Differential Regulation of Mesolimbic $\alpha3^*/\alpha6\beta2^*$ and $\alpha4\beta2^*$ Nicotinic Acetylcholine Receptor Sites and Function after Long-Term Oral Nicotine to Monkeys

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ABSTRACT

Because the mesolimbic dopamine system plays a critical role in nicotine addiction/reinforcement and because nicotinic receptors regulate dopamine release, we initiated a study to evaluate the long-term effects of nicotine (>6 months at the final dose) on nicotinic acetylcholine receptor (nAChR) sites and function in the nucleus accumbens of nonhuman primates. Nicotine was given in the drinking water as this mode of administration is long-term but intermittent, thus resembling smoking in this aspect. We determined the effects of nicotine treatment on function and binding of the $\alpha3/\alpha6\beta2^*$ and $\alpha4\beta2^*$ nAChRs subtypes in nucleus accumbens, a region directly implicated in the addictive effects of nicotine. To evaluate function, we measured nicotine and K$^+$-evoked $[^3H]$dopamine release from nucleus accumbens synaptosomes. Changes in $\alpha4\beta2^*$ and $\alpha3/\alpha6\beta2^*$ nAChRs were measured using $^{125}$I-epibatidine, $[^{125}]$A85380 [5-$[^{125}]$iodo-3(2(S)-azetidinylmethoxy) pyridine] and $^{129}$-α-conotoxin MII autoradiography. Chronic nicotine treatment, which led to plasma nicotine levels in the range of smokers, significantly increased nucleus accumbens $\alpha4\beta2^*$ nAChR sites and function compared with control. By contrast, this treatment did not significantly change $\alpha3/\alpha6\beta2^*$ nAChR sites or evoked dopamine release in this region compared with control. Thus, these data are distinct from previous results in striatum in which the same nicotine treatment paradigm decreased striatal $\alpha3/\alpha6\beta2^*$ nAChR sites and function. The finding that long-term nicotine treatment selectively modulates $\alpha4\beta2^*$ and not $\alpha3/\alpha6\beta2^*$ nAChR expression in primate nucleus accumbens is consistent with the results of studies in nicotinic receptor mutant mice implicating the $\alpha4\beta2^*$ nAChR subtype in nicotine-mediated addiction.

Nicotine, the primary reinforcing component in tobacco smoke, activates the mesolimbic dopamine system, as do most drugs of abuse (Buisson and Bertrand, 2002; Samaha and Robinson, 2005; Wonnacott et al., 2005). Nicotine-mediated stimulation of ventral tegmental dopaminergic neurons results in an increase in dopamine release from terminals located in the nucleus accumbens. Moreover, local administration of nicotine into the ventral tegmental area elicits an increase in dopamine efflux in the nucleus accumbens, which is blocked by nicotinic receptor antagonists, suggesting that this response is mediated via nicotinic acetylcholine receptors (nAChRs) (Corrigall et al., 1994).

Diverse populations of nAChRs are distributed throughout the mesolimbic dopamine pathway. $\alpha4\beta2^*$, $\alpha6\beta2^*$, and $\alpha7^*$ nAChR subtypes have been identified on dopamine cell bodies in the ventral tegmental area and nerve terminals in the nucleus accumbens (Le Novere et al., 1996; Quik et al., 2000, 2001; Klink et al., 2001; Champtiaux et al., 2003; Wooltorton et al., 2003). Although there are multiple nAChR populations in the mesolimbic dopamine system, the $\alpha4\beta2^*$ subtype seems primarily responsible for mediating nicotine’s reinforcing effects. This contention is based on studies using nAChR null mutant mice, which demonstrated a critical role for the $\beta2$ subunit in nicotine self-administration and conditioned place preference (Picciotto et al., 1998; Klink et al., 2001; Walters et al., 2006). Moreover, $\beta2$ subunit re-expression in the ventral tegmental area of $\beta2$ null mutant mice led to a

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; DOPAC, dihydroxyphenylacetic acid; $[^{125}]$A85380, 5-$[^{125}]$iodo-3(2(S)-azetidinylmethoxy) pyridine; ANOVA, analysis of variance.
recovery of nicotine self-administration (Maskos et al., 2005). The selective activation of α4* nAChRs in mice expressing a mutant form of the receptor indicates that receptors containing α4* are sufficient for nicotine-induced reward, tolerance, and sensitization (Tapper et al., 2004).

