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Cotinine Selectively Activates a Subpopulation of $\alpha 3/\alpha 6\beta 2^*$ Nicotinic Receptors in Monkey Striatum

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Abstract

The nicotine metabolite cotinine is an abundant long-lived bio-active compound that may contribute to the overall physiological effects of tobacco use. Although its mechanism of action in the central nervous system has not been extensively investigated, cotinine is known to evoke dopamine release in the nigrostriatal pathway through an interaction at nicotinic receptors (nAChRs). Because considerable evidence now demonstrates the presence of multiple nAChRs in the striatum, the present experiments were done to determine the subtypes through which cotinine exerts its effects in monkeys, a species that expresses similar densities of striatal $\alpha 4\beta 2^*$ (nAChR containing the $\alpha 4$ and $\beta 2$ subunits, but not $\alpha 3$ or $\alpha 6$) and $\alpha 3/\alpha 6\beta 2^*$ (nAChR composed of the $\alpha 3$ or $\alpha 6$ subunits and $\beta 2$) nAChRs. Competition binding studies showed that cotinine interacts with both $\alpha 4\beta 2^*$ and $\alpha 3/\alpha 6\beta 2^*$ nAChR subtypes in the caudate, with cotinine IC₅₀ values for inhibition of 5-[¹²⁵I]iodo-3-[2(S)-azetinylmethoxy]pyridine-2HCl ([¹²⁵I]A-85380) and [¹²⁵I]- α -conotoxinMII binding in the micromolar range. This interaction at the receptor level is of functional significance because cotinine stimulated both $\alpha 4\beta 2^*$ and $\alpha 3/\alpha 6\beta 2^*$ nAChR [³H]dopamine release from caudate synaptosomes. Our results unexpectedly showed that nicotine evokes [³H]dopamine release from two $\alpha 3/\alpha 6\beta 2^*$ nAChR populations, one of which was sensitive to cotinine and the other was not. This cotinine-insensitive subtype was only present in the medial caudate and was preferentially lost with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced nigrostriatal damage. In contrast, cotinine and nicotine elicited equivalent levels of $\alpha 4\beta 2^*$ nAChR-mediated dopamine release. These data demonstrate that cotinine functionally discriminates between two $\alpha 3/\alpha 6\beta 2^*$ nAChRs in monkey striatum, with the cotinine-insensitive $\alpha 3/\alpha 6\beta 2^*$ nAChR preferentially vulnerable to nigrostriatal damage.

Cotinine is one of the major metabolites of nicotine in a number of mammalian species, including humans (Fig. 1). Cotinine has a much longer pharmacokinetic half-life (15–19 h) than nicotine (2–3 h), which results in plasma cotinine levels 5 to 10-fold greater than those of the parent compound (Hukkanen et al., 2005). These observations raise the question of whether cotinine is pharmacologically active in vivo, thereby contributing to the overall effects of tobacco exposure. Indeed, numerous studies indicate that cotinine influences autonomic functions including heart rate, blood pressure, respiration, and hormone regulation (Borzelleca et al., 1962; Dominiak et al., 1985; Andersson et al., 1993; Buccafusco et al., 2007), and it also affects behavioral and cognitive task performance (Risner et al., 1985; Buccafusco and Terry, 2003; Terry et al., 2005).

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*denotes nicotinic receptors containing the indicated α and β subunit and possible additional subunits.

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The cotinine-induced changes described above seem to be independent of, and frequently opposite to, those of nicotine. However, like nicotine, they seem to be mediated by interaction with nicotinic acetylcholine receptors (nAChRs), pentameric ligand-gated ion channels composed of various combinations of α and β subunits. Cotinine displaces binding of various radiolabeled nAChR ligands to rat whole brain, hippocampal, and cortical membrane preparations, with a potency ~100-fold less than that of nicotine (Sloan et al., 1984; Anderson and Arneric, 1994; Vainio and Tuominen, 2001). This interaction is of functional relevance because cotinine has been shown to both activate and desensitize nAChRs. For instance, cotinine stimulates striatal dopamine (DA) release and noradrenaline release from bovine chromaffin cells (Vainio et al., 1998b; Dwoskin et al., 1999). On the other hand, cotinine pre-exposure leads to a subsequent inhibition of nAChR-mediated responses, an effect attributed to nAChR desensitization (Vainio et al., 1998a; Koh et al., 2003). These combined data suggest that, in addition to nicotine, cotinine may contribute to the overall effects of tobacco exposure.

