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Up-Regulation of Brain Nicotinic Acetylcholine Receptors in the Rat during Long-Term Self-Administration of Nicotine: Disproportionate Increase of the α6 Subunit

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ABSTRACT

In male rats continually self-administering nicotine (approximately 1.5 mg free base/kg/day), we found a significant increase of nicotinic acetylcholine receptors (nAChRs) labeled by epibatidine (Epb) in 11 brain areas. A large increase of high-affinity Epb binding sites was apparent in the ventral tegmentum/substantia nigra, nucleus tractus solitarii, nucleus accumbens, thalamus/subthalamus, parietal cortex, hypothalamus, and amygdala. A smaller but significant up-regulation of high-affinity Epb sites was seen in the piriform cortex, hippocampus, caudate/putamen, and cerebellar cortex. The up-regulation of nAChRs, shown by immunoadsorption and Western blotting, involved α4, α6, and β2 subunits. As a consequence of long-term self-administration of nicotine, the α6 immunoreactive (IR) binding of either labeled Epb or ^{125}I-α-conotoxin MII increased to a much greater extent than did α4 or β2 IR binding of Epb. In addition, the β2 IR binding of Epb was consistently enhanced to a greater extent than was α4. These findings may reflect a larger surface membrane retention of α6-containing and, to some degree, β2-containing nAChRs compared with α4-containing nAChRs during long-term self-administration of nicotine.

Nicotine, one of the most widely abused addictive alkaloids, activates a family of pentameric nicotinic acetylcholine receptors (nAChRs) known to transport cations. This affects the release of neurotransmitters (Gallardo and Leslie, 1998; Fu et al., 2000a) and influences diverse functions, including feeding, arousal, endocrine regulation, nociception, and aspects of cognition. Cell membrane nAChRs accumulate in various paradigms of long-term exposure to nicotine or other nAChR modulators. This occurs in the central nervous system of primates, including humans (Perry et al., 1999); of rodents, including mice (Marks et al., 1983; Pauly et al., 1996) and rats (Schwartz and Kellar, 1983; Mugnaini et al., 2002); as well as in mammalian cells expressing cloned nAChRs (Wang et al., 1998).

No substantial up-regulation of mRNAs encoding the major nAChR subunits was detected in the brains of rodents that exhibited major increases in nAChRs after long-term treatment with nicotine (Pauly et al., 1996; Mugnaini et al., 2002). The increases in nAChRs are thus not likely to depend primarily on enhanced de novo synthesis, but they could be linked to nAChR desensitization. A largely reversible partial desensitization of nAChRs in response to nicotine was documented in rodent brain (Damsma et al., 1989; Sharp and Matta, 1993). In view of the low nAChR endocytosis and recycling in homeotherms (Higgins and Berg, 1988) and the generally limited control of concentration of cell membrane constituents, aggregation or compartmentalization of nAChRs could lead to the accumulation of membrane receptors during long-term nicotine treatment.

In rodent forebrain, the principal nAChRs contain β2 subunits (β2* nAChRs) and α4 subunits (α4* nAChRs) (Flores et al., 1992); α7 receptors are also well represented (Kaiser and Wonnacott, 2000). In several areas, there is significant expression of α6* (Champtiaux et al., 2002; Zoli et al., 2002) and α3* (Azam et al., 2002) nAChRs. The release of dopamine by nicotine (Damsma et al., 1989; Fu et al., 2000a) seems to be associated with α6* (Champtiaux et al., 2002), α3* (Kulak

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; Bgtx, α-bungarotoxin; Cntx, α-conotoxin MII; Epb, epibatidine; VTA/SN, the ventral tegmentum area/substantia nigra; IR, immunoreactive; HEK, human embryonic kidney; β2* nAChR, nicotinic acetylcholine receptor containing β2 subunits; α4* nAChR, nicotinic acetylcholine receptor containing α4 subunits.

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et al., 2001), and α7 receptors (Fu et al., 2000b). Indeed, nicotine-induced locomotor activity, associated with enhanced striatal dopaminergic activity, is selectively decreased by α6* antisense oligonucleotides (Le Novere et al., 1999). Because the regulation of dopaminergic neurotransmission by acetylcholine may involve α6* receptors at the level of dopamine terminals (Champtiaux et al., 2003), an increase in this subtype of nAChR during long-term exposure to nicotine may alter dopamine release.

In cell lines, up-regulation of surface nAChRs by nicotine was shown to include both α3* (Meyer et al., 2001; Ridley et al., 2002) and α6*, as well as α4* (Nelson et al., 2003) and α7 receptors (Quik et al., 1996). Oocyte-expressed α3* receptors are less sensitive than the α4* receptors to down-regulation (Fenster et al., 1997) and desensitization (Hsu et al., 1996) by nicotinic agonists. This may also pertain to α6* receptors, because α3 and α6 subunits are structurally and pharmacologically similar (Champtiaux et al., 2002; Kulak et al., 2002). Also, α4* receptors may show a larger constitutive removal from cell membrane than α3* receptors (Cooper et al., 1999) and presumably α6* receptors. Thus, nAChRs containing different α subunits may not increase proportionally during long-term treatment with nicotine.

In view of the above findings, our experimental objectives were (1) to measure changes in nAChR expression related to long-term nicotine self-administration, especially in brain areas known for important interactions between nicotinic cholinergic and catecholaminergic neurotransmission and (2) to determine whether nicotine self-administration selectively up-regulates the nAChR subunits that are preferentially involved in the activation of catecholaminergic systems. Our results show a larger up-regulation of α6* (and also of β2*) relative to α4* nAChRs in several major brain areas of rats continually self-administering nicotine.

Materials and Methods

Chemicals and Antibodies. Chemicals were obtained from Sigma Chemical (St. Louis, MO) and Sigma/RBI (Natick, MA). Radioactive chemicals were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). α-Conotoxin MII was synthesized and labeled with ^{125}I by J. M. McIntosh. Mouse monoclonal antibody to bacterially expressed human α6 nAChR subunit (mAb350) and rat monoclonal antibodies against rat α4 (mAb299) and β2 (mAb295) subunits were raised by J. Luo and J. M. Lindstrom. To remove any α3 antibody-like immunoprotein, mAb350 was pretreated with human α3 nAChR subunit immobilized on CH Sepharose 4B (Kuryatov et al., 2000). This antibody does not cross-react with α4β2 or α3β2 nAChRs expressed in oocytes (Kuryatov et al., 2000). The polyclonal rabbit antibodies to bacterially expressed human α3 (sc-5590), α4 (sc-5591), α7 (sc-5544), and β2 (sc-11372) subunits were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). These antibodies were all raised against large (≥100 residues) C-terminal peptides from the respective subunits, and specific blocking peptides are not available. However, neither the monoclonal α6 antibody nor the polyclonal α4 and β2 antibodies produced significant immunoadsorption of α3β4 nAChRs expressed in HEK-293 cells (Xiao et al., 1998) (see *Results*). Also, saturating quantities of the polyclonal α7 antibody did not affect the immunoadsorption of any of the above antibodies; conversely, the immunoadsorption of ^{125}I-α-bungarotoxin bound to solubilized α7 receptors from four brain areas was not modified by saturating amounts of the above α3, α4, α6, or β2 antibodies (see *Results*).

Animals and Treatments. Male Lewis rats (2.5 months of age; initial body weight, 220–240 g) were obtained from Harlan (Indianapolis, IN), and male DBA mice (6–7 weeks old) were from the National Cancer Institute (Bethesda, MD). All treatments were in accord with the animal care protocols approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center at Memphis. Nicotine self-administration was performed according to our protocols published previously (Fu et al., 2001). Briefly, 7 d after acclimation to a reverse-light cycle and handling, all rats received jugular cannulae under xylazine-ketamine anesthesia and then were immediately housed in individual environmental enclosures without shaping, conditioning, or food deprivation. After an additional 3 d of recovery, rats were randomly assigned to treatment groups, and the jugular lines were filled either with nicotine bitartrate (4 mM in heparinized saline; 50 μl delivered over 0.81 s per 300 g of body weight in one self-administration) or saline without nicotine. The self-administration of nicotine proceeded as detailed previously (Fu et al., 2001). Stable nicotine self-administration, defined as 3 consecutive days showing more than 40 active lever presses per day at less than 15% variance, and with active (green-lit) lever-press counts significantly greater than those of inactive lever, was achieved within 6.5 ± 1.0 (mean ± S.E.M.) days. At that time, the approximate dosage of self-administered nicotine free base was 1.5 mg/kg/day (Fig. 1). After 18 days of self-administration, the animals were anesthetized with isoflurane before decapitation by guillotine. Brains were promptly excised and frozen in dry ice before storage for not more than 14 days at −80°C.

Tissue Collection. The rat or mouse forebrains were sliced (at −7°C) into 1-mm thick sections. Selected areas were then excised according to the coordinates of Paxinos and Watson (1986) and Franklin and Paxinos (1997), respectively. The following areas were collected from rat brains: parietal cortex, the piriform/entorhinal cortex, the caudate/putamen (from 0 to 2 mm anterior to bregma), nucleus accumbens (2 mm centered about anterior commissure and 0–2 mm anterior to bregma), amygdala (medial to entorhinal/piriform cortex, from approximately 3 to 6 mm posterior to bregma), hypothalamus (from optic chiasm to mammillary nuclei, 1–5 mm posterior to bregma), thalamus (including subthalamic area; approximately 2–3 mm inferior to corpus callosum and 3 mm lateral to midline, 1–6 mm posterior to bregma), hippocampus (from 4 to 6 mm posterior to bregma), ventral tegmental area/substantia nigra (VTA/SN; ventral horizontal cut, 4–6 mm posterior to bregma), the nucleus tractus solitarii area (1 mm inferior and 2 mm lateral to the fourth ventricle, 11–13 mm posterior to bregma), and rostral cerebellar cortex. With mouse brains, the thalamus/subthalamus was collected from 0.5 to 3 mm posterior to bregma, 1 to 2 mm inferior to corpus callosum, and approximately 2.5 mm lateral to midline. The hippocampus was taken from 3 to 4 mm posterior to bregma, and parietal/somatosensory cortex at ±1 mm from bregma. Excised tissues were immediately stored at −80°C and then processed to obtain particulates within 7 days.

