Increased Nicotinic Acetylcholine Receptor Protein Underlies Chronic Nicotine-Induced Up-Regulation of Nicotinic Agonist Binding Sites in Mouse Brain

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ABSTRACT

Chronic nicotine treatment elicits a brain region-selective increase in the number of high-affinity agonist binding sites, a phenomenon termed up-regulation. Nicotine-induced up-regulation of $\alpha 4\beta 2$ -nicotinic acetylcholine receptors (nAChRs) in cell cultures results from increased assembly and/or decreased degradation of nAChRs, leading to increased nAChR protein levels. To evaluate whether the increased binding in mouse brain results from an increase in nAChR subunit proteins, C57BL/6 mice were treated with nicotine by chronic intravenous infusion. Tissue sections were prepared, and binding of [¹²⁵I]3-((2S)-azetidylmethoxy)-5-iodo-pyridine (A85380) to $\beta 2^*$ -nAChR sites, [¹²⁵I]monoclonal antibody (mAb) 299 to $\alpha 4$ nAChR subunits, and [¹²⁵I]mAb 270 to $\beta 2$ nAChR subunits was determined by quantitative autoradiography. Chronic nicotine treatment dose-dependently increased binding of all three ligands. In regions that express $\alpha 4\beta 2$ -nAChR almost exclusively,

binding of all three ligands increased coordinately. However, in brain regions containing significant $\beta 2^*$ -nAChR without $\alpha 4$ subunits, relatively less increase in mAb 270 binding to $\beta 2$ subunits was observed. Signal intensity measured with the mAbs was lower than that with [¹²⁵I]A85380, perhaps because the small ligand penetrated deeply into the sections, whereas the much larger mAbs encountered permeability barriers. Immunoprecipitation of [¹²⁵I]epibatidine binding sites with mAb 270 in select regions of nicotine-treated mice was nearly quantitative, although somewhat less so with mAb 299, confirming that the mAbs effectively recognize their targets. The patterns of change measured using immunoprecipitation were comparable with those determined autoradiographically. Thus, increases in $\alpha 4\beta 2^*$ -nAChR binding sites after chronic nicotine treatment reflect increased nAChR protein.

Introduction

Chronic nicotine exposure elicits increases in high-affinity nicotinic receptor (nAChR) binding sites (termed up-regulation) in the brains of mice (Marks et al., 1983, 2004), rats (Schwartz and Kellar, 1983, 1985; Flores et al., 1992; Perry et

al., 2007; Moretti et al., 2010), and humans (Benwell et al., 1988; Breese et al., 1997; Perry et al., 1999). Immunoprecipitation of the binding sites after chronic nicotine treatment indicates that the $\alpha 4\beta 2^*$ -nAChR subtype accounts for most of the increased binding sites in rat brain (Flores et al., 1992). Although chronic nicotine exposure generally increases the density of $\alpha 4\beta 2^*$ -nAChR sites in rodent brain, the extent of the increase varies among brain regions (Marks et al., 1983, 2004; Pauly et al., 1991; Sanderson et al., 1993; Rowell and Li, 1997; Nguyen et al., 2003).

Early studies determined that chronic nicotine treatment elicits an increase in maximal ligand binding ($B_{\max}$) with no change in affinity (K_D) (Marks et al., 1983; Schwartz and Kellar, 1983), indicating that the increased binding resulted from an increase in nAChR protein. In cell cultures nicotine and other cholinergic ligands increase the assembly of nAChR subunits and decrease the turnover of mature nAChRs (Peng et al., 1994; Kuryatov et al., 2005; Sallette et

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ABBREVIATIONS: nAChR, neuronal nicotinic acetylcholine receptor; mAb, monoclonal antibody; I-A85380, 3-((2S)-azetidylmethoxy)-5-iodo-pyridine; PBS, phosphate-buffered saline; PEG, polyethylene glycol; ANOVA, analysis of variance.

al., 2005; Lester et al., 2009). However, an alternative hypothesis is that the increase in binding sites reflects a change in affinity of nAChR allowing the measurement of previously undetectable binding sites (Vallejo et al., 2005). This hypothesis predicts an increase in binding with little or no change in the amount of nAChR protein.

Immunochemical methods have been used to evaluate the effects of chronic nicotine treatment on $\alpha 4\beta 2^*$ -nAChR in brain and have established that the high affinity agonist binding sites that are increased by chronic nicotine treatment can be immunoprecipitated by antibodies to either the $\alpha 4$ or $\beta 2$ subunit (Flores et al., 1992; Moretti et al., 2010). While these studies clearly establish that the binding sites, including those up-regulated by chronic nicotine, are assembled from $\alpha 4$ and $\beta 2$ nAChR subunits, they do not establish whether chronic nicotine actually increases the amount of nAChR protein.

Evidence that supports the argument that chronic nicotine treatment increases the amount of subunit protein has been obtained in a study that used mice engineered to express an $\alpha 4$ subunit tagged with a fluorescent reporter incorporated into the cytoplasmic domain (Nashmi et al., 2007) and in a study that used quantitative Western blots to measure $\alpha 4\beta 2$ -nAChR subunit proteins in rat brain (Moretti et al., 2010). However, it has been difficult to extend the immunochemical analyses to provide resolution at a microscopic level, owing in part to the difficulty in using anti-nAChR antibodies in standard immunocytochemical procedures (Jones and Wonnacott, 2005; Moser et al., 2007). The use of ^{125}I -labeled monoclonal antibodies (mAbs) has overcome some of these difficulties for select nAChR subunits including $\beta 2$ with [^{125}I]mAb 270 (Swanson et al., 1987; Whiteaker et al., 2006) and $\alpha 4$ with [^{125}I]mAb 299 (Whiteaker et al., 2006).

We investigated the effect of chronic nicotine treatment on the expression of $\alpha 4\beta 2^*$ -nAChR using ligand binding with the $\beta 2$ -selective ligand [^{125}I]A85380 (3-[(2*S*)-2-azetidinylmethoxy]-5-iodopyridine) (Mukhin et al., 2000) and immunolabeling of $\alpha 4$ subunits with [^{125}I]mAb 299 and $\beta 2$ subunits with [^{125}I]mAb 270 (Whiteaker et al., 2006). The results demonstrate a region-selective, nicotine-dose-dependent increase not only in nAChR binding but also in antibody labeling. These results strongly support the hypothesis that the increase in high-affinity nAChR binding sites results from increases in assembled nAChR subunit proteins.

Materials and Methods

Materials. [^{125}I]epibatidine and [^{125}I]A85380 were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). NaCl, KCl, MgSO_4 , CaCl_2 , Na_2HPO_4 , NaH_2PO_4 , bovine serum albumin, polyethylene glycol (PEG), polyethylenimine, nicotine, and cytosine were obtained from Sigma-Aldrich (St. Louis, MO). Sucrose was obtained from Roche Diagnostics (Indianapolis, IN). HEPES and NaHEPES are products of BDH (Poole, Dorset, UK) and were obtained through VWR (West Chester, PA). Normal rat serum, protease-free bovine serum albumin, and rabbit anti-rat polyclonal antibody were obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove PA). Glass filters type B were products of Micro-Filtration Systems (Dublin, CA), and glass fiber filters type A/E were products of Pall Life Sciences (Port Washington, NY). Superfrost Plus microscope slides and silastic tubing (a product of Dow Corning, Midland, MI) were obtained from Thermo Fisher Scientific (Waltham, MA).

Mice. Animal production methods and experimental procedures using mice were reviewed and approved by the Animal Care and Utilization Committee at the University of Colorado, Boulder.

C57BL/6 mice were bred in the specific pathogen-free mouse colony at the Institute for Behavioral Genetics, University of Colorado, Boulder. Mice were weaned at 25 days of age and housed five per cage with like-sexed animals. $\beta 2$ Null mutant mice (Picciotto et al., 1995) were originally obtained from Marina Picciotto (Yale University, New Haven, CT). Heterozygous mice were mated to yield wild type (+/+), heterozygous (+/-), and homozygous (-/-) mutant mice. Genotypes were determined from tail clippings obtained from mice approximately 40 days old as described previously (Picciotto et al., 1995). Mice were housed in a vivarium maintained at 22°C and allowed free access to food and water. Lights were on from 7 AM to 7 PM.

Surgery. A cannula constructed of silastic tubing was inserted in the right jugular vein of each mouse (Marks et al., 1983). In brief, mice were anesthetized with pentobarbital (50 mg/kg) and chloral hydrate (100 mg/kg). An incision (approximately 1 cm) was made to expose the superficial right jugular vein. The silastic cannula (0.51 mm i.d., 0.94 mm o.d.) was inserted 8 mm into the right jugular vein through a small hole in the vein. The cannula, filled with isotonic saline containing 0.3% citric acid, was anchored to the underlying tissue with surgical thread, passed through the back in the mid-scapular region, and anchored to the skin with a wound clip. A head mount composed of dental cement, into which a small metal hook was inserted, was affixed to the skull. The mouse was injected with buprenorphine (0.1 mg/kg), placed in a clean cage, and warmed until wakening.

Nicotine Treatment. After recovery from surgery each mouse was transferred to an individual infusion chamber (15 × 15 × 25 cm, length × width × height), and its cannula was attached to medical-grade Tygon tubing connected to a 1-ml syringe mounted on an infusion pump (Harvard Apparatus Inc., Holliston, MA). Sterile saline was continuously infused at a rate of 35 $\mu\text{l/h}$. After 2 days of saline infusion, nicotine treatment was begun. Mice were divided into seven treatment groups that received the following nicotine doses: 0 (saline-infused control), 0.125, 0.25, 0.5, 1.0, 2.0, or 4.0 mg/kg/h. Nicotine solutions were prepared from liquid nicotine neutralized with HCl. All doses were free base. After 10 days of treatment with the indicated nicotine dose, the cannula of each mouse was disconnected from the Tygon tubing and checked for free fluid flow.

Tissue Preparation. A 2-h interval between discontinuation of nicotine treatment and sacrifice was used to allow nearly total metabolism of nicotine (Petersen et al., 1984).

For samples to be prepared for sectioning, each mouse was killed by cervical dislocation, and its brain was rapidly (<1 min) removed from the skull and quickly frozen by immersion in isopentane (-35°C) for 10 s. The frozen brain was wrapped in aluminum foil and stored at -70°C until sectioning.

Preparation of Tissue Sections. On the day of sectioning a brain was removed from the -70°C freezer and allowed to warm to the temperature of the cryostat (-14°C). The brain was subsequently mounted on the cryostat chuck with M-1 Embedding Matrix (Anatomical Pathology, Pittsburgh, PA). Subsequently, coronal sections (14 μm thick) were obtained using either a Leica CM 1850 cryostat/microtome (Leica, Nussloch, Germany) or an IEC Minotome (Damon Corp., Needham, MA) and thaw-mounted on Fisher Suprafrost/Plus microscope slides. A series of 10 sets of sections was prepared from each brain to allow comparison of results for several different experiments on adjacent or near-adjacent sections. Slides containing the brain sections were stored, desiccated at -70°C, until use.

[^{125}I]A85380 Autoradiography. Slides containing the tissue sections prepared from mice of each nicotine treatment group were warmed to room temperature in a desiccator. Slides were subsequently transferred to Bel-Art slide racks that have been modified to hold 50 slides and rehydrated by incubation at 22°C for 15 min in

isotonic buffer (144 mM NaCl, 2.2 mM KCl, 2.0 mM CaCl₂, 1.0 mM MgSO₄, 25 mM HEPES, pH 7.5). The racks containing the rehydrated slides were subsequently transferred to the isotonic buffer containing 200 pM I-A85380. [¹²⁵I]A85380, specific activity 2200 Ci/mmol was mixed with unlabeled 5I-A85380 to yield a final specific activity of 220 Ci/mmol (a 10-fold dilution). Samples were incubated for 2 h at 22°C. After the incubation the slides were redistributed to slide racks containing 25 slides and washed as follows (all solutions at 4°C): twice for 30 s in isotonic buffer, twice for 5 s in hypotonic buffer (0.1×), and twice for 5 s in 10 mM HEPES, pH 7.5. The samples were then air-dried with a stream of air and stored desiccated at room temperature in vacuum overnight before exposure initially to Packard Super Resolution Cyclone Storage Phosphor Screens (PerkinElmer Life and Analytical Sciences) to yield images for quantitation and subsequently to Kodak MR autoradiography film (Eastman Kodak, Rochester, NY) to yield higher resolution images for photography. Each Phosphor Screen was also simultaneously exposed to a series of tissue paste standards containing measured amounts of ¹²⁵I to allow quantitation of the image intensity. Tissue sections from β2 null mutant mice were used to establish blanks (Whiteaker et al., 2006).

