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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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ABSTRACT

Long-term 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, $\alpha4\beta2$ nAChRs were up-regulated in all brain regions, $\alpha6\beta2^*$ nAChRs were down-regulated in the nucleus accumbens (NAc) and caudate-putamen, and $\alpha7$ nAChRs were up-regulated in the caudal cerebral cortex (CCx); the up-regulation of $\alpha4\beta2\alpha5$ nAChRs in the CCx was also suggested. In the SA group, $\alpha4\beta2$ up-regulation was lower and limited to the CCx and NAc; there were no detectable changes in $\alpha6\beta2^*$ or $\alpha7$ nACRs. In the CCx of the MP rats, there was a close correlation between the increase in $\alpha4\beta2$ binding and $\alpha4$ and $\beta2$ subunit levels measured by means of Western blotting, demonstrating that the up-regulation was due to an increase in $\alpha4\beta2$ proteins. Western blotting also showed that the increase in the $\beta2$ subunit exceeded that of the $\alpha4$ subunit, suggesting that a change in $\alpha4\beta2$ 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.

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

The behavioral effects of nicotine may be due to nAChR activation or desensitization, because nicotine may affect neuronal function by stimulating nAChR-dependent cellular effects or by interrupting the nicotinic transmission of endogenous acetylcholine (Buisson and Bertrand, 2002; Picciotto et al., 2008). The various nAChR subtypes coexisting in the brain are not equally responsive to nicotine activation and desensitization and may be differently affected depending on where they are expressed in the neuronal circuits, leading to complex behavioral responses (for review, see Picciotto et al., 2008; 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 (for review, see Gen-

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; αCntxMII, α-conotoxin MII; SA, self-administration; MP, minipump; Epi, (±)-epibatidine; CPu, caudate-putamen; NAc, nucleus accumbens; VMB, ventral midbrain; SC, superior colliculus; CCx, caudal cerebral cortex; Ab, polyclonal antibody; αBgtx, α-Bungarotoxin; ANOVA, analysis of variance.

try 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 undergoing long-term treatment 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 Govind et al., 2009; Lester et al., 2009).

It was long believed 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 sensitization (for review, see Lester et al., 2009; Xiao et al., 2009). For example, Nashmi and Lester (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 α-conotoxin MII (αCntxMII) and play an important role in striatal dopamine release, locomotion, and nicotine self-administration (SA) (for review, see 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 long-term nicotine treatment by means of an osmotic minipump (MP) or drinking water decreases the number of striatal $\alpha 6^*$ receptors (Lai et al., 2005; Mugnaini et al., 2006; Perry et al., 2007). On the other hand, a6b2 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 administrated by osmotic MPs on $\alpha 6^*$ receptor regulation is the consequence of dissimilar nicotine pharmacokinetic profiles between the two models, diverse (operant versus non operant) behavioral 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 after long-term intravenous nicotine SA or long-term 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 (for review, see Livingstone and Wonnacott, 2009).

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

(±)-[^{3}H]epibatidine (Epi; specific activity, 50–66 Ci/mmol) was purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK), ^{125}I-α-bungarotoxin (αBgtx; specific activity, 200 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA), and nonradioactive ligands from Sigma-Aldrich (Milan, Italy). ^{125}I-α-Conotoxin MII (αCntxMII; specific activity, 200 Ci/mmol) was custom-synthesized by GE Healthcare. Nicotine bitartrate (Sigma-Aldrich, St. Louis, MO) used during SA sessions was dissolved in 5 I.U. of heparinized saline and then pH adjusted to 7.4 with NaOH. Nicotine doses were expressed as milligrams of free base per kilogram of body weight.

Apparatus for Nicotine Self-Administratio

Rats that underwent nicotine SA were tested in 12 operant chambers. Each chamber (30 cm wide × 25 cm deep × 32 cm high) (Med Associates Inc., St. Albans, VT) 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 2900-Hz tone module (Sonalert, Indianapolis, IN) 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., East Fairfield, VT) was connected via an external catheter consisting in a Tygon tubing (Norton Plastics Performance, Akron, OH) to a single-channel liquid swivel (Instech Laboratories Inc., Plymouth Meeting, PA) 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 Laboratories, Kent, UK), similar for age and housing, were used for all the experiments. In GlaxoSmithKline facilities, they were individually housed in a temperature-controlled environment with lights on from 6:00 AM to 6:00 PM. Water was continuously available, and animals were maintained at a constant body weight of 290 to 310 g. Animals were divided in three groups that were treated as described below.

Naive (Control) Group. Twenty-seven rats were handled and singly housed for approximately 3 months, after which they were euthanized for brain tissue microdissection.

Nicotine Self-Administration Group. Twenty-six rats were anesthetized with medetomidine (0.1 mg/kg i.m. Domitor; Pfizer Italia s.r.l., Rome, 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 benzylpenicillin and dihydrostreptomycin sulfate (Rubrocillina Veterinaria; Gellini International s.r.l., Aprilia, Italy) providing 72 h of protection and carprofen 5 mg/kg as analgesic (Rimadyl; Pfizer Italia s.r.l.). A silastic catheter was then implanted in in the rats' 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.) to