Isolation of Particulates. The isolation of particulates was done at 0 to 4°C, essentially as described previously by Marks et al. (1998). The tissue homogenate was made in ice-cold Epb assay buffer, using a motor-driven Teflon pestle at 800 rpm. The homogenate was sedimented for 12 min at 20,000g_{max}, and the pellets were resedimented before storage at −80°C.

The Epibatidine Binding Assay. The protocol described by Marks et al. (1998) was generally followed, including the same binding buffer. Particulates or dispersed cells were resedimented twice from this buffer before assays. For competition assays with ^{125}I-epibatidine (2170 Ci/mmol) (PerkinElmer Life and Analytical Sciences), 50 pM was used, yielding binding parameters similar to those obtained from saturation binding of ^{125}I-Epb or [^{3}H]Epb (see *Results*). To obtain numerically adequate binding, the input of [^{3}H]epibatidine (56 Ci/mmol) (PerkinElmer Life and Analytical Sciences) in competition assays was 500 pM. Duplicate assays at 0, 10, 30, 100, 300, and 1000 pM unlabeled Epb and at 30 μM (−)-nicotine (for nonspecific binding) were done for quantitative comparisons between animals self-administering nicotine versus saline. For saturation

assays, the ^{125}I- or ^{3}H-labeled Epb input was 10 to 1000 pM using 8 to 12 concentrations. The protein concentration was 0.025 mg/ml in ^{125}I-Epb assays and 0.1 mg/ml in [^{3}H]Epb assays. The assay volume was 0.40 ml, and reactions were incubated for 90 min at 23 to 24°C. Under these conditions, 95% saturation of 50 pM ^{125}I-Epb or 500 pM [^{3}H]Epb and 500 pM ^{125}I-Epb binding was achieved in less than 15 and 2 min, respectively. The time points for binding kinetics, measured by filtration, were 1, 3, 6, 10, 20, 30, 45, 60, and 90 min.

In kinetic as well as in some saturation experiments, the binding was terminated by filtration using Whatman GF/C filters (Whatman, Clifton, NJ) (presoaked in 0.3% polyethylenimine) and washing with ice-cold assay buffer. No consistent differences in binding were apparent between the centrifugation and filtration protocols. The competition and most saturation assays were terminated by spinning for 12 min at 20,000g_{max} at 4°C followed by rinsing with ice-cold assay buffer. The tube bottoms or filters containing bound ^{125}I-Epb were counted in a MicroMedic γ-scintillation counter (Valeant Pharmaceuticals International, Costa Mesa, CA). For counting of [^{3}H]Epb, the pellets were solubilized, or the filters were soaked overnight in 2% sodium dodecylsulfate/10 mM Tris-HCl, pH 8.8, followed by the addition of liquid scintillation solvent and then underwent counting in a Beckman LS-3801 counter (Beckman Coulter, Fullerton, CA). Receptors solubilized in 2% Triton X-100 were quantified by polyethylene glycol precipitation (Parker et al., 1998b).

The Binding of ^{125}I-Labeled α-Conotoxin MII and α-Bungarotoxin. α-Conotoxin MII (Cntx) was synthesized and monoiodinated (specific activity, 2170 Ci/mmol) as described previously (Whiteaker et al., 2000b), and ^{125}I-labeled α-bungarotoxin (Bgtx) (specific activity, 140 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences. Assay inputs of Cntx and Bgtx were 0.4 and 1.0 nM, respectively. Nonspecific binding for Cntx was defined at 10 μM (−)-cytisine plus 100 μM (−)-nicotine (because nicotine alone in some cases did not displace all binding that was sensitive to cytisine). For Bgtx, nonspecific binding was defined at 1 μM unlabeled Bgtx. The particulates were resedimented once from the binding buffer described by Whiteaker et al. (2000b) before incubation at 0.4 mg protein/ml protein for 2 (Cntx) and 5 (Bgtx) h at 24°C. The assay was terminated using Whatman GF/C filters presoaked in 1% polyethylenimine and rapid washing with ice-cold binding buffer. Nonspecific binding of Cntx and Bgtx was 40 to 60% of total binding.

Immunoadsorption of Nicotinic Receptors. At 2% Triton X-100 with inputs of solubilized protein lower than 0.2 mg/ml, the polyclonal rabbit antibodies to C-terminal portions of human α3, α4, and β2 nAChR subunits (expressed in *Escherichia coli*) produced saturating immunoadsorption of Epb-labeled nAChRs at immunoglobulin concentrations lower than 6 μg/ml; the mouse and rat monoclonal antibodies produced saturating immunoadsorption lower than 30 μg of immunoglobulin/ml. Particulates were processed at 0 to 4°C as described by Wang et al. (1998). Briefly, particulates were solubilized at 2% Triton X-100 and 0.4 to 0.5 mg protein/ml, and after 90 min in ice, they were centrifuged for 12 min at 20,000g_{max}. Tracers (1 nM [^{3}H]Epb, 200 pM ^{125}I-Epb, 500 pM ^{125}I-Cntx, or 1 nM ^{125}I-Bgtx) were added to the supernatant and incubated for 16 to 18 h at 4°C. Duplicate aliquots containing 25 μg of extracted protein were mixed, in the final volume of 0.20 ml, with the appropriate antibody, using 1, 3, and 6 μl of each antibody. This corresponded to 0.2, 0.6, and 1.2 μg of immunoglobulin for the polyclonal antibodies, or 1, 3, and 6 μg of immunoglobulin for the monoclonal α6 antibody, and produced a complete saturation of receptor immunoadsorption for all antibodies (see *Results*) (Fig. 5A). The mixtures were rotated with protein A/protein G agarose (Santa Cruz Biochemicals) for 8 h at 4°C. Correction for nonspecific adsorption was obtained by incubating the largest input of the respective antibody with 10 μM cytisine plus 100 μM (−)-nicotine for Epb or Cntx tracers and with 1 μM unlabeled Bgtx plus 100 μM nicotine for the Bgtx tracer. The nonspecific adsorption was always less than 2% of total agarose gel radioactivity with Epb tracers (with typical "signal-to-noise" ratios better than 100 for α3 and α6 and better than 1000 for α4 and β2 subunits), but it represented up to 30% of total gel radioactivity with ^{125}I-Cntx or ^{125}I-Bgtx tracer. The assays were terminated by dilution and sedimentation for 1 min at 1000g_{max} followed by three resedimentations

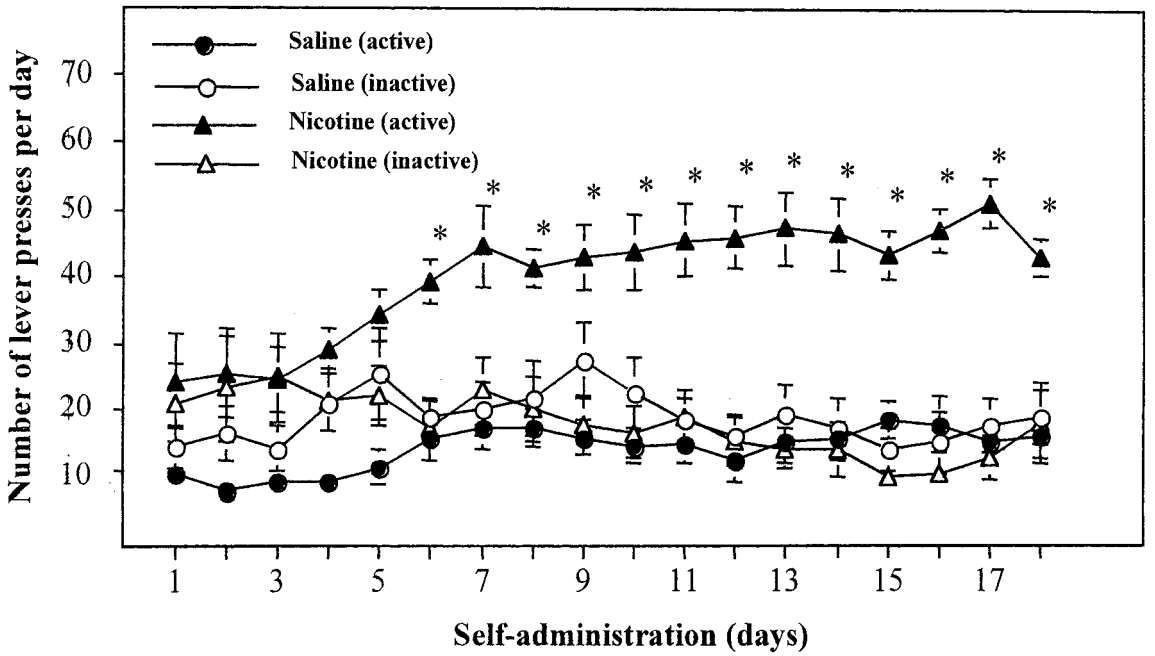

Fig. 1. A comparison of lever-pressing activity in self-administration of nicotine or saline by rats over 18 days after habituation. Six animals were compared in each group. The brains of these animals were used in receptor assays shown in Fig. 3. The mean numbers of lever presses per day (± S.E.M.) for the four data sets were analyzed by two-way analysis of variance (see *Materials and Methods*). The difference between treatments was highly significant (F = 51.6, $p < 0.001$), whereas the difference across the time points (F = 1.34, p = 0.164) or that in interaction between the type of treatment and the length of treatment (F = 1.29, p = 0.087) was not significant. The data were further compared in Scheffe's tests, and the differences significant at >95% confidence between the nicotine self-administering set and all other sets are indicated in the graph by asterisks.

from fresh assay buffer. [^{3}H]Epb-labeled pellets were dispersed in 2% sodium dodecyl sulfate/0.01 M Tris-HCl, pH 8.8, before liquid scintillation counting.