[¹²⁵I]mAb 270 and [¹²⁵I]mAb 299 Autoradiography. mAb 270 (Whiting and Lindstrom, 1987), which has been characterized as a β2 nAChR subunit-selective antibody (Conroy et al., 1992; Whiteaker et al., 2006) and mAb 299 (Whiting et al., 1987), which has been characterized as an α4 nAChR-selective antibody (Whiteaker et al., 2006), were radioiodinated by reaction of Na¹²⁵I with each mAb using a modified chloramine T method (Lindstrom et al., 1981). Specific activities were estimated to be 1370 Ci/mol for [¹²⁵I]mAb 270 and 1430 Ci/mmol for [¹²⁵I]mAb 299 at the time of the experiment by measuring the amount of radioactivity retained in the protein fraction after removal of free ¹²⁵I with a Bio-Rad 10EDG desalting column (Bio-Rad Laboratories, Hercules, CA).

Autoradiographic methods for ¹²⁵I-mAb binding were modified from the method of Swanson et al. (1987) as detailed previously (Whiteaker et al., 2006) and modified as follows. Slides containing the tissue sections prepared from mice of each nicotine treatment group were warmed to room temperature in a dessicator. Slides were subsequently transferred to Bel-Art slide racks modified to hold 50 slides and rehydrated by incubation at 22°C for 15 min in phosphate-buffered saline (PBS; 100 mM NaCl and 10 mM sodium phosphate buffer, pH 7.5) that included 10 μM phenylmethylsulfonyl fluoride. After rehydration, the slides were transferred to PBS containing 10 mM NaN₃, 10% (v/v) normal rat serum, and 5% (w/v) protease-free bovine serum albumin in addition to the radiolabeled mAbs. Final antibody concentrations were 0.7 nM for [¹²⁵I]mAb 270 and 0.3 nM for [¹²⁵I]mAb 299. These concentrations are near the K_D values for the mAbs and provide optimal signal-to-noise ratios (Whiteaker et al., 2006). Trays containing the 50 slides were incubated for 48 h at 4°C in slide boxes containing 100 ml of mAb containing buffer so that incubation conditions were maintained as uniform as possible. After the 48-h incubation, slides were washed using four 30-min incubations with PBS at 22°C. The samples were then air-dried and stored desiccated at room temperature in vacuum overnight. Samples were initially exposed to Packard Super Resolution Cyclone Storage Phosphor Screens to yield images for quantitation. Each Phosphor Screen was also simultaneously exposed to a series of tissue paste standards containing measured amounts of ¹²⁵I to allow quantitation of the image intensity. Tissue sections from β2 null mutant mice were used to establish blanks.

Samples were subsequently exposed to Kodak MR autoradiography film to yield higher resolution images for photography.

Quantitation. Tissue paste samples prepared from whole brain homogenates were used to construct standard curves. The Phosphor Screens yielded a linear relationship between signal intensity and tissue radioactivity content over several orders of magnitude. The regression line calculated for the standard curve was used to convert the measured value of pixels/mm² to the cpm/mg wet weight. Signal

intensity in fmol/mg wet weight was estimated from the specific activity of each ligand. Brain regions were identified using the mouse brain atlas (Franklin and Paxinos, 1997) as a guide. Several measurements were made in each brain region of each mouse, and the average of these measurements defined the signal intensity for each region.

Immunoprecipitation of Solubilized [¹²⁵I]-Epibatidine Binding Sites. Preliminary experiments indicated that the binding of [¹²⁵I]A85380 was significantly reduced by solubilization in Triton X-100 and this reduction depended on detergent concentration. Subsequently, [¹²⁵I]epibatidine, the binding of which is unaffected by Triton X-100 solubilization, was used to measure nAChR binding sites in the immunoprecipitation studies using a modification of a previously published method (Brown et al., 2007).

Mice were chronically infused with either saline (controls), 0.25 mg/kg/h nicotine, 1.0 mg/kg/h nicotine, or 4.0 mg/kg/h nicotine (doses expressed as free base) for 10 days. After each cannula was checked for free flow, a 2-h interval between discontinuation of nicotine treatment and sacrifice was used to allow significant metabolism of nicotine (Petersen et al., 1984). For samples to be prepared for immunoprecipitation, each mouse was killed by cervical dislocation and its brain was rapidly removed from the skull and placed on an ice-cold platform. Olfactory tubercles, hippocampus, striatum, cerebral cortex, and thalamus were dissected. Each brain region was placed in ice-cold, hypotonic buffer (14 mM NaCl, 0.22 mM KCl, 0.2 mM CaCl₂, 0.1 mM MgSO₄, 2.5 mM HEPES, pH 7.5) and homogenized using a glass/Teflon tissue grinder. The homogenates were subsequently centrifuged at 20,000g for 20 min. The supernatant was discarded, and the pellet was resuspended in fresh hypotonic buffer, which was again centrifuged at 20,000g for 20 min. This resuspension/centrifugation cycle was repeated three more times after which the washed pellets were stored at -70°C under fresh hypotonic buffer until assay.

On each assay day, samples for a single brain region were thawed, the pellets were resuspended in the overlying buffer, and the samples were centrifuged at 20,000g for 20 min. The resulting pellet was resuspended in PBS (composition: 136.9 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄). Particulate protein was solubilized by incubation of the sample with 2% Triton X-100 in PBS at room temperature for 1 h. After this incubation the samples were centrifuged at 20,000g for 10 min to remove insoluble material, and the supernatants were used in subsequent experiments.

Binding of [¹²⁵I]epibatidine to solubilized sites was measured by incubating the Triton X-100 supernatant overnight at 4°C with 200 pM [¹²⁵I]epibatidine in a 100-μl final volume of PBS containing 1% Triton X-100. Blanks were established by including 100 μM cytosine in some samples. When the incubation was complete, 100 μl of 40% polyethylene glycol was added to each sample, which was then shaken for 2 min to precipitate the solubilized protein including nAChR to which [¹²⁵I]epibatidine was bound. Samples were diluted with 500 μl of ice-cold wash buffer, and the precipitated protein was collected by filtration onto glass fiber filters that had been soaked in 0.5% polyethylenimine [two filters: top filter type B (MicroFiltration Systems); bottom filter, type A/E (Pall Life Sciences)] using an Inotech Cell Harvester (Inotech Biosystems, Rockville, MD). Samples were subsequently washed five times with ice-cold wash buffer. The washed filters were transferred to glass 12 × 75-mm culture tubes and ¹²⁵I was counted at 80% efficiency on a Packard Cobra Gamma Counter (PerkinElmer Life and Analytical Sciences).

Before measuring immunoprecipitation of [¹²⁵I]epibatidine binding sites from multiple brain regions, experiments were conducted to determine the mAb affinity and extent of immunoprecipitation achieved with mAb 299 and mAb 270. Precipitation curves for the concentration dependence of mAb 299 and mAb 270 were constructed using hippocampal extracts prepared from mice treated with saline or 4.0 mg/kg/h nicotine. For mAb 299, the concentrations that achieved half-maximal specific immunoprecipitation were 0.02 ± 0.01 and 0.03 ± 0.01 μg/ml and total specific immunocapture

was 20.9 ± 1.4 and 30.3 ± 1.1 fmol/mg protein for saline- and nicotine-treated mice, respectively. For mAb 270, the concentrations that achieved half-maximal specific immunoprecipitation were 0.62 ± 0.21 and 0.97 ± 0.21 $\mu\text{g/ml}$ and total specific immunocapture was 22.6 ± 2.1 and 34.8 ± 2.2 fmol/mg protein for saline- and nicotine-treated mice, respectively. For both mAbs maximal specific immunoprecipitation revealed a significant difference between saline- and nicotine-treated mice (for mAb 299, $t_8 = 5.32$, $p < 0.05$; for mAb 270, $t_8 = 3.98$, $p < 0.05$), but no significant difference in antibody affinity was detected between the two groups.

Immunoprecipitation of $\beta 2^*$ -nAChR with mAb 270 and $\alpha 4^*$ -nAChRs with mAb 299, which were raised in rats, was achieved using a rabbit antibody directed against rat IgG to form an insoluble protein precipitate from nAChRs labeled with ^{125}I -epibatidine. Final mAb concentrations were 5 $\mu\text{g/ml}$ mAb 270 or 1 $\mu\text{g/ml}$ mAb 299. These concentrations had been determined to be saturating from experiments designed to determine the affinity of the antibodies using hippocampal tissue of mice treated with saline or 4.0 mg/kg/h nicotine. Solubilized protein was incubated in 100 μl of buffer containing 200 pM ^{125}I -epibatidine, 1% Triton X-100 PBS, and 3% normal rat serum in addition to the appropriate mAb. Nonspecific ^{125}I -epibatidine labeling was determined by including 100 μM cytosine in the incubation. Samples were incubated overnight at 4°C . After the overnight incubation, the insoluble protein complex was formed by the addition of 50 μl of rabbit-anti-rat IgG (1 mg/ml in PBS), and incubation at 4°C for 60 min. Immediately before capture, the samples were diluted with 1 ml of ice-cold 1% Triton X-100 in PBS to reduce nonspecific capture of labeled nAChRs. The precipitates were isolated by filtration onto 0.5% polyethylenimine-soaked glass fiber filters (top filter type B, bottom filter type A/E) and washed three times with ice-cold PBS. The washed filters were transferred to glass 12×75 -mm culture tubes, and ^{125}I was counted at 80% efficiency on a Packard Cobra Gamma Counter.

Protein. Protein was measured using the method of Lowry et al. (1951).

Statistical Analyses. The SPSS statistical package (SPSS Inc., Chicago, IL) was used for all analyses. One-way ANOVAs were used to examine the effects of nicotine treatment on binding site density for each ligand in each brain region. Linear regression analysis was used to compare the signal intensity in the various brain regions for mAb 270 and mAb 299 to that of A85380 at each infusion dose. One-way ANOVAs were also used to examine the effect of nicotine treatment on ^{125}I -epibatidine binding sites in tissue homogenates, Triton X-100-solubilized sites, and solubilized sites precipitated by either mAb 270 or mAb 299.

Results

Binding of [^{125}I]A85380 Is Closely Correlated to Binding of [^{125}I]mAb 270 and [^{125}I]mAb 299 in Mouse Brain. The relationships between signal intensity for binding of [^{125}I]mAb 270 to $\beta 2$ nAChR subunits or [^{125}I] mAb 299 to $\alpha 4$ nAChR subunits and [^{125}I]A85380 binding to $\beta 2^*$ -nAChR sites were quantitated by autoradiography in 38 brain regions of saline-treated mice and are illustrated in Fig. 1.

The scattergram in Fig. 1, left illustrates the linear relationship and high correlation between [^{125}I]mAb 270 and [^{125}I]A85380 binding ($r = 0.94$). No systematic deviation of the data points from the regression line was noted. Such a relationship is not unexpected inasmuch as I-A85380 has been identified as a $\beta 2^*$ -nAChR-selective ligand (Mukhin et al., 2000).

The scattergram in Fig. 1, right illustrates the relationship between [^{125}I] mAb 299 and [^{125}I]A85380 binding. Although there is a strong relationship between these two parameters, the correlation ($r = 0.88$) is less robust than that between [^{125}I]mAb 270 and [^{125}I]A85380 binding. This less robust correlation undoubtedly reflects the presence of [^{125}I]A85380 binding to $\beta 2^*$ -nAChR that are not to $\alpha 4\beta 2^*$ -nAChR. When brain regions known to express such non- $\alpha 4\beta 2$ -nAChR sites [including medial habenula, interpeduncular nucleus, superior colliculus (superficial gray), dorsolateral and ventral lateral geniculate nuclei, caudate putamen, nucleus accumbens, and olfactory tubercle (Whiteaker et al., 2006)] are removed, a very robust correlation between [^{125}I] mAb 299 and [^{125}I]A85380 binding is obtained ($r = 0.99$). Several of the regions that deviate from the line are labeled.

Chronic Nicotine Administration Elicits a Saturable, Dose-Dependent Increase in [^{125}I]A85380 Binding to $\beta 2^*$ -nAChR Sites in Many Brain Regions. The effects of chronic nicotine infusion on the density of [^{125}I]A85380 binding sites in 38 brain regions measured by autoradiography are summarized in Table 1.