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

After the recovery period, nicotine SA was initiated under a fixed ratio (FR) 1 schedule of reinforcement in operant chambers in which two levers were exposed. Rats were connected to the external catheter, and each active lever press led to a 22-μl infusion of nicotine (0.03 mg/kg/infusion) delivered over 1 s, the extinction of the house light (60 s), and illumination of the stimulus light located above the active lever (1 s), as well as a 1-s sounding of the Sonalert device. The last three events represent nicotine-associated conditioned stimuli. Each infusion was followed by a 60-s time-out 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 h had elapsed, whichever occurred first. When the 25-infusion 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 h. Once animals had reached stable responding under this schedule, the length of the session was limited to 1 h. An animal was considered to have achieved stable responding when it made a similar number of responses on the active lever for 3 consecutive days (± 10%). Rats underwent this latter schedule for at least 15 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 of ligature 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 10 days, and then sucrose SA was initiated under an FR1 schedule of reinforcement in operant chambers in which two levers were exposed. Each active lever press led to the presentation of 20 μl of 10% sucrose delivered by liquid dipper over 10 s, the extinction of the house light (60 s), and illumination of the stimulus light located above the active lever (1 s), as well as a 1-s sounding of the Sonalert device. Each sucrose presentation was followed by a 60-s time-out 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 h 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 h. Once animals had reached stable responding under this schedule, the length of the session was decreased to 1 h. An animal was considered to have achieved stable responding when it made a similar number of responses on the active lever for 3 consecutive days (± 10%). They underwent this latter schedule for at least 15 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 anesthetized by inhalation of a mixture of air and O_2 containing 2.5% isoflurane (Forane; Abbott S.p.A., Campoverde, Italy). While the rats were anesthetized, a large subcutaneous pocket for the pump was created in the dorsal thoracic area. Osmotic minipumps (2-ML2; Alzet, Cupertino, CA) with a pumping rate of 5 μl/h saline were implanted subcutaneously. Before subcutaneous implantation, pumps were filled with sterile water and placed in the same solution in a 37°C water bath overnight to equilibrate saline release. Rats were subcutaneously infused with saline for 14 consecutive daysand killed immediately after treatment. Soon after euthanasia, the brain was removed and microdissected into the brain regions of interest.

Nicotine Osmotic MPs. Nineteen rats were anesthetized as described above. While the rats were anesthetized, a large subcutaneous pocket for the pump was created in the dorsal thoracic area. Alzet osmotic minipumps were implanted as described above. Before subcutaneous implantation, pumps were filled with nicotine solution the concentration of which was calculated to provide a dose of 3 mg/kg/day nicotine free base. The solution was placed in sterile saline in a 37°C water bath overnight to equilibrate nicotine release to ensure steady-state delivery after implantation. Rats were subcutaneously infused with 3 mg/kg/day nicotine for 14 consecutive days and killed immediately after treatment. Soon after euthanasia, the brain was removed and microdissected into the brain regions of interest.

Brain Tissue Dissection

Rats were decapitated, their eyes and brains 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, 1998). Immediately after dissection, these brain regions were precooled with dry ice (−35°C), frozen in isopentane, 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 and/or intracytoplasmic loop regions of rat, human, or mouse subunit sequences and affinity-purified as described previously (Zoli et al., 2002). Most of the Abs have been described previously (Zoli et al., 2002; Gotti et al., 2005a,b). The amino acid sequences 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 immu-

TABLE 1

Species, localization, and amino acid sequences of the peptides used to produce the nAChR subunit-specific polyclonal 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.

Subunit	Species	Localization	Peptide sequence
α2	Human	CYT	CHPLRLKLSPSYHWLESNVDAEEREV
α3	Human	CYT	TRPTSNEGNAQKPRPLYGAELSNLNC
α4	Rat	CYT	PTSSPTSLKARPSQLPVSDQASPC
α4	Rat	COOH	PPWLAAC
α5	Rat	CYT	DRYFTQREEAESGAGPKSRNTLEAALD
α5	Rat	COOH	cgPVHIGNTIK
α6(1)	Rat	CYT	GVKDPKTHTKRPAKVKFTHRKEPKLLKE
α6(2)	Rat	CYT	CHKSSEIAPGKRLSQQPAQWTENSEHPPDV
β2	Human	CYT	RQREREGAGALFFREAPGADSCTy
β2	Rat	COOH	cgLHPDHSAPSSK
β3	Rat	CYT	CGKESDTAVRGKVSGKRKQTPASD
β3	Rat	COOH	cgPALKMWIHRFH
β4	Rat	CYT	VSSHTAGLPRDARLRSSGRFREDLQEALEGc

CYT, cytoplasmic loop; COOH, C terminus.

noprecipitation or immunopurification experiments or Western blotting using nAChRs from different areas of the central nervous system 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,b). 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 homogenized in 10 ml of 50 mM sodium phosphate, pH 7.4, 1 M NaCl, 2 mM EDTA, 2 mM EGTA, and 2 mM phenylmethylsulfonyl fluoride using a homogenizer, and the homogenates were diluted and centrifuged for 1.5 h at 60,000*g*. The total membrane homogenization, dilution, and centrifugation procedures were repeated, after which the pellets were collected, rapidly rinsed with 50 mM Tris HCl, pH 7, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 2 mM phenylmethylsulfonyl fluoride and then resuspended in the same buffer containing a 20 μg/ml mixture 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 60,000*g*, recovered, and an aliquot of the supernatants was collected for protein measurement using the BCA protein assay (Pierce Chemical, Rockford, IL) with bovine serum albumin as the standard.