Immunoblotting of Nicotinic Receptors. The washed particulates were solubilized and extracted at 4°C in radioimmunoprecipitation assay buffer (Santa Cruz Biochemicals), the extracts were cleared (30 min at 30,000g_{max} and 4°C), and the supernatants were mixed with an equal volume of a denaturation buffer (Bio-Rad, Hercules, CA), heated for 30 min at 98°C, and then resedimented as described above. Aliquots of the supernatant were electrophoresed in polyacrylamide gels, electroeluted onto nitrocellulose, and saturated with a blotting buffer (Blotto B; Bio-Rad). The membranes were then incubated for 16 h at 4°C in 0.14 M NaCl/10 mM Tris-HCl, pH 7.4, containing 2 μg/ml of one of the rabbit polyclonal antibodies (Santa Cruz Biochemicals) to human α3, α4, or β2 nAChR subunits or 6 μg/ml of the mouse monoclonal antibody to human α6 subunit. After washing and a 90-min exposure to goat anti-rabbit or anti-mouse immunoglobulins coupled to horseradish peroxidase (1:2000 to 1:5000 dilution) and final washing, membranes were exposed to Luminol bioluminescent reagent (Santa Cruz Biochemicals), apposed to Kodak BR Biomax film (Eastman Kodak, Rochester, NY), and the negatives were scanned.

Data Analysis. To analyze the frequency of nicotine and saline self-administration, the mean number of lever presses per day were compared by two-way analysis of variance, followed where appropriate by Scheffe's test, using SPSS statistical software, version 11.0 (SPSS Inc., Chicago, IL). Evaluation of immunoadsorption with multiple data-point profiles was done by nonlinear fitting to Michaelis-Menten equation (Parker and Waud, 1971) followed by t tests on means of the binding parameters. Grain density in scans of Western blot profiles was evaluated using OptiQuant software (PerkinElmer Life and Analytical Sciences). The means of receptor binding parameters were compared in two-tailed Student's or Dunnett's t tests (Zar, 1984). The quantitative comparisons on six competition points (see The Epibatidine Binding Assay section under *Materials and Methods*) were done with least-square Scatchard fits, because nonlinear Scatchard fitting with only six concentration points tends to result in large variations; the least-squares Scatchard linearization using analytical second derivatives is routine in similar evaluations (Segel, 1974; Marks et al., 1998). The nonlinear curve fits generated by either the LIGAND program (Munson and Rodbard, 1980) or the curve-fitting facility of the SigmaPlot program (version 8.0; SPSS Inc.) were used to check for multiple components in competition profiles with eight or more concentration points. Hill slopes (n_H) were calculated using Scatchard estimates of B_{max} in the logarithmic Hill equation (Segel, 1974). Sequence comparisons were done in FASTA programs (Pearson, 2000).

Results

Active and Inactive Lever Presses during Nicotine or Saline Self-Administration. The self-administration profiles for the two groups of rats used for receptor assays is shown in Fig. 1. The number of active lever presses resulting in nicotine self-administration was significantly greater than for any other group from the 6th day of treatment and remained at 40 to 50 active lever presses per day through day 18. In contrast, the number of active lever presses for control rats self-administering saline varied between 20 and 25 presses per day. There was a highly significant difference in active lever presses between treatment groups (analysis of variance, $p < 0.001$) (Fig. 1), and analysis by day from the 6th day on also showed a significant effect of nicotine (p value range of 0.001 to 0.05) (Fig. 1). The number of inactive lever presses, which had no programmed consequence, was similar for both nicotine and saline self-administration and never exceeded 15 per day. Analyses showed no difference in inactive lever presses by treatment group or between active lever presses in the saline group and inactive lever presses in either treatment group (see legend to Fig. 1).

Homogeneity of High-Affinity Epibatidine Binding in Discrete Rat Forebrain Areas. Previous reports indicated the presence of two distinct high-affinity (in the K_d range of 10–400 pM) components of epibatidine binding in particulates from whole rat forebrain (Houghtling et al., 1995) but not in a number of forebrain areas of the mouse (Marks et al., 1998). Differences related to multiple high-affinity components of agonist binding, if present in discrete brain areas of the rat, may confound comparisons of receptor numbers between brain areas or between treatment groups.

The saturation binding of epibatidine (in the range of 10–1000 pM) to particulates from several rat brain areas is shown for three rat forebrain areas in Table 1 and Fig. 2. The K_d values were in the range of 20 to 35 pM with either ^{125}I-Epb or [^{3}H]Epb, and the binding profiles could not be numerically split [using the LIGAND program (Munson and Rodbard, 1980) or the curve-fitting facility of the SPSS SigmaPlot program] in more than one component. With particulates from several brain areas, solubilization at 2% Triton X-100 and assay at 0.1 to 0.5% final of the detergent lowered the binding affinity of [^{3}H]epibatidine uniformly approximately 3-fold without a significant change of B_{max}; data for rat thalamic extract are shown in Fig. 2. As would be expected from the lack of change in the binding capacity, the solubilization also did not result in additional binding components discernible in the 10 to 1000 pM range. The observed change in K_d is not major and should relate to a less compact conformation of the binding site. The robust preservation of nAChR binding capacity is of critical importance for appro-

TABLE 1

Parameters for saturation binding of ^{125}I-epibatidine and [^{3}H]epibatidine to particulates from three rat forebrain areas

The binding parameters are Scatchard estimates from three saturation assays for each brain region and ligand (using eight different concentrations ranging from 10 to 1000 pM of either radionuclide), shown as means ± S.E.M. In all cases, fitting to a single specific component in the LIGAND program (Munson and Rodbard, 1980) produced lower residual variance and much higher F values in F-runs testing, relative to fits assuming two specific binding components. At 1000 pM, the nonspecific binding [defined using 30 μM (−)-nicotine] did not exceed 12% of the total binding for particulates from any brain region (also see Fig. 2).

Tissue	^{125}I Tracer			^{3}H Tracer		
	K_d	B_{max}	n_H	K_d	B_{max}	n_H
Control rats						
Hypothalamus	27.8 ± 3.3	25.4 ± 1.8	1.09 ± 0.12	25.9 ± 2	24.5 ± 1.4	1.15 ± 0.11
Parietal cortex	31.8 ± 6.1	38.1 ± 4	1.16 ± 0.13	30.6 ± 3.7	45 ± 3.3	1.07 ± 0.08
Thalamus	22.1 ± 1.4	60.3 ± 2.8	0.94 ± 0.12	20.9 ± 2.8	52.2 ± 5.3	1.05 ± 0.07
Nicotine self-administering						
Parietal cortex	28.2 ± 3.5	55.5 ± 7.4	1.22 ± 0.15	34.5 ± 4.1	64.9 ± 4.5	1.19 ± 0.14
Thalamus	25.2 ± 4.3	99.1 ± 7.5	1.21 ± 0.16	27.7 ± 7.6	113 ± 10.8	1.18 ± 0.12

priate immunoassays of the solubilized receptor. It is of interest to note that the number of epibatidine binding sites in the mouse brain was similar to those found in the rat brain in two regions (data not shown), hypothalamus and hippocampus, but it was 40% lower than the rat number for thalamus/subthalamus (Fig. 2) and was approximately 75% lower for the mouse parietal cortex [K_d = 34.1 ± 2.7 pM and B_{max} of only 10.4 ± 1.4 fmol/mg protein (n = 3) compared with 38.1 fmol/mg protein in the rat parietal cortex] (Table 1).

By examining the particulates from three forebrain areas (hypothalamus, parietal cortex, and thalamus/subthalamus) of control rats and from two areas (parietal cortex and thalamus/subthalamus) of nicotine-self administering rats, however, we were unable to find significant area-related or treatment-dependent differences in the saturation binding K_d values for either ^{125}I-epibatidine or [^{3}H]epibatidine within the concentration range of 10 to 1000 pM (Table 1). Furthermore, the affinity of saturation binding obtained for both radioligands did not differ significantly over the range of 10 to 1000 pM with particulates from any of the areas. The Hill slopes (n_H), calculated using the logarithmic Hill equation (Segel, 1974), were in the range of 0.8 to 1.2 (Table 1), indicating an absence of multiple components in the binding of either ^{125}I-epibatidine or [^{3}H]epibatidine at up to 1 nM. The B_{max} estimates obtained in saturation assays were also similar for the same brain area with both ^{125}I-Epb and [^{3}H]Epb (Table 1). With rat thalamic tissue, dissociation of either ^{125}I-Epb or [^{3}H]Epb at 24°C after saturation at 500 nM was characterized by a fast component (half-periods of 17 ± 2.5 and 19 ± 4 min, respectively; n = 3 for each) constituting 75 to 80% of the binding and a very slow additional decrease. Analyses of ln(bound/equilibrium bound) versus time were essentially linear ($r^2 > 0.95$), indicating a single specific component of dissociation (Whiteaker et al., 1998).