Statistically significant increases in [^{125}I]A85380 binding were observed in 22 of these 38 regions after chronic nicotine treatment. Increases were observed in olfactory bulbs, each of the five cortical areas quantitated, hypothalamus, zona incerta, deep layers of superior colliculus, in-

Fig. 1. Relative [^{125}I]mAb 270, [^{125}I]mAb 299, and [^{125}I]A85380 binding is very similar in most brain regions. Scattergrams were constructed comparing the density of [^{125}I]A85380 with that of [^{125}I]mAb 270 (left) and [^{125}I]mAb 299 (right). Each point is the mean $\pm$ S.E.M. of the data for saline-infused mice that is summarized in Tables 1, 2, and 3. The regression line ($r = 0.94$) for [^{125}I]A85380/[^{125}I]mAb 270 has been calculated for all data points. The regression line ($r = 0.99$) for [^{125}I]A85380/[^{125}I]mAb 299 has been calculated omitting the points for brain regions for which nAChR other than $\alpha 4\beta 2^*$ -nAChR have been identified (Whiteaker et al., 2006). VLG, ventrolateral geniculate nucleus; mHab, medial habenula; SC, superior colliculus; sg, superficial gray; DLG, dorsolateral geniculate nucleus; IPN, interpeduncular nucleus.

TABLE 1

[¹²⁵I]A85380 labeling of brain regions after chronic nicotine treatment

Density of [¹²⁵I]A85380 labeling (fmol/mg wet weight) was measured by quantitative autoradiography as described under *Materials and Methods* in brain regions of mice chronically treated with the indicated doses of nicotine. Each value represents the mean $\pm$ S.E.M. of 6 to 10 individual mice. Results of each individual one-way ANOVA are also given. Values differing significantly from those of saline-infused mice ($P < 0.05$, Duncan's test) are indicated in bold.

Brain Region	Saline	Nicotine						ANOVA Results
		0.125 mg/kg/h	0.25 mg/kg/h	0.5 mg/kg/h	1.0 mg/kg/h	2.0 mg/kg/h	4.0 mg/kg/h	
Olfactory bulbs	0.85 $\pm$ 0.08	1.28 $\pm$ 0.12	1.71 $\pm$ 0.27	1.81 $\pm$ 0.29	1.55 $\pm$ 0.22	1.79 $\pm$ 0.15	2.06 $\pm$ 0.26	$F_{6,56} = 4.96, P = 0.0004$
Accessory olfactory n.	2.47 $\pm$ 0.18	3.66 $\pm$ 0.30	4.44 $\pm$ 0.58	4.87 $\pm$ 0.39	4.71 $\pm$ 0.70	5.34 $\pm$ 0.46	6.05 $\pm$ 0.67	$F_{6,56} = 6.67, P < 0.0001$
Frontal cortex	2.22 $\pm$ 0.16	3.22 $\pm$ 0.35	3.67 $\pm$ 0.64	3.63 $\pm$ 0.31	3.58 $\pm$ 0.52	4.29 $\pm$ 0.39	4.34 $\pm$ 0.63	$F_{6,56} = 3.42, P = 0.006$
Orbital cortex	4.96 $\pm$ 0.27	6.64 $\pm$ 0.55	7.50 $\pm$ 0.80	7.54 $\pm$ 0.47	8.09 $\pm$ 0.92	7.94 $\pm$ 0.52	8.72 $\pm$ 0.89	$F_{6,56} = 4.44, P = 0.001$
Cingulate cortex	6.66 $\pm$ 0.43	8.20 $\pm$ 0.88	9.13 $\pm$ 0.96	9.61 $\pm$ 0.70	9.93 $\pm$ 0.97	9.84 $\pm$ 0.61	9.95 $\pm$ 0.66	$F_{6,56} = 3.38, P = 0.0065$
Cerebral cortex, outer	2.41 $\pm$ 0.14	3.02 $\pm$ 0.28	3.71 $\pm$ 0.38	3.67 $\pm$ 0.55	4.27 $\pm$ 0.39	3.80 $\pm$ 0.24	4.08 $\pm$ 0.32	$F_{6,56} = 4.53, P = 0.0008$
Cerebral cortex, inner	4.52 $\pm$ 0.28	5.86 $\pm$ 0.45	6.63 $\pm$ 0.68	6.88 $\pm$ 0.49	7.41 $\pm$ 0.75	7.26 $\pm$ 0.49	7.37 $\pm$ 0.60	$F_{6,56} = 4.71, P = 0.0006$
Amygdala	1.58 $\pm$ 0.10	2.10 $\pm$ 0.19	2.88 $\pm$ 0.57	2.45 $\pm$ 0.16	2.88 $\pm$ 0.28	2.77 $\pm$ 0.22	2.83 $\pm$ 0.30	$F_{6,56} = 4.16, P = 0.0016$
Septum	3.68 $\pm$ 0.24	4.54 $\pm$ 0.44	5.08 $\pm$ 0.50	5.24 $\pm$ 0.35	5.60 $\pm$ 0.74	5.68 $\pm$ 0.44	5.83 $\pm$ 0.59	$F_{6,56} = 2.94, P = 0.015$
Hippocampus	2.22 $\pm$ 0.15	2.91 $\pm$ 0.23	3.55 $\pm$ 0.54	3.28 $\pm$ 0.25	3.96 $\pm$ 0.51	3.52 $\pm$ 0.26	3.61 $\pm$ 0.37	$F_{6,56} = 3.44, P = 0.0059$
Subiculum	10.95 $\pm$ 0.66	13.59 $\pm$ 1.02	13.89 $\pm$ 1.41	14.00 $\pm$ 1.40	14.10 $\pm$ 0.96	14.71 $\pm$ 1.03	14.38 $\pm$ 1.35	$F_{6,56} = 1.68, P > 0.05$, N.S.
Anteriodorsal thalamic n.	20.60 $\pm$ 1.40	24.29 $\pm$ 2.03	25.83 $\pm$ 2.68	24.47 $\pm$ 1.97	24.64 $\pm$ 2.79	24.44 $\pm$ 1.82	25.00 $\pm$ 2.14	$F_{6,56} = 0.76, P > 0.05$, N.S.
Laterodorsal thalamic n.	16.55 $\pm$ 0.85	19.63 $\pm$ 1.56	19.72 $\pm$ 2.01	19.78 $\pm$ 1.55	20.01 $\pm$ 2.34	18.63 $\pm$ 1.23	20.04 $\pm$ 1.47	$F_{6,56} = 0.79, P > 0.05$, N.S.
Mediodorsal thalamic n.	17.09 $\pm$ 1.03	19.71 $\pm$ 1.72	20.16 $\pm$ 1.87	19.95 $\pm$ 1.66	20.75 $\pm$ 2.34	20.11 $\pm$ 1.61	20.12 $\pm$ 1.68	$F_{6,56} = 0.62, P > 0.05$, N.S.
Ventrolateral thalamic n.	13.71 $\pm$ 0.88	15.27 $\pm$ 1.31	16.56 $\pm$ 1.62	16.11 $\pm$ 1.05	17.43 $\pm$ 2.10	16.55 $\pm$ 1.15	16.29 $\pm$ 1.21	$F_{6,56} = 0.92, P > 0.05$, N.S.
Hypothalamus	3.30 $\pm$ 0.24	4.67 $\pm$ 0.40	5.33 $\pm$ 0.87	5.31 $\pm$ 0.50	6.15 $\pm$ 0.46	5.86 $\pm$ 0.50	6.30 $\pm$ 0.63	$F_{6,56} = 5.01, P = 0.0004$
Zona incerta	7.76 $\pm$ 0.47	9.50 $\pm$ 0.71	10.70 $\pm$ 1.39	10.73 $\pm$ 0.64	11.68 $\pm$ 1.04	11.38 $\pm$ 0.88	11.32 $\pm$ 0.81	$F_{6,56} = 3.19, P = 0.0091$
Ventrolateral geniculate n.	14.50 $\pm$ 1.02	16.12 $\pm$ 1.30	16.34 $\pm$ 1.79	16.21 $\pm$ 1.20	17.19 $\pm$ 1.99	15.61 $\pm$ 1.28	15.12 $\pm$ 1.03	$F_{6,56} = 0.44, P > 0.05$, N.S.
Dorsolateral geniculate n.	20.76 $\pm$ 1.35	23.28 $\pm$ 2.01	22.74 $\pm$ 2.04	23.99 $\pm$ 1.67	24.71 $\pm$ 2.54	22.15 $\pm$ 2.05	22.80 $\pm$ 1.99	$F_{6,56} = 0.48, P > 0.05$, N.S.
Superior colliculus, superficial gray	13.20 $\pm$ 0.77	15.22 $\pm$ 1.34	15.91 $\pm$ 1.79	16.31 $\pm$ 1.45	15.89 $\pm$ 1.27	17.33 $\pm$ 1.37	16.06 $\pm$ 1.46	$F_{6,56} = 1.16, P > 0.05$, N.S.
Superior colliculus, deeper layers	9.87 $\pm$ 0.58	11.71 $\pm$ 1.09	12.31 $\pm$ 1.23	13.00 $\pm$ 0.87	13.13 $\pm$ 1.00	14.10 $\pm$ 1.04	13.82 $\pm$ 1.14	$F_{6,56} = 2.65, P = 0.025$
Medial geniculate n.	11.13 $\pm$ 0.78	12.93 $\pm$ 1.05	13.72 $\pm$ 1.36	14.46 $\pm$ 0.87	13.79 $\pm$ 1.00	14.87 $\pm$ 1.34	14.28 $\pm$ 1.15	$F_{6,56} = 1.65, P > 0.05$, N.S.
Inferior colliculus, dorsal cortex	4.56 $\pm$ 0.24	5.71 $\pm$ 0.84	6.44 $\pm$ 0.71	7.34 $\pm$ 0.63	7.26 $\pm$ 0.57	7.55 $\pm$ 0.54	7.38 $\pm$ 0.42	$F_{6,56} = 6.07, P = 0.0001$
Inferior colliculus, core	1.99 $\pm$ 0.19	2.82 $\pm$ 0.11	3.34 $\pm$ 0.44	3.68 $\pm$ 0.29	3.60 $\pm$ 0.33	3.73 $\pm$ 0.28	3.67 $\pm$ 0.24	$F_{6,56} = 6.71, P < 0.0001$
Inferior colliculus, exterior cortex	3.23 $\pm$ 0.19	3.98 $\pm$ 0.32	5.30 $\pm$ 0.50	5.39 $\pm$ 0.36	5.20 $\pm$ 0.46	5.63 $\pm$ 0.46	5.28 $\pm$ 0.34	$F_{6,56} = 6.45, P < 0.0001$
Caudate putamen	5.42 $\pm$ 0.30	6.55 $\pm$ 0.59	6.95 $\pm$ 0.70	7.03 $\pm$ 0.47	6.23 $\pm$ 0.97	7.25 $\pm$ 0.54	7.33 $\pm$ 0.72	$F_{6,56} = 1.41, P > 0.05$, N.S.
Nucleus accumbens	3.96 $\pm$ 0.24	4.64 $\pm$ 0.46	5.09 $\pm$ 0.51	5.11 $\pm$ 0.37	5.30 $\pm$ 0.50	5.10 $\pm$ 0.42	5.54 $\pm$ 0.53	$F_{6,56} = 1.73, P > 0.05$, N.S.
Olfactory tubercle	3.32 $\pm$ 0.17	4.22 $\pm$ 0.43	4.78 $\pm$ 0.48	4.68 $\pm$ 0.35	5.06 $\pm$ 0.47	4.74 $\pm$ 0.40	5.28 $\pm$ 0.52	$F_{6,56} = 3.10, P = 0.010$
Substantia nigra, pars compacta	9.20 $\pm$ 0.57	10.97 $\pm$ 0.87	10.87 $\pm$ 1.12	11.60 $\pm$ 0.81	11.52 $\pm$ 0.87	11.57 $\pm$ 0.90	11.86 $\pm$ 1.00	$F_{6,56} = 1.33, P > 0.05$, N.S.
Ventral tegmental area	10.10 $\pm$ 0.86	11.80 $\pm$ 0.98	12.34 $\pm$ 1.43	13.10 $\pm$ 1.06	13.20 $\pm$ 1.19	12.91 $\pm$ 0.98	13.52 $\pm$ 1.16	$F_{6,56} = 1.42, P > 0.05$, N.S.
Medial habenula	16.51 $\pm$ 0.88	17.92 $\pm$ 1.56	18.91 $\pm$ 1.79	20.28 $\pm$ 1.91	19.37 $\pm$ 1.75	19.24 $\pm$ 1.48	20.20 $\pm$ 1.61	$F_{6,56} = 0.85, P > 0.05$, N.S.
Interpeduncular n.	34.00 $\pm$ 2.53	36.08 $\pm$ 2.99	36.11 $\pm$ 3.34	36.67 $\pm$ 2.45	35.20 $\pm$ 2.68	37.01 $\pm$ 2.84	35.28 $\pm$ 2.90	$F_{6,56} = 0.15, P > 0.05$, N.S.
Periaquiductal gray	6.51 $\pm$ 0.40	7.74 $\pm$ 0.62	8.55 $\pm$ 1.10	9.16 $\pm$ 0.70	9.76 $\pm$ 0.71	10.07 $\pm$ 0.88	9.86 $\pm$ 0.81	$F_{6,56} = 3.63, P = 0.0041$
Deep mesencephalic n.	6.85 $\pm$ 0.38	8.17 $\pm$ 0.53	9.05 $\pm$ 0.90	9.59 $\pm$ 0.73	9.94 $\pm$ 0.72	10.06 $\pm$ 0.84	9.63 $\pm$ 0.66	$F_{6,56} = 3.44, P = 0.0058$
Dorsal tegmental nucleus	8.48 $\pm$ 0.49	10.30 $\pm$ 0.80	11.01 $\pm$ 1.21	12.38 $\pm$ 1.02	12.45 $\pm$ 0.87	12.54 $\pm$ 0.93	11.51 $\pm$ 0.44	$F_{6,56} = 3.78, P = 0.0031$
Pons	3.68 $\pm$ 0.23	4.62 $\pm$ 0.37	5.24 $\pm$ 0.55	5.65 $\pm$ 0.52	5.60 $\pm$ 0.47	5.71 $\pm$ 0.50	5.54 $\pm$ 0.27	$F_{6,56} = 3.75, P = 0.0033$
Pontine nucleus	5.71 $\pm$ 0.37	7.45 $\pm$ 0.62	7.88 $\pm$ 0.89	8.32 $\pm$ 0.70	8.55 $\pm$ 0.72	8.81 $\pm$ 0.69	8.79 $\pm$ 0.55	$F_{6,56} = 3.65, P = 0.0041$
Cerebellum	0.56 $\pm$ 0.06	0.86 $\pm$ 0.10	0.97 $\pm$ 0.17	0.97 $\pm$ 0.14	1.04 $\pm$ 0.14	0.98 $\pm$ 0.13	1.02 $\pm$ 0.15	$F_{6,56} = 2.24, P = 0.052$