nAChRs are important modulators of dopaminergic function in the striatum, with several subtypes implicated in DA release. This includes the $\alpha 4\beta 2^*$ (non- $\alpha 3/\alpha 6$ -containing) receptor population (* denotes the possible presence of additional subunits), which is present on DA terminals and other neurons in the striatum, and the $\alpha 3/\alpha 6\beta 2^*$ subtype, which is located exclusively on the nigrostriatal dopaminergic terminals (Le Novère et al., 1996; Champtiaux et al., 2002; Perry et al., 2002; Zoli et al., 2002; Salminen et al., 2004). As mentioned, cotinine evokes [^3H]dopamine release from rat striatal slices via nAChR activation (Dwoskin et al., 1999). However, the subtypes through which cotinine elicits dopamine release are not yet known. Such knowledge is important because accumulating studies indicate that the $\alpha 4\beta 2^*$ and $\alpha 3/\alpha 6\beta 2^*$ subtypes are differentially modified after nigrostriatal damage, nicotine administration, and other drug treatments (Quik and McIntosh, 2006). In addition, there seems to be a unique regulation of $\alpha 3/\alpha 6\beta 2^*$ receptor subtypes with nicotine exposure and nigrostriatal degeneration (Bordia et al., 2007; Khwaja et al., 2007; Perry et al., 2007). Therefore, the present experiments were done to examine the nAChR subtype specificity of cotinine using functional [^3H]DA release assays and ligand-binding autoradiography in monkey striatum. Monkeys were used because they have similar levels of $\alpha 4\beta 2^*$ and $\alpha 3/\alpha 6\beta 2^*$ nAChRs in striatum, unlike rodents that express low levels of $\alpha 3/\alpha 6\beta 2^*$ receptors, making it difficult to study this subtype. To distinguish between the $\alpha 3/\alpha 6\beta 2^*$ and $\alpha 4\beta 2^*$ nAChR subtypes, we have used the nAChR antagonist α -conotoxinMII. Accumulating evidence shows that this toxin is selective for $\alpha 3\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs across species, including rodents, monkeys, and humans (Champtiaux et al., 2002; Zoli et al., 2002; Quik and McIntosh, 2006; Bordia et al., 2007). Our results show that cotinine can discriminate between two $\alpha 3/\alpha 6\beta 2^*$ nAChR subtypes, one of which is particularly vulnerable to nigrostriatal damage.

Materials and Methods

Animals

Female adult squirrel monkeys (*Saimiri sciureus*) weighing between 0.5 and 0.7 kg were purchased from Worldwide Primates (Miami, FL). They were housed in a room with a 13/11-h light/dark cycle with food given once daily and water ad libitum. After a 1-month quarantine according to California State regulations, the monkeys received s.c. injections with saline ($n = 6$) or 1×1.75 mg/kg MPTP in saline ($n = 8$). They were euthanized 2 or 6 weeks later according to the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. The animals were first given an i.p. injection with euthanasia solution (390 mg of sodium pentobarbital and 50 mg of phenytoin sodium/milliliter), followed by a 2.2 ml/kg i.v. injection of the same solution. Because the results from the two MPTP groups were similar, the data were pooled. All care and treatments were

carried out in accordance with the Institute of Laboratory Animal Resources (1996) and were approved by the Institutional Animal Care and Use Committee at the Parkinson's Institute.

Tissue Preparation

The brains were rapidly removed and sectioned along the midline (McCallum et al., 2005). One half was placed in a Plexiglas mold and sliced into 6-mm-thick blocks, which were quick-frozen on glass slides in isopentane on dry ice, and stored at -80°C for future use. These blocks were sectioned ($20\text{ }\mu\text{m}$) on a cryostat (Leica Microsystems Inc., Deerfield, IL), mounted, air-dried on Superfrost Plus slides (Fisher Scientific Co., Pittsburgh, PA), and stored at -80°C for autoradiography experiments. The second half of the brain was sliced into 2-mm blocks. The medial and lateral caudate were dissected from the blocks at level A15 to A13.5 mm anterior to bregma (Emmers and Akert, 1963) and used immediately for DA release assays.