Estimates of epibatidine binding parameters obtained in saturation assays using ^{125}I-Epb and [^{3}H]Epb (Table 1) were in good agreement with those obtained in competition of 50 pM ^{125}I-Epb by unlabeled Epb at six concentrations in the range of 10 to 1000 pM (Fig. 3 and Table 2). This is to be expected, because only one binding component was detected with either radioligand within the concentration range used. Extension of the Epb concentration range showed a small α7 site-related low-affinity component (K_d = 7–12 nM) that could be eliminated by 1 μM α-bungarotoxin (Zoli et al., 2002; results not shown). Using nonlinear Scatchard fitting (Munson and Rodbard, 1980), no significant second component could be detected in any of the competition profiles in the range of 10 to 1000 pM of competing Epb. In all cases, the Hill slopes (n_H) for the competition studies were in the range of 0.8 to 1.2 (data not shown), again indicating the absence of subpopulations with significantly different affinities.

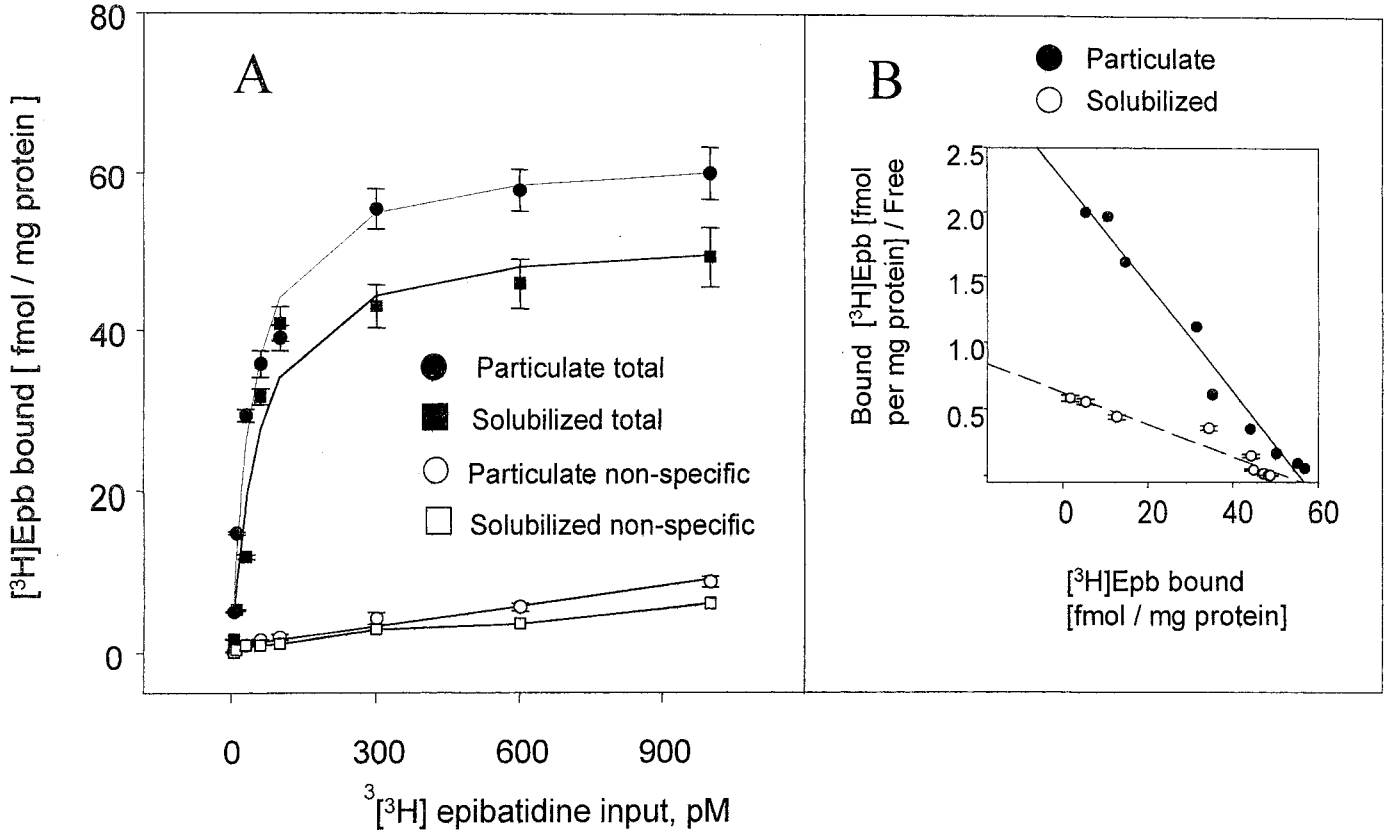

Fig. 2. Saturation binding of [^{3}H]epibatidine to rat thalamic particulates and to Triton X-100 extract of the particulates. A, total and nonspecific binding of [^{3}H]epibatidine (5–1000 pM) to particulates and to Triton X-100 extract. The nonlinear exponential, hyperbolic, and sigmoid, or LIGAND program (Munson and Rodbard, 1980) nonlinear Scatchard fitting in both cases rejected two-component models. The nonspecific binding at 1000 pM [^{3}H]Epb was approximately 12% of the total binding for each condition. B, Scatchard plots of the data from A (corrected for the nonspecific binding). With particulate receptors, the K_d was 24.7 ± 1.9 pM, B_{max} was 55.5 ± 2.3 fmol/mg protein, and n_H value was 1.08 ± 0.09. With receptors solubilized at 2% Triton X-100, the respective values were 83.7 ± 4.3, 51.9 ± 3.7, and 0.96 ± 0.08. The results are averages of three saturation assays for each condition. For other details, see *Materials and Methods*.

Up-Regulation of Epibatidine Binding Sites by Long-Term Self-Administration of Nicotine. Increased numbers of Epb-labeled nicotinic receptors compared with paired controls were found in animals continually self-administering nicotine in all brain areas examined (Fig. 3 and Table 2). The ranges of B_{max} increase relative to controls showed no overlap in 8 of 11 areas examined. Studies of an additional six pairs of animals, self-administering the same dose of nicotine, showed a substantial and uniform increase in the number of Epb sites in all areas examined (i.e., thalamus/subthalamus, hypothalamus, parietal cortex, and VTA/SN; data not shown).

Table 2 shows that the largest increase of ^{125}I-Epb sites was found with particulates from the region containing the nucleus tractus solitarii area and from VTA/SN, followed by those from nucleus accumbens. A large increase also was present in thalamus/subthalamus, parietal cortex, hypothalamus and amygdala. A significant but smaller increase was evident in piriform cortex, caudate/putamen, and hippocampus (Fig. 3 and Table 2). A small yet significant increase was also found with particulates from cerebellar cortex. These increases were routinely confirmed by saturation assays using concentrations of [^{3}H]Epb in the range of 10 to 1000 pM; the results for thalamus/subthalamus and parietal cortex are shown in Table 1.

Cytisine Sensitivity of the Binding of Either Epibatidine or α-Conotoxin MII Is Decreased in Rats Continually self-administering nicotine. The α3*, α4*, and α6* nicotinic receptors all bind epibatidine with high affinity (Kaiser and Wonnacott, 2000; Zoli et al., 2002) but differ in the sensitivity of Epb binding to cytisine. This nicotinic agonist strongly competes Epb binding to α4*β2* sites (Flores et al., 1997). However, cytisine is a much weaker competitor of Epb binding to nAChRs containing α subunits structurally similar to rat α6, including chick α6* receptors (Vailati et al., 1999) (77% sequence identity with rat α6 subunit) and rat α3* receptors (Xiao et al., 1998) (66% sequence identity with rat α6 subunit). Cytisine was also shown to differentiate Epb binding sites in areas of mouse brain (Marks et al., 1998), which may to some degree be related to the content of α6*