N.S., not significant.

ferior colliculus, and several midbrain and hindbrain nuclei. Maximal increases ranged from 40% in the deep layers of superior colliculus to 144% in the accessory olfactory nucleus.

No significant differences in [¹²⁵I]A85380 binding were observed in 16 of the 38 regions quantitated. These regions included anteriodorsal, laterodorsal, mediodorsal, and ventrolateral thalamic nuclei, dosolateral, ventrolateral, and medial geniculate nuclei, most parts of the basal ganglia including caudate putamen, nucleus accumbens, substantia nigra pars compacta and ventral tegmental area (with the olfactory tubercle being an exception), and the medial habenula and interpeduncular nucleus. Although changes in these brain regions were not significant, binding site density tended to show modest increases (9% in interpeduncular nucleus to 40% in nucleus accumbens).

The differential effect of chronic nicotine treatment on the up-regulation of nicotinic binding sites in mouse brain regions observed here is comparable with that seen with other nicotinic cholinergic ligands in mice (Marks et al., 1983, 2004; Pauly et al., 1991) and rats (Sanderson et al., 1993; Nguyen et al., 2003).

Chronic Nicotine Administration Elicits a Saturable, Dose-Dependent Increase in [¹²⁵I]mAb 299 Binding to α 4-nAChR Subunits in Many Brain Regions. The effects of chronic nicotine infusion on the density of [¹²⁵I]mAb 299 binding sites in 38 brain regions measured by autoradiography are summarized in Table 2.

Statistically significant increases in [¹²⁵I]mAb 299 binding were observed in 30 of the 38 regions quantitated. Significant increases in [¹²⁵I]mAb 299 binding were noted in each of the regions for which significant nicotine-induced increases in [¹²⁵I]A85380 binding were observed, including olfactory bulbs, each of the five cortical areas, hypothalamus, zona incerta, deep layers of superior colliculus, inferior colliculus, and several midbrain and hindbrain nuclei. In addition, statistically significant increases in [¹²⁵I]mAb 299 binding were observed in each of the areas of the basal ganglia: caudate putamen, nucleus accumbens, olfactory tubercle, substantia nigra pars compacta, and ventral tegmental area, as well as the subiculum and the medial geniculate nucleus. Maximal increases ranged from 35% in the medial geniculate nucleus to 250% in the olfactory bulbs.

No statistically significant changes in [¹²⁵I]mAb 299 binding were noted in 8 of the 38 regions assayed. All eight of these regions also showed no significant changes in [¹²⁵I]A85380 binding. No significant effects of nicotine treatment were detected in anteriodorsal, laterodorsal, mediodorsal, or ventrolateral thalamic nuclei, the ventrolateral or dorsolateral geniculate nuclei, or the medial habenula or interpeduncular nucleus. Although not significant, [¹²⁵I]mAb 299 binding did tend to increase in these regions (5% in anteriodorsal thalamic nucleus to 30% in ventrolateral geniculate nucleus).

Chronic Nicotine Administration Elicits a Saturable, Dose-Dependent Increase in [¹²⁵I]mAb 270 Binding to β 2 nAChR Subunits in Many Brain Areas. The effects of

TABLE 2

[¹²⁵I]mAb299 labeling of brain regions after chronic nicotine treatment

Density of [¹²⁵I]mAb299 labeling (fmol/mg wet weight) was measured by quantitative autoradiography as described under *Materials and Methods* in brain regions of mice chronically treated with the indicated doses of nicotine. Each value represents the mean $\pm$ S.E.M. of 6 to 10 individual mice. Results of each individual one-way ANOVA are also given. Values differing significantly from those of saline infused mice ($P < 0.05$, Duncan's test) are indicated in bold.

Brain Region	Saline	Nicotine						ANOVA Results
		0.125 mg/kg/h	0.25 mg/kg/h	0.5 mg/kg/h	1.0 mg/kg/h	2.0 mg/kg/h	4.0 mg/kg/h	
Olfactory bulbs	0.062 $\pm$ 0.015	0.092 $\pm$ 0.010	0.082 $\pm$ 0.019	0.130 $\pm$ 0.024	0.182 $\pm$ 0.022	0.168 $\pm$ 0.019	0.217 $\pm$ 0.037	$F_{6,55} = 6.45, P < 0.001$
Accessory olfactory N.	0.284 $\pm$ 0.032	0.437 $\pm$ 0.043	0.440 $\pm$ 0.00040	0.488 $\pm$ 0.043	0.586 $\pm$ 0.048	0.589 $\pm$ 0.031	0.681 $\pm$ 0.057	$F_{6,55} = 9.48, P < 0.001$
Frontal cortex	0.266 $\pm$ 0.025	0.322 $\pm$ 0.035	0.376 $\pm$ 0.070	0.367 $\pm$ 0.034	0.433 $\pm$ 0.043	0.458 $\pm$ 0.031	0.441 $\pm$ 0.052	$F_{6,55} = 2.51, P < 0.05$
Orbital cortex	0.593 $\pm$ 0.038	0.802 $\pm$ 0.053	0.798 $\pm$ 0.119	0.815 $\pm$ 0.066	0.915 $\pm$ 0.065	0.932 $\pm$ 0.050	1.025 $\pm$ 0.109	$F_{6,55} = 3.18, P < 0.01$
Cingulate cortex	0.852 $\pm$ 0.065	0.924 $\pm$ 0.086	0.876 $\pm$ 0.076	1.138 $\pm$ 0.117	1.129 $\pm$ 0.083	1.178 $\pm$ 0.084	1.146 $\pm$ 0.085	$F_{6,55} = 2.75, P < 0.05$
Cerebral cortex, outer	0.315 $\pm$ 0.025	0.368 $\pm$ 0.025	0.298 $\pm$ 0.028	0.358 $\pm$ 0.028	0.511 $\pm$ 0.041	0.454 $\pm$ 0.034	0.476 $\pm$ 0.031	$F_{6,55} = 7.10, P < 0.001$
Cerebral cortex, inner	0.549 $\pm$ 0.044	0.651 $\pm$ 0.047	0.568 $\pm$ 0.045	0.693 $\pm$ 0.045	0.849 $\pm$ 0.057	0.846 $\pm$ 0.059	0.839 $\pm$ 0.053	$F_{6,54} = 6.91, P < 0.001$
Amygdala	0.194 $\pm$ 0.022	0.246 $\pm$ 0.018	0.230 $\pm$ 0.026	0.285 $\pm$ 0.028	0.358 $\pm$ 0.037	0.354 $\pm$ 0.029	0.353 $\pm$ 0.021	$F_{6,54} = 6.38, P < 0.001$
Septum	0.293 $\pm$ 0.026	0.380 $\pm$ 0.037	0.363 $\pm$ 0.029	0.399 $\pm$ 0.039	0.478 $\pm$ 0.047	0.427 $\pm$ 0.025	0.441 $\pm$ 0.034	$F_{6,55} = 3.04, P < 0.05$
Hippocampus	0.252 $\pm$ 0.028	0.296 $\pm$ 0.027	0.269 $\pm$ 0.025	0.320 $\pm$ 0.031	0.372 $\pm$ 0.027	0.367 $\pm$ 0.026	0.353 $\pm$ 0.021	$F_{6,55} = 3.75, P < 0.01$
Subiculum	1.215 $\pm$ 0.091	1.333 $\pm$ 0.091	1.226 $\pm$ 0.087	1.402 $\pm$ 0.083	1.500 $\pm$ 0.183	1.691 $\pm$ 0.109	1.507 $\pm$ 0.088	$F_{6,55} = 2.40, P < 0.05$
Anterodorsal thalamic n.	2.461 $\pm$ 0.110	2.541 $\pm$ 0.129	2.225 $\pm$ 0.137	2.231 $\pm$ 0.191	2.596 $\pm$ 0.136	2.428 $\pm$ 0.149	2.444 $\pm$ 0.161	$F_{6,55} = 0.75, P > 0.05, N.S.$
Laterodorsal thalamic n.	2.021 $\pm$ 0.116	2.089 $\pm$ 0.122	1.818 $\pm$ 0.119	1.971 $\pm$ 0.121	2.052 $\pm$ 0.123	2.129 $\pm$ 0.132	2.269 $\pm$ 0.231	$F_{6,55} = 0.96, P > 0.05, N.S.$
Mediodorsal thalamic n.	1.986 $\pm$ 0.115	1.995 $\pm$ 0.117	1.699 $\pm$ 0.130	1.929 $\pm$ 0.113	2.013 $\pm$ 0.113	2.025 $\pm$ 0.129	2.126 $\pm$ 0.157	$F_{6,55} = 1.12, P > 0.05, N.S.$
Ventrolateral thalamic n.	1.399 $\pm$ 0.081	1.411 $\pm$ 0.063	1.275 $\pm$ 0.087	1.433 $\pm$ 0.087	1.576 $\pm$ 0.093	1.530 $\pm$ 0.093	1.551 $\pm$ 0.110	$F_{6,55} = 1.38, P > 0.05, N.S.$
Hypothalamus	0.329 $\pm$ 0.046	0.397 $\pm$ 0.020	0.395 $\pm$ 0.035	0.444 $\pm$ 0.035	0.544 $\pm$ 0.043	0.562 $\pm$ 0.059	0.548 $\pm$ 0.038	$F_{6,55} = 4.77, P < 0.001$
Zona incerta	0.756 $\pm$ 0.051	0.913 $\pm$ 0.056	0.786 $\pm$ 0.064	1.074 $\pm$ 0.061	1.048 $\pm$ 0.063	1.133 $\pm$ 0.077	1.071 $\pm$ 0.055	$F_{6,55} = 5.68, P < 0.001$
Ventrolateral geniculate n.	0.709 $\pm$ 0.056	0.741 $\pm$ 0.0047	0.665 $\pm$ 0.031	0.790 $\pm$ 0.058	0.922 $\pm$ 0.054	0.887 $\pm$ 0.053	0.867 $\pm$ 0.041	$F_{6,55} = 3.92, P < 0.01$
Dorsolateral geniculate n.	1.406 $\pm$ 0.112	1.534 $\pm$ 0.138	1.236 $\pm$ 0.078	1.480 $\pm$ 0.099	1.642 $\pm$ 0.122	1.685 $\pm$ 0.100	1.411 $\pm$ 0.147	$F_{6,55} = 1.80, P > 0.05, N.S.$
Superior colliculus, outer	0.961 $\pm$ 0.056	1.143 $\pm$ 0.077	0.987 $\pm$ 0.056	1.185 $\pm$ 0.091	1.371 $\pm$ 0.100	1.379 $\pm$ 0.086	1.354 $\pm$ 0.062	$F_{6,55} = 5.38, P < 0.001$
Superior colliculus, deeper	0.803 $\pm$ 0.053	1.032 $\pm$ 0.070	0.867 $\pm$ 0.047	1.017 $\pm$ 0.073	1.113 $\pm$ 0.165	1.137 $\pm$ 0.149	1.248 $\pm$ 0.066	$F_{6,55} = 2.44, P < 0.05$
Medial geniculate n.	1.125 $\pm$ 0.054	1.421 $\pm$ 0.108	1.135 $\pm$ 0.078	1.302 $\pm$ 0.161	1.517 $\pm$ 0.096	1.512 $\pm$ 0.109	1.452 $\pm$ 0.070	$F_{6,55} = 2.77, P < 0.05$
Inferior colliculus, dorsal	0.474 $\pm$ 0.043	0.552 $\pm$ 0.030	0.514 $\pm$ 0.039	0.671 $\pm$ 0.058	0.724 $\pm$ 0.076	0.790 $\pm$ 0.061	0.710 $\pm$ 0.034	$F_{6,55} = 5.40, P < 0.001$
Inferior colliculus, core	0.196 $\pm$ 0.019	0.228 $\pm$ 0.019	0.215 $\pm$ 0.025	0.283 $\pm$ 0.033	0.321 $\pm$ 0.039	0.335 $\pm$ 0.036	0.295 $\pm$ 0.026	$F_{6,55} = 3.49, P < 0.01$
Inferior colliculus, external	0.340 $\pm$ 0.032	0.416 $\pm$ 0.026	0.365 $\pm$ 0.033	0.499 $\pm$ 0.039	0.568 $\pm$ 0.060	0.608 $\pm$ 0.063	0.549 $\pm$ 0.022	$F_{6,55} = 6.14, P < 0.001$
Caudate putamen	0.459 $\pm$ 0.034	0.576 $\pm$ 0.047	0.544 $\pm$ 0.049	0.600 $\pm$ 0.045	0.720 $\pm$ 0.066	0.654 $\pm$ 0.032	0.691 $\pm$ 0.067	$F_{6,55} = 3.22, P < 0.01$
Nucleus accumbens	0.267 $\pm$ 0.023	0.337 $\pm$ 0.031	0.341 $\pm$ 0.036	0.377 $\pm$ 0.031	0.460 $\pm$ 0.045	0.416 $\pm$ 0.032	0.414 $\pm$ 0.032	$F_{6,55} = 3.70, P < 0.01$
Olfactory tubercles	0.252 $\pm$ 0.025	0.350 $\pm$ 0.034	0.341 $\pm$ 0.042	0.365 $\pm$ 0.027	0.472 $\pm$ 0.045	0.443 $\pm$ 0.034	0.451 $\pm$ 0.031	$F_{6,55} = 5.08, P < 0.001$
Substantia nigra, pars compacta	0.639 $\pm$ 0.034	0.788 $\pm$ 0.064	0.671 $\pm$ 0.045	0.785 $\pm$ 0.052	0.928 $\pm$ 0.072	0.953 $\pm$ 0.070	0.877 $\pm$ 0.052	$F_{6,55} = 4.64, P < 0.001$
Ventral tegmental area	0.684 $\pm$ 0.044	0.834 $\pm$ 0.058	0.730 $\pm$ 0.034	0.832 $\pm$ 0.044	0.996 $\pm$ 0.072	0.994 $\pm$ 0.067	0.932 $\pm$ 0.056	$F_{6,55} = 5.03, P < 0.001$
Medial habenula	0.664 $\pm$ 0.0042	0.675 $\pm$ 0.052	0.687 $\pm$ 0.036	0.730 $\pm$ 0.043	0.834 $\pm$ 0.052	0.836 $\pm$ 0.066	0.805 $\pm$ 0.067	$F_{6,55} = 2.07, P > 0.05, N.S.$
Interpeduncular nucleus	1.976 $\pm$ 0.122	2.190 $\pm$ 0.175	1.544 $\pm$ 0.142	1.884 $\pm$ 0.150	1.876 $\pm$ 0.117	2.067 $\pm$ 0.155	1.776 $\pm$ 0.142	$F_{6,55} = 2.18, P > 0.05, N.S.$
Periaquiductal grey	0.632 $\pm$ 0.043	0.815 $\pm$ 0.051	0.732 $\pm$ 0.040	0.852 $\pm$ 0.054	0.973 $\pm$ 0.080	1.018 $\pm$ 0.081	1.017 $\pm$ 0.053	$F_{6,55} = 6.46, P < 0.001$
Deep mesencephalic area	0.588 $\pm$ 0.047	0.719 $\pm$ 0.048	0.624 $\pm$ 0.031	0.802 $\pm$ 0.059	0.877 $\pm$ 0.067	0.953 $\pm$ 0.076	0.863 $\pm$ 0.038	$F_{6,55} = 6.20, P < 0.001$
Dorsal tegmental n.	0.862 $\pm$ 0.074	0.969 $\pm$ 0.053	0.872 $\pm$ 0.050	1.066 $\pm$ 0.081	1.185 $\pm$ 0.092	1.229 $\pm$ 0.156	1.120 $\pm$ 0.058	$F_{6,55} = 3.51, P < 0.001$
Pons	0.307 $\pm$ 0.027	0.405 $\pm$ 0.032	0.326 $\pm$ 0.030	0.434 $\pm$ 0.033	0.505 $\pm$ 0.039	0.532 $\pm$ 0.059	0.452 $\pm$ 0.031	$F_{6,55} = 5.17, P < 0.001$
Pontine nucleus	0.564 $\pm$ 0.030	0.697 $\pm$ 0.045	0.633 $\pm$ 0.084	0.747 $\pm$ 0.081	0.874 $\pm$ 0.079	0.821 $\pm$ 0.083	0.861 $\pm$ 0.035	$F_{6,55} = 3.12, P < 0.05$
Cerebellum	0.020 $\pm$ 0.009	0.029 $\pm$ 0.008	0.000 $\pm$ 0.010	0.038 $\pm$ 0.014	0.062 $\pm$ 0.015	0.049 $\pm$ 0.014	0.043 $\pm$ 0.011	$F_{6,55} = 2.92, P < 0.05$