Most studies concerning the reinforcing effects of nicotine use acute administration of nicotine. However, because smoking is a chronic behavior that leads to long-term adaptive changes in the brain, knowledge of the chronic changes is essential for understanding smoking behavior and implementing effective treatments for smoking cessation. One consequence of long-term nicotine exposure is an altered regulation of central nervous system nicotinic receptors in both animal models and smokers. The α4β2* nAChR population is increased in rodent brain, with chronic nicotine treatment, and is also elevated in the brains of smokers (Marks et al., 1983; Schwartz and Kellar, 1983; Benwell et al., 1988). Other neuronal nAChR subtypes seem to respond in a different manner with nicotine administration, with a down-regulation of α6β2* nAChRs (Nguyen et al., 2003; Lai et al., 2005; Mugnaini et al., 2006) and no change in α3* subtypes (Ulrich et al., 1997; Davila-Garcia et al., 2003).

These differential changes in nAChR subtypes with long-term nicotine treatment may lead to complex effects on receptor-mediated activity, particularly in brain regions in which several nAChR subtypes regulate the same function. For instance, striatal dopamine release is modulated by both α4β2* and α6β2* nAChRs in rodents (Champtiaux et al., 2003; Salminen et al., 2004b) and in monkeys (McCallum et al., 2006). Recent studies have shown that binding sites and function of these two distinct subtypes are differentially regulated in striatum, with long-term nicotine exposure resulting in an increase in α4β2* and a simultaneous decrease in α6β2* nAChR sites and function (Salminen et al., 2004a; Lai et al., 2005; McCallum et al., 2006; Mugnaini et al., 2006).

The objective of the present study was to investigate whether chronic nicotine administration resulted in a similarly complex regulation of nAChR binding sites and function in the nucleus accumbens, a region critically involved in regulating the reinforcing effects of nicotine. To approach this, we measured both α4β2* and α3α6β2* nAChR-mediated function and binding sites after nicotine treatment in the nucleus accumbens of nonhuman primates, an animal model with a close behavioral resemblance to man. Results show that only α4β2* and not α3α6β2* nAChR sites and function were altered with chronic nicotine treatment, suggesting that α4β2* nAChRs play a more prominent role in modulating the effects of long-term nicotine treatment in nucleus accumbens.

**Materials and Methods**

**Animals and Treatments.** The experiments with drug-naive female squirrel monkeys (n = 13) (Saimiri sciureus) were performed as described previously (McCallum et al., 2006), according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at the Parkinson’s Institute. Monkeys, ranging in weight from 0.5 to 0.7 kg, were obtained from Osage Research Primates (Osage Beach, MO) and from the Primate Research Laboratory (University of South Alabama, Mobile, AL). The animals’ history, together with their general appearance, suggested that they were in late adulthood (10–15 years). They were immediately quarantined in accordance with California State regulations. The animals were housed in a room with a 13:11-h light/dark cycle and given food once daily (at quantities to maintain body weight) with free access to water. Quarantine was followed by a 1-month acclimation period. The animals were subsequently given either 1% saccharin (n = 7) or nicotine (free base) plus 1% saccharin (n = 6) in their drinking water, with both groups run in parallel. In brief, nicotine was increased by 50 μg/ml-increments over a 3-month period up to 650 μg/ml, a concentration at which the animals were maintained for an additional 6 to 8 months. This treatment regimen resulted in plasma nicotine and cotinine levels of 8.3 ± 2.6 ng/ml (n = 6) and 275 ± 84.3 ng/ml (n = 6), respectively, as reported previously (McCallum et al., 2006). Nicotine levels were within the reported range (5–50 ng/ml) detected in smokers (Hukkanen et al., 2005). Fluid intake was not significantly different between the two groups. Nicotine was removed from the drinking water 24 h before death. The animals were euthanized according to the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association by injecting 1.5 ml of euthanasia solution intraperitoneally (390 mg of sodium pentobarbital and 50 mg of phenytoin sodium/ml) followed by 1.5 ml/kg of the same solution intravenously.

**Tissue Preparation.** The brains were removed from the skull and divided along the midline with a dissecting knife. One half was placed in a Plexiglas monkey brain mold and sectioned into 6-mm blocks, which were each quickly frozen on a glass slide in isopentane on dry ice and stored at −80°C for autoradiographic studies. The other half was cut into 2-mm-thick blocks. The nucleus accumbens was hand-dissected from block A15-A14 for the measurement of dopamine and metabolites and preparation of synaptosomes for the [3H]dopamine uptake and release experiments.