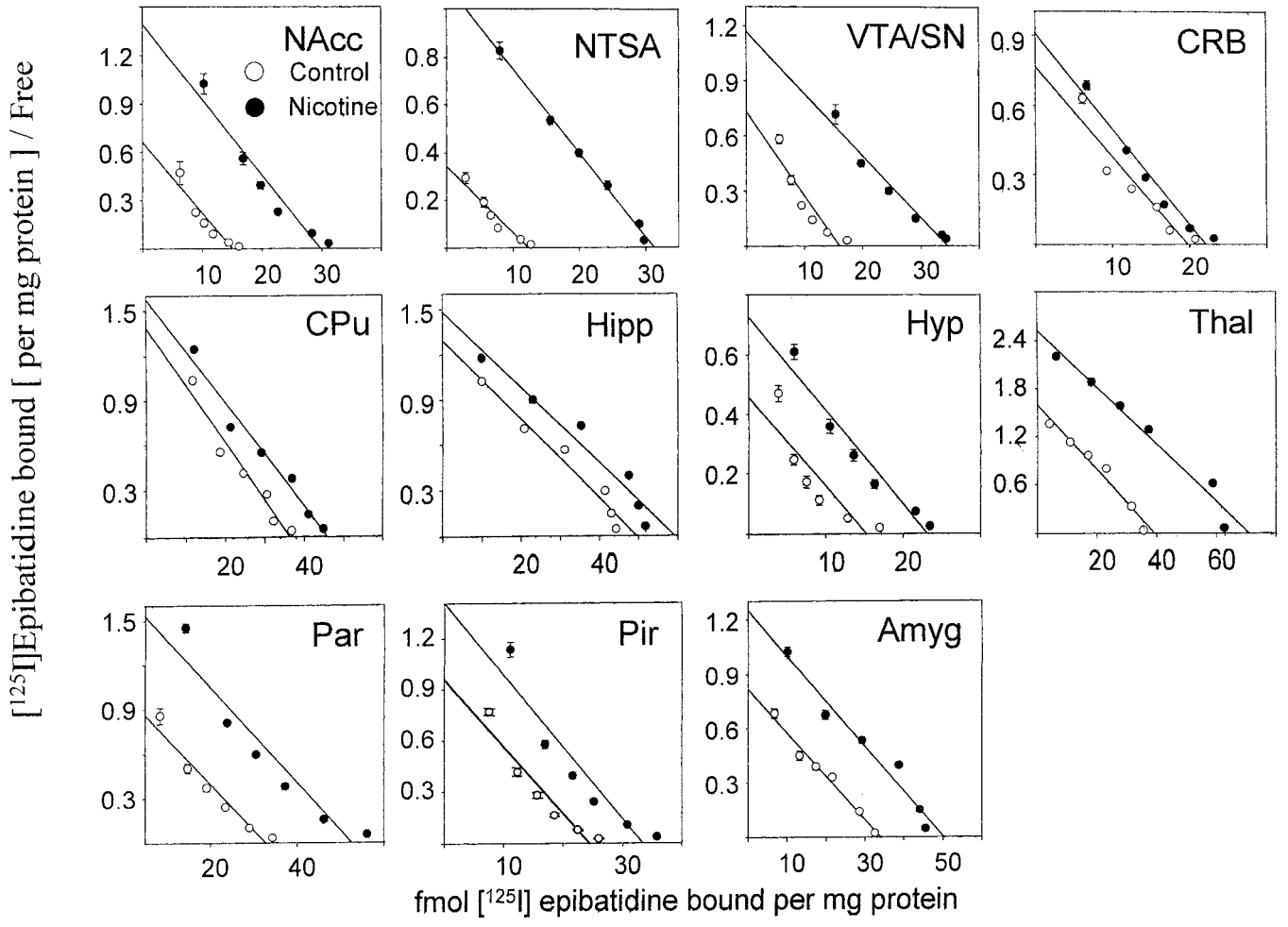

Fig. 3. Scatchard binding parameters for ^{125}I-epibatidine binding to particulates from brain areas of rats continually self-administering (−)-nicotine (~1.5 mg/kg/day) and the corresponding control rats. The results are averages of separate competition assays on six individual animals for each paradigm and brain area, competing 50 pM of the radioligand with six different concentrations (in the range of 10–1000 pM) of nonlabeled Epb. The K_d range was 21 to 43 pM. In *t* tests, the only significant K_d value difference from control rats was found for VTA/SN area. The Hill slope coefficients (n_H) taken from Scatchard B_{max} values ranged from 0.85 to 1.25 (data not shown). *, significance versus the corresponding control mean B_{max} in *t* tests (*, $p < 0.05$; **, $p < 0.01$). Very similar B_{max} values were observed in assays using [^{3}H]epibatidine (see Table 1). For other details see *Materials and Methods*. NAcc, nucleus accumbens; NTSA, area of the nucleus tractus solitarii; CPu, the caudate/putamen area; Par, the parietal cortex; Pir, the piriform/entorhinal cortex; Thal, thalamus/subthalamus; Hipp, hippocampus; Hyp, hypothalamus; Amyg, amygdala; CRB, the cerebellar cortex.

receptors (Champtiaux et al., 2002). As seen with four tissues in Table 3, a significant increase in resistance to cytisine in rats continuously self-injecting nicotine was found especially for [^{3}H]Epb binding to particulates from nucleus accumbens and also from thalamus/subthalamus and parietal cortex. The increase over control rats was especially pronounced at the highest molarity of cytisine tested. Moreover, the resistance apparently increased with the molarity of cytisine. The linear regression coefficient (r^2) for cytisine concentration versus Epb binding was 0.93 to 0.98 ($p = 0.05$–0.001) with particulates from the above areas. A lesser increase in resistance of [^{3}H]Epb binding to cytisine and a lack of regression were found with hippocampal particulates.

The binding of the $\alpha3/\alpha6$ subunit-selective nicotinic antagonist ^{125}I-α-conotoxin MII as displaced by cytisine (Whiteaker et al., 2000a) was also compared in four brain areas from rats continually self-administering nicotine versus saline. As expected, this binding was much lower than that of epibatidine but showed a large up-regulation after long-term nicotine self-administration, especially in nucleus accumbens and thalamus/subthalamus (Table 4). The sensitivity of ^{125}I-Cntx binding to cytisine dramatically decreased in nucleus accumbens and thalamic/subthalamic particulates of animals self-administering nicotine, in which the displacement relative to control decreased steadily with increasing molarity of cytisine. This also was present with particulates from parietal cortex. In contrast, the decline in cytisine sensitivity was significant for hippocampal particulates only at the highest concentration of cytisine (Table 4). However, the linear regression was significant ($r^2 > 0.9$, $p < 0.05$) in all cases.

A recent autoradiographic study indicated a high density of α-conotoxin MII binding sites in mouse dorsal thalamic areas, which includes the habenular nuclei, as well as a moderate density in the subthalamic region (Champtiaux et al., 2002). Because we also found a relatively high density of nAChRs in rat thalamus, we compared the binding of ^{125}I-Cntx in three thalamic subsections of rat and mouse. As seen in Table 5, the specific binding of this neurotoxin was consistently higher in rat thalamic areas compared with those of the mouse, especially in the subthalamic area. In both species, the Cntx binding to particulates from dorsal thalamic area was significantly larger than that in the other two thalamic areas.

Characterization of the Subunit Composition of nAChRs Using Antibodies Specific for the Major Subunits. The identities of subunits as well as the differences in subunit abundance between the long-term nicotine versus saline self-administration groups were verified by immunoblotting. Western blots prepared with extracts of thalamic particulates using the rabbit polyclonal antibodies to human $\alpha4$ or $\beta2$ subunit or the mouse monoclonal antibody to human $\alpha6$ subunit showed a single major component linearly increasing in quantity with increase in receptor protein ($r^2 > 0.95$, $p < 0.05$ in all cases) (Fig. 4). As expected, the immunoreactivity was considerably greater in animals self-administering nicotine than in those receiving saline (Fig. 4). For low-abundance proteins such as $\alpha3$ or $\alpha6$ nAChR subunits, only semiquantitative comparisons could be made by immunoblotting; however, the binding of high-affinity radioligands to nAChRs, followed by immunoadsorption, requires much lower protein input yet provides a precise quantification for all subunits.

Published studies have demonstrated the specificity of monoclonal $\alpha6$ antibody by showing that neither $\alpha3\beta2$ nor $\alpha4\beta2$ nAChRs expressed in oocytes were immunoadsorbed (Kuryatov et al., 2000). In contrast, large amounts of $\alpha6\beta2$ were immunoadsorbed by $\alpha6$ monoclonal antibody, and none were immunoadsorbed by antisera to $\alpha3$ or $\alpha4$ subunits. In addition, the rat $\alpha3$ subunit expressed in HEK-293 cells as $\alpha3\beta4$ nAChRs (Xiao et al., 1998) was detected by the polyclonal $\alpha3$ antibody at 118 ± 4 fmol ^{125}I-epibatidine immunoadsorbed per mg input protein ($n = 6$), whereas the monoclonal $\alpha6$ antibody only immunoadsorbed 4.1 ± 0.5% of this amount of radioactivity. Moreover, saturating quantities of the polyclonal $\alpha4$, $\alpha7$, and $\beta2$ antibodies immunoadsorbed 3.6 ± 0.15, 1.7 ± 0.4, and 5.8 ± 1.0%, respectively, of the above $\alpha3$ antibody immunoreactivity ($n = 3$). The polyclonal antibody to human $\alpha7$ subunit immunoadsorbed 12 ± 2.4 fmol ^{125}I-Bgtx/mg protein of rat thalamus; virtually no immunoadsorption ensued upon ^{125}I-Bgtx-labeled receptor incubation with saturating amounts of the $\alpha4$, $\alpha6$, and $\beta2$ antibodies. These results and the previous report (Kuryatov

TABLE 2

Parameters of ^{125}I-epibatidine binding to particulates from brain areas of rats continuously self-administering nicotine compared with the corresponding control rats

Particulates from six rats self-administering nicotine and six paired control rats were individually assayed for competition of the binding of 50 pM ^{125}I-epibatidine by 0, 10, 30, 100, 300, and 1000 pM of unlabeled Epb and by 30 μM (−)-nicotine (used to define the nonspecific binding). The parameters are estimates from Scatchard plots (bound/free versus bound). Because the particulate protein input was only 25 μg/ml, the highest bound/free ratios were lower than 0.25 in all profiles, and there were no significant effects on the apparent affinity caused by ligand depletion seen with [^{3}H]nAChR ligands because of their low specific activity (Houghtling et al., 1995; Marks et al., 1998). The mean percentage increase values for nicotine-treated over control rats are followed by levels of significance in t tests (*, $p < 0.05$, **, $p < 0.01$). The Scatchard plots are presented graphically in Fig. 3. The slopes of logarithmic Hill plots (n_H) were in the range of 0.8 to 1.2, indicating the absence of multiple affinity components in the binding over the Epb molarity range used.