N.S., not significant.

chronic nicotine infusion on the density of [¹²⁵I]mAb 270 binding sites in 36 brain regions measured by autoradiography are summarized in Table 3.

Statistically significant increases in [¹²⁵I]mAb 270 binding were observed in 15 of the 36 regions quantitated (owing to lower signal to noise encountered with [¹²⁵I]mAb 270 resolution did not permit accurate distinction of three regions of inferior colliculus, so binding was measured in the whole nucleus, hence the difference in region number from the other two ligands). The areas for which significant effects of nicotine treatment were observed was a subset of those found significant for [¹²⁵I]A85380 and [¹²⁵I]mAb 299 and included accessory olfactory nucleus, most cortical areas, hypothalamus, inferior colliculus, substantia nigra pars compacta, ventral tegmental area, and several midbrain and hindbrain nuclei. Maximal increases ranged from 20% in the subiculum to 140% in the olfactory bulbs.

No statistically significant effects of chronic nicotine treatment on [¹²⁵I]mAb 270 binding were observed in 21 of the 36 brain regions measured. Consistent with the results for [¹²⁵I]A85380 and [¹²⁵I]mAb 299 binding, [¹²⁵I]mAb 270 binding in the thalamus, geniculate nuclei, medial habenula, and interpeduncular nucleus were not significantly affected by chronic nicotine treatment. As also was the case with the other two ligands, although not significant, the [¹²⁵I]mAb 270 binding tended to increase with chronic nicotine treatment.

Illustration that Responses to Chronic Nicotine Treatment of [¹²⁵I]A85380 ($\beta 2^*$ -nAChR Binding Sites), [¹²⁵I]mAb 299 ($\alpha 4$ nAChR Subunit Protein), and [¹²⁵I]mAb 270 ($\beta 2$ nAChR Subunit Protein) Binding Differ among Brain Areas. The results presented in Tables 1 to 3 summarize the effects of chronic nicotine treatment on the binding site densities for [¹²⁵I]A85380, [¹²⁵I]mAb 299, and [¹²⁵I]mAb 270, but do not easily allow a direct comparison of the effects of chronic treatment on these ligands. The illustrations presented in Figs. 2, 3, and 4 provide such a comparison at three different anatomical levels. In each figure, binding of [¹²⁵I]A85380, [¹²⁵I]mAb 299, and [¹²⁵I]mAb 270 to representative sections from a saline-infused mouse are shown. The effects of chronic nicotine treatment on each of the ligands in four regions at each level are illustrated graphically. Within each graph the mean $\pm$ S.E.M. for each ligand is shown as well as a curve describing the average nicotine dose dependence for the three ligands. All data are normalized to the binding site densities for saline-infused mice.

Figure 2 illustrates the effects of chronic nicotine treatment at the level of the nucleus accumbens (approximately +0.7 mm Bregma). Saturable increases in overall binding site density are observed for each brain area. The responses of [¹²⁵I]A85380, [¹²⁵I]mAb 299, and mAb 270 binding to chronic nicotine infusion in the cingulate cortex are similar and do not deviate markedly from the curve des-

TABLE 3

[¹²⁵I]mAb270 labeling of brain regions after chronic nicotine treatment

Density of [¹²⁵I]mAb270 labeling (fmol/mg wet weight) was measured by quantitative autoradiography as described under *Materials and Methods* in brain regions of mice chronically treated with the indicated doses of nicotine. Each value represents the mean $\pm$ S.E.M. of 6 to 10 individual mice. Results of each individual one-way ANOVA are also given. Values differing significantly from those of saline-infused mice ($P < 0.05$, Duncan's test) are indicated in bold.

