenylmethanesulfonyl fluoride; SA, self administration; TO, time out.

## ABSTRACT

Chronic nicotine exposure changes neuronal acetylcholine nicotinic receptor (nAChR) subtype expression in the brains of smokers and experimental animals. The aim of this study was to investigate nicotine-induced changes in nAChR expression in two models commonly used to describe the effects of nicotine in animals: operant (two-lever presses) intravenous self-administration (SA), and passive subcutaneous nicotine administration via an osmotic minipump (MP). In the MP group,  $\alpha 4\beta 2$  nAChRs were up-regulated in all brain regions,  $\alpha 6\beta 2^*$  nAChRs were down-regulated in the nucleus accumbens (NAc) and caudate-putamen (CPu), and  $\alpha 7$  nAChRs were up-regulated in the caudal cerebral cortex (CCx); the up-regulation of  $\alpha 4\beta 2\alpha 5$  nAChRs in the CCx was also suggested. In the SA group,  $\alpha 4\beta 2$  up-regulation was lower and limited to the CCx and NAc; there were no detectable changes in  $\alpha 6\beta 2^*$  or  $\alpha 7$  nAChRs. In the CCx of the MP rats, there was a close correlation between the increase in  $\alpha 4\beta 2$  binding and  $\alpha 4$  and  $\beta 2$  subunit levels measured by means of Western blotting, demonstrating that the up-regulation was due to an increase in  $\alpha 4\beta 2$  proteins. Western blotting also showed that the increase in the  $\beta 2$  subunit exceeded that of the  $\alpha 4$  subunit, suggesting that a change in  $\alpha 4\beta 2$  stoichiometry may occur *in vivo* as has been shown *in vitro*. These results show that nicotine has an area-specific effect on receptor subtypes, regardless of its administration route, but the effect is quantitatively greater in the case of MP administration.

## INTRODUCTION

Nicotine is the most widely used drug of abuse and has a number of important behavioural effects on the central nervous system (CNS) as a result of its interactions with neuronal nicotinic receptors (nAChRs), a very heterogeneous class of ligand-gated cation channels.

The behavioural effects of nicotine may be due to nAChR activation or desensitisation, because nicotine may affect neuronal function by stimulating nAChR-dependent cellular effects or by interrupting the nicotinic transmission of endogenous acetylcholine (ACh) (Picciotto et al., 2008; Buisson and Bertrand, 2002). The various nAChR subtypes co-existing in the brain are not equally responsive to nicotine activation and desensitisation and may be differently affected depending on where they are expressed in the neuronal circuits, leading to complex behavioural responses (reviewed by Picciotto et al., 2008 and Govind et al., 2009).

It has been shown that prolonged exposure to nicotine leads to the increased expression (up-regulation) of nAChRs. *In vitro* studies of cells transfected with nAChR subtypes have shown that nicotine increases the number of homomeric and various heteromeric nAChR subtypes, although the kinetics and concentration-dependence of the up-regulation varies among subtypes (reviewed in Gentry and Lukas, 2002; Gaimarri et al., 2007; Picciotto et al., 2008). *In vivo* studies of the brains of human smokers (Perry et al., 1999) or animals chronically treated with nicotine (Marks et al., 1992) have shown that nicotine triggers nAChR up-regulation without affecting  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$  or  $\beta 2$  nAChR subunit mRNA levels, thus indicating that post-transcriptional mechanisms are responsible. The most up-regulated receptor in primates and rodents is the  $\alpha 4\beta 2$  subtype for which at least six different post-transcriptional mechanisms have been proposed (for review see Lester et al., 2009 and Govind et al., 2009).

It was long thought that the up-regulation of nAChRs is an epiphenomenon of nicotine addiction, but recent studies clearly indicate that it may affect nAChR-dependent cell functions and thus contribute to some of the main biological effects of nicotine addiction, such as tolerance, locomotor and cognitive sensitisation (reviewed in Lester et al., 2009 and Xiao et

al., 2009). For example, Nashmi et al. (2007) have recently observed a cell specific nicotine-induced up-regulation of GFP-tagged  $\alpha 4$  subunits that correlates with an increase in functional response as measured by means of electrophysiological recordings.

Much attention has recently been given to  $\alpha 6^*$  receptors, which bind  $\alpha$ conotoxin MII ( $\alpha$ CntxMII) and play an important role in striatal dopamine release, locomotion and nicotine self-administration (SA) (reviewed in Gotti et al., 2009) but the effects of prolonged nicotine exposure on  $\alpha 6^*$  receptor expression have not yet been elucidated. Recent studies have shown that intravenous nicotine SA leads to an increase in the number of  $\alpha 6^*$  receptors in rats (Parker et al., 2004), but chronic nicotine treatment by means of an osmotic minipump (MP) or drinking water decreases the number of striatal  $\alpha 6^*$  receptors (Mugnaini et al., 2006; Perry et al., 2007; Lai et al., 2005). On the other hand,  $\alpha 6\beta 2$  receptors in transfected cells are strongly up-regulated by nicotine (Tumkosit et al., 2006; Walsh et al., 2008).

It is still not known if the discrepancy between the *in vivo* effects of nicotine by SA and those of nicotine administered by osmotic MPs on  $\alpha 6^*$  receptor regulation is the consequence of dissimilar nicotine pharmacokinetic profiles between the two models, diverse (operant vs. non operant) behavioural paradigms, or differences in the way the receptors are quantified. Moreover, there is still much debate concerning the *in vivo* effect of nicotine on  $\alpha 4\beta 2$  up-regulation, and very little is known about the regulation of other nAChR subtypes.

The aim of this study was to determine possible changes in the expression and subunit composition of native nAChRs following long-term intravenous nicotine SA or chronic nicotine treatment administered subcutaneously by means of an implanted osmotic MPs. We focused on the mesostriatal pathway which mediates many of the reinforcing properties of nicotine and expresses a large variety of nAChR subtypes (reviewed in Livingstone and Wonnacott, 2009).

In order to overcome possible methodological differences, we used a combination of two different techniques (receptor binding and immunoprecipitation) to measure receptor levels. In the case of  $\alpha 4\beta 2$  subtype expressed in the caudal cortex, the levels of the  $\alpha 4$  and  $\beta 2$  subunits were quantified also by means of Western blotting.

## MATERIALS AND METHODS

### Materials

(+/-)<sup>3</sup>H-Epi (Epi, s.a. 50-66 Ci/mmol) was purchased from GE Healthcare (Bucks, UK), <sup>125</sup>I- $\alpha$ Bungarotoxin ( $\alpha$ Bgtx, s.a. 200 Ci/mmol) from PerkinElmer (Boston, USA) and non-radioactive ligands from Sigma-Aldrich (Italy). <sup>125</sup>I- $\alpha$ Conotoxin MII (CntxMII, s.a. 200 Ci/mmol) was custom synthesised by GE Healthcare. Nicotine bitartrate (Sigma-Aldrich, St. Louis, USA) used during SA sessions was dissolved in 5 U.I. heparinised saline and then pH adjusted to 7.4 with NaOH. Nicotine doses were expressed as mg of free base/kg of body weight.

### Apparatus for nicotine self-administration

Rats that underwent nicotine SA were tested in 12 operant chambers. Each chamber (30 cm wide  $\times$  25 cm deep  $\times$  32 cm high) (Med Associates Inc., St. Albans, VT, USA) was placed within a sound and light-attenuating box equipped with a ventilation fan that supplied background white noise. The chamber's floor consisted of a metallic grid. Two walls, as well as the ceiling, were in Perspex, whereas the two lateral ones were made of stainless steel; one of these metal walls was equipped with two retractable levers 4.5 cm wide, 12 cm apart and 6 cm from the grid floor. A 2.5-W, 24-V cue light was placed above each lever. A sonalert 2900-Hz tone module to allow the production of an acoustic stimulus at 65 dB was located 23 cm above the left lever. On the opposite wall, a 2.5-W, 24-V white house-light was located 27 cm above the grid floor.

Outside each box, an infusion pump (Model PHM-100VS, Med Associates Inc.) was connected via an external catheter consisting in a tygon tubing (Norton Plastics Performance, Akron, OH, USA) to a single-channel liquid swivel (Instech laboratories Inc., Plymouth Meeting, PA, USA) mounted on a counterbalanced moving arm fixed at its base to the side of

the chamber. The swivel was connected to the rat's implanted catheter via another length of tygon tubing protected by a metallic spring.

### **Computer control and data collection**

Data acquisition and operant-schedule parameters were controlled by a Med-PC software (Med Associates Inc.) running on one PC-microcomputer connected to the chambers via interface modules (Med Associates Inc.).

### **Self administration**

Male Lister Hooded rats (Charles River, England), similar for age and housing, were used for all the experiments. In GSK facilities, they were individually housed in a temperature-controlled environment with lights on from 06.00 to 18.00 hours. Water was continuously available and animals were maintained at a constant body weight of 290 - 310 g.