Area	K_d Control	K_d Nicotine	B_{max} Control	B_{max} Nicotine	B_{max} Increase by Nicotine
	pM	*pM*	*fmol/mg particulate protein*	*fmol/mg particulate protein*	*%*
Nucleus accumbens	22.8 ± 1.46	21.5 ± 0.91	15.0 ± 0.75	29.8 ± 0.95	99**
VTA/SN	22.0 ± 1.54	29.5 ± 0.92	16.1 ± 0.81	34.6 ± 0.85	115**
Nucleus tractus solitarii	35.7 ± 1.67	28.3 ± 0.29	12.2 ± 0.39	31.2 ± 0.23	156**
Caudate/putamen	26.2 ± 1.01	29.0 ± 0.85	36.2 ± 1.1	45.8 ± 0.97	27*
Hippocampus	38.2 ± 1.23	39.9 ± 1.51	49.5 ± 1.10	59.2 ± 1.49	20*
Hypothalamus	33.4 ± 3.1	31.7 ± 1.28	15.3 ± 0.96	23.1 ± 0.66	51**
Thalamus	24.7 ± 0.75	28.2 ± 0.85	39.5 ± 0.72	71.2 ± 1.3	80**
Parietal cortex	32.2 ± 1.31	31.1 ± 1.63	33.0 ± 1.0	52.8 ± 2.1	60**
Piriform cortex	25.5 ± 1.28	23.7 ± 1.41	24.5 ± 0.92	33.6 ± 1.51	37*
Amygdala	41.4 ± 0.91	40.2 ± 1.33	34.2 ± 0.52	50.5 ± 1.17	48**
Cerebellar cortex	25.8 ± 1.43	24.3 ± 0.78	19.9 ± 0.81	22.3 ± 0.55	12*

et al., 2000) are in accord, showing strong selectivity of the antibodies used to detect the respective subunits.

As shown in Fig. 5A for rat thalamic [^{3}H]Epb-labeled receptors reacting with four nAChR subunit-specific antibodies, the immunoadsorption of Epb binding to Triton X-100–solubilized receptor was saturated within the range of antibody input used in the assays. As expected, the immunoadsorbed β2 binding was by far the most abundant in all brain areas examined (Figs. 5 and 6) followed by α4 binding. The Epb binding ratios for immunoadsorbed β2/α4 were 3 to 3.5:1 with rabbit polyclonal anti-human antibodies. Using the monoclonal rat anti-rat antibodies, the β2/α4 ratios were 2.5 to 3 (data not shown). The immunoadsorption of [^{3}H]Epb directed by the mouse monoclonal antibody to human α6 receptor was approximately four to six times less than that produced by either rabbit polyclonal (Fig. 5B) or mouse monoclonal (not shown) antibody to the human α4 subunit. The immunoadsorption induced by rabbit polyclonal antibody to human α3 subunit was lower than α6 immunoadsorption in thalamus (Figs. 5B and 6) and three other areas tested (Fig. 6).

The Abundance of α3, α4, α6, and β2 Subunits Compared by Immunoadsorption of Radiolabeled Receptors. Figure 6 shows the immunoadsorption of ^{125}I-Epb-labeled receptors from four brain areas of long-term nicotine versus saline self-administering rats using antibodies against α3, α4, α6, and β2 nAChR subunits. Radiolabeled α3* subunit nAChRs were immunoadsorbed at levels lower than 2 fmol Epb/mg protein in all areas examined. Nicotine self-administration was associated with up-regulation of these α3* receptors only in VTA/SN and nucleus accumbens. In addition to these areas, the binding to α4 subunit-containing AChRs was significantly increased in thalamus/subthalamus. The immunoadsorbed binding corresponding to nAChRs containing the β2 subunit was strongly and significantly elevated in all areas. Finally, the immunoadsorbed α6 subunit, labeled by either ^{125}I-Epb or ^{125}I-Cntx, was significantly up-regulated in all areas.

The binding ratios of immunoadsorbed nAChR subunits from nicotine compared with saline self-administering rats are shown in Fig. 7. From the preceding data (Fig. 6), the binding ratio of nAChR α6 subunit was strongly increased in

TABLE 3

Cytisine sensitivity of ^{125}I-epibatidine binding in four forebrain areas of rats continuously self-administering nicotine and of control rats

Particulates from four individual animals were compared in each group. The binding values are given in fmol/mg particulate protein at [^{3}H]epibatidine input of 500 pM, corrected for the binding at 30 μM (−)-nicotine. The percentage increase for nicotine self-administering over the corresponding control group is shown below the binding data at each concentration of cytisine. Asterisks indicate the significance (*, $p < 0.05$; **, $p < 0.01$) of differences between the mean values for nicotine self-administering versus control groups in t tests.

Area	Binding without Cytisine		Binding at 5 nM Cytisine		Binding at 50 nM Cytisine	
	Control	Nicotine	Control	Nicotine	Control	Nicotine
Nucleus accumbens	13.4 ± 2	22.6 ± 3.4*	9.06 ± 0.8	16.6 ± 1.2*	7.6 ± 1.2	13.2 ± 1.1**
% Increase		68.7 ± 10.3		83.2 ± 6		73.7 ± 8.3
Thalamus	62.6 ± 5.5	95.6 ± 5.4*	41.7 ± 3.7	66.2 ± 4.3*	16.8 ± 1.2	28.7 ± 2.4**
% Increase		52.7 ± 3		58.8 ± 3.8		70.8 ± 5.9
Parietal cortex	35.9 ± 1.9	52.8 ± 1.4**	24.1 ± 0.8	37.5 ± 1.8**	11.3 ± 0.5	20.3 ± 0.6**
% Increase		47.1 ± 1.2		55.6 ± 2.7		79.6 ± 2.4
Hippocampus	50.1 ± 0.43	61.6 ± 2.7*	19.3 ± 0.8	23.7 ± 0.68*	8.71 ± 0.63	10.6 ± 0.46*
% Increase		23 ± 1		22.8 ± 0.7		21.7 ± 0.9

TABLE 4

Cytisine sensitivity of ^{125}I-α-conotoxin MII binding in four forebrain areas of rats continuously self-administering nicotine and of control rats

Particulates from four individual animals were compared in each group. The binding values are given in fmol/mg particulate protein at 0.5 nM ^{125}I-Cntx, corrected for the binding at 10 μM (+)-cytisine plus 100 μM (−)-nicotine. The percentage increase for nicotine self-administering over the corresponding control group is shown below the binding data at each concentration of cytisine. Asterisks indicate the significance (*, $p < 0.05$; **, $p < 0.01$) of differences between the mean values for nicotine self-administering vs. control groups in t tests.

Area	Binding without Cytisine		Binding at 25 nM Cytisine		Binding at 250 nM Cytisine	
	Control	Nicotine	Control	Nicotine	Control	Nicotine
Nucleus accumbens	3.8 ± 0.15	6.3 ± 0.36**	2.3 ± 0.14	4.8 + /-0.3**	0.92 ± 0.14	2.7 ± 0.3**
% Increase		64.8 ± 3.7		107.5 ± 7.4		188.5 ± 23
Thalamus	5.1 ± 0.3	13.7 ± 0.8 **	3.7 ± 0.31	12.3 ± 0.8**	1.83 ± 0.31	7.9 ± 0.72**
% Increase		167.6 ± 9.8		232 ± 15		332 ± 30
Parietal cortex	1.7 ± 0.05	2.08 ± 0.12 *	1.0 ± 0.049	1.36 ± 0.12*	0.33 ± 0.05	0.55 ± 0.11*
% Increase		20.9 ± 1.2		36 ± 3.1		64.6 ± 2.9
Hippocampus	2.93 ± 0.3	3.34 ± 0.13	2.04 ± 0.37	2.46 ± 0.12	0.678 ± 0.24	1.08 ± 0.11*
% Increase		14 ± 0.5		20.6 ± 1		59 ± 6.1

TABLE 5

A comparison of ^{125}I-α-conotoxin MII binding in three thalamic areas of control rats and mice

The thalamic cuts (see *Materials and Methods*) were further sectioned into approximately 10% of the lateral thalamic, 60% of the dorsal thalamic, and 30% of the subthalamic material. The binding of ^{125}I-Cntx to particulates (total and nonspecific; $n = 6$ for each) was performed as described in Table 4. The results are expressed as fmol ^{125}I-Cntx specifically bound per mg particulate protein. The binding to rat particulates was for each area significantly higher (*, $p < 0.05$ in t tests) than in the corresponding mouse particulates. The binding to particulates from dorsal hypothalamic area was in both species significantly larger (†, $p < 0.05$ in t tests) than that for the other two areas of the species.

Animal	Dorsal Thalamic Area	Subthalamic Area	Lateral Thalamic Area
Rat	6.3 ± 0.32*†	4.6 ± 0.13*	4.1 ± 0.38*
Mouse	4.3 ± 0.22 †	2.2 ± 0.24	2.6 ± 0.24

thalamus/subthalamus, caudate/putamen, and nucleus accumbens of nicotine self-administering rats (Fig. 7). In all four areas, this ratio was consistently higher (at least at the 95% confidence level in Dunnett's *t* test) than the ratio for the α4 subunit. The mean ratios of α6 subunits were also significantly greater than those for β2 in thalamus/subthalamus and caudate/putamen (Fig. 7). In these two areas, the ratio also was consistently and significantly greater for the β2 compared with the α4 subunit, indicating a relatively greater change of the β2 subunit during nicotine self-administration.