Brain Region	Saline	Nicotine						ANOVA Results
		0.125 mg/kg/h	0.25 mg/kg/h	0.5 mg/kg/h	1.0 mg/kg/h	2.0 mg/kg/h	4.0 mg/kg/h	
Olfactory bulbs	0.031 $\pm$ 0.012	0.019 $\pm$ 0.015	0.036 $\pm$ 0.016	0.046 $\pm$ 0.017	0.069 $\pm$ 0.018	0.073 $\pm$ 0.013	0.075 $\pm$ 0.018	$F_{6,86} = 1.90, P > 0.05$, N.S.
Accessory olfactory N.	0.096 $\pm$ 0.016	0.110 $\pm$ 0.024	0.123 $\pm$ 0.020	0.148 $\pm$ 0.021	0.156 $\pm$ 0.024	0.170 $\pm$ 0.018	0.186 $\pm$ 0.022	$F_{6,86} = 2.37, P < 0.05$
Frontal cortex	0.129 $\pm$ 0.015	0.118 $\pm$ 0.018	0.130 $\pm$ 0.019	0.166 $\pm$ 0.018	0.191 $\pm$ 0.022	0.184 $\pm$ 0.017	0.202 $\pm$ 0.028	$F_{6,86} = 2.82, P < 0.05$
Orbital cortex	0.213 $\pm$ 0.019	0.221 $\pm$ 0.024	0.215 $\pm$ 0.017	0.259 $\pm$ 0.022	0.256 $\pm$ 0.027	0.273 $\pm$ 0.025	0.300 $\pm$ 0.026	$F_{6,86} = 1.80, P > 0.05$, N.S.
Cingulate cortex	0.168 $\pm$ 0.016	0.192 $\pm$ 0.014	0.208 $\pm$ 0.020	0.217 $\pm$ 0.017	0.236 $\pm$ 0.027	0.247 $\pm$ 0.022	0.249 $\pm$ 0.022	$F_{6,86} = 2.20, P = 0.05$
Cerebral cortex, outer	0.105 $\pm$ 0.018	0.107 $\pm$ 0.012	0.112 $\pm$ 0.017	0.140 $\pm$ 0.015	0.141 $\pm$ 0.017	0.154 $\pm$ 0.017	0.177 $\pm$ 0.019	$F_{6,86} = 2.45, P < 0.05$
Cerebral cortex, inner	0.156 $\pm$ 0.020	0.170 $\pm$ 0.011	0.173 $\pm$ 0.022	0.215 $\pm$ 0.017	0.220 $\pm$ 0.023	0.232 $\pm$ 0.021	0.260 $\pm$ 0.022	$F_{6,86} = 3.56, P < 0.01$
Amygdala	0.096 $\pm$ 0.016	0.108 $\pm$ 0.018	0.111 $\pm$ 0.018	0.146 $\pm$ 0.015	0.161 $\pm$ 0.022	0.151 $\pm$ 0.017	0.157 $\pm$ 0.023	$F_{6,86} = 2.14, P = 0.057$
Septum	0.117 $\pm$ 0.014	0.132 $\pm$ 0.023	0.132 $\pm$ 0.016	0.162 $\pm$ 0.018	0.159 $\pm$ 0.022	0.166 $\pm$ 0.018	0.180 $\pm$ 0.020	$F_{6,86} = 1.42, P > 0.05$, N.S.
Hippocampus	0.102 $\pm$ 0.024	0.130 $\pm$ 0.013	0.132 $\pm$ 0.020	0.158 $\pm$ 0.017	0.166 $\pm$ 0.020	0.163 $\pm$ 0.020	0.180 $\pm$ 0.023	$F_{6,86} = 1.86, P > 0.05$, N.S.
Subiculum	0.326 $\pm$ 0.026	0.296 $\pm$ 0.017	0.300 $\pm$ 0.019	0.324 $\pm$ 0.020	0.360 $\pm$ 0.022	0.365 $\pm$ 0.014	0.392 $\pm$ 0.031	$F_{6,86} = 2.38, P < 0.05$
Anterodorsal thalamic n.	0.426 $\pm$ 0.024	0.390 $\pm$ 0.034	0.393 $\pm$ 0.026	0.436 $\pm$ 0.034	0.452 $\pm$ 0.043	0.424 $\pm$ 0.033	0.477 $\pm$ 0.041	$F_{6,86} = 0.71, P > 0.05$, N.S.
Lateralodorsal thalamic n.	0.401 $\pm$ 0.022	0.422 $\pm$ 0.040	0.374 $\pm$ 0.033	0.450 $\pm$ 0.042	0.466 $\pm$ 0.051	0.431 $\pm$ 0.034	0.466 $\pm$ 0.032	$F_{6,86} = 1.86, P > 0.05$, N.S.
Mediodorsal thalamic n.	0.394 $\pm$ 0.025	0.437 $\pm$ 0.037	0.379 $\pm$ 0.039	0.437 $\pm$ 0.040	0.453 $\pm$ 0.052	0.0429 $\pm$ 0.034	0.476 $\pm$ 0.033	$F_{6,86} = 0.67, P > 0.05$, N.S.
Ventrolateral thalamic n.	0.316 $\pm$ 0.021	0.362 $\pm$ 0.029	0.310 $\pm$ 0.032	0.364 $\pm$ 0.032	0.354 $\pm$ 0.046	0.374 $\pm$ 0.031	0.476 $\pm$ 0.021	$F_{6,86} = 0.69, P > 0.05$, N.S.
Hypothalamus	0.128 $\pm$ 0.014	0.143 $\pm$ 0.026	0.145 $\pm$ 0.019	0.185 $\pm$ 0.021	0.188 $\pm$ 0.028	0.204 $\pm$ 0.022	0.240 $\pm$ 0.020	$F_{6,86} = 3.09, P < 0.01$
Zona incerta	0.234 $\pm$ 0.015	0.246 $\pm$ 0.022	0.243 $\pm$ 0.020	0.282 $\pm$ 0.022	0.329 $\pm$ 0.035	0.315 $\pm$ 0.025	0.297 $\pm$ 0.035	$F_{6,86} = 2.20, P = 0.05$
Ventrolateral geniculate n.	0.457 $\pm$ 0.034	0.445 $\pm$ 0.048	0.425 $\pm$ 0.031	0.447 $\pm$ 0.033	0.495 $\pm$ 0.043	0.454 $\pm$ 0.038	0.470 $\pm$ 0.049	$F_{6,86} = 0.29, P > 0.05$, N.S.
Dorsolateral geniculate n.	0.550 $\pm$ 0.034	0.546 $\pm$ 0.055	0.507 $\pm$ 0.039	0.552 $\pm$ 0.039	0.556 $\pm$ 0.054	0.550 $\pm$ 0.046	0.556 $\pm$ 0.056	$F_{6,86} = 1.12, P > 0.05$, N.S.
Superior colliculus, outer	0.564 $\pm$ 0.039	0.501 $\pm$ 0.042	0.533 $\pm$ 0.040	0.538 $\pm$ 0.043	0.603 $\pm$ 0.053	0.601 $\pm$ 0.048	0.671 $\pm$ 0.065	$F_{6,86} = 1.26, P > 0.05$, N.S.
Superior colliculus, deeper	0.369 $\pm$ 0.031	0.342 $\pm$ 0.036	0.367 $\pm$ 0.027	0.381 $\pm$ 0.035	0.436 $\pm$ 0.049	0.435 $\pm$ 0.037	0.474 $\pm$ 0.055	$F_{6,86} = 1.36, P > 0.05$
Medial geniculate n.	0.359 $\pm$ 0.026	0.355 $\pm$ 0.024	0.374 $\pm$ 0.025	0.377 $\pm$ 0.027	0.417 $\pm$ 0.035	0.362 $\pm$ 0.038	0.438 $\pm$ 0.035	$F_{6,86} = 1.00, P > 0.05$
Inferior colliculus	0.157 $\pm$ 0.020	0.137 $\pm$ 0.013	0.170 $\pm$ 0.017	0.186 $\pm$ 0.022	0.243 $\pm$ 0.027	0.247 $\pm$ 0.023	0.280 $\pm$ 0.029	$F_{6,86} = 5.26, P < 0.001$
Caudate putamen	0.212 $\pm$ 0.014	0.216 $\pm$ 0.021	0.225 $\pm$ 0.019	0.243 $\pm$ 0.016	0.246 $\pm$ 0.022	0.256 $\pm$ 0.021	0.275 $\pm$ 0.023	$F_{6,86} = 1.24, P > 0.05$, N.S.
Nucleus accumbens	0.183 $\pm$ 0.016	0.175 $\pm$ 0.019	0.182 $\pm$ 0.021	0.201 $\pm$ 0.017	0.200 $\pm$ 0.020	0.216 $\pm$ 0.024	0.226 $\pm$ 0.027	$F_{6,86} = 0.76, P > 0.05$, N.S.
Olfactory tubercles	0.133 $\pm$ 0.014	0.137 $\pm$ 0.017	0.141 $\pm$ 0.016	0.157 $\pm$ 0.015	0.183 $\pm$ 0.021	0.192 $\pm$ 0.021	0.197 $\pm$ 0.022	$F_{6,86} = 2.11, P = 0.06$, N.S.
Substantia nigra, compacta	0.241 $\pm$ 0.018	0.217 $\pm$ 0.019	0.249 $\pm$ 0.016	0.283 $\pm$ 0.023	0.306 $\pm$ 0.027	0.319 $\pm$ 0.023	0.311 $\pm$ 0.029	$F_{6,86} = 2.83, P < 0.05$
Ventral tegmental area	0.257 $\pm$ 0.019	0.221 $\pm$ 0.018	0.248 $\pm$ 0.016	0.280 $\pm$ 0.022	0.350 $\pm$ 0.042	0.322 $\pm$ 0.025	0.318 $\pm$ 0.032	$F_{6,86} = 3.02, P < 0.01$
Medial habenula	0.402 $\pm$ 0.022	0.430 $\pm$ 0.029	0.428 $\pm$ 0.037	0.462 $\pm$ 0.034	0.472 $\pm$ 0.030	0.477 $\pm$ 0.031	0.527 $\pm$ 0.048	$F_{6,86} = 1.47, P > 0.05$, N.S.
Interpeduncular nucleus	0.729 $\pm$ 0.067	0.773 $\pm$ 0.069	0.669 $\pm$ 0.049	0.695 $\pm$ 0.049	0.752 $\pm$ 0.055	0.720 $\pm$ 0.051	0.764 $\pm$ 0.051	$F_{6,86} = 0.39, P > 0.05$, N.S.
Periaquiductal grey	0.174 $\pm$ 0.014	0.206 $\pm$ 0.036	0.211 $\pm$ 0.020	0.235 $\pm$ 0.029	0.265 $\pm$ 0.029	0.265 $\pm$ 0.020	0.311 $\pm$ 0.031	$F_{6,86} = 2.84, P < 0.05$
Deep mesencephalic area	0.180 $\pm$ 0.011	0.193 $\pm$ 0.031	0.196 $\pm$ 0.019	0.235 $\pm$ 0.025	0.236 $\pm$ 0.017	0.261 $\pm$ 0.013	0.269 $\pm$ 0.018	$F_{6,86} = 3.07, P < 0.01$
Dorsal tegmental n.	0.312 $\pm$ 0.032	0.276 $\pm$ 0.028	0.317 $\pm$ 0.030	0.308 $\pm$ 0.033	0.353 $\pm$ 0.037	0.396 $\pm$ 0.042	0.449 $\pm$ 0.051	$F_{6,86} = 2.46, P < 0.05$
Pons	0.125 $\pm$ 0.017	0.140 $\pm$ 0.013	0.142 $\pm$ 0.014	0.157 $\pm$ 0.020	0.182 $\pm$ 0.026	0.0186 $\pm$ 0.017	0.191 $\pm$ 0.021	$F_{6,86} = 1.78, P > 0.05$, N.S.
Pontine nucleus	0.115 $\pm$ 0.020	0.128 $\pm$ 0.030	0.119 $\pm$ 0.023	0.136 $\pm$ 0.027	0.190 $\pm$ 0.023	0.193 $\pm$ 0.019	0.174 $\pm$ 0.024	$F_{6,86} = 2.02, P = 0.072$, N.S.
Cerebellum	0.033 $\pm$ 0.015	0.050 $\pm$ 0.017	0.056 $\pm$ 0.018	0.069 $\pm$ 0.012	0.069 $\pm$ 0.024	0.071 $\pm$ 0.016	0.094 $\pm$ 0.029	$F_{6,86} = 1.01, P > 0.05$, N.S.

N.S., not significant.

cribing the overall effect of chronic nicotine treatment. In contrast, the density of [¹²⁵I]mAb 299 sites tends to be higher than the average line at low to intermediate nicotine doses in caudate putamen, nucleus accumbens, and olfactory tubercles.

Figure 3 illustrates the effects of chronic nicotine treatment at the level of the thalamus and medial habenula (approximately -1.6 mm Bregma). Saturable increases in overall binding are observed in the outer and inner cortical layers and hypothalamus, whereas no significant effect of chronic nicotine treatment was noted in mediodorsal thalamus. In each of these regions, the effects of chronic nicotine treatment on [¹²⁵I]A85380, [¹²⁵I]mAb 299, and mAb 270 binding are similar and these responses do not differ markedly from the curves describing the average dose dependence.

Figure 4 illustrates the effects of chronic nicotine treatment at the level of the superior colliculus and the interpeduncular nucleus (approximately -2.6 mm Bregma). Saturable increases in overall binding site densities were noted for periaquiductal gray and deep mesencephalic nucleus, with a less robust change observed in the superficial gray area of the superior colliculus. No significant effect of nicotine treatment was observed for any ligand in the interpeduncular nucleus. The effect of chronic nicotine treatment on [¹²⁵I]A85380, [¹²⁵I]mAb 299, and mAb 270 binding in periaquiductal gray, deep mesencephalic nucleus, and interpeduncular nucleus is similar and does not differ markedly from the average nicotine dose dependence in these regions. In contrast to the responses in these three regions, but similar to the patterns observed for caudate putamen, nucleus accumbens, and olfactory tubercle (Fig. 2), the density of [¹²⁵I]mAb 299 sites tends to be higher than the average line,

whereas the density of [¹²⁵I]mAb 270 binding tends to be lower than the average line at low to intermediate nicotine doses in the superior colliculus, superficial gray.

The Relative Amounts of [¹²⁵I]A85380 Binding Sites and the Relative Binding Site Densities of [¹²⁵I]mAb 270 and [¹²⁵I] mAb 299 Respond Similarly to Chronic Nicotine Treatment. As an additional analysis of effects of chronic nicotine treatment, scattergrams have been constructed comparing the density of [¹²⁵I]mAb 299 binding or [¹²⁵I]mAb 270 binding with density of [¹²⁵I]A85380 binding at each chronic nicotine infusion dose in the brain regions that had significant effects of treatment on [¹²⁵I]A85380 binding.

Figure 5 presents the scattergrams comparing the density of [¹²⁵I]A85380 sites to the density of [¹²⁵I]mAb 299 sites. A significant linear relationship between [¹²⁵I]mAb 299 and [¹²⁵I]A85380 was observed for all seven nicotine infusion doses ($r = 0.91$ – 0.97). In addition, the slopes of the regression lines were not significantly different for any dose. It should be noted, however, that the slopes of the lines were approximately 10, indicating that under the conditions of the experiment [¹²⁵I]A85380 labeled approximately 10-fold as many sites as did [¹²⁵I]mAb 299.