[^3H]DA Release

Striatal synaptosomes were prepared and [^3H]DA release assays were performed as described previously (McCallum et al., 2005). Fresh striatal tissue ($\sim 15\text{ mg}$ per region) was homogenized in 2 ml of cold homogenization buffer (0.32 M sucrose and 5 mM HEPES, pH 7.5) and centrifuged at $12,000g$ for 20 min. P1 pellets were resuspended in 0.8 ml uptake buffer (128 mM NaCl, 2.4 mM KCl, 3.2 mM CaCl_2 , 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 25 mM HEPES, pH 7.5, 10 mM glucose, 1 mM ascorbic acid, and 0.01 mM pargyline) and incubated for 10 min at 37°C . [^3H]DA ($4\text{ }\mu\text{Ci}$ for a final concentration of 100 nM DA; PerkinElmer Life Analytical Sciences, Waltham, MA) was then added, and the tissue was incubated for another 5 min. Eighty-microliter aliquots of synaptosomes (approximately 0.5–2 mg of tissue) was placed on 5-mm diameter A/E glass-fiber filters (Gelman Instrument Co., Ann Arbor, MI) and perfused with uptake buffer containing 0.1% bovine serum albumin and 10 μM nomifensine for 10 min at a rate of 1 ml/min before fraction collection was initiated. Tissue was then stimulated with either (–)-nicotine hydrogen tartrate salt (0.03–30 μM), (–)-cotinine (3 μM –3 mM), or 20 mM K^+ buffer for 18 s. When α -conotoxinMII (50 nM) (J. M. McIntosh) was used, tissue was pre-exposed to the antagonist for 3 min before agonist exposure (Cartier et al., 1996). For each tissue aliquot, 15 fractions (18 s) were collected, including basal release before and after stimulation. Econosafe scintillation cocktail (Research Products International, Mt. Prospect, IL) was added to the fractions, and radioactivity was counted on a Beckman Coulter LS6500 scintillation counter (Fullerton, CA).

[^{125}I]RTI-121 Autoradiography

[^{125}I]RTI-121 (PerkinElmer Life Analytical Sciences) was used to measure binding to the DA transporter as described previously (McCallum et al., 2005). Twenty-micrometer-thick striatal sections were thawed and preincubated for $2 \times 15\text{ min}$ in 50 mM Tris-HCl buffer, pH 7.4, containing 120 mM NaCl and 5 mM KCl at room temperature. Sections were then incubated for 2 h in the same buffer plus 50 pM [^{125}I]RTI-121, 0.025% bovine serum albumin, and 1 μM fluoxetine. Addition of 100 μM nomifensine to the incubation buffer was used to define nonspecific binding. Incubation was followed by $4 \times 15\text{-min}$ washes in 4°C preincubation buffer. Sections were then dipped in ice-cold water, air dried, and exposed to Kodak BioMax MR film (VWR, West Chester, PA) with ^{125}I microscale standards (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

¹²⁵I- α -ConotoxinMII Autoradiography

¹²⁵I- α -ConotoxinMII (J. M. McIntosh) was synthesized and radiolabeled as described previously (Whiteaker et al., 2000), and binding was performed as reported previously (McCallum et al., 2005). HEPES buffer (20 mM), containing 144 mM NaCl, 1.5 mM KCl, 2 mM CaCl₂, and 1 mM MgSO₄, pH 7.5, was used throughout the experiment. Thawed sections were preincubated at room temperature for 15 min in buffer plus 0.1% bovine serum albumin and 1 mM phenylmethylsulfonyl fluoride. Cotinine (0.1 nM–10 mM) was included in the preincubation and incubation buffers for the competition studies. Nicotine (100 μ M) was used to define nonspecific binding and was added to both preincubation and incubation buffers, when used. Sections were then incubated for 1 h in buffer that included 0.5 nM ¹²⁵I- α -conotoxinMII, 0.5% bovine serum albumin, 5 mM EDTA, 5 mM EGTA, and 1 μ l/ml each of aprotinin, leupeptin, and pepstatin A. Sections were washed with buffer for 10 min at room temperature and then at 4°C, followed by a 2 $\times$ 10-min wash in 4°C 0.1 $\times$ buffer, and dipped in 4°C water twice. They were air-dried and exposed to Kodak BioMax MR film with ¹²⁵I standards for several days.