Animals were divided in three groups that were treated as followed:*Naïve (control) group*: twenty seven rats were handled and singly housed for about three months, after which they were euthanized for brain tissue micro-dissection.*Nicotine self administration group*: twenty six rats were anaesthetized with medetomidine (0.1 mg/kg, intramuscular (i.m.); Domitor, Pfizer Italia s.r.l., Roma-Latina, Italy), followed by a combination of tiletamine and zolazepam (40 mg/kg i.m. Zoletil 100, Laboratoires Virbac, Carros, France). Animals also received subcutaneous injections of 0.30 ml of an antibiotic suspension of benzathine benzylpenicilline and dihydrostreptomycine sulphate (Rubrocillina Veterinaria, Gellini International s.r.l. Latina, Italy) providing 72 hours of protection and carprofen 5 mg/kg as analgesic (Rimadyl, Pfizer Italia s.r.l. Roma-Latina, Italy). They were then implanted with a silastic catheter in the right jugular vein, and the mesh end of the catheter was sutured subcutaneously on the dorsum. Immediately after completion of the surgery, rats received atipamezole hydrochloride 0.25 mg/kg i.m. (Antisedan, Pfizer Italia s.r.l. Roma-Latina, Italy) to facilitate recovery from



anaesthesia. During the 10-day recovery period, rats were injected intravenously (i.v.) with 0.1 ml of a solution containing 4 IU/ml heparin (Liquemin, Roche S.p.A., Milano, Italy) twice a week.

After the recovery period, nicotine SA was initiated under a FR1 schedule of reinforcement in the operant chambers where two levers were exposed. Rats were connected to the external catheter and each active lever press led to a 22  $\mu$ l infusion of nicotine (0.03 mg/kg/infusion) delivered over 1 sec, the extinction of the house light (60 sec) and illumination of the stimulus light located above the active lever (1 sec), as well as 1-sec sounding of the Sonalert device, the last three events representing nicotine-associated conditioned stimuli (CS). Each infusion was followed by a 60-sec time-out (TO) during which responses were recorded but did not lead to additional infusions. Each session lasted until rats had received 25 infusions of nicotine or 3 hours had elapsed, whichever occurred first. When the 25-infusions criterion was met, the response requirement was increased to FR2. Under this schedule two presses were necessary to lead to the complex of events described above. Each session lasted for 2 hours. Once animals had reached stable responding under this schedule, the length of the session was limited to 1 hour. An animal was considered to have achieved stable responding when it made a similar number of responses on the active lever for three consecutive days ( $\pm$  10%). Rats underwent this latter schedule for at least fifteen sessions. Immediately after the end of the last session they were sacrificed for the brain dissection.

*Oral sucrose self administration group:* twenty-six rats underwent a sham-operation consisting in the ligation of the jugular vein according to the procedure described for nicotine self-administering rats except for the catheter implantation. Rats were allowed to recover for ten days, and then sucrose SA was initiated under a FR1 schedule of reinforcement in operant chambers where two levers were exposed. Each active lever press led to the presentation of 20  $\mu$ l of 10% sucrose delivered by liquid dipper over 10 sec, the extinction of the house light (60 sec) and

illumination of the stimulus light located above the active lever (1 sec), as well as 1-sec sounding of the Sonalert device. Each sucrose presentation was followed by a 60-sec TO during which responses were recorded but did not lead to additional sucrose deliveries. Each session lasted until rats had received 25 presentations of sucrose or 3 hours had elapsed, whichever occurred first. When the 25-presentation criterion was met, the response requirement was increased to FR2. Under this schedule two presses were necessary to lead to the complex of events described above. Each session lasted for 2 hours. Once animals had reached stable responding under this schedule, the length of the session was decreased to 1 hour. An animal was considered to have achieved stable responding when it made a similar number of responses on the active lever for three consecutive days ( $\pm 10\%$ ). They underwent this latter schedule for at least fifteen sessions. Immediately after the end of the last session they were sacrificed for the brain dissection.

### **Osmotic minipumps**

*Saline osmotic MPs (control):* nineteen rats were anaesthetized by inhalation of a mixture of air and O<sub>2</sub> containing 2.5% isoflurane (Forane<sup>®</sup>, Abbott S.p.A., Italy) and implanted subcutaneously (s.c.), with Alzet Osmotic Minipumps (model 2-ML2, Alzet, USA) with a pumping rate of 5  $\mu$ l/h delivering saline. Before s.c. implantation, pumps were filled with sterile water and placed in the same solution in a 37°C water bath overnight to equilibrate saline release. Under anaesthesia, a large subcutaneous pocket for the pump was created in the dorsal thoracic area of the rats to host the osmotic MP. Rats were infused s.c. for 14 consecutive days with saline and killed immediately after treatment. Soon after euthanasia, the brain was removed and micro-dissected into the brain regions of interest.

*Nicotine osmotic MPs:* nineteen rats were implanted subcutaneously, under isoflurane anaesthesia, with Alzet Osmotic Minipumps (model 2-ML2) with a pumping rate of 5  $\mu$ l/h delivering saline. Before s.c. implantation, pumps were filled with nicotine solution which

concentration was calculated to provide a dose of 3 mg/kg/day nicotine free base and placed in sterile saline in a 37°C water bath overnight to equilibrate nicotine release to ensure steady-state delivery after implantation. Under anaesthesia, a large subcutaneous pocket for the pump was created in the dorsal thoracic area of the rats to host the osmotic MP. Rats were infused s.c. for 14 consecutive days with 3 mg/kg/day of nicotine and killed immediately after treatment. Soon after euthanasia, the brain was removed and micro-dissected into the brain regions of interest.

### **Brain tissue dissection**

Rats were decapitated, their eyes and brain quickly removed and the following brain areas carefully dissected on ice: caudate–putamen (CPu), the nucleus accumbens (NAc), the ventral midbrain (VMB), the superior colliculus (SC), and the caudal cerebral cortex (CCx). The CCx was defined as the cerebral cortex between -4.80 and -6.04 mm from Bregma in stereotaxic coordinates (Paxinos and Watson, *The Rat Brain in Stereotaxic Coordinates*. Academic Press, San Diego, 1998). Immediately after dissection, the above mentioned brain regions were frozen in dry ice pre-cooled isopentane (-35 °C) and stored at -80 °C.

### **Antibody production and characterization**

The subunit-specific polyclonal antibodies (Abs) used were produced in rabbit against peptides derived from the C-terminal (CooH) and/or intracytoplasmic loop (Cyt) regions of rat (R), human (H) or mouse (M) subunit sequences and affinity purified as previously described (Zoli et al., 2002). Most of the Abs have been previously described (Zoli et al., 2002; Gotti et al., 2005a; Gotti et al., 2005b) and the aminoacid sequence of the peptides used to produce the ABs used in this work are reported in Table 1.

Antibody specificity was checked by means of quantitative immunoprecipitation or immunopurification experiments or Western blotting using nAChRs from different areas of the

CNS of wild-type (+/+) and null mutant (-/-) mice, which allowed selection of Abs specific for the subunit of interest, and established the immunoprecipitation capacity of each Ab (Zoli et al., 2002; Gotti et al., 2005a; Gotti et al., 2005b). For the full characterization of nAChR subunit antibodies see Supplementary Table 1 in Grady *et al.* (2009).

### **Preparation of membranes and 2% Triton X-100 extracts**

In every experiment, the tissues from three rats from each experimental group were pooled and homogenised in 10 ml of 50 mM Na phosphate, pH 7.4, 1 M NaCl, 2 mM EDTA, 2 mM EGTA and 2 mM PMSF using an homogeniser, and the homogenates were diluted and centrifuged for 1.5 h at 60,000g. The total membrane homogenisation, dilution and centrifugation procedures were repeated, after which the pellets were collected, rapidly rinsed with 50mM Tris HCl, pH 7, 120 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 2.5 mM CaCl<sub>2</sub> and 2 mM PMSF, and then resuspended in the same buffer containing a mixture of 20 µg/ml of each of the following protease inhibitors: leupeptin, bestatin, pepstatin A and aprotinin. Triton X-100 at a final concentration of 2% was added to the washed membranes, which were extracted for 2 h at 4°C. The extracts were centrifuged for 1.5 h at 60,000g, recovered, and an aliquot of the supernatants was collected for protein measurement using the BCA protein assay (Pierce) with bovine serum albumin as the standard.

### **Binding studies**

<sup>125</sup>I-*α*Bungarotoxin. <sup>125</sup>I-*α*Bgtx binding experiments were performed by incubating membranes from each experimental group with a saturating concentration (5nM) of <sup>125</sup>I-*α*Bgtx at 20°C. For <sup>125</sup>I-*α*Bgtx, 2 mg/ml bovine serum albumin was added to the suspension buffer. Specific radioligand binding was defined as total binding minus non-specific binding determined in the presence of 1 µM cold *α*Bgtx.