Binding Studies

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

^{125}I-α-Conotoxin MII. Binding experiments were performed by incubating tissue membranes from each experimental group for 3 h of 0.5 nM ^{125}I-αCntxMII at 20°C. For ^{125}I-CntxMII, 2 mg/ml bovine serum albumin was added to the suspension buffer. Specific radioligand binding was defined as total binding minus nonspecific binding determined in the presence of 1 μM unlabeled αCntxMII or 250 nM unlabeled 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 sodium phosphate, pH 7.4, plus 50 mM NaCl, and the filters were counted in a gamma counter.

[^{3}H]Epibatidine. To ensure that the α7-containing subtypes did not contribute to [^{3}H]Epi binding (Marks et al., 2006), both membrane and solubilized receptors (present in the extract and immunoprecipitation experiments) were first incubated for 3 h with 2 μM αBgtx, which specifically binds to α7-nAChR (and thus prevents [^{3}H]Epi from 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 [^{3}H]Epi at 4°C. Nonspecific binding (averaging 5–10% of total binding) was determined in parallel samples containing 100 nM unlabeled 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 (sodium phosphate, 10 mM, pH 7.4, and 50 mM NaCl), and counted in a beta counter.

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

Immunoprecipitation of [^{3}H]Epibatidine-Labeled Receptors by Subunit-Specific Antibodies

The tissue extracts were preincubated with 2 μM αBgtx, labeled with 2 nM [^{3}H]Epi, and incubated overnight with a saturating concentration of affinity-purified anti-subunit IgG (20–30 μg; Sigma, St Louis). 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 [^{3}H]Epi-labeled receptors immunoprecipitated by the antibodies (taking the amount present in the Triton X-100 extract solution before immunoprecipitation as 100%) or as femtomoles of immunoprecipitated receptors per milligram of protein.

Immunoblotting and Densitometric Quantification of Western Blot Bands

The analysis of the $\alpha4\beta2$ subtype by Western blotting was performed as described previously (Gotti et al., 2008; Grady et al., 2009). In brief, 10 μg of 2% Triton X-100 membrane extracts obtained from CCx of MP saline and nicotine rats were diluted 1:1 (v/v) with Laemmli buffer and then underwent SDS-polyacrylamide gel electrophoresis using 9% acrylamide. After SDS-polyacrylamide gel electrophoresis, the proteins were electrophoretically transferred to nitrocellulose membranes with 0.45-mm pores (Schleicher and Schüll, Dassel, Germany). The blots were blocked overnight in 5% nonfat milk in Tris-buffered saline, washed in a buffer containing 5% nonfat milk and 0.3% Tween 20 in Tris-buffered saline, incubated for 2 h with the primary antibody (1–2.5 mg/ml), and then incubated with the appropriate peroxidase-conjugated secondary antibody. After another series of washes, peroxidase was detected using a chemiluminescent substrate (Pierce, Rockford, IL).

The quantification of the signal intensity of the Western blot bands was performed as described previously (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 ± S.E.M. of four separate experiments for each antibody.

Statistical Analysis

Statistical analysis of the expression of [^{3}H]Epi, ^{125}I-αBgtx, and ^{125}I-αCntxMII receptors as well as the subunit content of the expressed [^{3}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 with the use of a paired *t* test. The software for statistical analysis was Prism 4 (GraphPad Software, San Diego, CA).

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 the SA experiment, the number of active lever presses per session was 55.7 ± 4.2 (mean values of the last three sessions before the sacrifice), significantly greater than the number of inactive lever presses (10.9 ± 1.3; Student's *t* test, $p < 0.001$). The mean value of intravenous nicotine intake was 0.82 ± 0.06 mg/kg/day (mean of the last three sessions; expressed as nicotine free base) that were attributable to 27.3 ± 2.1 infusions/day.

In the sucrose self-administering rats, the number of sucrose deliveries was 56.7 ± 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 ± 1.4, significantly greater than the number of inactive lever presses (0.7 ± 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 SC of control and treated rats were analyzed using binding studies with radioactive ligands selective for heteromeric nAChRs in general ([^{3}H]Epi), homomeric α7-nAChRs (^{125}I-αBgtx), and α6*-nAChRs (^{125}I-αCntxMII). The naive (untreated) group and the saline-treated group were the control groups in the SA and the MP experiments, respectively.

[^{3}H]Epi Binding to Membrane nAChRs. In naive and saline-treated control rats, the highest levels of [^{3}H]Epi bound receptors (expressed as femtomoles per milligram of protein) were found in the SC (151.9 ± 10.2 and 144.2 ± 15.1, respectively), followed by CPu (97.0 ± 9.2; 99.6 ± 7.6), CCx (66.2 ± 1.9; 78.8 ± 2.9), NAc (60.2 ± 7.9; 48.0 ± 1.8), and VMB (57.9 ± 6.9; 50.9 ± 2.2) (Table 2).

The different SA treatments (sucrose or nicotine) did not significantly alter the level of [^{3}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 post hoc comparisons showed that binding in the nicotine-treated rats was significantly higher than in the nicotine-naive ($P < 0.001$) or sucrose-treated rats ($P < 0.01$). Nicotine treatment via MP significantly increased [^{3}H]Epi binding, with respect to saline, in the CPu (paired t test saline versus nicotine: $P = 0.0213$), NAc ($P < 0.0001$), SC ($P = 0.0060$), and CCx ($P < 0.0001$) but not in the VMB.

^{125}I-αBgtx Binding to Membrane nAChRs. In naive and saline-treated controls, the highest levels of ^{125}I-αBgtx-bound receptors (expressed as femtomoles per milligram of protein) were found in the SC (50.4 ± 11.5 and 67.3 ± 7.1, respectively) followed by CCx (31.7 ± 1.3; 31.8 ± 4.3) and, at similar levels, the VMB (16.9 ± 1.2; 21.5 ± 1.4) and NAc (20.0 ± 2.4; 16.0 ± 3.4). The levels in the CPu (9.3 ± 1.6; 5.1 ± 0.7) were much lower (Table 3).