[¹²⁵I]A-85380 Autoradiography

[¹²⁵I]A-85380 binding was performed as described previously (Kulak et al., 2002a). In brief, thawed sections were incubated for 60 min at room temperature in 50 mM Tris buffer (120 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, and 1 mM MgCl₂, pH 7.5) containing 200 pM [¹²⁵I]A-85380. Cotinine (1 nM–1 mM) was included in the incubation buffer for the competition studies. Nicotine (100 μ M) was used to define nonspecific binding. Sections were washed for 2 $\times$ 5 min in 4°C buffer on ice and 1 $\times$ 10 s in ice-cold ddH₂O. They were air-dried and exposed to Kodak BioMax MR film with ¹²⁵I standards for several days.

Data Analysis

[³H]DA release was quantified as described previously (McCallum et al., 2005). Release was plotted as cpm versus fraction number using a curve-fitting algorithm in SigmaPlot 5.0 for MS-DOS (SPSS Inc., Chicago, IL). Fractions before and after the peak were selected to calculate basal release by plotting values as a single exponential decay function. Baseline was subtracted out, and fractions above 10% of baseline were added to obtain evoked release. This value was then normalized to wet tissue weight per filter to obtain cpm/mg tissue. $\alpha 4\beta 2^*$ and $\alpha 3/\alpha 6\beta 2^*$ nAChR components of release were discriminated by the addition of α -conotoxinMII to perfusion buffer. Release remaining in the presence of α -conotoxinMII was mediated by $\alpha 4\beta 2^*$ nAChRs. The $\alpha 3/\alpha 6\beta 2^*$ nAChR-mediated component was determined by subtraction of the $\alpha 4\beta 2^*$ component from total release. $R_{\max}$ and EC₅₀ values for dose-response curves were calculated by nonlinear regression equations in GraphPad Prism (GraphPad Software Inc., San Diego, CA).

The ImageQuant program (GE Healthcare) was used to obtain optical density values from autoradiographic films. Specific binding was calculated by subtracting background tissue levels from total binding, and these values were converted to femtomole per milligram of tissue using standard curves generated from ¹²⁵I radioactivity standards. Sample optical density readings were within the linear range of the film.

Statistical Analysis

Statistical comparisons were conducted using two-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests. A level of $p < 0.05$ was considered significant. All analyses were performed using GraphPad Prism. Data represent the mean $\pm$ S.E.M. of the indicated number of animals. Values for each animal represent the average from two or more independent experiments.

Results

Cotinine Binds to $\alpha 3/\alpha 6\beta 2^*$ and $\alpha 4\beta 2^*$ nAChRs in Caudate

To confirm that cotinine is interacting with striatal nAChRs and to determine the subtypes that bind cotinine, we characterized cotinine inhibition of [125 I]- α -conotoxinMII and [125 I]A-85380 binding in the medial and lateral caudate. We used these two radioligands because [125 I]- α -conotoxinMII binds to $\alpha 3/\alpha 6\beta 2^*$ nAChRs, whereas [125 I]A-85380 binds to both $\alpha 3/\alpha 6\beta 2^*$ and $\alpha 4\beta 2^*$ receptors (Kulak et al., 2002a,b), the major nAChR subtypes in the striatum.

Cotinine blocked [125 I]- α -conotoxinMII binding in a concentration-dependent manner in both striatal regions, with complete inhibition observed at 10^{-3} M, suggesting that cotinine binds to all $\alpha 3/\alpha 6\beta 2^*$ receptor subtypes (Fig. 2A). The IC_{50} values for cotinine inhibition of [125 I]- α -conotoxinMII binding were similar in the medial [3.588 (1.08 – 4.51) μ M] and lateral [3.101 (1.11–2.68) μ M] caudate (Hill slopes = -0.88 and -0.87 , respectively). Thus, cotinine is nearly 1000-fold less potent than nicotine ($IC_{50} = 5.7 \pm 0.4$ nM) at striatal [125 I]- α -conotoxinMII binding sites (Quik et al., 2001).