Figure 6 presents the scattergrams comparing the density of [¹²⁵I]A85380 sites with the density of [¹²⁵I]mAb 270 sites. A significant linear relationship between [¹²⁵I]mAb 270 and [¹²⁵I]A85380 was observed for all seven infusion doses ($r = 0.80$ – 0.91). These data were more variable than those for the [¹²⁵I]mAb 299/[¹²⁵I]A85380 correlations, and the slopes of the lines calculated at the seven infusion doses were not constant. The lines for nicotine doses of 0.125, 0.25, 0.50, 1.0, and 2.0 mg/kg/h were steeper than those at either the saline

Fig. 2. $[^{125}\text{I}]\text{A85380}$, $[^{125}\text{I}]\text{mAb 299}$, and $[^{125}\text{I}]\text{mAb 270}$ binding sites show regional differences to chronic nicotine treatment for sections at approximately +0.7 mm Bregma. Relative binding for each probe was calculated by normalizing to the binding site density for saline-treated mice. Data points represent mean $\pm$ S.E.M. for 7 to 10 mice at each nicotine infusion dose for $[^{125}\text{I}]\text{A85380}$ (●), $[^{125}\text{I}]\text{mAb 299}$ (□), and $[^{125}\text{I}]\text{mAb 270}$ (△). Curves are nonlinear least-squares curve fits of all the data as described under *Materials and Methods*. CgCx, cingulate cortex; OT, olfactory tubercle; CPu, caudate putamen; nAcc, nucleus accumbens. Shown are representative sections from saline-treated mice.

or 4.0 mg/kg/h doses, indicating that nicotine treatment has a larger effect on the number of $[^{125}\text{I}]\text{A85380}$ binding sites than it did on $[^{125}\text{I}]\text{mAb 270}$ binding sites. In addition, the slopes of $[^{125}\text{I}]\text{mAb 270}/[^{125}\text{I}]\text{A85380}$ ranged between 27 and 39, indicating that $[^{125}\text{I}]\text{A85380}$ was labeling in the range of 30 times as many sites as was $[^{125}\text{I}]\text{mAb 270}$.

Immunoprecipitation of nAChR Solubilized from Brain Regions Parallels the Nicotine-Induced Increases in the Amounts of nAChRs Assayed Autoradiographically. The results presented above strongly indicate that $[^{125}\text{I}]\text{A85380}$ binding increases in parallel with the $\alpha 4$ -nAChR and $\beta 2$ -nAChR protein measured by binding of $[^{125}\text{I}]\text{mAb 299}$ and $[^{125}\text{I}]\text{mAb 270}$, respectively. However, the slopes of the scattergrams relating ligand binding to subunit density differ significantly from unity. This result may merely be a consequence of the differences in assay conditions such as the fact that mAb concentrations are subsaturating whereas $[^{125}\text{I}]\text{A85380}$ concentration is saturating, the specific activity of functional mAbs may have been overestimated, and lower tissue and cell membrane permeability of mAbs occurred. However, the difference may reflect some inability of the mAb to recognize all mouse $\alpha 4$ -nAChR or $\beta 2$ -nAChR subunits. To address this possibility, the ability of

mAb 299 and mAb 270 to immunoprecipitate detergent-solubilized ligand binding sites in control and nicotine-treated mouse brain regions was assessed.

Immunoprecipitation was examined in cortical, hippocampal, striatal, and thalamic homogenates of mice chronically treated with 0 (control), 0.25, 1.0, or 4.0 mg/kg/h nicotine. These regions and nicotine treatment doses were chosen to include brain areas that show relatively robust responses to nicotine (cortex and hippocampus) or show little response to nicotine (thalamus). Striatum and olfactory tubercles were also included because these brain regions contain non- $\alpha 4\beta 2$ -nAChR binding sites. Table 4 presents these data.

Precipitation of $[^{125}\text{I}]\text{epibatidine}$ binding sites by mAb 270 in the saline-treated mice was virtually quantitative in four of the brain regions (mean $\pm$ S.E.M. of the Triton X-100-solubilized $[^{125}\text{I}]\text{epibatidine}$ binding sites: $99 \pm 12\%$ in cortex, $116 \pm 8\%$ in hippocampus, $98 \pm 5\%$ in thalamus, and $100 \pm 19\%$ in striatum), but was less complete in olfactory tubercles ($67 \pm 5\%$). mAb 299 was less efficient in precipitating these sites (mean $\pm$ S.E.M. of the Triton X-100-solubilized $[^{125}\text{I}]\text{epibatidine}$ binding sites: $75 \pm 10\%$ in cortex, $90 \pm 6\%$ in hippocampus, $86 \pm 4\%$ in thalamus, $57 \pm 14\%$ in striatum, and $57 \pm 4\%$ in olfactory tubercles).

Response to Chronic Nicotine About -1.6 mm Bregma

Fig. 3. $[^{125}\text{I}]\text{A85380}$, $[^{125}\text{I}]\text{mAb 299}$, and $[^{125}\text{I}]\text{mAb 270}$ binding sites show regional differences to chronic nicotine treatment for sections at approximately -1.6 mm Bregma. Relative binding for each probe was calculated by normalizing to the binding site density for saline-treated mice. Data points represent mean $\pm$ S.E.M. for 7 to 10 mice at each nicotine infusion dose for $[^{125}\text{I}]\text{A85380}$ ($\bullet$), $[^{125}\text{I}]\text{mAb 299}$ ($\square$), and $[^{125}\text{I}]\text{mAb 270}$ ($\triangle$). Curves are nonlinear least-squares curve fits of all the data as described under *Materials and Method*. outCx, cerebral cortex, outer layers; inCx, cerebral cortex, deeper layers; MD, mediodorsal thalamic nucleus; Ht, hypothalamus. Shown are representative sections from saline-treated mice.

Results of the one-way ANOVAs analyzing the effects of chronic nicotine treatment are summarized in Table 4. Significant effects of chronic nicotine treatment were noted for total Triton X-100-solubilized $[^{125}\text{I}]\text{epibatidine}$ binding sites, precipitated by mAb 270 and binding sites precipitated by mAb 299 in cortex, hippocampus, and olfactory tubercles. No significant effects of chronic nicotine treatment were observed for any measure in thalamus. For striatum, the effects of chronic nicotine treatment on $[^{125}\text{I}]\text{epibatidine}$ or $[^{125}\text{I}]\text{mAb 270}$ binding were not statistically significant, in contrast to the significant effects on $[^{125}\text{I}]\text{mAb 299}$ binding.

The relative changes in $[^{125}\text{I}]\text{epibatidine}$ binding sites precipitated by PEG, mAb 270, and mAb 299 as a function of nicotine treatment are shown in Fig. 7. In addition, the curve calculated for the effect of nicotine treatment on the PEG-precipitated $[^{125}\text{I}]\text{epibatidine}$ binding sites is shown. The relative changes in $[^{125}\text{I}]\text{epibatidine}$ binding sites precipitated by PEG, mAb 270, and mAb 299 in cortex (increases), hippocampus (increases), and thalamus (no changes) were very similar in both the extent of increase and the nicotine dependence of the response. In contrast, the relative changes in response to nicotine of the $[^{125}\text{I}]\text{epibatidine}$ binding sites

precipitated by PEG, mAb 270, and mAb 299 in olfactory tubercles and striatum differed. In olfactory tubercles, the pattern of increase in $[^{125}\text{I}]\text{epibatidine}$ binding sites precipitated by PEG, mAb 270, and mAb 299 was similar, but the relative number of sites precipitated by the two antibodies was slightly higher than the relative increase in the total, PEG-precipitable counts. In striatum, the pattern and relative increase in $[^{125}\text{I}]\text{epibatidine}$ binding sites precipitated by PEG and mAb 299 were similar. Relatively fewer sites were precipitated by mAb 270 after treatment with 0.25 or 1.0 mg/kg/h nicotine, but precipitation of sites by mAb 270 in striatum of mice treated with 4.0 mg/kg/h nicotine was similar to that for PEG and mAb 299.

Discussion

We have demonstrated, using an extensive analysis of region-specific up-regulation of nAChRs in mouse brain, that chronic nicotine treatment elicits corresponding increases in $\beta 2^*$ -nAChR binding sites and $\alpha 4$ -nAChR and $\beta 2$ -nAChR subunit proteins measured with mAb binding. These results strongly support the hypothesis that nicotine-induced up-

Fig. 4. $[^{125}\text{I}]\text{A85380}$, $[^{125}\text{I}]\text{mAb 299}$, and $[^{125}\text{I}]\text{mAb 270}$ binding sites show regional differences to chronic nicotine treatment for sections at approximately -2.6 mm Bregma. Relative binding for each probe was calculated by normalizing to the binding site density for saline-treated mice. Data points represent mean $\pm$ S.E.M. for 7 to 10 mice at each nicotine infusion dose for $[^{125}\text{I}]\text{A85380}$ ($\bullet$), $[^{125}\text{I}]\text{mAb 299}$ ($\square$), and $[^{125}\text{I}]\text{mAb 270}$ ($\triangle$). Curves are nonlinear least-squares curve fits of all the data as described under *Materials and Method*. SC, superior colliculus; sg, superficial gray; IPN, interpeduncular nucleus; PAG, periaquiductal gray; DpMe, deep mesencephalic area. Shown are representative sections from saline-treated mice.

regulation of nicotinic ligand binding arises from increases in mature assembled nAChR protein. These results are expected from experiments with cell cultures that show that nicotine exposure increases the assembly of $\alpha\beta 2$ -nAChR from subunit pools and decreases the rate of turnover of mature nAChRs on the cell surface (Kuryatov et al., 2005; Sallette et al., 2005; Lester et al., 2009).

The region-selective changes in the binding of $[^{125}\text{I}]\text{A85380}$, which is a $\beta 2^*$ -nAChR subtype-selective ligand (Mukhin et al., 2000), are consistent with previous autoradiographic studies using other ligands that bind to $\alpha\beta 2^*$ -nAChR (Pauly et al., 1991; Sparks and Pauly, 1999; Nguyen et al., 2003). Significant increases in $[^{125}\text{I}]\text{A85380}$ binding were noted in 22 of the 38 brain regions. Those brain regions resistant to nicotine-induced increase in $[^{125}\text{I}]\text{A85380}$ binding have been reported to be relatively unaffected by nicotine treatment when other ligands have been used to measure these sites (Pauly et al., 1991; Sparks and Pauly, 1999; Nguyen et al., 2003).

Most of the binding sites that increase after chronic nicotine treatment can be immunoprecipitated by antibodies to either the $\alpha 4$ - or $\beta 2$ -nAChR subunit (Flores et al., 1992; Mao

et al., 2008; Moretti et al., 2010) consistent with the observation that nAChRs remaining in $\beta 2$ knockout mice are resistant to the effects of chronic nicotine treatment (McCallum et al., 2006). Although immunoprecipitation experiments provide important information about the subunit compositions of nAChR subtypes and illustrate regional differences in nAChR regulation, they lack the anatomical resolution achieved with autoradiography or immunocytochemistry. Unfortunately, standard immunocytochemical methods with antibodies directed at nAChR subunits have been problematic (Jones and Wonnacott, 2005; Moser et al., 2007). However, $[^{125}\text{I}]\text{mAb 270}$, which binds to the $\beta 2$ -nAChR subunit, labels nAChR in unfixed rodent brain with regional distributions paralleling those of high-affinity agonist binding sites (Swanson et al., 1987). Furthermore, studies with null mutant mice demonstrate that $[^{125}\text{I}]\text{mAb 270}$ and $[^{125}\text{I}]\text{mAb 299}$ binding is mouse brain is specific to appropriate nAChR subunits (Whiteaker et al., 2006). This study demonstrated that both mAbs recognize assembled nAChR in brain sections rather than individual subunit proteins because of the loss of labeling after deletion of either the $\alpha 4$ or $\beta 2$ gene. However, both mAbs recognize unassembled or partially as-

Fig. 5. Binding site densities for [125 I]A85380 and [125 I]mAb 299 are highly correlated after chronic treatment with each of seven nicotine doses. Scattergrams comparing the density of [125 I]A85380 binding sites and [125 I]mAb 299 were constructed for the brain regions for which significant effects of nicotine treatment on [125 I]A85380 binding was observed. Each point is the mean $\pm$ S.E.M. of data from 7 to 10 individual mice at each chronic infusion dose. Lines are the best linear fits of the data. The slope $\pm$ S.E.M. of these regression lines as a function of nicotine infusion dose is also shown.

Fig. 6. Binding site densities for [125 I]A85380 and [125 I]mAb 270 are highly correlated after chronic treatment with each of seven nicotine doses. Scattergrams comparing the density of [125 I]A85380 binding sites and [125 I]mAb 270 were constructed for the brain regions for which significant effects of nicotine treatment on [125 I]A85380 binding was observed. Each point is the mean $\pm$ S.E.M. of data from 7 to 10 individual mice at each chronic infusion dose. Lines are the best linear fits of the data. The slope $\pm$ S.E.M. of these regression lines as a function of nicotine infusion dose is also shown.