There were no significant differences in ^{125}I-αBgtx binding levels between the various SA treatments in the regions considered. In the MP group, ^{125}I-αBgtx binding in the CCx was higher in the rats receiving nicotine than in those re-

TABLE 2

Levels of [^{3}H]epibatidine binding to membranes and 2% Triton X-100 extracts in different brain areas of naive 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 CCx, CPu, NAc,VMB, and SC. The statistical analysis of the expression of [^{3}H]Epi receptors were carried out by one-way ANOVA, followed by Bonferroni post hoc comparisons for naive 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.

Tissue	Naive	Sucrose SA	Nicotine SA	Fold Increase Nicotine/Naive	Saline MP	Nicotine	Fold Increase Nicotine/Saline
	fmol/mg of protein				*fmol/mg of protein*		
[^{3}H]Epibatidine binding to membranes							
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**
[^{3}H]Epibatidine binding to extracts							
CCx	88.0 ± 5.8	95.9 ± 3.7	120.0 ± 4.7	1.36**	83.5 ± 3.0	150.6 ± 7.8	1.80***
CPu	160.6 ± 19.1	157.5 ± 318.16	179.4 ± 18.6	1.11	133.8 ± 13.0	184.8 ± 27.0	1.38*
NAc	89.4 ± 8.0	93.2 ± 6.27	123.2 ± 9.7	1.37*	89.7 ± 7.9	139.3 ± 5.45	1.55**
VMB	77.5 ± 10.80	76.9 ± 6.9	99.3 ± 4.2	1.28	80.8 ± 2.9	107.3 ± 7.7	1.32*
SC	187.7 ± 23.8	186.6 ± 11.7	191.9 ± 9.3	1.02	181.8 ± 0.1	231.4 ± 2.5	1.28*

* $P < 0.05$, significantly different from naive in the SA group or saline in the MP group.
** $P < 0.01$, significantly different from naive in the SA group or saline in the MP group.
*** $P < 0.001$, significantly different from naive in the SA group or saline in the MP group.

TABLE 3

Levels of ^{125}I -α-bungarotoxin and ^{125}I-α-conotoxin MII binding in different brain areas (CCx, CPu, NAc, VMB, and SC) of naïve rats, sucrose and nicotine self-administering rats, and in rats infused with saline or nicotine by means of MP. The statistical analysis of the expression of [^{3}H]Epi receptors were carried out by one-way ANOVA, followed by Bonferroni post hoc comparisons for naive 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.

Tissue	Naïve	Sucrose SA	Nicotine SA	Fold Increase Nicotine/Naive	Saline MP	Nicotine	Fold Increase Nicotine/Saline
	fmol/mg of protein				*fmol/mg of protein*		
^{125}I-α-Bungarotoxin to membranes							
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}I-α-Conotoxin MII to membranes							
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*
NAc	3.4 ± 0.9	2.3 ± 0.7	2.6 ± 0.7	0.76	2.2 ± 0.7	0.9 ± 0.4	0.4*
VMB	0.88 ± 0.2	0.90 ± 0.05	0.96 ± 0.16	1.09	0.83 ± 0.13	0.93 ± 0.63	1.12
SC	11.9 ± 4.6	12.5 ± 4.6	8.5 ± 2.4	0.71	15.2 ± 2.7	15.3 ± 4.3	1.00

* $P < 0.05$, significantly different from naive in the SA group or saline in the MP group.

ceiving saline (paired t test, $P = 0.0255$). There were no significant differences in the other areas.

^{125}I-αCntxMII Binding to Membrane nAChRs. In naive and saline-treated control rats, the highest levels of ^{125}I-αCntxMII binding were found in the SC (11.9 ± 4.6 and 15.2 ± 2.7, respectively), followed by the CPu (7.0 ± 0.8; 6.2 ± 0.7), NAc (3.4 ± 0.9; 2.2 ± 0.7) and VMB (0.88 ± 0.2; 0.83 ± 0.1). No specific binding was detected in the CCx. Results are shown in Table 3.

One-way ANOVA revealed no statistically significant difference between the nicotine-naive rats and rats self-administering sucrose or nicotine. In contrast, MP administered nicotine significantly decreased ^{125}I-αCntxMII binding in the NAc (paired t tests, $P = 0.0125$) and CPu ($P = 0.0298$) compared with saline, but there were no significant differences in the VMB or SC.

[^{3}H]Epi Binding to 2% Triton X-100 Extracts. In agreement with the membrane binding data, the level of [^{3}H]Epi receptors in the 2% Triton X-100 extracts of the control animals [that is, untreated (naive) in the SA experiment and treated with saline in the MP experiment] were highest in the SC (187.7 ± 23.8 and 181.8 ± 0.1, respectively) followed by the CPu (160.6 ± 19.1; 133.8 ± 13,0), NAc (89.4 ± 8.0; 89.7 ± 7.9), CCx (88.0 ± 5.8; 83.5 ± 3.0), and VMB (77.5 ± 10.8; 80.8 ± 2.9).