Cotinine also inhibited [125 I]A-85380 binding, with a complete block at 10^{-3} M. These data indicate that cotinine interacts with both $\alpha 3/\alpha 6\beta 2^*$ and $\alpha 4\beta 2^*$ receptor subtypes (Fig. 2B). The IC_{50} for cotinine inhibition of [125 I]A-85380 binding was 78.7 μ M (48.7–127.1 μ M) in the medial caudate and 64.8 μ M (32.4–129.5 μ M) in the lateral caudate, approximately 10,000-fold less potent than nicotine (7.53 nM) (Kulak et al., 2002a). The Hill slope for the medial caudate was -0.65 , possibly suggesting negative cooperativity or a heterogeneous population of receptors. However, a nonlinear regression one-site versus two-site comparison analysis indicated the presence of only one binding site. The Hill slope in the lateral caudate was -0.99 .

Cotinine Selectively Activates Only a Subpopulation of $\alpha 3/\alpha 6\beta 2^*$ nAChRs in the Medial and Lateral Caudate

We next examined the functional effects of cotinine on nAChR-evoked [3 H]DA release from medial and lateral caudate synaptosomes. The $\alpha 3/\alpha 6\beta 2^*$ -specific antagonist α -conotoxinMII was used to discriminate between $\alpha 3/\alpha 6\beta 2^*$ and $\alpha 4\beta 2^*$ nAChR-evoked release. Release remaining in the presence of α -conotoxinMII was defined as that mediated by $\alpha 4\beta 2^*$ nAChRs, whereas $\alpha 3/\alpha 6\beta 2^*$ nAChR-mediated release was defined as the difference between total and α -conotoxin-MII-resistant ($\alpha 4\beta 2^*$) release.

Cotinine and nicotine dose-response curves revealed that cotinine was less potent than nicotine at $\alpha 3/\alpha 6\beta 2^*$ receptors, with cotinine EC_{50} values ~ 200 -fold greater than those for nicotine in both the medial and lateral caudate (Table 1; Fig. 3). Cotinine was also less efficacious than nicotine in the medial although not lateral caudate. Cotinine-stimulated $\alpha 3/\alpha 6\beta 2^*$ nAChR-mediated [3 H]DA release was only approximately half that of nicotine in the medial caudate ($p < 0.001$; Fig. 3, A and C; Table 3). In contrast, cotinine and nicotine yielded similar levels of release in the lateral caudate, with a maximal dose of each ligand (R_{max}) eliciting ~ 6000 cpm/mg of tissue [3 H]DA release (5659 ± 681.4 cotinine, 6669 ± 533.9 nicotine) (Fig. 3B; Table 3). It is noteworthy that this value is comparable to maximal cotinine-evoked [3 H]DA release in the medial caudate (7612 ± 1422 cpm/mg tissue) (Fig. 3, A and C).

These combined data suggest that there are two $\alpha 3/\alpha 6\beta 2^*$ nAChR subpopulations that mediate DA release, only one of which is activated by cotinine. The cotinine-insensitive subtype seems to be present only in the medial caudate, whereas the cotinine-sensitive population mediates DA release in both the medial and lateral caudate.

Cotinine Does Not Discriminate between $\alpha 4\beta 2^*$ nAChR Subtypes Mediating [^3H]DA Release in the Medial or Lateral Caudate

As with $\alpha 3/\alpha 6\beta 2^*$ receptors, cotinine was less potent than nicotine in eliciting $\alpha 4\beta 2^*$ subtype-mediated [^3H]DA release in the medial and lateral caudate (Table 1). However, in both regions, cotinine and nicotine stimulated similar levels of [^3H]DA release (Fig. 4; Table 4). Consistent with previous studies, greater release was observed in the medial compared with lateral caudate (McCallum et al., 2005,2006).