TABLE 4

Comparison of [125 I]epibatidine binding in Triton X-100 extracts and samples immunoprecipitated with mAb270 or mAb299 in five brain regions of mice chronically treated with nicotine

[125 I]epibatidine binding (fmol/mg protein) was measured in brain samples that had been extracted with 2% Triton X-100 and recovered by precipitation with either PEG (20%), mAb270 (5 μ g/ml), or mAb299 (1 μ g/ml) as indicated. Values are mean $\pm$ S.E.M. for five (saline and 0.25 mg/kg/h nicotine) or six (1.0 and 4.0 mg/kg/h nicotine) mice. Results from the one-way ANOVAs are also summarized. Values differing significantly from saline-infused mice ($P < 0.05$, Duncan's test after one-way ANOVA) are indicated in bold.

Brain Region/Sample	Saline	Nicotine			ANOVA Results
		0.25 mg/kg/h	1.0 mg/kg/h	4.0 mg/kg/h	
Cortex					
PEG	35.5 $\pm$ 4.1	35.7 $\pm$ 1.3	44.7 $\pm$ 1.6	54.2 $\pm$ 6.0	$F_{3,20} = 5.69, P < 0.01$
mAb 270	35.3 $\pm$ 1.7	35.3 $\pm$ 0.3	43.8 $\pm$ 1.6	50.2 $\pm$ 4.1	$F_{3,21} = 7.97, P < 0.001$
mAb 299	26.7 $\pm$ 1.3	28.3 $\pm$ 0.5	33.6 $\pm$ 0.8	41.8 $\pm$ 4.0	$F_{3,21} = 8.82, P < 0.001$
Hippocampus					
PEG	25.1 $\pm$ 0.9	28.5 $\pm$ 1.3	31.2 $\pm$ 1.7	34.2 $\pm$ 1.8	$F_{3,21} = 6.32, P < 0.01$
mAb 270	29.3 $\pm$ 1.6	29.2 $\pm$ 1.9	31.9 $\pm$ 2.9	37.9 $\pm$ 2.5	$F_{3,21} = 3.29, P < 0.05$
mAb 299	22.5 $\pm$ 1.3	25.2 $\pm$ 1.5	29.2 $\pm$ 2.1	33.9 $\pm$ 1.6	$F_{3,21} = 8.48, P < 0.001$
Olfactory tubercles					
PEG	44.2 $\pm$ 2.3	52.0 $\pm$ 4.4	72.0 $\pm$ 4.4	66.9 $\pm$ 6.3	$F_{3,18} = 7.77, P < 0.01$
mAb 270	29.8 $\pm$ 1.7	36.7 $\pm$ 3.3	54.6 $\pm$ 3.7	52.5 $\pm$ 3.7	$F_{3,18} = 12.51, P < 0.001$
mAb 299	25.0 $\pm$ 1.0	28.9 $\pm$ 2.2	44.9 $\pm$ 2.4	45.1 $\pm$ 2.9	$F_{3,18} = 19.86, P < 0.001$
Striatum					
PEG	56.8 $\pm$ 6.5	86.5 $\pm$ 10.7	91.7 $\pm$ 17.4	101.6 $\pm$ 10.5	$F_{3,18} = 2.56, P > 0.05$
mAb 270	57.0 $\pm$ 9.4	64.8 $\pm$ 4.4	67.0 $\pm$ 10.9	96.2 $\pm$ 11.3	$F_{3,18} = 3.07, P = 0.05$
mAb 299	32.5 $\pm$ 5.8	52.4 $\pm$ 3.7	58.3 $\pm$ 12.0	74.6 $\pm$ 5.1	$F_{3,18} = 4.87, P < 0.05$
Thalamus					
PEG	225.7 $\pm$ 6.9	239.6 $\pm$ 46.3	245.4 $\pm$ 17.4	276.8 $\pm$ 36.4	$F_{3,21} = 0.50, P > 0.05$
mAb 270	220.9 $\pm$ 9.8	225.2 $\pm$ 46.0	210.1 $\pm$ 28.1	259.3 $\pm$ 13.9	$F_{3,21} = 0.62, P > 0.05$
mAb 299	193.4 $\pm$ 8.1	216.3 $\pm$ 42.9	198.1 $\pm$ 33.9	204.3 $\pm$ 25.7	$F_{3,21} = 0.10, P > 0.05$

sembled subunits in detergent extracts. This difference in subunit labeling between tissue samples and extracts could occur because the mAbs only label mature nAChR expressed on the cell surface because mAbs cannot cross cell membranes. Alternatively, rapid subunit degradation may occur in the absence of a partner subunit with which to assemble.

Because [125 I]A85380 labels $\beta 2^*$ -nAChR, we found a robust and highly significant correlation between [125 I]A85380 and [125 I] mAb 270 binding in saline-treated control mice. The relationship between [125 I]A85380 and [125 I]mAb 299 binding was less robust with several brain regions expressing significantly more [125 I]A85380 binding than predicted by [125 I]mAb 299 binding. However, brain regions that deviated from the regression line exhibit residual [125 I]mAb 270 binding in the brains of $\alpha 4$ null mutant mice, indicating the presence of $\beta 2^*$ -nAChRs that do not include the $\alpha 4$ subunit (Whiteaker et al., 2006). When those regions that express significant $\beta 2^*$ -nAChR that do not contain the $\alpha 4$ subunit were omitted, we observed a robust correlation between [125 I]A85380 and [125 I]mAb 299 binding.

We found that chronic nicotine treatment increases the number of [125 I]A85380 binding sites, as well as the number of [125 I]mAb 270 and [125 I]mAb 299 binding sites. The effects of chronic nicotine treatment varied across brain regions. When only those regions that showed significant nicotine-induced increases in [125 I]A85380 binding were analyzed, highly significant correlations between [125 I]A85380 and [125 I]mAb 299 binding site densities and between [125 I]A85380 and [125 I]mAb 270 binding site densities were observed for each nicotine dose. This result supports the conclusion that the increases in ligand binding density reflect increases in nAChR subunit protein. This conclusion is consistent with the observations that chronic nicotine treatment increases the expression of fluorescently labeled $\alpha 4$ -nAChR subunit protein (Nashmi et al., 2007) and increases $\alpha 4$ - and $\beta 2$ -nAChR subunit protein measured with quantitative Western blots (Moretti et al., 2010).

The regression coefficients for the [125 I]A85380/[125 I]mAb 299 lines tend to be higher than those for the [125 I]A85380/[125 I]mAb 270 lines. These less robust correlations may reflect in part the somewhat higher variability encountered in measuring the [125 I]mAb 270 signal, but it is possible that they arise from a fundamental difference in regional regulation of the two subunit proteins. The slopes of the [125 I]A85380/[125 I]mAb 299 regression lines (Fig. 5) are unaffected by the chronic nicotine dose, suggesting a stable relationship between ligand binding sites and $\alpha 4$ subunit protein levels. The slopes of the [125 I]A85380/[125 I]mAb 270 regression lines (Fig. 6) differ among the nicotine treatment doses with steeper slopes observed after treatment with the lower nicotine doses; the slopes tend to decrease as nicotine treatment dose increases. Whether these differences in the relationship between $\alpha 4$ and $\beta 2$ subunits and [125 I]A85380 binding reflects a nicotine-induced change in subunit stoichiometry remains to be determined.

Quantitative differences in the response of [125 I]A85380, [125 I]mAb 299, and [125 I]mAb 270 binding sites to chronic nicotine treatment are noticeable in individual brain regions (as illustrated in Figs. 2, 3, and 4). For example in cortical and thalamic areas in which [125 I]A85380 binds virtually exclusively to $\alpha 4\beta 2^*$ -nAChR sites, relative binding of [125 I]A85380, [125 I]mAb 299, and [125 I]mAb 270 show very similar responses to chronic nicotine treatment. In contrast, in caudate putamen, nucleus accumbens, olfactory tubercles, and superficial gray area of superior colliculus, in which additional $\beta 2^*$ -nAChR (such as $\alpha 3\beta 2^*$ - or $\alpha 6\beta 2^*$ -nAChR) are expressed, [125 I]mAb 270 binding shows a less robust response to chronic nicotine treatment at the lower chronic treatment doses than does either [125 I]A85380 and [125 I]mAb 299 binding. $\alpha 4\beta 2^*$ -nAChR shows more robust up-regulation after chronic nicotine treatment than other $\beta 2^*$ -nAChR subtypes (Xiao and Kellar, 2004; Perry et al., 2007; Perez et al., 2008; Moretti et al., 2010). Thus, the relatively smaller in-

Fig. 7. Immunoprecipitation of [125 I]epibatidine binding sites in select brain regions of mice chronically treated with nicotine parallels the patterns observed autoradiographically. Membrane preparations from five brain regions of mice treated with saline and 0.25, 1.0, or 4.0 mg/kg/h nicotine were solubilized with 2% Triton X-100. Samples were incubated with 200 pM [125 I]epibatidine, and solubilized binding sites were collected by incubation with 20% PEG (●) to quantitatively precipitate the binding sites, with mAb 299 followed by rabbit anti-rat serum (□) to immunoprecipitate sites containing the $\alpha 4$ -nAChR subunit or mAb 270 followed by rabbit anti-rat serum (△) to immunoprecipitate sites containing the $\beta 2$ -nAChR subunit. Each data point represents the mean $\pm$ S.E.M. of five to six individual mice treated with one of the four nicotine doses. The data have been normalized to the binding measured for saline-treated mice. The curves are the nonlinear least-squares curve fits for the PEG-precipitated counts.

crease in [125 I]mAb 270 binding after chronic nicotine treatment in brain regions expressing (non- $\alpha 4$) $\beta 2^*$ -nAChR subtypes is consistent with the relative resistance of the latter subtypes to nicotine-induced up-regulation.

Although the relative changes in binding of the three probes was similar, total binding of the three probes differed markedly. [125 I]mAb 299 labeled approximately 1/10 as many sites as [125 I]A85380, and [125 I]mAb 270 labeled approximately 1/30 as many sites as [125 I]A85380. Technical differences between ligand binding and antibody labeling could account for these differences, including, but not limited to, the use of nonsaturating mAb concentrations (this accounts for approximately a factor of two of the differences), differences in penetration of the probes into the tissue (the relatively small and membrane-permeable [125 I]A85380 is more likely to penetrate fully into the sections than are the mAbs

that are nearly 1000-fold larger and membrane impermeable), and/or inaccurate definition of specific activity of the functional mAbs owing to possible degradation of the mAb between its synthesis and use in experiment.

Immunoprecipitation of [125 I]epibatidine binding sites in several brain regions of saline- and nicotine-treated mice was measured to assess the ability of mAb 299 and mAb 270 to recognize all $\alpha 4\beta 2^*$ -nAChRs. [125 I]epibatidine was used to label the sites because solubilization in Triton X-100 significantly reduced affinity for [125 I]A85380. Both mAb 270 and mAb 299 effectively immunoprecipitated [125 I]epibatidine binding sites in each of the five regions assayed. Immunoprecipitation by mAb 270 was virtually quantitative, whereas that with mAb 299 was less complete. Immunoprecipitation results paralleled those observed autoradiographically. In cortex, hippocampus, and thalamus the relative changes in total [125 I]epibatidine binding sites and those precipitated by mAb 270 and mAb 299 elicited by nicotine treatment were very similar. However, mAb 270 precipitated relatively fewer striatal [125 I]epibatidine binding sites from nicotine-treated mice than did mAb 299. This result is analogous to that determined autoradiographically. In olfactory tubercle, both mAb 270 and mAb 299 precipitated relatively more [125 I]epibatidine binding sites after chronic nicotine treatment than did PEG. This probably occurred because olfactory tubercle contains a relatively high proportion of epibatidine sites that are non- $\beta 2^*$ -nAChR (Marks et al., 2000) that are not labeled with [125 I]A85380.

In summary, we have demonstrated that chronic nicotine treatment has very similar effects on the expression of [125 I]A85380 (total $\beta 2^*$ -nAChR binding sites), [125 I]mAb 299 ($\alpha 4$ -nAChR subunit protein), and [125 I]mAb 270 ($\beta 2$ subunit protein), in both brain regions that respond to chronic nicotine treatment and those that are resistant to the drug-induced increase in ligand binding. Immunoprecipitation studies confirm that mAb 270 and mAb 299 recognize ligand binding sites in samples prepared from several brain regions of mice treated with nicotine. Taken together, these results demonstrate that the increases in $\alpha 4\beta 2^*$ -nAChR binding sites after chronic nicotine treatment of mice result from an increase in the number of nAChRs containing $\alpha 4$ and $\beta 2$ subunit proteins.

Authorship Contributions

Participated in research design: Marks, McClure-Begley, and Whiteaker.

Conducted experiments: Marks, McClure-Begley, Whiteaker, Salminen, and Brown.

Contributed new reagents or analytic tools: Cooper and Lindstrom.

Performed data analysis: Marks, McClure-Begley, Salminen, and Brown.

Wrote or contributed to the writing of the manuscript: Marks, McClure-Begley, Whiteaker, Salminen, Collins, and Lindstrom.

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