**Determination of Dopamine and Its Metabolites.** Approximately 5 mg of tissue was dissected from the nucleus accumbens from each animal and extracted into 250 μl of 0.4 N perchloric acid. Dopamine and its metabolites, homovanillic acid and dihydroxyphenylacetic acid (DOPAC), were determined using high-pressure liquid chromatography coupled to electrochemical detection (Quik et al., 2001). The rate of dopamine turnover was calculated as the ratio of dopamine to its metabolites (dopamine/DOPAC + homovanillic acid).

**Uptake and Release of [3H]Dopamine.** The nucleus accumbens (10–15 mg wet weight) was homogenized in 2 ml of ice-cold 0.32 M sucrose buffered with 5 mM HEPES, pH 7.5. Synaptosomes were prepared, and [3H]dopamine uptake measured as described previously (Grady et al., 2002; McCallum et al., 2005, 2006). In brief, synaptosomes (250 μg of tissue/ml) were preincubated at 37°C for 10 min in buffer containing 128 mM NaCl, 2.4 mM KCl, 3.2 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25 mM HEPES, pH 7.5, 10 mM glucose, 1 mM ascorbic acid, and 0.01 mM pargyline. This was followed by incubation (2 or 10 min) in the same buffer also containing 0.5 μCi of [3H]dopamine (final concentration of 100 nM). Uptake was terminated by the addition of ice-cold buffer followed by vacuumfiltration through type A/E glass fiber filters. After washing, radioactivity on the filters was determined using liquid scintillation counting.

[3H]Dopamine release was done by preincubation (37°C) of another aliquot of the same synaptosomal preparation as above for 10 min followed by a 5-min incubation with 100 nM [3H]dopamine (Grady et al., 2002; McCallum et al., 2005, 2006). An aliquot of synaptosomes (~0.5–1 mg of tissue) was placed onto A/E glass fiber filters. After a 10-min baseline period, release was stimulated by an 18-s exposure to nicotine or K+ . Filters were perfused with or without 50 nM α-conotoxin MII for 3 min just before nicotine exposure to determine α4β2* and α3/α6β2* nAChR-mediated release. Fifteen 18-s fractions were collected. Radioactivity was determined by liquid scintillation counting.

[3H]Dopamine release was quantified as described previously (McCallum et al., 2005) by plotting release as fraction number against counts per minute, using a curve-fitting algorithm of SigmaPlot 5.0
for MicroSoft Disk Operating System (MS-DOS) (Jandel Scientific, San Rafael, CA) to fit the data. Basal release was assessed from fractions just before and after the stimulated release. Release was normalized to the tissue wet weight to obtain units of counts per minute/milligram of tissue. \( R_{\text{max}} \) and EC50 values were obtained from dose-response curves by fitting data to a nonlinear regression equation in SigmaPlot 2001 for Windows (SPSS Inc., Chicago, IL).

**Receptor Autoradiography.** Sections (20 \( \mu \text{m} \)) were cut using a cryostat at \(-15^\circ\text{C} \). After thaw-mounting onto Superfrost Plus slides, sections were air-dried and stored at \(-80^\circ\text{C} \). Nonspecific binding was determined using 100 \( \mu \text{M} \) nicotine or 100 \( \mu \text{M} \) epibatidine. After each experiment, slides were exposed to Kodak MR film (Eastman Kodak Rochester, NY) with \(^{125}\text{I}-\alpha\)-conotoxin MII autoradiography was performed as described previously (Quik et al., 2001). Thawed sections were preincubated at 22°C for 15 min in 20 mM HEPES buffer, pH 7.5, containing 144 mM NaCl, 1.5 mM KCl, 2 mM CaCl2, 1 mM MgSO4, 0.1% bovine serum albumin, and 1 mM phenylmethylsulfonyl fluoride. They were then incubated for 1 h with 0.5 \( \text{nM} \) \(^{125}\text{I}-\alpha\)-conotoxin MII in buffer with 0.5% bovine serum albumin, 5 mM EDTA, 5 mM EGTA, and 10 \( \mu \text{g/ml} \) each of aprotinin, leupeptin, and pepstatin A, followed by washing in HEPES buffer.