<sup>125</sup>I-*αconotoxin MII*. Binding experiments were performed by incubating tissue membranes from each experimental group for three hours 0.5 nM <sup>125</sup>I-*αCnTxMII* at 20°C. For <sup>125</sup>I-*αCnTxMII*, 2 mg/ml bovine serum albumin was added to the suspension buffer. Specific radioligand binding was defined as total binding minus non-specific binding determined in the presence of 1 μM cold *αCnTxMII* or 250 nM cold Epi. At the end of the incubation, the samples were filtered on a GFC filter soaked in 0.5% polyethylenimine and washed with 15 ml of 10 mM Na phosphate pH 7.4 plus 50 mM NaCl, and the filters counted in a γ counter.

<sup>3</sup>H-*Epibatidine*. In order to ensure that the *α7*-containing subtypes did not contribute to <sup>3</sup>H-Epi binding (Marks et al. 2006), both membrane as well as in solubilized receptors (present in the extract and immunoprecipitation experiments) were first incubated for 3 hours with 2 μM *αBgtx* which specifically binds to *α7*-nAChR (and thus prevents <sup>3</sup>H-Epi binding to these sites).

Binding to the homogenates obtained from the different brain areas of the different groups was carried out overnight by incubating aliquots of the membrane with 2 nM <sup>3</sup>H-Epi at 4°C. Non-specific binding (averaging 5-10% of total binding) was determined in parallel samples containing 100 nM unlabelled Epi. At the end of the incubation, the samples were filtered on a GFC filter soaked in 0.5% polyethylenimine, washed with 15 ml of buffer (Na phosphate, 10 mM, pH 7.4; NaCl, 50 mM) and counted in a beta counter.

The Triton X-100 extracts were labeled with 2 nM <sup>3</sup>H-Epi. Tissue extract binding was performed using DE52 ion-exchange resin (Whatman, Maidstone, Kent,UK) as previously described (Gotti et al., 2005a).

### **Immunoprecipitation of <sup>3</sup>H-Epibatidine-labelled receptors by subunit-specific antibodies**

The tissue extracts were preincubated with 2 μM *αBgtx*, labelled with 2 nM <sup>3</sup>H-Epi, and incubated overnight with a saturating concentration of affinity purified anti-subunit IgG (20-30 μg) (Sigma, St Louis, USA). The immunoprecipitation was recovered by incubating the samples

with beads containing bound anti-rabbit goat IgG (Technogenetics, Milan, Italy). The level of antibody immunoprecipitation was expressed as the percentage of <sup>3</sup>H-Epi-labelled receptors immunoprecipitated by the antibodies (taking the amount present in the Triton X-100 extract solution before immunoprecipitation as 100%) or as fmol of immunoprecipitated receptors/mg of protein.

### **Immunoblotting and densitometric quantification of western blot bands**

The analysis of the  $\alpha 4\beta 2$  subtype by Western blotting was performed as previously described (Gotti et al., 2008; Grady et al., 2009). Briefly 10  $\mu$ g of 2% triton X-100 extracts membranes obtained from CCx of MP saline and nicotine rats were diluted 1:1(vol:vol) with Laemmli buffer and then underwent sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 9% acrylamide. After SDS-PAGE, the proteins were electrophoretically transferred to nitrocellulose membranes with 0.45 mm pores (Schleicher and Schuell, Dassel, Germany). The blots were blocked overnight in 5% non-fat milk in Tris buffered saline (TBS), washed in a buffer containing 5% non fat-milk and 0.3% Tween 20 in TBS, incubated for 2 hours with the primary antibody (1–2.5 mg/ml), and then incubated with the appropriate peroxidase conjugated secondary Abs. After another series of washes, peroxidase was detected using a chemiluminescent substrate (Pierce, Rockford, IL, USA).

The quantification of the signal intensity of the Western blot bands was performed as previously described (Gotti et al., 2008; Grady et al., 2009). The optical density ratio was calculated by taking the optical density of the control saline as 1. The values are the mean  $\pm$  SEM of four separate experiments for each antibody.

### **Statistical analysis**

Statistical analysis of the expression of <sup>3</sup>H-Epi, <sup>125</sup>I- $\alpha$ Bgtx and <sup>125</sup>I- $\alpha$ CntxMII receptors as well as the subunit content of the expressed <sup>3</sup>H-Epi receptors in the different experimental

conditions in the different areas of SA rats were carried out by one way analysis of variance (ANOVA), followed by Bonferroni post hoc comparisons.

In the case of rats that received nicotine by MP, comparison was made using a paired t test.

The software for statistical analysis was GraphPad Prism 4.

## RESULTS

### Nicotine and sucrose self-administration

Rats that were allowed to self-administer nicotine acquired responding for nicotine without prior food training or priming. In this group, at the end of SA experiment, the number of active lever presses per session was  $55.7 \pm 4.2$  (mean values of the last three sessions before the sacrifice), significantly greater than the number of inactive lever presses ( $10.9 \pm 1.3$ ; Student's t-test  $p < 0.001$ ). The mean value of i.v. nicotine intake was  $0.82 \pm 0.06$  mg/kg/day (mean of the last three sessions; expressed as nicotine free base) that were attributable to  $27.3 \pm 2.1$  infusions/day.

In the sucrose self-administering rats, the number of sucrose deliveries was  $56.7 \pm 0.6$  (mean of the daily means of reinforcers obtained during the last three sessions). The number of active lever presses per session was  $115.9 \pm 1.4$ , significantly greater than the number of inactive lever presses ( $0.7 \pm 0.4$ ; Student's t-test  $p < 0.001$ ).

### Membrane binding of nicotinic ligands in control and treated rats

Tissues from the CCx, NAc, CPu, VMB and the SC of control and treated rats were analysed using binding studies with radioactive ligands selective for heteromeric nAChRs in general ( $^3\text{H}$ -Epi), homomeric  $\alpha 7$ -nAChRs ( $^{125}\text{I}\alpha\text{Bgtx}$ ) and  $\alpha 6^*$ -nAChRs ( $^{125}\text{I}\alpha\text{CntxMII}$ ).

The naïve (untreated) group and the saline-treated group were the control groups in the SA and the MP experiment, respectively.

#### $^3\text{H}$ -Epi binding to membrane nAChRs

In naïve and saline-treated controls, the highest levels of  $^3\text{H}$ -Epi bound receptors (expressed as fmol/mg of protein) were found in the SC ( $151.9 \pm 10.2$  and  $144.2 \pm 15.1$ , respectively), followed by CPu ( $97.0 \pm 9.2$ ;  $99.6 \pm 7.6$ ), CCx ( $66.2 \pm 1.9$ ;  $78.8 \pm 2.9$ ), NAc ( $60.2 \pm 7.9$ ;  $48.0 \pm 1.8$ ) and VMB ( $57.9 \pm 6.9$ ;  $50.9 \pm 2.2$ ) (Table 2).

The different SA treatments (sucrose or nicotine) did not significantly alter the level of  $^3\text{H}$ -Epi bound receptors in the SC, CPu, NAc or VMB ( $P > 0.05$  one way ANOVA), but significantly increased the levels in the CCx ( $P < 0.0001$ ); Bonferroni's post hoc comparisons showed that binding in the nicotine-treated rats was significantly higher than in the nicotine-naïve ( $P < 0.001$ ) or sucrose treated rats ( $P < 0.01$ ).

Nicotine treatment via MP significantly increased  $^3\text{H}$ -Epi binding, with respect to saline, in the CPu (paired t test saline vs nicotine:  $P = 0.0213$ ), NAc ( $P < 0.0001$ ), SC ( $P = 0.0060$ ), and CCx ( $P < 0.0001$ ), but not in the VMB.

#### $^{125}\text{I}$ - $\alpha$ Bgtx binding to membrane nAChRs

In naïve and saline-treated controls, the highest levels of  $^{125}\text{I}$ - $\alpha$  Bgtx bound receptors (expressed as fmol/mg of protein) were found in the SC ( $50.4 \pm 11.5$  and  $67.3 \pm 7.1$ , respectively) followed by CCx ( $31.7 \pm 1.3$ ;  $31.8 \pm 4.3$ ) and, at similar levels, the VMB ( $16.9 \pm 1.2$ ;  $21.5 \pm 1.4$ ) and NAc ( $20.0 \pm 2.4$ ;  $16.0 \pm 3.4$ ); the levels in the CPu ( $9.3 \pm 1.6$ ;  $5.1 \pm 0.7$ ) were much lower (Table 3).

There were no significant differences in  $^{125}\text{I}$ - $\alpha$  Bgtx binding levels between the various SA treatments in the regions considered.

In the MP group,  $^{125}\text{I}$ - $\alpha$  Bgtx binding in the CCx was higher in the rats receiving nicotine than in those receiving saline (paired t test  $P = 0.0255$ ). There were no significant differences in the other areas.