The Cotinine-Insensitive $\alpha 3/\alpha 6\beta 2^*$ nAChR Subpopulation in the Medial Caudate Is Preferentially Lost with MPTP Lesioning

We subsequently evaluated the effects of nigrostriatal damage on $\alpha 3/\alpha 6\beta 2^*$ nAChR-mediated [^3H]DA release. MPTP administration resulted in a significant decrease in DA transporter values in the medial caudate (Table 2). This was accompanied by a significant reduction in nicotine-stimulated [^3H]DA release, with a 44% decrease in $R_{\max}$ ($p < 0.05$; Figs. 5A and 6B; Table 3), similar to previously reported results (McCallum et al., 2006). In contrast, cotinine-evoked release was similar to control after nigrostriatal damage (Figs. 5B and 6B; Table 3). It is noteworthy that the nicotine-stimulated release was reduced to a level similar to cotinine-evoked release in the control and MPTP-lesioned conditions (Figs. 5, A and B, and 6B; Table 3). These data suggest that dopaminergic terminals in the medial caudate expressing the cotinine-insensitive $\alpha 3/\alpha 6\beta 2^*$ nAChR subtype are preferentially lost with MPTP-induced nigrostriatal degeneration. This loss of cotinine-insensitive release was accompanied by a 36% decrease in ^{125}I - α -conotoxinMII binding. These results suggest that approximately one third of the $\alpha 3/\alpha 6\beta 2^*$ nAChRs in the medial caudate are selectively vulnerable to nigrostriatal damage (Fig. 6, A and B). However, although this $\alpha 3/\alpha 6\beta 2^*$ nAChR subtype binds cotinine, it does not seem to be involved in cotinine-mediated DA release because release is similar in the control and lesioned medial caudate.

Nicotine- and Cotinine-Evoked $\alpha 3/\alpha 6\beta 2^*$ nAChR-Mediated [^3H]DA Release in the Lateral Caudate Is Reduced with Nigrostriatal Damage

Nicotine- and cotinine-evoked $\alpha 3/\alpha 6\beta 2^*$ nAChR-mediated [^3H]DA release from lateral caudate synaptosomes were both reduced to approximately the same extent by MPTP treatment (Figs. 5, C and D, and 6D; Table 3). $R_{\max}$ values were decreased by 49% for nicotine ($p < 0.001$) and 47% for cotinine ($p < 0.01$) (Fig. 6D; Table 3). These data suggest that DA terminals in the lateral caudate that express cotinine-sensitive $\alpha 3/\alpha 6\beta 2^*$ nAChRs are vulnerable to nigrostriatal damage, unlike in the medial caudate where they remain unaffected by MPTP treatment. This decrease in DA release is accompanied by a similar loss in the dopamine transporter and ^{125}I - α -conotoxinMII binding sites in the lateral caudate, which are reduced to 42 and 33% of control levels, respectively (Table 2; Fig. 6C).

MPTP Lesioning Differentially Affects Nicotine- and Cotinine-Evoked $\alpha 4\beta 2^*$ nAChR-Mediated [^3H]DA Release in Lateral but Not Medial Caudate

In the medial caudate, both nicotine- and cotinine-elicited release mediated by $\alpha 4\beta 2^*$ nAChRs remained unchanged after MPTP administration (Fig. 7, A and B; Table 4). In contrast, although nicotine- and cotinine-stimulated [^3H]DA release is equivalent in the control lateral caudate, cotinine-stimulated release is completely lost after nigrostriatal damage (Fig. 7D; Table 4), whereas nicotine-evoked release is only reduced by 46% ($p < 0.01$, $R_{\max}$ values) (Fig. 7C).

Discussion

The present results are the first to show that cotinine, a long-lasting nicotine metabolite, functionally discriminates between two $\alpha 3/\alpha 6\beta 2^*$ nAChR populations on nigrostriatal dopamine terminals in the nonhuman primate striatum. The presence of these two $\alpha 3/\alpha 6\beta 2^*$ subtypes was region-specific, with both the cotinine-sensitive and cotinine-insensitive receptors present in the medial caudate but only the cotinine-sensitive population in the lateral caudate. Lesion studies suggest that the dopaminergic terminals that express these cotinine-insensitive receptors are preferentially vulnerable to nigrostriatal damage.