\(^{125}\text{I}]\text{[3H\text{D}opamine (30–60 Ci/mmol} \) binding to brain sections was done at 22°C for 60 min. The buffer consisted of 50 mM Tris, pH 7.5, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, and 1.0 mM MgCl2, with or without 100 \( \mu \text{M} \) epibatidine. Sections were washed in buffer as described previously (Kulak et al., 2002).

For \(^{125}\text{I}]\text{epibatidine binding, slides were preincubated at 22°C for 30 min in buffer containing 50 mM Tris, pH 7.5, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, and 1.0 mM MgCl2. This was followed by a 40-min incubation with 0.015 \( \text{nM} \) \(^{125}\text{I}-\text{epibatidine in the presence or absence of } \alpha\)-conotoxin MII (100 \( \text{nM} \). Sections were washed as described previously (McCallum et al., 2006). Optical density measurements from the autoradiograms were determined using ImageQuant (Amersham Biosciences Inc., Piscataway, NJ). They were converted to nCi/mg tissue using standard curves generated from \(^{125}\text{I} \) standards, with optical density readings within the linear range of the film. The receptor binding values for any one animal were averaged from two to four independent experiments for each radioligand.

**Data Analyses.** Statistical analyses were done with the Prism program (GraphPad Software, Inc., San Diego, CA). Student’s unpaired \( t \) test was used for comparison between the two different treatment groups, as indicated in the legends. Two-way repeated measures analysis of variance (ANOVA), followed by Bonferroni post hoc tests, was used for comparison of dose-response curves for the two treatment groups, with the independent variables being treatment versus nicotine concentration used in the release assay. Values are expressed as the mean \( \pm \) S.E.M. of the indicated number of animals. Values were considered statistically significantly different when \( p < 0.05 \).

**Materials.** \([3\text{H\text{Dopamine (30–60 Ci/mmol}} \) and \(^{125}\text{I}]\text{epibatidine (2200 Ci/mmol} \) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). \(^{125}\text{I} \) standards were from Amersham Biosciences Inc. Saccharin and nicotine were from Sigma (St. Louis, MO); A/E glass-fiber filters were from Gelman, Inc. (Ann Arbor, MI), Ecosafe cocktail was from RPI (Mt. Prospect, IL). Kodak MR film was from Amersham Biosciences Inc., and Superfrost Plus slides were from Fisher (Pittsburgh, PA).

### Results

**Effect of Long-Term Oral Nicotine Treatment on the Level of Dopamine and Metabolites in Nucleus Accumbens.** Experiments were first done to investigate the effect of chronic nicotine treatment on the levels of dopamine and its metabolites DOPAC and homovanillic acid in nucleus accumbens tissue from control and nicotine-treated monkeys (Table 1). Although there was no effect of nicotine treatment on dopamine levels in accumbens \((p > 0.05)\), the metabolite homovanillic acid but not DOPAC was significantly decreased \((p < 0.01)\). This resulted in a slightly higher, although not significant, rate of dopamine turnover in the brains of nicotine-treated animals as reflected in the dopamine to metabolites ratio (Table 1).

**Effect of Long-Term Oral Nicotine Treatment on Synaptosomal \([3\text{H\text{Dopamine Uptake.}}\) Since nicotine exposure altered \([3\text{H\text{dopamine uptake characteristics in striatum (McCallum et al., 2006), we determined both maximal and rate of } [3\text{H\text{dopamine uptake into synaptosomes prepared from nucleus accumbens of treated and control animals (Table 2). Nicotine treatment had no effect on either measure in nucleus accumbens. Maximal uptake of } [3\text{H\text{dopamine, expressed as femtomoles/microgram of tissue, was determined by incubating synaptosomes with } [3\text{H\text{dopamine at equilibrium (10 min); it was similar in both control and nicotine-treated animals. The rate of } [3\text{H\text{dopamine uptake (femtomole/microgram of tissue/minute)}\) was determined after a 2-min incubation period when uptake was half-maximal; no differences were observed between the two treatment groups (Table 2).**