#### $^{125}\text{I}$ - $\alpha$ CntxMII binding to membrane nAChRs

In naïve and saline-treated controls, the highest levels of  $^{125}\text{I}$ - $\alpha$ CntxMII binding were found in the SC ( $11.9 \pm 4.6$ ;  $15.2 \pm 2.7$  respectively), followed by the CPu ( $7.0 \pm 0.8$ ;  $6.2 \pm 0.7$ ), NAc



( $3.4\pm 0.9$ ,  $2.2\pm 0.7$ ) and VMB ( $0.88\pm 0.2$ ;  $0.83\pm 0.1$ ). No specific binding was detected in the CCx. Results are shown in Table 3.

One way ANOVA did not reveal any statistically significant difference between the nicotine-naïve rats and rats self-administering sucrose or nicotine. In contrast, MP administered nicotine significantly decreased  $^{125}\text{I}$ - $\alpha\text{CntxMII}$  binding in the NAc (paired t tests,  $P=0.0125$ ) and CPu ( $P=0.0298$ ) in comparison with saline, but there were no significant differences in the VMB or SC.

#### $^3\text{H}$ - Epi binding to 2% Triton x-100 extracts

In agreement with the membrane binding data, the level of  $^3\text{H}$ -Epi receptors in the 2 %Triton X-100 extracts of the control animals (that is untreated - naïve - in the SA experiment, and treated with saline in the MP experiment) were highest in the SC ( $187.7\pm 23.8$  and  $181.8\pm 0.1$ , respectively) followed by the CPu ( $160.6\pm 19.1$ ;  $133.8\pm 13.0$ ), NAc ( $89.4\pm 8.0$ ;  $89.7\pm 7.9$ ), CCx ( $88.0\pm 5.8$ ;  $83.5\pm 3.0$ ) and VMB ( $77.5\pm 10.8$ ;  $80.8\pm 2.9$ ).

The different SA treatments (sucrose or nicotine) did not significantly alter the level of  $^3\text{H}$ -Epi receptors in the SC, CPu, and VMB ( $P>0.05$ , one way ANOVA). In the NAc,  $^3\text{H}$ -Epi binding was significantly higher in the nicotine treated rats, with respect to naïve (one-way ANOVA, followed by a post hoc Bonferroni test,  $P < 0.05$ ). In the CCx, binding levels were significantly higher in the nicotine SA rats than in the sucrose SA ( $P < 0.05$ ) and the naïve rats ( $P<0.01$ ; one-way ANOVA, followed by a post hoc Bonferroni test).

In the MP group, nicotine significantly increased the expression of  $^3\text{H}$ -Epi receptors in the CPu (paired t test saline vs nicotine:  $P=0.0460$ ), NAc ( $P=0.0025$ ), SC ( $P=0.0480$ ), CCx ( $P=0.0002$ ) and in the VMB ( $P=0.0490$ ).

## Quantitative immunoprecipitation of <sup>3</sup>H-Epi bound nAChRs

As our binding studies in both membrane-bound and solubilised nAChRs clearly showed that nicotine affects the expression of nAChR subtypes depending on the type of treatment and the brain area, we analysed the <sup>3</sup>H-Epi labelled receptors by means of immunoprecipitation experiments using subunit specific Abs.

Table 1 shows aminoacid sequences of the peptides used to produce the subunit-specific Abs used in the quantitative immunoprecipitation. In each experiment, for each subunit other than  $\alpha 2$ ,  $\alpha 3$  and  $\beta 4$ , we always separately immunoprecipitated the receptors labelled by <sup>3</sup>H-Epi using two Abs directed against the two different epitopes of the same subunit.

Figure 2 shows the results of the immunoprecipitation experiments of labelled <sup>3</sup>H-Epi receptors using anti- $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\beta 2$ ,  $\beta 3$  and  $\beta 4$  Abs. The values shown for the  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\beta 2$  and  $\beta 3$  subunits are the average of the values obtained using the two Abs.

### Caudal cortex

The large majority of the <sup>3</sup>H-Epi receptors in this particular area of the cortex contained the  $\alpha 4\beta 2$  subtype and 10-15% also contained the  $\alpha 5$  subunits. These results are consistent with results previously published by us and other groups (Mao et al., 2007).

There was no difference in the level of  $\alpha 4\beta 2^*$  receptors between naïve and sucrose-treated rats, but levels measured in the nicotine SA group were significantly higher than the naïve and the sucrose SA group (one way ANOVA followed by post hoc Bonferroni test  $P < 0.05$ ) (Figure 2A).

In line with the extract binding data, MP nicotine treatment greatly increased (~1.7 fold) the levels of  $\alpha 4\beta 2^*$  nAChRs with respect to saline, an increase that was much higher than that induced in the same area by self-administered nicotine (~ 1.2 fold).

Moreover, we found that MP nicotine also increased (1.7 fold) the level of  $\alpha 5$  subunit in a statistically significant manner, whereas no significant increase in  $\alpha 5$  was detected in the nicotine SA group.

### Nucleus Accumbens and Caudate Putamen

Recent published data from our group have shown that the subtype compositions of nAChRs expressed in the NAc resemble those expressed in the dorsal striatum (CPu) (Gotti et al., 2010), although the total amount of nAChRs in the NAc is lower. In particular, we found that the most expressed subtypes are the  $\alpha 4\beta 2$ ,  $\alpha 4\alpha 5\beta 2$ ,  $\alpha 6\beta 2$ ,  $\alpha 6\beta 2\beta 3$  and  $\alpha 4\alpha 6\beta 2\beta 3$  subtypes.

Analysis of the subunit content of the nAChRs in the NAc of the rats undergoing nicotine SA showed the up-regulation of  $\alpha 4$  (one way ANOVA, Bonferroni's post test  $P < 0.05$ ) with no change in  $\alpha 6$ -containing receptors. In the MP treated rats, there was a statistically significant increase in the receptors containing the  $\alpha 4$  (paired t test,  $P = 0.0073$ ) and  $\beta 2$  ( $P = 0.0005$ ) subunits as well as a decrease in those containing the  $\alpha 6$  ( $P = 0.0055$ ) and  $\beta 3$  ( $P = 0.0145$ ) subunits. The decrease in  $\alpha 6^*$  receptor levels (-52%) matched the decrease in  $^{125}\text{I}-\alpha\text{CntxMII}$  binding (-60%).

Nicotine SA had no effects on the expression of nAChRs in the CPu, whereas nicotine administered by MP increased the level of the receptors containing the  $\alpha 4$  (paired t test,  $P = 0.0228$ ) and  $\beta 2$  ( $P = 0.0313$ ) subunits and decreased the level of  $\alpha 6$  ( $P = 0.0393$ ) and  $\beta 3$  ( $P = 0.0125$ ) containing receptors. As in the case of the NAc, the decrease in  $\alpha 6$  level in the CPu (-37%) matched the decrease in  $^{125}\text{I}-\alpha\text{CntxMII}$  membrane binding (-26%) (Figure 2B and 2C).

### Ventral Midbrain

In agreement with previous in situ-hybridisation (Klink et al., 2001) and immunoprecipitation studies of the same area (Gotti et al., 2010), nAChR subunits were very heterogeneously

expressed in the VMB: 50-60% of the nAChRs contained the  $\alpha 4$  and  $\beta 2$  subunits, 10-15% the  $\alpha 3$  and  $\beta 4$  subunits, 20% the  $\alpha 5$  subunit, and 10-15% the  $\alpha 6$  and  $\beta 3$  subunits.

The present immunoprecipitation experiments did not reveal any significant difference in the level of nAChR subunits between the naïve rats and the rats self-administering sucrose or nicotine. Instead, in MP treated rats there was a statistically significant increase in  $\beta 2$ -containing receptors, with respect to saline (paired t test saline vs nicotine  $P=0.0161$ ), paralleled by a trend to an increase in  $\alpha 4$ -containing receptors ( $P=0.09$ ) (Figure 2D).

### Superior colliculus

The visual pathway expresses  $\alpha 6$  receptors at both the retinal cell body and the retino collicular and retino geniculate nerve terminal level (Gotti et al., 2005a). As a control of the effect of nicotine on the expression of  $\alpha 6^*$  receptors in the visual pathway, we analysed the retino collicular terminal region in SC. As reported above, nicotine MP administration, but not SA, significantly increased the expression of  $^3\text{H}$ -Epi receptors (paired t test, in membranes  $P=0.0004$  and in extracts  $P=0.0480$ , see Table 2).

The SC subunit content determined in the control rats by means of immunoprecipitation experiments, was in line with that observed in a previous study (Gotti et al., 2005b) and we determined that major expressed subtypes are the  $\alpha 4\beta 2$ ,  $\alpha 4\alpha 5\beta 2$ ,  $\alpha 6\beta 2$ ,  $\alpha 6\beta 2\beta 3$ ,  $\alpha 4\alpha 6\beta 2\beta 3$  and  $\alpha 3\beta 2^*$  subtypes.