The different SA treatments (sucrose or nicotine) did not significantly alter the level of [^{3}H]Epi receptors in the SC, CPu, and VMB ($P > 0.05$, one-way ANOVA). In the NAc, [^{3}H]Epi binding was significantly higher in the nicotine-treated rats, with respect to naive (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 naive 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}H]Epi receptors in the CPu (paired t test saline versus 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 [^{3}H]Epi-Bound nAChRs

Because our binding studies in both membrane-bound and solubilized nAChRs clearly showed that nicotine affects the expression of nAChR subtypes depending on the type of treatment and the brain area, we analyzed the [^{3}H]Epi-labeled receptors by means of immunoprecipitation experiments using subunit-specific Abs.

Table 1 shows amino acid sequences of the peptides used to produce the subunit-specific Abs used in the quantitative immunoprecipitation. In each experiment, for each subunit other than α2, α3, and β4, we always separately immunoprecipitated the receptors labeled by [^{3}H]Epi using two Abs directed against the two different epitopes of the same subunit.

Figure 1 shows the results of the immunoprecipitation experiments of labeled [^{3}H]Epi receptors using anti-α2, α3, α4, α5, α6, β2, β3, and β4 Abs. The values shown for the α4, α5, α6, β2, and β3 subunits are the average of the values obtained using the two Abs.

Caudal Cortex. The large majority of the [^{3}H]Epi-labeled receptors in this particular area of the cortex contained the $\alpha4\beta2$ subtype and 10 to 15% also contained the α5 subunits. These results are consistent with results previously published by us and other groups (Mao et al., 2008). There was no difference in the level of $\alpha4\beta2$* receptors between naive and sucrose-treated rats, but levels measured in the nicotine SA group were significantly higher than the naive and the sucrose SA group (one-way ANOVA followed by post hoc Bonferroni test, $P < 0.05$) (Fig. 2A).

In line with the extract binding data, MP nicotine treatment greatly increased (~1.7-fold) the levels of $\alpha4\beta2$* 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 α5 subunit in a statistically significant manner, whereas no significant increase in α5 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 were $\alpha4\beta2$, $\alpha4\alpha5\beta2$, $\alpha6\beta2$, $\alpha6\beta2\beta3$, and $\alpha4\alpha6\beta2\beta3$.

Analysis of the subunit content of the nAChRs in the NAc of the rats undergoing nicotine SA showed the up-regulation of α4 (one-way ANOVA; Bonferroni post test, $P < 0.05$) with no change in α6-containing receptors. In the MP-treated rats, there was a statistically significant increase in the receptors containing the α4 (paired t test, $P = 0.0073$) and β2 ($P = 0.0005$) subunits as well as a decrease in those containing the α6 ($P = 0.0055$) and β3 ($P = 0.0145$) subunits. The decrease in α6* receptor levels (−52%) matched the decrease in ^{125}I-αCntxMII 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 α4 (paired t test, $P = 0.0228$) and β2 ($P = 0.0313$) subunits and decreased the level of α6- ($P = 0.0393$) and β3- ($P = 0.0125$) containing receptors. As in the case of the NAc, the decrease in α6 level in the CPu (−37%) matched the decrease in ^{125}I-αCntxMII membrane binding (−26%) (Fig. 2, B and C).

Ventral Midbrain. In agreement with previous in situ hybridization (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 to 60% of the nAChRs contained the α4 and β2 subunits, 10 to 15% contained the α3 and β4 subunits, 20% contained the α5 subunit, and 10 to 15% contained the α6 and β3 subunits.

The present immunoprecipitation experiments revealed no significant difference in the level of nAChR subunits between the naive rats and the rats self-administering sucrose or nicotine. Instead, in MP-treated rats, there was a statistically significant increase in β2-containing receptors with respect to saline (paired t test, saline versus nicotine, $P = 0.0161$), paralleled by a trend to an increase in α4-containing receptors ($P = 0.09$) (Fig. 2D).

Superior Colliculus. The visual pathway expresses α6 receptors at both the retinal cell body and the retinocollicular and retinogeniculate nerve terminal levels (Gotti et al., 2005a). As a control of the effect of nicotine on the expression of α6* receptors in the visual pathway, we analyzed the retinocollicular terminal region in SC. As reported above, nicotine MP administration, but not SA, significantly in-