**Long-Term Oral Nicotine Treatment Increases \(\alpha4\beta2* \) but Not \(\alpha3\alpha6\beta2* \) Receptor-Mediated \([3\text{H\text{Dopamine Release in Monkey Nucleus Accumbens.}}\) We next determined the effect of long-term nicotine treatment on nicotine-evoked \([3\text{H\text{dopamine release from synaptosomes prepared from the nucleus accumbens of control and nicotine-treated monkeys (Fig. 1). We differentiated accumbal dopamine release mediated by \(\alpha4\beta2* \text{nAChRs from that component mediated by } \alpha3\alpha6\beta2* \text{nAChRs using the selective \(\alpha3/\alpha6\beta2* \) antagonist, } \alpha\)-conotoxin MII. In control animals, the proportion of release mediated by \(\alpha3/\alpha6\beta2* \text{nAChRs (75%) was appreciably greater than that mediated by } \alpha4\beta2* \text{nAChRs (25%), with similar proportions in nicotine-treated monkeys (Fig. 1). This differs from the relative proportions of release observed in the rodent nucleus accumbens for which**

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Dopamine</th>
<th>DOPAC</th>
<th>Homovanillic Acid</th>
<th>Turnover</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>77.15 ± 4.59</td>
<td>20.30 ± 3.39</td>
<td>52.18 ± 2.05</td>
<td>1.10 ± 0.10</td>
</tr>
<tr>
<td>Nicotine</td>
<td>3</td>
<td>80.67 ± 7.99</td>
<td>12.08 ± 3.52</td>
<td>39.86 ± 2.52**</td>
<td>1.63 ± 0.36</td>
</tr>
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</table>

Significance of difference from control using Student’s \( t \) test; **\( P < 0.01 \).
<50% is mediated by α-conotoxin MII-sensitive nAChRs (Grady et al., 2002).

The EC50 values for nicotine-evoked dopamine release from nucleus accumbens were not significantly different (p > 0.05) for α-conotoxin MII-sensitive (α3/α6β2* nAChR-mediated) release compared with α-conotoxin MII-resistant (α4β2* nAChR-mediated) release. In control animals, the EC50 value (±S.E.M.) for α3/α6β2*-mediated release was 0.33 ± 0.16 μM, whereas the EC50 for the α4β2*-mediated component was 1.13 ± 0.39 μM (n = 7). However, there was a significant difference (p < 0.05) in nicotine-treated animals, with EC50 values of 0.26 ± 0.09 versus 1.04 ± 0.24 μM; n = 5, respectively.

Previously, we had reported that long-term nicotine treatment to monkeys resulted in differential effects on nAChR subtype-evoked dopamine release in monkey striatum, such that the α4β2* nAChR-mediated component was increased and that portion of release mediated by α3/α6β2* nAChRs was decreased (McCallum et al., 2006). In nucleus accumbens, however, a different pattern emerged following oral nicotine treatment. As in striatum, the component of dopamine release mediated by α4β2* nAChRs was increased in the nucleus accumbens of nicotine-treated monkeys compared with controls (Fig. 1, top panels). In treated animals, the Rmax for nicotine-evoked release was 2927 ± 185 cpm/mg (n = 5), a 27% increase compared with the Rmax value for nicotine-evoked dopamine release in control animals (2142 ± 192 cpm/mg; n = 7; p < 0.05) (Fig. 1).

Whereas α4β2* nAChR-mediated release was increased in nucleus accumbens, long-term nicotine treatment was associated with no change in the Rmax of α-conotoxin MII-sensitive dopamine release or that mediated by α3/α6β2* nAChRs. In fact, a small (14%) but nonsignificant increase in the Rmax value obtained from nicotine-treated animals (8077 ± 641; n = 5) was observed compared with the Rmax calculated for dopamine release in control animals (6924 ± 762; n = 7) (Fig. 1, bottom panels). These findings are in contrast to the significant declines in this component of dopamine release observed in primate and rodent striatum from nicotine-treated animals (Salminen et al., 2004a; Lai et al., 2005; McCallum et al., 2006).

**Effect of Long-Term Nicotine Treatment on Baseline and K⁺-Evoked Dopamine Release.** Long-term nicotine exposure may have affected basal release and thus modulated the increase in nicotine-evoked release observed for the α4β2*-mediated component. To rule out this possibility, basal release was calculated by taking the mean of several fractions of release before and after the stimulated release in the presence and absence of α-conotoxin MII. There was no effect of nicotine treatment on basal release, with comparable
values between nicotine-treated and control animals (p > 0.05; Table 3). Likewise, there was no significant change in baseline release in the presence of α-conotoxin MII in either treatment group (p < 0.05; Table 3).