Immunoprecipitation experiments showed that in MP-treated rats the only up-regulated subtype was that containing the  $\alpha 4$  (paired t test  $P=0.0420$ ) and  $\beta 2$  subunits ( $P=0.0079$ ), whereas there was no change in the levels of receptors containing the  $\alpha 6$  and  $\beta 3$  subunits (Figure 2E), to confirm results with  $^{125}\text{I}$ - $\alpha$ CntxMII membrane binding experiments (see Table 3).

In line with previous experiments (Gotti et al., 2005b), we found that about 10-15 % of SC nAChRs contained the  $\alpha 3\beta 2^*$  subtype, which was shown to be strongly up-regulated by nicotine

*in vitro* (Salette et al., 2004; Kuryatov et al., 2005; Riganti et al., 2005). In the present *in vivo* experiment, however, this subtype was not up-regulated in rats self-administering nicotine or treated with nicotine via MP.

### **Western blotting analysis of caudal cortex extracts**

To further demonstrate nicotine-induced nAChR regulation, we quantified this effect with a different technique, for the case which revealed the highest level of up-regulation (the MP nicotine vs saline induced up-regulation in the CCx).

The results of our binding and immunoprecipitation experiments using 2% Triton X-100 extracts clearly indicated that there was a strong up-regulation of the  $\alpha 4^*$ ,  $\beta 2^*$  and  $\alpha 5^*$  nAChRs (presumably  $\alpha 4\alpha 5\beta 2$  subtype) in the CCx of the nicotine MP-injected rats. In particular, the immunoprecipitation ratio between  $\alpha 4$  levels in the nicotine-treated rats and saline-treated controls was almost identical to that between the  $\beta 2$  levels in the two groups ( $\alpha 4=1.75\pm 0.06$ ;  $\beta 2=1.70\pm 0.05$ ) (Figure 2A). However, when we loaded the same amount of proteins (10-15  $\mu$ g) of 2% Triton extract from the saline- and nicotine-treated rats the Western Blotting ratio of the  $\alpha 4$  subunit in the two groups was almost identical to the immunoprecipitation ratio ( $1.68\pm 0.06$ ), whereas the Western Blotting ratio of the  $\beta 2$  subunit was significantly higher ( $2.27\pm 0.18$ ) (paired t test,  $P=0.011$ ). The immunoprecipitation and WB results confirmed that nicotine-induced increase in the number of  $^3\text{H}$ -Epi binding sites corresponded to an increase in protein levels. This is indeed important as up-regulation measured by radiolabelled agonists has been questioned (Vallejo al., 2005).

## DISCUSSION (1500 words)

The main finding of this study is that MP-administered nicotine robustly up-regulates  $^3\text{H-Epi}$  binding in all brain areas, whereas SA nicotine leads to less up-regulation limited fewer regions. The increase in  $^3\text{H-Epi}$  binding was paralleled by an increase in the number of  $\alpha 4$  and  $\beta 2$  subunits, thus indicating very different up-regulation of  $\alpha 4\beta 2$  receptors across brain regions (CCx>NAc~CPu>VMB~SC). Furthermore, MP-administered nicotine decreased  $\alpha 6\beta 2(\beta 3)^*$  receptor levels in the NAc and CPu (as shown by the reduction in  $^{125}\text{I-}\alpha\text{Cntx MII}$  binding and the decrease in the number of  $\alpha 6$  and  $\beta 3$  subunits); and increased  $\alpha 7$  receptor levels in the CCx, as shown by  $^{125}\text{I-}\alpha\text{Bgtx}$  binding. Interestingly, MP-administered nicotine did not affect  $\alpha 6^*$  receptors in the SC, a region in which they are highly expressed. Finally, SA nicotine significantly increased the levels of  $\alpha 4\beta 2$  receptors only in the CCx and NAc, and did not affect  $\alpha 6\beta 2^*$  or  $\alpha 7$  receptor levels in any of the other regions.

These differences may be explained by the different doses and pharmacokinetic profiles of nicotine in the two models, as has been reported by Ulrich et al. (1997) and Rowell and Li (1997). Previous studies performed under identical conditions have shown that the serum of rats undergoing MP treatment has a constant nicotine concentration of 32 ng/ml (0.2  $\mu\text{M}$ ; Mugnaini et al 2006), whereas that of rats immediately after an SA session has a concentration of 84 ng/ml (0.5  $\mu\text{M}$ ), which decreases to 10 ng/ml (0.06  $\mu\text{M}$ ) after three hours (unpublished results). The estimated serum nicotine concentration area under the curve (AUC) over 24 hours is about 700-800 ng h/ml in MP rats and 100-200 ng h/ml in SA rats (unpublished results). The greater daily nicotine exposure of MP rats may explain why  $\alpha 4\beta 2$  up-regulation was more pronounced in the MP experiment, and why  $\alpha 6\beta 2^*$  down-regulation and  $\alpha 7$  up-regulation were undetectable in the SA experiment. Alternatively, a nicotine concentration constantly above a certain threshold value in the MP experiment may desensitise  $\alpha 4\beta 2$  receptors and/or decrease their degradation

and/or turnover (Kuryatov et al., 2005; Rezvani et al., 2007); in the case of SA, the decrease in serum nicotine levels over the day may allow the receptors to recover from desensitisation and/or differently affect subunit degradation and/or turnover. The hypothesis that a significant level of up-regulation requires serum nicotine levels above a certain threshold for a certain period of time is supported by the finding that the same dose of nicotine induces up-regulation when given in two daily administrations, but not when given in four or eight administrations (Rowell and Li, 1997).

The effect of MP-administered nicotine on the expression of  $\alpha 4\beta 2$ nAChRs in the CCx was much greater than that previously reported in other cortical areas (Nguyen et al., 2004, Mugnaini et al., 2006, Perry et al., 2007, Doura et al., 2008), which is in line with the fact that the caudal parts of the cerebral cortex, such as the visual cortex and auditory cortex, showed higher up-regulation. We also detected a significant increase in the  $\alpha 4\alpha 5\beta 2$  subtype, unlike Mao et al (2008) who found that these receptors are resistant to up-regulation even after the administration of a higher nicotine dose than that used by us (6 vs 3 mg/kg/day). This may be because they did not specifically test the CCx (the cortical area showing the highest level of up-regulation in our study) as we believe that the increase is an area-specific effect of nicotine.

In line with the results of previous studies (Mugnaini et al., 2006; Lai et al., 2005; Perry et al., 2007), MP-administered nicotine increased the overall expression of  $^3\text{H}$ -Epi-labelled receptors in the NAc, CPu and VTA, but there was a large increase in the number of  $\alpha 4\beta 2^*$  receptors and a decrease in the number of  $\alpha 6\beta 2^*$  receptors in the NAc and CPu. The co-existence of oppositely regulated receptor subtypes may explain why autoradiographic studies have found little or no change in [ $^{125}\text{I}$ ]Epi binding in these regions (Nguyen et al., 2004; Mugnaini et al., 2006).

SA nicotine increased  $\alpha 4\beta 2$  subtype levels only in the NAc, and had no effect on the  $\alpha 6\beta 2^*$  subtype in any of the three considered areas; these results are different from those of Parker et

al. (2004), who found increased levels of  $\alpha 6^*$  receptors and  $\alpha$ CntxMII binding in SA rats. The reason for this is not known, but it is worth pointing out that our rats self-administered  $0.81 \pm 0.01$  mg/kg/day of nicotine in a one-hour session, whereas Parker's rats self-administered 1.5 mg/kg/day over 23 hours, probably leading to more constant nicotine levels; in addition, we used Lister Hooded rats, whereas Parker et al. (2004) used Lewis rats. It is therefore possible that the differences between our findings and those of Parker et al. were due to different nicotine pharmacokinetics in the two SA protocols, differences between rat strains (as reviewed by Matta et al., 2007) or unknown technical issues (such as the specificity of the anti- $\alpha 6$  Abs or  $^{125}$ I- $\alpha$ CntxMII binding).

Unlike Perry et al. (2007), we found that the decreased level of  $\alpha 6$  receptors in the NAc and CPu of rats receiving MP-administered nicotine was paralleled by a decrease in the level of the  $\beta 3$  subunit. This is not surprising as we have previously shown that more than 70% of the  $\alpha 6^*$  receptors in the striatum contain the  $\beta 3$  subunit, and that the  $\beta 3$ -null mutation selectively reduces striatal  $\alpha 6^*$ nAChR expression by 76% in comparison with  $\beta 3^{+/+}$  controls (Gotti et al., 2005a).

As previously reported by others (Mugnaini et al., 2006; Perry et al., 2007), the least sensitive area to the effects of nicotine was the SC, in which SA led to no change in receptor subtypes and MP administration increased the levels of the  $\alpha 4\beta 2$  subtype but had no effect on the  $\alpha 6^*$  subtype.