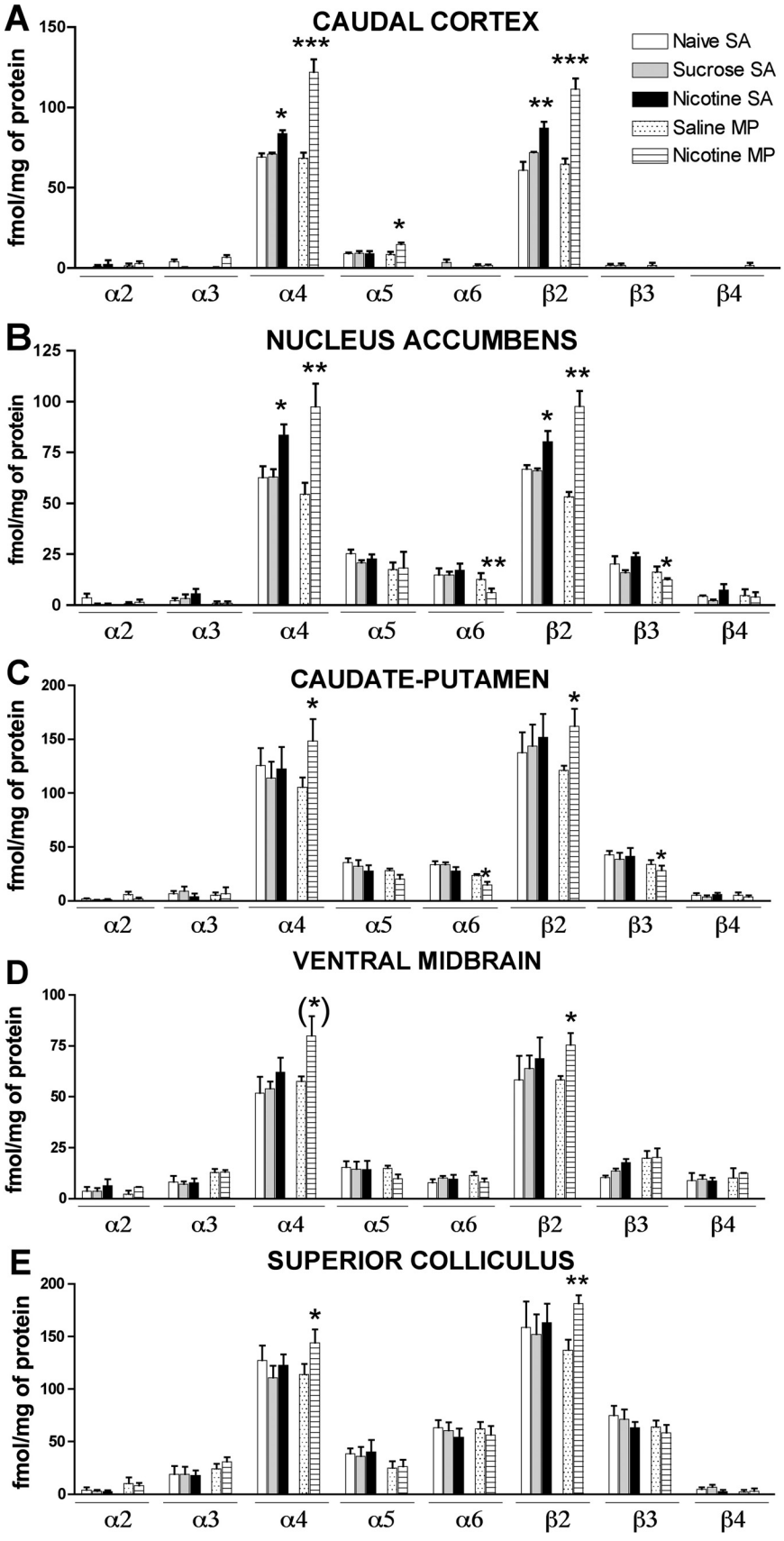

Fig. 1. Immunoprecipitation analysis of the subunit content of extracts prepared from different brain areas. Extracts of the caudal cortex (A), nucleus accumbens (B), caudate-putamen (C), ventral midbrain (D), and superior colliculus (E) prepared from naive rats, rats self-administering sucrose or nicotine, and rats infused with saline or nicotine by means of MP were labeled with 2 nM [^{3}H]Epi and immunoprecipitated using saturating concentrations (20–30 μg) of anti-subunit Abs as described under *Materials and Methods*. Antibodies directed against two separate peptides of the same subunits were used in the case of the α4, α5, α6, β2, and β3 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 femtomoles of immunoprecipitated labeled [^{3}H]Epi nAChR per milligram of protein. Mean values ± S.E.M. of three to four experiments. For statistical comparison, data were analyzed using one-way ANOVA followed by Bonferroni post hoc comparison in the case of brain areas from the SA group (naive 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). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significantly different from naive (in the SA group) or saline (in the MP group).

creased the expression of [^{3}H]Epi-labeled 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 $\alpha4\beta2$, $\alpha4\alpha5\beta2$, $\alpha6\beta2$, $\alpha6\beta2\beta3$, $\alpha4\alpha6\beta2\beta3$, and $\alpha3\beta2$*. Immunoprecipitation experiments showed that in MP-treated rats, the only up-regulated subtype was that containing the $\alpha4$ (paired t test, $P = 0.0420$) and $\beta2$ subunits ($P = 0.0079$), whereas there was no change in the levels of receptors containing the $\alpha6$ and $\beta3$ subunits (Fig. 2E), to confirm results with ^{125}I-αCntxMII membrane-binding experiments (see Table 3).

In line with previous experiments (Gotti et al., 2005b), we found that approximately 10 to 15% of SC nAChRs contained the $\alpha3\beta2$* subtype, which was shown to be strongly up-regulated by nicotine in vitro (Sallette 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 that revealed the highest level of up-regulation (the MP nicotine versus 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 $\alpha4$*, $\beta2$*, and $\alpha5$* nAChRs (presumably $\alpha4\alpha5\beta2$ subtype) in the CCx of the nicotine MP-injected rats. In particular, the immunoprecipitation ratio between $\alpha4$ levels in the nicotine-treated rats and saline-treated control rats was almost identical to that between the $\beta2$ levels in the two groups ($\alpha4 = 1.75 \pm 0.06$; $\beta2 = 1.70 \pm 0.05$) (Fig. 2A). However, when we loaded the same amount of proteins (10–15 μg) of 2% Triton extract from the saline- and nicotine-treated rats, the Western Blotting ratio of the $\alpha4$ subunit in the two groups was almost identical to the immunoprecipitation ratio (1.68 ± 0.06), whereas the Western Blotting ratio of the $\beta2$ subunit was significantly higher (2.27 ± 0.18) (paired t test, $P = 0.011$). The immunoprecipitation and WB results confirmed that nicotine-induced increase in the number of [^{3}H]Epi binding sites corresponded to an increase in protein levels. This is indeed important, because up-regulation measured by radiolabeled agonists has been questioned (Vallejo et al., 2005).