Next, we evaluated the effect of extended oral nicotine treatment on [3H]dopamine release evoked by 20 mM K+. No differences in mean K+-evoked dopamine release were observed between the two treatment groups (p > 0.05; Table 3). This suggests that the observed changes in nicotine-evoked release with long-term nicotine treatment were selectively mediated via nAChR activation and were not the result of a generalized effect of chronic nicotine treatment on the exocytotic machinery.

Long-Term Nicotine Treatment Increases α4β2* but Not α3α6β2* nAChR Sites in Monkey Nucleus Accumbens. Nicotinic receptor sites in the nucleus accumbens were measured using [125I]A85380, which labels α4β2* through α6* nAChRs, and [125I]A85380, which binds to β2* subtypes. To distinguish between α4β2* and α3α6β2* nAChRs using these ligands, unlabeled α-conotoxin MII was added during the binding experiments to allow for the measurement of both α-conotoxin MII-resistant (α4β2*) and α-conotoxin MII-sensitive (α3α6β2*) [125I]-epibatidine and [125I]A85380 binding sites. In addition, α3α6β2* nAChR sites were measured directly using [125I]-α-conotoxin MII.

When α-conotoxin MII-resistant sites (α4β2* nAChRs) were examined in the nucleus accumbens of nicotine-treated and control monkeys, significant increases in binding were found after long-term nicotine treatment (Fig. 2, top panels). α-Conotoxin MII-resistant [125I]-epibatidine sites in monkeys given oral nicotine increased by 33% compared with controls (p < 0.05). Likewise, α-conotoxin MII-resistant [125I]A85380 sites showed a substantial increase of 72% in nicotine-treated animals compared with controls (p < 0.001). The α4β2* sites measured using [125I]A85380 were significantly greater (p < 0.01) than those determined using [125I]-epibatidine in nucleus accumbens of animals chronically treated with oral nicotine. In contrast to the up-regulation of α4β2* nAChRs in nucleus accumbens of nicotine-treated animals, there was no significant change in α3α6β2* nAChR sites (Fig. 2, bottom panels). There were no differences in [125I]-α-conotoxin MII binding in the nucleus accumbens between the treatment groups, with the number of α3α6β2* binding sites in nicotine-treated monkeys being 102% of control (p > 0.05). Likewise, α-conotoxin MII-sensitive nAChR binding sites identified using either [125I]A85380 or [125I]-epibatidine were similar in the nucleus accumbens of control and nicotine-treated monkeys. Therefore, the number of α3α6β2* nAChRs in the nucleus accumbens was not altered by long-term nicotine treatment, consistent with the lack of change in α3α6β2* nAChR-evoked dopamine release (Fig. 1).

**Discussion**

The present results are the first to show the consequences of extended nicotine treatment (~1 year) delivered in the drinking water on nicotinic receptor sites and function in nonhuman primate nucleus accumbens, a region subject to a