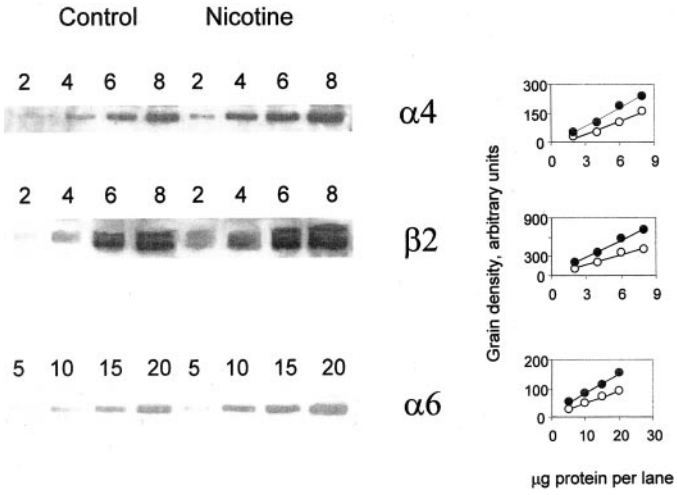

Fig. 4. Representative immunoblots of rat thalamic α4, α6, and β2 subunits from rats continually self-administering nicotine versus saline (control). Tissue from three control or nicotine self-administering animals was pooled for the assay. After electrophoretic separation and blotting, the transfers were reacted with the corresponding antibodies (see *Materials and Methods*). Protein input per lane (in μg) is indicated above the corresponding profiles. Molecular masses, from covalently dyed standards, were approximately 60 kDa for α4 and 55 kDa for α6 and β2. No other major bands were present near the indicated molecular mass range of the corresponding subunit bands. As seen in graphs on the right, grain densities followed a linear ($r^2 > 0.95$ in all cases) change with amount of input protein for each subunit and indicated consistently greater immunoreactivity in blots from rats continually self-administering nicotine.

Discussion

The results of this study demonstrate consistent up-regulation of nAChRs in multiple regions of rat brain during long-term self-administration of nicotine. This is in keeping with both the earliest (Schwartz and Kellar, 1983) and the most recent (Mugnaini et al., 2002) findings on the regulation of nAChRs by nicotine in rat brain, as well as with extensive studies in the mouse (Pauly et al., 1996; Whiteaker et al., 2000b).

The increase of nAChRs by long-term nicotine administration in the rat may strongly depend on the dosage and the mode of delivery of the alkaloid and on the rat strain used. Thus, intermittent subcutaneous application of nicotine in Sprague-Dawley rat at 4 mg/kg/day produced only 15 to 25% increase of [^{3}H]cytisine binding (Jacobs et al., 2002). In Wistar rats, continuous infusion of nicotine at 3 mg/kg/day produced an average 46% increase of [^{3}H]nicotine binding in the majority of brain areas examined (Mugnaini et al., 2002). In the present study, Lewis rats (used because of their propensity to self-administer nicotine) continually self-administering nicotine at approximately 1.5 mg/kg/day showed an average 63% increase of ^{125}I-epibatidine binding in the brain areas examined.

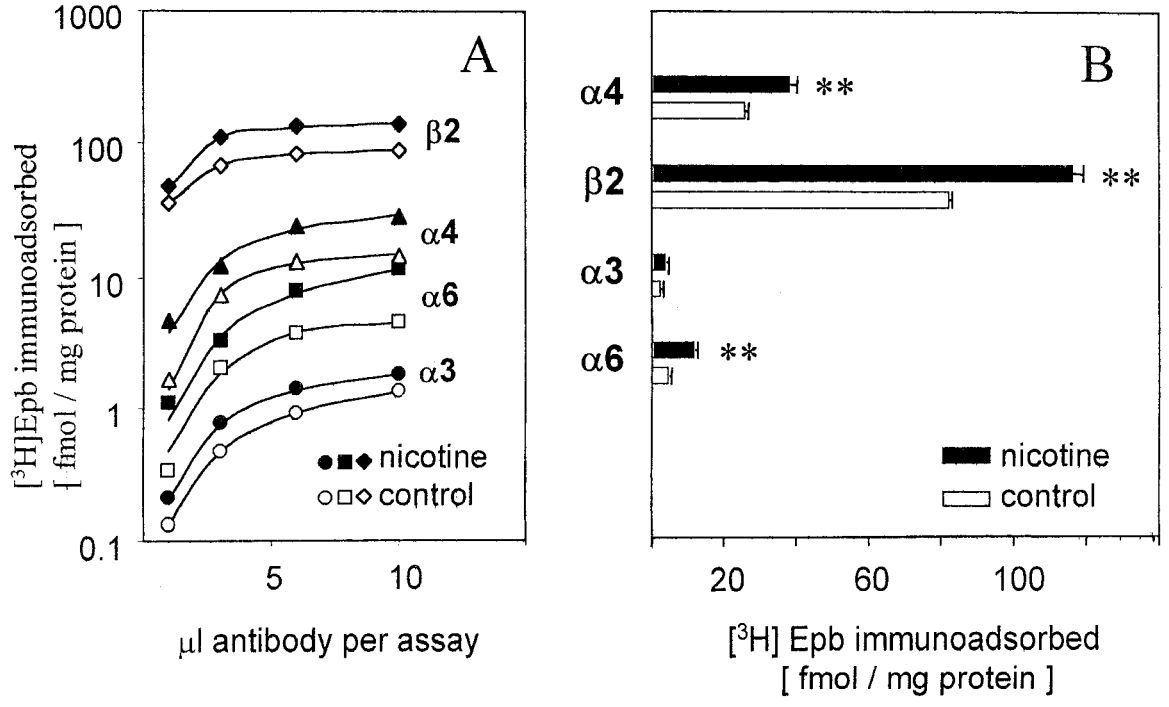

Fig. 5. Subunit-selective immunoadsorption of [^{3}H]epibatidine bound to nAChRs in thalamic tissue from control rats and rats continually self-administering nicotine. A, a semilogarithmic plot showing the saturation of [^{3}H]Epb immunoadsorption with four subunit-selective antibodies. Rabbit polyclonal antibodies to human α3, α4, and β2 nAChR subunit and mouse monoclonal antibody to human α6 subunit were used at indicated volumes of input per assay. Data represent means of triplicate assays. Standard errors (data not shown) were in all cases less than 10% of the corresponding means. For other details see *Materials and Methods*. B, maximum immunoadsorption of nAChRs by subunit-selective antibodies (in fmol Epb bound/mg protein input) as estimated from nonlinear hyperbolic fits (see *Materials and Methods*) of the data presented in Fig. 5A. Differences in *t* tests between control and nicotine self-administering animal means are indicated by asterisks (**, $p < 0.01$).

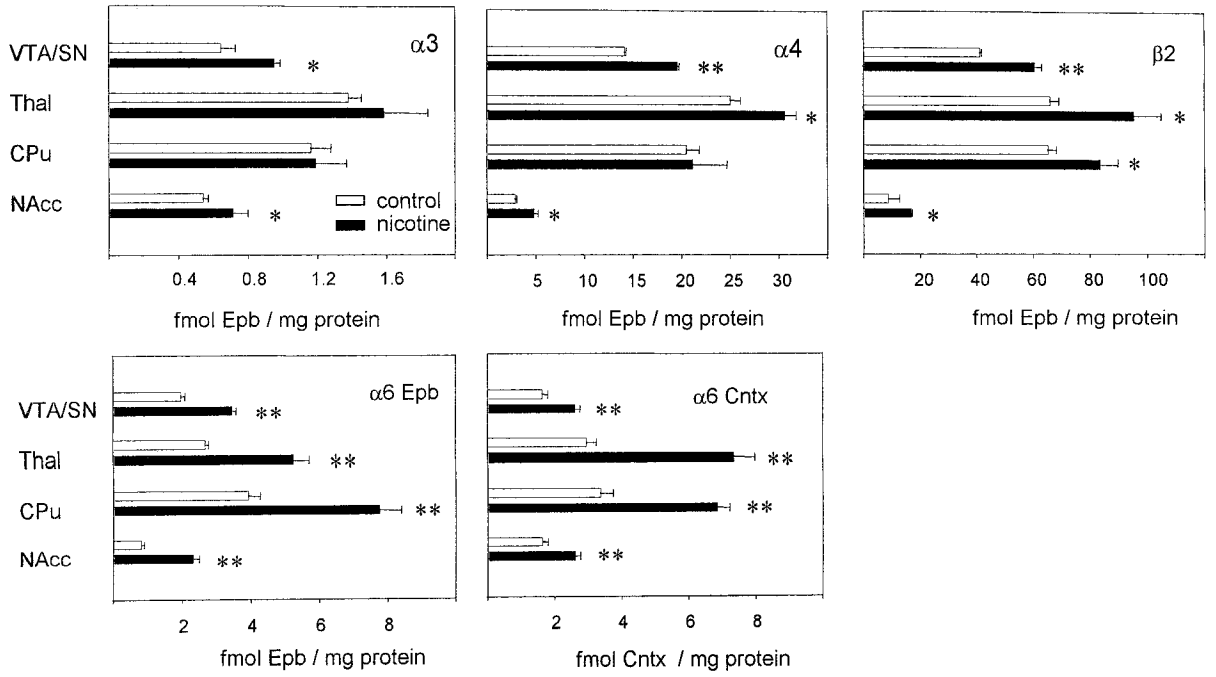

Fig. 6. Immunoadsorption of ^{125}I-epibatidine- or ^{125}I-α-conotoxin MII-labeled nicotinic receptors from four brain areas of control rats and of rats continually self-administering nicotine. Polyclonal rabbit antibodies to human α3, α4, and β2 nicotinic subunits and monoclonal mouse antibody to human α6 nicotinic receptor subunit were used as detailed under *Materials and Methods*. The results are the average maximum binding estimates using single-component hyperbolic fitting (Parker and Waud, 1971) on data from six control and six nicotine-injected animals. The mean maximum binding values were compared in *t* tests (significance levels: *, $p < 0.05$; **, $p < 0.01$). Thal, thalamus/subthalamus; NAcc, nucleus accumbens; CPu, the caudate/putamen area.