It is not known why MP-administered nicotine up-regulates  $\alpha 4\beta 2^*$ nAChRs, but down-regulates  $\alpha 6^*$  receptors in midbrain dopaminergic neurons. *In situ* hybridisation, single-cell PCR and lesion studies have all shown that such neurons contain a mixture of  $\alpha 4$ ,  $\alpha 6$ ,  $\beta 2$  and  $\beta 3$  subunits (Klink et al., 2001; Lai et al., 2005; Gotti et al., 2010), and many immunoprecipitation experiments (including ours) have revealed the same subunits at terminal level in the NAc and CPu. It can be hypothesised that, if the number of  $\beta 2$  subunits is limited in these neurons,  $\alpha 4$  and  $\alpha 6$  subunits



compete for assembly in the endoplasmic reticulum. By acting as a preferential chaperone of  $\alpha 4\beta 2$  receptors (Salette et al., 2004; Kuryatov et al., 2005), nicotine may favour their formation in MP-treated rats by decreasing the pool of  $\beta 2$  subunits available for assembly with the  $\alpha 6$  subunit and consequently the number of  $\alpha 6\beta 2^*$ -nAChRs, whereas the more limited up-regulation of the  $\alpha 4\beta 2$  subtype in SA rats may lead to more  $\beta 2$  subunits being available for  $\alpha 6$  subunits and thus maintain the number of  $\alpha 6\beta 2^*$ -nAChRs. This mechanism is also suggested by the findings of *in vitro* studies in which the nicotine concentration necessary to up-regulate  $\alpha 4\beta 2$  receptors ( $EC_{50}=35$  nM) is much lower than that needed to up-regulate the  $\alpha 6\beta 3\beta 2$  subtype ( $EC_{50}=890$  nM; Tumkosit et al., 2006; Walsh et al., 2008). Moreover, the decrease in nAChR degradation during prolonged nicotine exposure (Revzani et al., 2007) may contribute to the up-regulation, although it is not yet known if this has different effects on the  $\alpha 4\beta 2$  and  $\alpha 6\beta 2$  subtypes.

In the case of the SC, it is not known whether  $\alpha 4$ ,  $\alpha 6$ ,  $\beta 2$  and  $\beta 3$  subunits are present in the same retinal ganglionic cells. However, if they are co-expressed, the much smaller nicotine-induced increase in  $\alpha 4^*$  levels in the SC than in the NAc (17% vs 78%) may explain the lack of competition between  $\alpha 4$  and  $\alpha 6$  subunits and the unchanged levels of the  $\alpha 6\beta 2^*$  subtype.

One important finding of our immunoprecipitation experiments is the identical nicotine-induced up-regulation of  $\alpha 4$  and  $\beta 2$  subunits in the CCx (measured as the ratio between the increase induced by nicotine and that induced by saline), whereas Western blotting showed the same increase in  $\alpha 4$  subunits but a significantly greater increase in  $\beta 2$  subunits. The non-denaturing immunoprecipitation protocol evaluates the ability of anti- $\alpha 4$  and anti- $\beta 2$  Abs to immunoprecipitate  $^3\text{H}$ -Epi-bound receptors regardless of their stoichiometry, but Western blotting can pick up variations in subunit content. The difference in the up-regulation of  $\beta 2$ -containing receptors measured by the two methods is therefore compatible with a nicotine-induced change in stoichiometry, as previously demonstrated in heterologous systems in which

the  $\alpha 4\beta 2$  subtype may exist in two different stoichiometries,  $(\alpha 4)_3(\beta 2)_2$  and  $(\alpha 4)_2(\beta 2)_3$ , with different functional and pharmacological properties (Nelson et al., 2003; Moroni et al., 2006). It has also been shown that chronic nicotine exposure up-regulates the expression of  $(\alpha 4)_2(\beta 2)_3$  stoichiometry (Kuryatov et al 2005).

The demonstration that nicotine can regulate the stoichiometries of natively expressed  $\alpha 4\beta 2$ -nAChRs may be important in pathophysiological states because it has been shown in heterologous systems that nicotine can normalise the intracellular subunit stoichiometry of nAChRs carrying mutations linked to autosomal dominant nocturnal frontal lobe epilepsy (Son et al., 2009).

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## **FOOTNOTES :**

Milena Moretti( MM), Manolo Mugnaini (MM), Michela Tessari (MT), contributed equally to this work.

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## LEGENDS FOR FIGURES

### **Figure 1: Immunoprecipitation analysis of the subunit content of extracts prepared from different brain areas.**

Extracts of the caudal cortex (A), nucleus accumbens (B), caudate-putamen (C), ventral midbrain (D) and superior colliculus (E) prepared from naïve rats, rats self-administering sucrose or nicotine, and rats infused with saline or nicotine by means of MP were labelled with 2nM  $^3\text{H-Epi}$  and immunoprecipitated using saturating concentrations (20-30  $\mu\text{g}$ ) of anti-subunit Abs as described in Materials and Methods. Antibodies directed against two separate peptides of the same subunits were used in the case of the  $\alpha_4$ ,  $\alpha_5$ ,  $\alpha_6$ ,  $\beta_2$  and  $\beta_3$  subunits. The amount immunoprecipitated by each antibody was subtracted from the value obtained in control samples containing an identical concentration of normal rabbit IgG, and the results are expressed as fmol of immunoprecipitated labelled  $^3\text{H-Epi}$  nAChR/mg of protein. Mean values  $\pm$  SEM of 3-4 experiments. For statistical comparison, data were analysed using one way ANOVA followed by Bonferroni's post-hoc comparison in the case of brain areas from the SA group (naïve rats, sucrose and nicotine self-administering rats) or a paired t test in the case of brain areas from the MP group (saline and nicotine).

### **Figure 2: Characterization of the $\alpha_4\beta_2$ subunit content in caudal cortex extracts obtained from rats treated with saline or nicotine administrated by MP.**

A) Mean values  $\pm$  SEM of the immunoprecipitation ratios of the anti- $\alpha_4$  and anti- $\beta_2$  antibodies obtained in the 2% Triton X-100 extracts of CCx taken from rats treated with of saline or nicotine by MP. The results were obtained from four separate immunoprecipitation experiments using two separate Abs directed against the Cyt and the Cooh peptides of the  $\alpha_4$  and  $\beta_2$  subunits as described in Materials and Methods.

B) WB analysis of CCx extracts taken from rats receiving saline or nicotine. 10 -15  $\mu$ g of proteins of the 2% Triton X-100 were separated on 9% acrylamide SDS gels, electrotransferred to nitrocellulose, probed with 1-2.5 $\mu$ g/ml of the indicated primary Abs, and then incubated with the secondary Ab (anti-rabbit conjugated to peroxidase, dilution 1:40000). The bound Abs were revealed using a chemiluminescent substrate (Pierce,Rockford,IL,USA)

The anti- $\alpha$ 4 Ab recognised a band of 68-70 kDa (corresponding to the expected size of the  $\alpha$ 4 subunit) and the anti- $\beta$ 2 Ab recognised a band of 52kDa (corresponding to the expected size of the  $\beta$ 2 subunit). The same blots were first incubated with anti- $\alpha$ 4 Abs, and then stripped and incubated with anti- $\beta$ 2 Abs or vice versa.

C) Optical density ratios of the  $\alpha$ 4 and  $\beta$ 2 subunits in caudal cortex extracts obtained from rats treated with saline or nicotine administrated by MP. The developed films were acquired as described in Materials and Methods and the images were analysed using NIH Image J software (National Technical Information Service, Springfield, VA,USA). The pixel values of all of the images were transformed to optical density values by the program using the calibrated curve obtained by acquiring the calibrated tablet using the same parameters as those used for the images.

Values are expressed as mean  $\pm$  SEM obtained from the ratios between the optical densities of the  $\alpha$ 4 (left) and  $\beta$ 2 (right) subunits in the extracts taken from rats treated with saline or nicotine administrated by MP.

## TABLES

**Table 1**

Amino acid sequences of the peptides used to produce the subunit-specific antibodies. Capital letters represent the amino acids in subunit sequence; lower case letters indicate the extra-sequence amino acids introduced to enable specific coupling to carrier protein.

**Table 1. Species, localisation and amino acid sequences of the peptides used to produce the nAChR subunit-specific polyclonal antibodies**

Subunit	Species	Localisation	Peptide sequence
$\alpha 2$	HUMAN	CYT	CHPLRLKLSPSYHWLESNVDAEEREV
$\alpha 3$	HUMAN	CYT	TRPTSNEGNAQKPRPLYGAELSNLNC
$\alpha 4$	RAT	CYT	PTSSPTSLKARPSQLPVSDQASPC
$\alpha 4$	RAT	COOH	PPWLAAC
$\alpha 5$	RAT	CYT	DRYFTQREEAESGAGPKSRNTLEAALD
$\alpha 5$	RAT	COOH	cgPVHIGNTIK
$\alpha 6$ (1)	RAT	CYT	GVKDPKTHTKRPAKVKFTHRKEPKLLKE
$\alpha 6$ (2)	RAT	CYT	CHKSEIAPGKRLSQQPAQWTENSEHPPDV
$\beta 2$	HUMAN	CYT	RQREREGAGALFFREAPGADSCTy
$\beta 2$	RAT	COOH	cgLHPDHSAPSSK
$\beta 3$	RAT	CYT	CGKESDTAVRGKVSGKRKQTPASD
$\beta 3$	RAT	COOH	cgPALKMWIHRFH
$\beta 4$	RAT	CYT	VSSHTAGLPRDARLRSSGRFREDLQEALEGc

## TABLES

**Table 2:** Levels of  $^3\text{H}$ -Epibatidine binding to membranes (upper) and 2% Triton X-100 extracts (lower) (expressed as fmol/mg of protein) in different brain areas of naïve rats, rat self-administering sucrose or nicotine and rats infused with saline or nicotine by means of MP.