**TABLE 3**

<table>
<thead>
<tr>
<th>Dopamine Release</th>
<th>Monkey Treatment</th>
<th>Control</th>
<th>Nicotine</th>
</tr>
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<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td>895 ± 70 (4)</td>
<td>769 ± 55 (4)</td>
</tr>
<tr>
<td>Baseline + α-conotoxin MII</td>
<td></td>
<td>917 ± 66 (3)</td>
<td>821 ± 92 (4)</td>
</tr>
<tr>
<td>K+-stimulated</td>
<td></td>
<td>12005 ± 1450 (7)</td>
<td>11779 ± 1978 (5)</td>
</tr>
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Fig. 2. Nicotine treatment increases α4β2*-nAChR sites in monkey nucleus accumbens with no change in the α3α6β2* nAChR subtype. Monkeys were administered nicotine as described under Materials and Methods. α4β2* nAChR binding sites (Ctx-res) were determined by measuring binding of [125I]-epibatidine (epi, top left panel) and [125I]A85380 (top right panel) in the presence of α-conotoxin MII (10⁻⁷ M); α3α6β2* nAChR binding sites (Ctx-sens) were determined by measuring the difference between total binding and binding in the presence of α-conotoxin MII (10⁻⁷ M) for [125I]-epibatidine (bottom left panel) and [125I]A85380 (bottom middle panel). Binding of [125I]-α-conotoxin MII is shown in the bottom right panel. Values represent the mean ± S.E.M. of five to seven animals. Significance of difference from control using Student’s t test, *p < 0.05; ***, p < 0.001.
complex regulation by multiple nAChR subtypes (Quik et al., 2000, 2001; Klink et al., 2001; Champtiaux et al., 2003; Wooltorton et al., 2003). Moreover, our examination of the nAChR regulation of the nucleus accumbens, a critical region in nicotine-mediated addiction, is distinct from similar studies in rodents in which the focus is frequently on other dopaminergic brain regions. We found that long-term nicotine administration resulted in an up-regulation of α4β2* nAChRs in the nucleus accumbens that was associated with a corresponding increase in α4β2* receptor-evoked [3H]dopamine release. In contrast, long-term nicotine treatment did not change nucleus accumbens α3/δβ2* receptor binding or receptor-evoked dopamine release. Since α4β2* and α3/δβ2* are the predominant nAChR populations on dopamine terminals in the nucleus accumbens, these data indicate that the primary receptor influenced by chronic nicotine treatment in this region is the α4β2* population. This preferential up-regulation of α4β2* nAChRs may suggest that this subtype is more important in nicotine-mediated reward in non-human primates. This interpretation is consistent with previous work in nAChR mutant mice, suggesting that α4β2* nAChRs are important in nicotine addiction (Picciotto et al., 1998; Klink et al., 2001; Tapper et al., 2004; Maskos et al., 2005; Walters et al., 2006). Enhanced expression of α4β2* nAChRs on presynaptic dopaminergic terminals may lead to an elevated release of dopamine in the mesolimbic system, a cellular mechanism that may underlie nicotine reinforcement.

The question arises regarding the molecular basis for the differential regulation of the α4* and α6* nAChR subtypes by nicotine in the mesolimbic compared with the nigrostriatal dopaminergic system. Evidence from experiments in cell culture suggests that nicotine can act as a pharmacological chaperone to cause up-regulation by inducing an active or desensitized receptor conformation (most likely of high affinity) that assembles more efficiently (Sallette et al., 2004; Kuryatov et al., 2005). In the mesolimbic system there may be no limiting amount of any subunit. Thus, nicotine may enhance assembly of high-affinity α4β2* nAChRs without decreasing the formation of other nAChRs, such as the α3/α6* subtypes that may not desensitize and thus be of lower affinity. In the nigrostriatal pathway, the amount of β2 subunit may be limiting such that nicotine exposure could up-regulate assembly of the high-affinity α4β2* nAChRs only at the expense of the lower affinity α3/α6* nAChRs.

In our studies, we used α-conotoxin MII-resistant 125I-epibatidine or [125I]A85380 binding to identify α4β2* receptors. This was based on previous work showing that 125I-epibatidine binds to α2-α6* nAChRs (Davila-Garcia et al., 1997), whereas α-conotoxin MII interacts with α3/α6β2* subtypes (Quik and McIntosh, 2006). 125I-Epibatidine sites measured in the presence of α-conotoxin MII would therefore most likely represent α4β2* subtypes since the two major subtypes in this region are the α4β2* and α3/α6β2* (Quik et al., 2001; Kulak et al., 2002). Likewise, because [125I]A85380 labels β2* nAChRs (Kulak et al., 2002) and α-conotoxin MII inhibits binding to α3/α6β2* subtypes, α-conotoxin MII-resistant [125I]A85380 binding sites provide an index of α4β2* nAChRs. Unexpectedly, although both α-conotoxin MII-resistant 125I-epibatidine and [125I]A85380 binding detect α4β2* subtypes, nicotine treatment led to an appreciably greater increase in the sites identified with [125I]A85380 compared with 125I-epibatidine. These results are consistent with those in rodent striatum (Lai et al., 2005), which also demonstrate a larger increase in α4β2* sites identified with [125I]A85380. These data most likely suggest that 125I-[125I]A85380 and 125I-epibatidine bind to different α4β2* nAChR populations containing α2 and/or α3 subunits in monkey striatum (Quik et al., 2005) and α5 and/or β4 subunits in rodent striatum (Zoli et al., 2002). Previous work also suggests that [125I]A85380 and 125I-epibatidine bind to different nAChR subtypes in spinal cord, although their composition remains unknown (Khan et al., 2001).