Discussion

The main finding of this study is that MP-administered nicotine robustly up-regulates [^{3}H]Epi binding in all brain areas, whereas SA nicotine leads to less up-regulation limited fewer regions. The increase in [^{3}H]Epi binding was paralleled by an increase in the number of $\alpha4$ and $\beta2$ subunits, thus indicating very different up-regulation of $\alpha4\beta2$ receptors across brain regions (CCx > NAc ~ CPu > VMB ~ SC). Furthermore, MP-administered nicotine decreased $\alpha6\beta2(\beta3)$* receptor levels in the NAc and CPu (as shown by the reduction in ^{125}I-αCntx MII binding and the decrease in the number of $\alpha6$ and $\beta3$ subunits) and increased $\alpha7$ receptor levels in the CCx (as shown by ^{125}I-αBgtx binding). It is noteworthy that MP-administered nicotine did not affect $\alpha6$* receptors in the SC, a region in which they are highly expressed. Finally, SA nicotine significantly increased the levels of $\alpha4\beta2$ receptors only in the CCx and NAc and did not affect $\alpha6\beta2$* or $\alpha7$ receptor levels in any other region.

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 μM; Mugnaini et al., 2006), whereas that of rats immediately after an SA session has a concentration of 84 ng/ml (0.5 μM), which decreases to 10 ng/ml (0.06 μM) after 3 h (M. Tessari, unpublished data). The estimated serum nicotine concentration area under

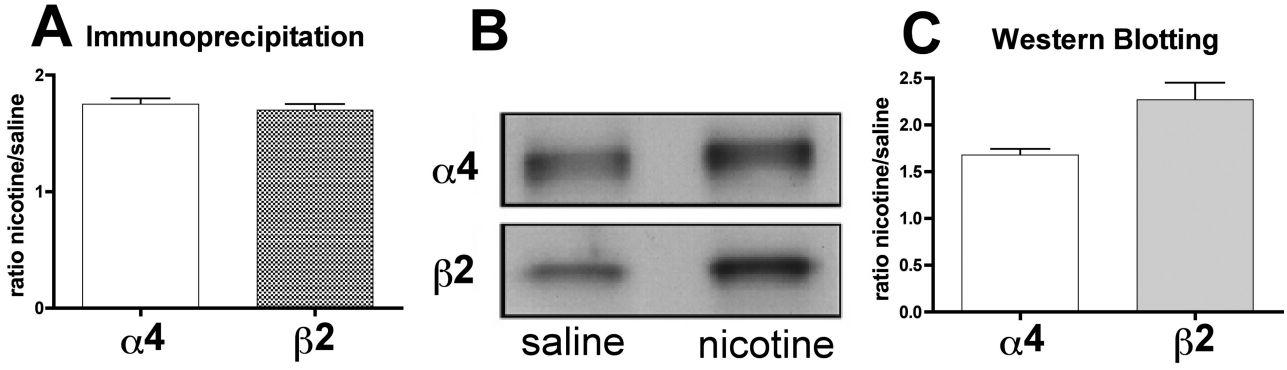

Fig. 2. Characterization of the $\alpha4\beta2$ subunit content in caudal cortex extracts obtained from rats treated with saline or nicotine administrated by MP. A, mean values ± S.E.M. of the immunoprecipitation ratios of the anti-$\alpha4$ and anti-$\beta2$ antibodies obtained in the 2% Triton X-100 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 C-terminal and intracytoplasmic loop peptides of the $\alpha4$ and $\beta2$ subunits as described under *Materials and Methods*. B, WB analysis of CCx extracts taken from rats receiving saline or nicotine. Ten to 15 μg of proteins of the 2% Triton X-100 were separated on 9% acrylamide SDS gels, electrotransferred to nitrocellulose, probed with 1 to 2.5 μg/ml indicated primary Abs, and then incubated with the secondary Ab (anti-rabbit conjugated to peroxidase, dilution 1:40,000). The bound Abs were revealed using a chemiluminescent substrate (Pierce). The anti-$\alpha4$ Ab recognized a band of 68 to 70 kDa (corresponding to the expected size of the $\alpha4$ subunit) and the anti-$\beta2$ Ab recognized a band of 52 kDa (corresponding to the expected size of the $\beta2$ subunit). The same blots were first incubated with anti-$\alpha4$ Abs and then stripped and incubated with anti-$\beta2$ Abs or vice versa. C, optical density ratios of the $\alpha4$ and $\beta2$ subunits in caudal cortex extracts obtained from rats treated with saline or nicotine administrated by MP. The developed films were acquired as described under *Materials and Methods*, and the images were analyzed using NIH Image J software (http://rsbweb.nih.gov/ij/). 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 ± S.E.M. obtained from the ratios between the optical densities of the $\alpha4$ (left) and $\beta2$ (right) subunits in the extracts taken from rats treated with saline or nicotine administrated by MP.

the curve over 24 h is approximately 700 to 800 ng/h/ml in MP rats and 100 to 200 ng/h/ml in SA rats (M. Tessari, unpublished data). The greater daily nicotine exposure of MP rats may explain why $\alpha4\beta2$ up-regulation was more pronounced in the MP experiment and why $\alpha6\beta2$* down-regulation and $\alpha7$ up-regulation were undetectable in the SA experiment. Alternatively, a nicotine concentration constantly above a certain threshold value in the MP experiment may desensitize $\alpha4\beta2$ 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 desensitization 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 $\alpha4\beta2$ 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 $\alpha4\alpha5\beta2$ 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 versus 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); we believe that the increase is an area-specific effect of nicotine.