The membranes and extracts were prepared from caudal cerebral cortex (CCx), caudate–putamen (CPu) the nucleus accumbens (NAc), the ventral midbrain (VMB) and the superior colliculus (SC).

The statistical analysis of the expression of  $^3\text{H}$ -Epi<sub>1</sub> receptors were carried out by one way analysis of variance (ANOVA), followed Bonferroni post hoc comparisons for naïve rats, rat self-administering sucrose or nicotine and paired t test in the case of brain areas of the saline or nicotine MP infused rats \*, \*\*, \*\*\*, significantly different from naïve, in the SA group, or saline, in the MP group ( $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ , respectively).

### $^3\text{H}$ -Epibatidine binding to membranes

Tissue	Naïve	Sucrose SA	Nicotine SA	Fold increase Nicotine / Naïve	Saline MP	Nicotine	Fold increase Nicotine/Saline
CCx	66.2±1.9	72.8±3.00	93.4± 4.3	1.41***	78.8±2.9	152.8± 8.3	1.95***
CPu	97.0±9.2	95.2± 8.1	109.30±9.2	1.12	99.6± 7.6	139.5± 19.6	1.40*
NAc	60.2±7.9	58.3±7.6	75.4± 10.6	1.25	48.0±1.8	75.7±5.4	1.57***
VMB	57.9±6.9	66.0±9.2	66.9± 4.6	1.15	50.9±2.2	59.5±4.1	1.16
SC	151.9± 10.2	162.7± 12.9	156.1± 15.7	1.02	144.2± 15.1	179.6± 19.4	1.19**

**<sup>3</sup>H-Epiibatidine binding to extracts**

<b>Tissue</b>	<b>Naïve</b>	<b>Sucrose SA</b>	<b>Nicotine SA</b>	<b>Fold increase Nicotine / Naïve</b>	<b>Saline MP</b>	<b>Nicotine</b>	<b>Fold increase Nicotine/Saline</b>
<b>CCx</b>	88.0±5.8	95.9±3.7	120.0±4.7	1.36**	83.5±3.0	150.6± 7.8	1.80***
<b>CPu</b>	160.6±19.1	157.5 18.16	179.4±18.6	1.11	133.8±13.0	184.8±27.0	1.38*
<b>NAc</b>	89.4± 8.0	93.2±6.27	123.2±9.7	1.37*	89.7±7.9	139.3± 5.45	1.55**
<b>VMB</b>	77.5±10.80	76.9±6.9	99.3±4.2	1.28	80.8±2.9	107.3± 7.7	1.32*
<b>SC</b>	187.7±23.8	186.6±11.7	191.9±9.3	1.02	181.8±0.1	231.4± 2.5	1.28*

**Table 3:** Levels of  $^{125}\text{I}$ - $\alpha$ Bungarotoxin (upper) and  $^{125}\text{I}$ - $\alpha$ Conotoxin MII (lower) binding in different brain areas (CCx, CPu, NAc, VMB and SC), of naïve, sucrose and nicotine self-administering rats and in rats infused with saline or nicotine by means of MP (expressed as fmol/mg of protein). The statistical analysis was performed as described in Table 2