The pronounced ability of nicotine to release dopamine during long-term in vivo treatment (Carboni et al., 2000) may be related to a disproportionate increase of specific nAChR subunits. In nAChRs that are located in brain areas possessing abundant dopaminergic terminals, such as nucleus accumbens (Champtiaux et al., 2003), these could be the α6* receptors. We indeed found the largest relative increase of α6* in two areas rich in cell bodies and terminals releasing this catecholamine (VTA/SN and nucleus accumbens). In the same areas, we also found a strong and concordant up-regulation of the binding of α-conotoxin MII, a partial agonist of dopamine release (Grady et al., 2002) and an α6*-selective nAChR ligand. The amount of immunoreactive α3* receptors detected in particulates from these areas was much lower than that of α6* receptors. Most of the dopaminergic neurons in areas that in our experiments showed strong up-regulation of α6* ligand binding and immunoreactivity are known to express relatively high levels of the α6* nAChRs (Champtiaux et al., 2003).

Our experiments consistently detected a single high-affinity binding component for epibatidine in the rat brain areas studied. This is predictable from the similarity of the affinity ranges for epibatidine at α3*, α4*, α6* (Parker et al., 1998a; Xiao et al., 1998; Zoli et al., 2002; Champtiaux et al., 2003), and even chimeric α6α4* receptors (Evans et al., 2003), which would produce an apparent integration of small differences in affinity. A single high-affinity component of Epb binding was found in mouse brain (Marks et al., 1998), whereas the same study showed large sensitivity differentials across brain areas for cytisine, an agonist preferring α4* AChRs (Flores et al., 1997) and less active at β2* (Houlihan et al., 2001) receptors. Two high-affinity binding components for Epb in rat forebrain were found by Houghtling et al. (1995); however, they used conditions of homogenization and assay that were quite different from those used by Marks et al. (1998) and in the present study.

We found increased resistance to cytisine for either [^{3}H]epibatidine or ^{125}I-α-conotoxin MII binding in rats continually self-administering nicotine. This was present not

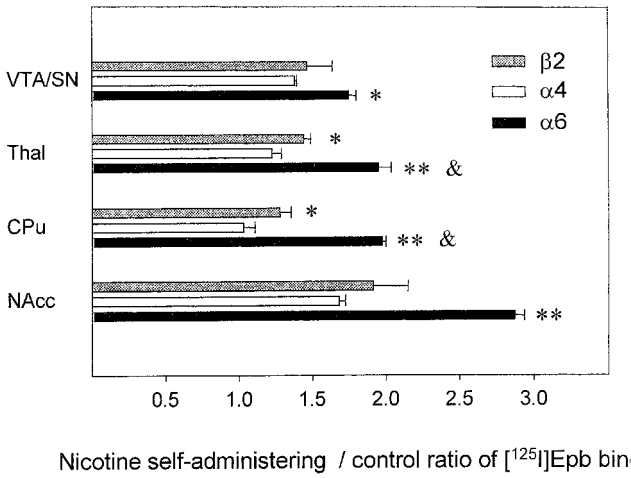

Fig. 7. Ratios of immunoadsorbed ^{125}I-Epb binding for α4, α6, and β2 nAChR subunits of rats self-administering nicotine relative to control animals. The results are average ratios of data shown in Fig. 4 for immunoadsorption of nAChR ^{125}I-Epb binding that reacted with antibodies to the indicated nAChR subunits. The mean ratios for control and nicotine self-administering animals in each area were compared using Dunnett's *t* test after positive analysis of variance (significance levels: *, $p < 0.05$; **, $p < 0.01$ for the difference between the mean α6 or β2 and α4 ratio; &, $p < 0.05$ for the difference between the mean α6 and β2 ratio). Thal, thalamus/subthalamus; NAcc, nucleus accumbens; CPu, the caudate/putamen area.

only in the dopaminergically active nucleus accumbens but also in the parietal cortex and hippocampus. Taken from a relatively low sensitivity of β2* nAChRs to cytisine (Houlihan et al., 2001), this nAChR agonist might also be less active in competing Epb binding to AChRs containing two or more β2 subunits. Surface nAChRs containing three β2 subunits are preferentially increased by nicotine in cells expressing cloned α4β2 receptors (Nelson et al., 2003). Such nAChRs could accumulate in rats continually self-administering nicotine. By analogy with its poor labeling of cell surface $\alpha4_2\beta2_3$ nAChRs expressed in HEK-293 cells (Zhang and Steinbach, 2003), cytisine also may not readily access a fraction of brain particulate nAChRs. The nAChRs not readily available for cytisine binding could be the more membrane-immersed or aggregated receptors, accumulating in animals self-administering nicotine because of channel desensitization or other factors. The structure of cytisine is rigid and more conformationally restricted than that of epibatidine (Barlow and Johnson, 1989; Wei et al., 2003). Consequently, cytisine may not access the binding sites of closely grouped or partially compartmentalized nAChRs. This could produce multiple components of competition with the binding of epibatidine (Marks et al., 1998) and result in partial agonism (Houlihan et al., 2001).

Up-regulation of surface nAChRs caused by long-term nicotine treatment could be related to desensitization, as shown for α4β2 receptors in oocytes (Fenster et al., 1999). Desensitization of nAChRs may be associated with a partial sequestration or compartmentalization, which is found with some rhodopsin-like receptors (Beaumont et al., 1998; Parker et al., 2002). The large second intracellular domain found in all sequenced α4 subunits (approximately 270 amino acid residues), being twice the size of the corresponding domain of any other nAChR subunit, is likely to induce an asymmetry in assembled holoreceptor pentamers. This segment also contains an alternating-cysteine CXCXC sequence (X = amino acid other than Cys), not present in any other nAChR subunit, which can be expected to interact with chaperones and assist the association of α4* nAChRs with intracellular membranes. The above features are likely to induce a larger compartmentalization of α4 subunits relative to other α subunits.

Surface α4* receptors are difficult to maintain in cell lines at 37°C, apparently because of a strong inward vectorial drive under physiological conditions, but can be maintained below 37°C (Cooper et al., 1999). Recent work shows that at 29°C (i.e., below the expected phase transition of major membrane phospholipids) (Martin and MacDonald, 1976) all nicotinic receptor subunits can be surface-expressed and up-regulated by nicotine in cell lines (Nelson et al., 2003; J. M. Lindstrom, unpublished observations). Also, cell-surface $\alpha4_2\beta2_3$ receptors are up-regulated by nicotine treatment more than $\alpha4_3\beta2_2$ receptors (Nelson et al., 2003). In contrast, α3* receptors are well-retained in plasma membrane at 37°C and have been stably expressed by many researchers. The 65% sequence homology of rat α6 and α3 subunits [for sequences, see the Swiss-Protein files P43143 (rat α6) and P04757 (rat α3)] indicates that surface α6* receptors are also likely to be stably exteriorized at 37°C, as has indeed been found in HEK-293 cells (Evans et al., 2003). The above studies are all compatible with our finding of a disproportionate up-regulation, relative to α4* receptors, of α6* nAChRs, and to some degree also of β2* nAChRs, in rats continually self-administering nicotine.

Because long-term nicotine treatment can up-regulate α3* receptors in a cell line (Wang et al., 1998), the structurally similar α6* receptors are likely to accumulate during nicotine treatment, as found in our experiments. The surface retention of nAChRs may be promoted by nicotine-induced depolymerization of F-actin (Cheek and Burgoyne, 1987). A decrease in microfilament proteins induced by long-term nicotine administration (observed in ventral tegmentum by Sbarbati et al., 2002) may also improve the surface retention of nAChRs. Nicotine also promotes the transfer of phospholipid-interacting annexins into the filament matrix (Sagot et al., 1997), which might increase surface embedding of nicotinic and other receptors. An accumulation of receptor-organizing proteins akin to the neuromuscular synapse proteins rapsyn or agrin (which slow the turnover of the muscle nicotinic receptor) (Phillips et al., 1997) could increase the surface retention of nAChRs. However, scaffoldings of this type have thus far not been defined in the brain.

Up-regulation of nAChRs during long-term nicotine treatment could be related to the aforementioned nicotine-induced decrease of receptor interaction with the microfilament/cytoskeletal system. The up-regulation of nAChRs containing specific subunits, and in particular the α4* receptors, could be counteracted by sequestration and/or compartmentalization. For a significant fraction of nAChRs, micromechanical changes related to altered cytoskeletal interactions may contribute to a decrease in receptor-channel sensitivity to agonists without a large change in the binding affinity, a state usually identified as desensitization. The extent, duration, and occurrence of desensitization could, however, differ according to the subunit composition of nAChRs. By analogy with α3* receptors (Fenster et al., 1999), the α6* receptors could be more resistant to desensitization by nicotine and presumably easier to recover. The observed larger up-regulation of α6* nAChRs relative to α4* receptors in rats continually self-administering nicotine may help maintain, or even facilitate, the release of neurotransmitters, especially dopamine.

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Correction to "Up-regulation of brain nicotinic acetylcholine receptors in the rat during long-term self-administration of nicotine: disproportionate increase of the α6 subunit"

In the above article [Parker SL, Fu Y, McAllen K, Luo J, McIntosh JM, Lindstrom JM, and Sharp BM (2004) *Mol Pharmacol* **65:**611–622], an author's name was spelled incorrectly. The correct spelling is Jie Luo.

The authors regret this error and apologize for any confusion or inconvenience it may have caused.