In line with the results of previous studies (Lai et al., 2005; Mugnaini et al., 2006; Perry et al., 2007), MP-administered nicotine increased the overall expression of [^{3}H]Epi-labeled receptors in the NAc, CPu and VTA, but there was a large increase in the number of $\alpha4\beta2$* receptors and a decrease in the number of $\alpha6\beta2$* receptors in the NAc and CPu. The coexistence of oppositely regulated receptor subtypes may explain why autoradiographic studies have found little or no change in ^{125}I-Epi binding in these regions (Nguyen et al., 2004; Mugnaini et al., 2006).

SA nicotine increased $\alpha4\beta2$ subtype levels only in the NAc and had no effect on the $\alpha6\beta2$* subtype in any of the three considered areas; these results are different from those of Parker et al. (2004), who found increased levels of $\alpha6$* receptors and αCntxMII 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 1-h session, whereas Parker's rats self-administered 1.5 mg/kg/day over 23 h, 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 theirs were due to different nicotine pharmacokinetics in the two SA protocols, differences between rat strains (for review, see Matta et al., 2007), or unknown technical issues (such as the specificity of the anti-$\alpha6$ Abs or ^{125}I-αCntxMII binding).

Unlike Perry et al. (2007), we found that the decreased level of $\alpha6$ receptors in the NAc and CPu of rats receiving MP-administered nicotine was paralleled by a decrease in the level of the $\beta3$ subunit. This is not surprising because we have shown previously that more than 70% of the $\alpha6$* receptors in the striatum contain the $\beta3$ subunit and that the $\beta3$-null mutation selectively reduces striatal $\alpha6$* nAChR expression by 76% compared with $\beta3(+/+)$ controls (Gotti et al., 2005a).

As reported previously (Mugnaini et al., 2006; Perry et al., 2007), the area least sensitive 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 $\alpha4\beta2$ subtype but had no effect on the $\alpha6$* subtype.

It is not known why MP-administered nicotine up-regulates $\alpha4\beta2$* nAChRs but down-regulates $\alpha6$* receptors in midbrain dopaminergic neurons. In situ hybridization, single-cell polymerase chain reaction, and lesion studies have all shown that such neurons contain a mixture of $\alpha4$, $\alpha6$, $\beta2$ and $\beta3$ subunits (Klink et al., 2001; Lai 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 hypothesized that, if the number of $\beta2$ subunits is limited in these neurons, $\alpha4$ and $\alpha6$ subunits compete for assembly in the endoplasmic reticulum. By acting as a preferential chaperone of $\alpha4\beta2$ receptors (Sallette et al., 2004; Kuryatov et al.,2005), nicotine may favor their formation in MP-treated rats by decreasing the pool of $\beta2$ subunits available for assembly with the $\alpha6$ subunit and consequently the number of $\alpha6\beta2$*nAChRs, whereas the more limited up-regulation of the $\alpha4\beta2$ subtype in SA rats may lead to more $\beta2$ subunits being available for $\alpha6$ subunits and thus maintain the number of $\alpha6\beta2$*-nAChRs. This mechanism is also suggested by the findings of in vitro studies in which the nicotine concentration necessary to up-regulate $\alpha4\beta2$ receptors (EC_{50} = 35 nM) is much lower than that needed to up-regulate the $\alpha6\beta3\beta2$ subtype (EC_{50} = 890 nM; Tumkosit et al., 2006; Walsh et al., 2008). Moreover, the decrease in nAChR degradation during prolonged nicotine exposure (Rezvani et al., 2007) may contribute to up-regulation, although it is not yet known if this has different effects on the $\alpha4\beta2$ and $\alpha6\beta2$ subtypes.

In the case of the SC, it is not known whether $\alpha4$, $\alpha6$, $\beta2$, and $\beta3$ subunits are present in the same retinal ganglionic cells. However, if they are coexpressed, the much smaller nicotine-induced increase in $\alpha4$* levels in the SC than in the NAc (17% versus 78%) may explain the lack of competition between $\alpha4$ and $\alpha6$ subunits and the unchanged levels of the $\alpha6\beta2$* subtype.

One important finding of our immunoprecipitation experiments is the identical nicotine-induced up-regulation of $\alpha4$ and $\beta2$ 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 $\alpha4$ subunits but a significantly greater increase in $\beta2$ subunits. The nondenaturating immunoprecipitation protocol evaluates the ability of anti-$\alpha4$ and anti-$\beta2$ Abs to immunoprecipitate [^{3}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 $\beta2$-containing receptors measured by the two methods is therefore compatible with a nicotine-induced change in stoichiometry, as demonstrated previously in heterologous systems in which the $\alpha4\beta2$ subtype may exist in two different stoichiometries,

$(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$, with different functional and pharmacological properties (Nelson et al., 2003; Moroni et al., 2006). It has also been shown that long-term nicotine exposure up-regulates the expression of $(\alpha4)_2(\beta2)_3$ stoichiometry (Kuryatov et al., 2005).

The demonstration that nicotine can regulate the stoichiometries of natively expressed $\alpha4\beta2$-nAChRs may be important in pathophysiological states because it has been shown in heterologous systems that nicotine can normalize the intracellular subunit stoichiometry of nAChRs carrying mutations linked to autosomal dominant nocturnal frontal lobe epilepsy (Son et al., 2009).

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