### $^{125}\text{I}$ - $\alpha$ Bungarotoxin to membranes

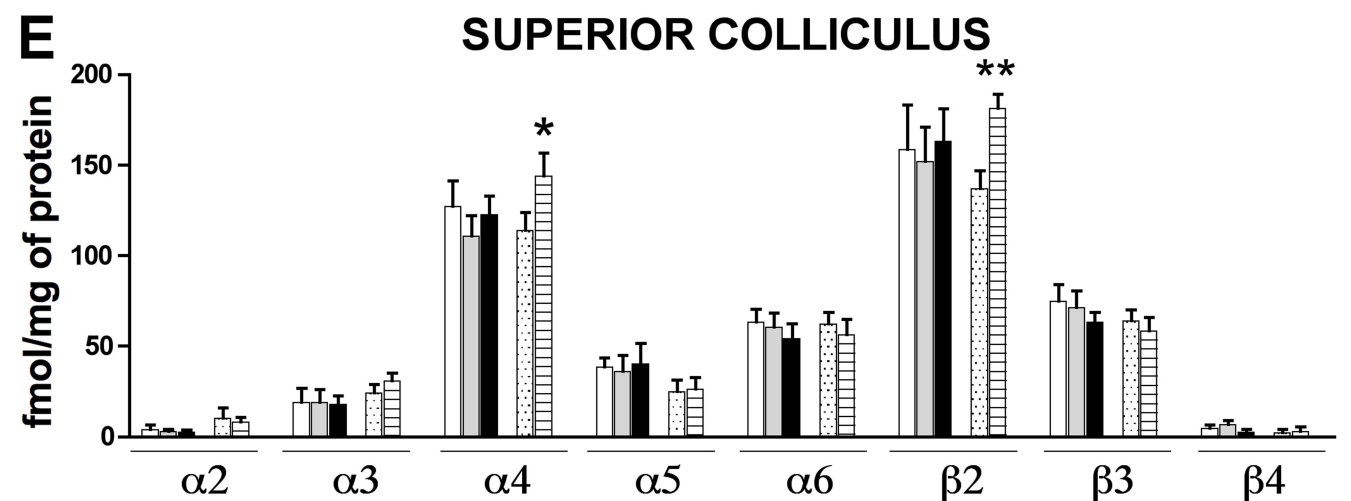
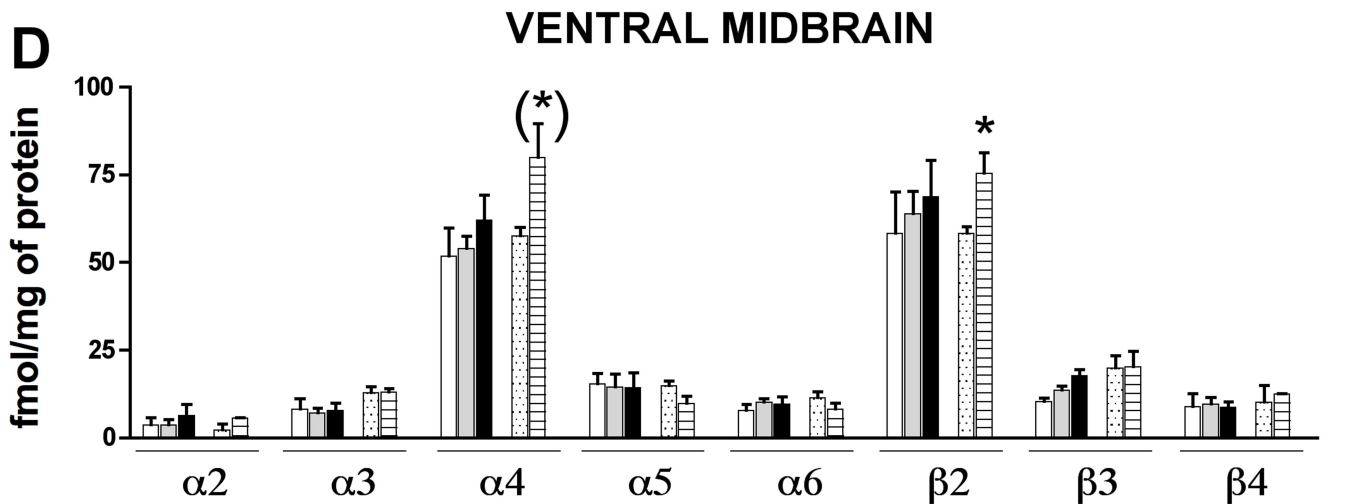
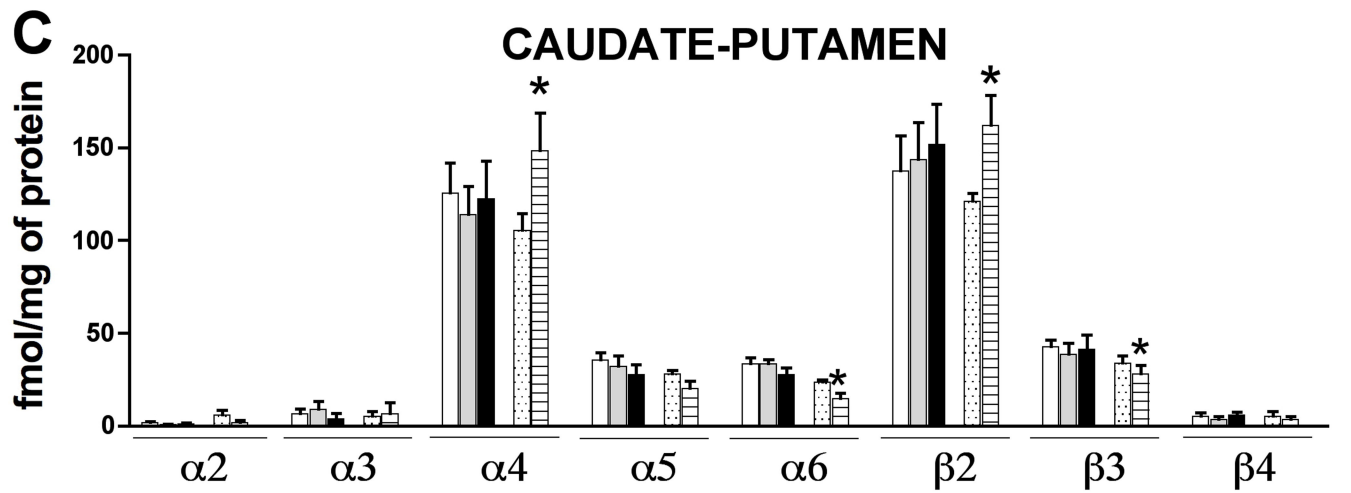
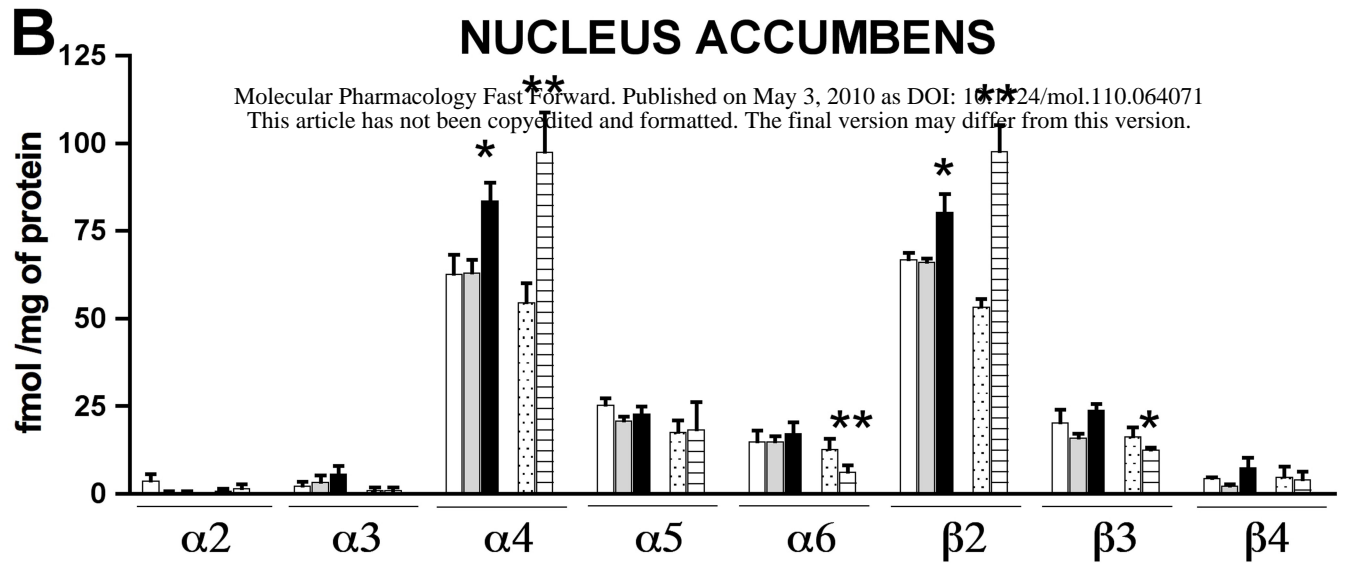
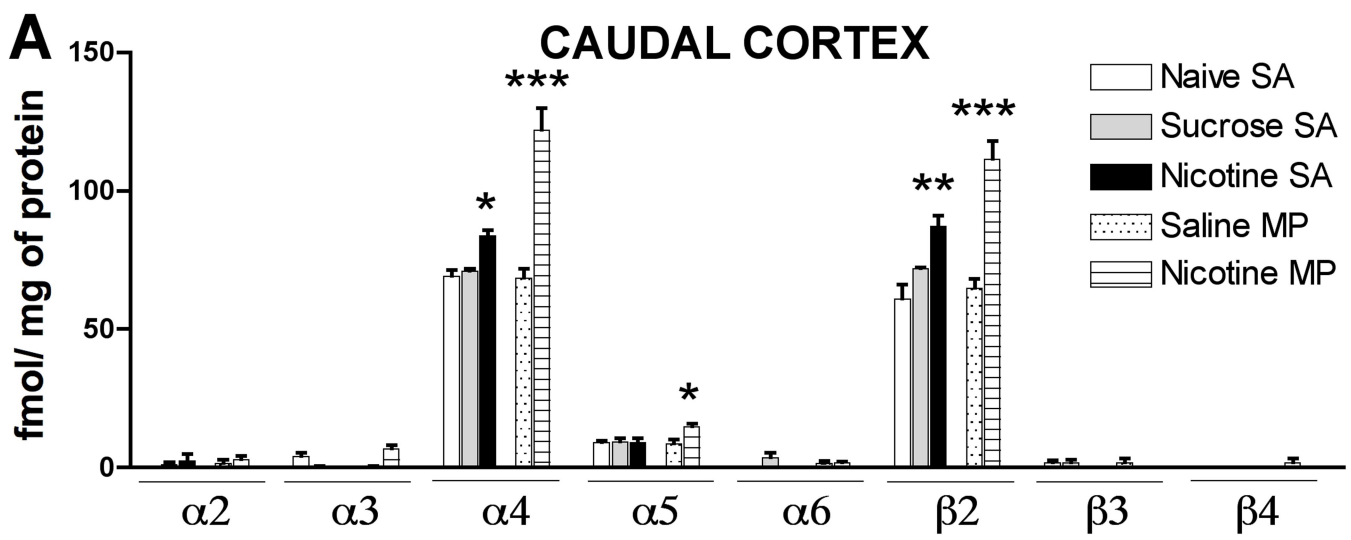
Tissue	Naïve	Sucrose SA	Nicotine SA	Fold increase Nicotine / Naïve	Saline MP	Nicotine	Fold increase Nicotine/Saline
CCx	31.7±1.3	29.7±2.7	31.4±3.3	0.99	31.8±4.4	41.5±7.1	1.30*
CPu	9.3±1.6	9.2±3.1	8.9±2.6	0.96	5.1±0.7	5.7±0.8	1.11
NAc	20.0±2.4	15.5±6.20	16.5±1.4	0.83	16.0±3.4	16.9±2.2	1.05
VMB	16.9±1.3	16.0± 1.9	16.9±1.9	1.00	21.5±1.4	20.8±1.7	0.97
SC	50.4±11.5	41.8±5.7	55.5±10.7	1.10	67.3±7.1	78.3± 7.1	1.16

### $^{125}\text{I}$ - $\alpha$ Conotoxin MII to membranes

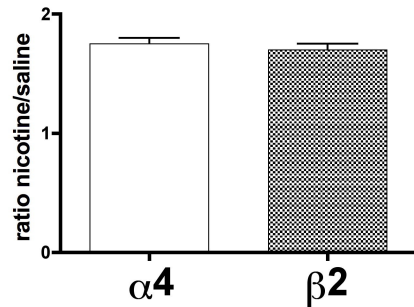
Tissue	Naïve	Sucrose SA	Nicotine SA	Fold increase Nicotine / Naïve	Saline MP	Nicotine	Fold increase Nicotine/Saline
CPu	7.0±0.8	6.3±0.7	5.1±0.8	0.73	6.2±0.7	4.6±0.8	0.74*



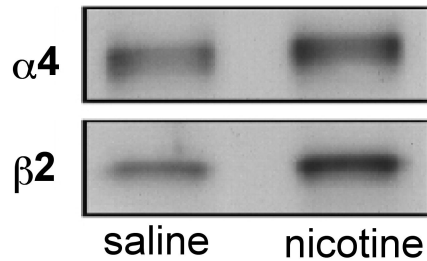
<b>NAc</b>	3.4±0.9	2.3±0.7	2.6±0.7	0.76	2.2±0.7	0.9±0.4	0.4*
<b>VMB</b>	0.88±0.2	0.90±0.05	0.96±0.16,9	1.09	0.83±0.13	0.93±0.63	1.12



### **A** Immunoprecipitation



### **B**



### **C** Western Blotting