Our results showing that α3/α6β2* nAChR sites and function in the mesolimbic dopaminergic system were not altered by long-term nicotine exposure were somewhat surprising because this subtype mediates a large proportion of nicotine-evoked release (75%) in monkey nucleus accumbens. One possibility is that α3/α6β2* nAChRs in the nucleus accumbens are more resistant to modification resulting from chronic drug exposure than are α4β2* nAChRs. Such a hypothesis is consistent with evidence that α3/α6β2* nAChRs are more resistant to nicotine-induced up-regulation than are α4* nAChRs in rats (Ulrich et al., 1997; Davila-Garcia et al., 2003; Nguyen et al., 2003). These observations do not rule out the possibility that the α3α6β2* subtype is important in nicotine-induced addictive processes but that its involvement in this process is not reflected by an up- or down-regulation of receptor sites or function.

Not only are α3/α6β2* and α4β2* nAChRs in the nucleus accumbens controlled in a different manner with long-term nicotine treatment but, in addition, regulation of α3/α6β2* nAChRs with nicotine exposure is different in the nucleus accumbens compared with striatum (summarized in Table 4). Although nicotine treatment did not change α3/α6β2* nAChR binding or nicotine-mediated dopamine release in the nucleus accumbens, this same treatment down-regulated both α3/α6β2* nAChR sites and function in monkey striatum (McCallum et al., 2006). In contrast, α4β2* nAChR number and function are up-regulated in both striatum and nucleus accumbens with nicotine treatment. A point of note is that changes in receptor binding and function correlate well in both nucleus accumbens and striatum when considered separately for the two subtypes, that is, α3α6β2* versus α4β2* nAChRs. This close correspondence is no longer observed in either region when changes in total nAChR sites are compared with total nicotine-evoked function after nicotine treat-

<table>
<thead>
<tr>
<th>Subtype Region</th>
<th>No Change in nAChR-Mediated DA Release</th>
<th>No Change in nAChR Binding Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>α4β2* Striatum*</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>α3α6β2* Striatum*</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>α2α5 Striatum*</td>
<td>No change</td>
<td>No change</td>
</tr>
</tbody>
</table>

* Taken from McCallum et al. (2006).
ment (Table 4), most likely because the change in receptor subtype selective sites or function with the greatest magnitude predominates.

Differential changes in mesolimbic and nigrostriatal nAChR sites and function in response to nicotine administration that we observed in a synaptosomal preparation have also been observed in vivo. For instance, microdialysis studies in rodents have shown that nicotine causes a greater output of dopamine in the nucleus accumbens compared with striatum (Di Chiara and Imperato, 1988; Benwell and Balfour, 1997; Janhunen and Ahtee, 2004). Janhunen and Ahtee (2004) observed differential effects on striatal and accumbal extracellular dopamine with epibatidine, such that epibatidine produced a smaller output of dopamine in the nucleus accumbens compared with the striatum, whereas the effects of nicotine on dopamine output were similar in these two regions. These differences in nicotine-mediated dopamine output observed using microdialysis data might be due to the presence of varying nAChR subtypes with unique characteristics in the nucleus accumbens compared with the striatum. For instance, the α3/α6β2* nAChR populations present in the nucleus accumbens may be slower to desensitize following nicotine exposure than their counterparts in the striatum. This may have relevance for the development of new pharmaceuticals for smoking cessation.

To our knowledge, changes in α3/α6β2* nAChR-mediated function in nucleus accumbens after nicotine treatment have not been determined in rodent brain, although α3/α6β2* nAChR sites and function in the mouse striatum have previously been shown to decrease with chronic nicotine treatment (Salminen et al., 2004a; Lai et al., 2005). Receptor changes in rodent striatum following long-term nicotine treatment have been somewhat inconsistent, with studies reporting both increases and decreases in α3/α6β2* nAChRs (Nguyen et al., 2003; Parker et al., 2004; Mugnaini et al., 2006). These discrepancies among studies may be attributed to species differences with respect to the composition and proportion of these receptors expressed in the nucleus accumbens, as well as the methods used for identification of α3/α6β2* nAChRs.

In the present results, we show that chronic nicotine treatment selectively up-regulates α4β2* nAChR sites and function in the nucleus accumbens of nonhuman primates, whereas in striatum, the up-regulation of α4β2* nAChR sites is accompanied by a down-regulation of α3/α6β2* sites and function. As the nucleus accumbens is a critical component of the reward pathway, these findings may indicate a greater involvement of α4β2* nAChRs in nicotine-mediated reward systems than α3/α6β2* nAChRs.

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