Evidence for the presence of two pharmacologically distinct $\alpha 3/\alpha 6\beta 2^*$ nAChRs in the medial caudate stems from the results of our functional studies. These demonstrated that cotinine and nicotine differentially stimulate $\alpha 3/\alpha 6\beta 2^*$ nAChR-mediated DA release in the medial caudate, with cotinine-evoked release only ~50% of the nicotine-evoked release. In contrast, in the lateral caudate, these two agonists were equally efficacious, and the level of release was similar to cotinine-stimulated release in the medial caudate. These combined data suggest that a cotinine-insensitive subpopulation of $\alpha 3/\alpha 6\beta 2^*$ nAChRs is expressed in the medial but not lateral caudate. Our previous data demonstrated a higher expression of $\alpha 3/\alpha 6\beta 2^*$ nAChRs in monkey medial compared with the lateral caudate (Quik et al., 2001; McCallum et al., 2005, 2006). These cotinine-insensitive receptors may at least partly account for the higher levels of $\alpha 3/\alpha 6\beta 2^*$ nAChR binding sites and nicotine-evoked [^3H]DA release in monkey medial caudate.

The use of selective lesioning is frequently used as a means to identify the specific cellular locations of molecular structures. Such studies have contributed to the finding that striatal $\alpha 3/\alpha 6\beta 2^*$ nAChRs are present exclusively on DA terminals, whereas $\alpha 4\beta 2^*$ nAChRs are on both dopaminergic terminals and other neurons in the striatum (Schwartz et al., 1984; Clarke and Pert, 1985; Quik et al., 2001; McCallum et al., 2005, 2006). Therefore, we determined $\alpha 3/\alpha 6\beta 2^*$ nAChR expression and function in the striatum of monkeys lesioned with the selective dopaminergic neurotoxin MPTP. The results showed that nicotine-evoked $\alpha 3/\alpha 6\beta 2^*$ nAChR-mediated DA release in the medial caudate was decreased after MPTP lesioning as previously observed (McCallum et al., 2005, 2006). However, unexpectedly, cotinine-evoked release was not affected by nigrostriatal damage. It is interesting to note that the lesion reduced nicotine-stimulated release in the medial caudate to a level similar to cotinine-evoked release in the control and MPTP-lesioned conditions. These data provide further support for the presence of two distinct $\alpha 3/\alpha 6\beta 2^*$ nAChRs on dopaminergic terminals in monkey medial caudate. In addition, these data indicate that cotinine-insensitive $\alpha 3/\alpha 6\beta 2^*$ nAChRs are selectively lost with nigrostriatal damage. One possible explanation for these results is that DA terminals expressing these nAChRs are particularly vulnerable to nigrostriatal degeneration. If this interpretation is correct, this unique subtype could serve as a selective marker for these nigrostriatal fibers in the medial caudate. Alternatively, because striatal dopaminergic measures have been shown to recover after an acute lesion, a lesion-induced decline in cotinine-sensitive $\alpha 3/\alpha 6\beta 2^*$ nAChRs may have been masked by axonal sprouting and a selective re-expression of this subtype. However, this seems unlikely given the fact that the cotinine-sensitive subtype was unaffected at both postlesion time points tested (2 and 6 weeks).

The lesion studies also revealed that the functional effects of the cotinine-insensitive $\alpha 3/\alpha 6\beta 2^*$ receptors are greater than would be expected based on their relative numbers in the medial caudate. The 50% decline in DA release attributed to a loss of cotinine-insensitive receptors was accompanied by only a 36% reduction in ^{125}I - α -conotoxinMII binding, suggesting that approximately one third of all $\alpha 3/\alpha 6\beta 2^*$ nAChRs in the medial caudate are responsible for mediating half of the $\alpha 3/\alpha 6\beta 2^*$ nAChR-evoked DA release. In addition, all

of these cotinine-insensitive receptors seem to be located on only ~20% of DA terminals in the medial caudate, as demonstrated by the decline in dopamine transporter levels after nigrostriatal damage.

In contrast to the preservation of cotinine-sensitive $\alpha 3/\alpha 6\beta 2^*$ nAChR-mediated DA release in the medial caudate after lesioning, a decrease in release was observed in the lateral caudate. This decline may relate to the well known enhanced vulnerability of nigrostriatal dopaminergic neurons projecting to the dorsolateral striatum (Kish et al., 1988; Moratalla et al., 1992). The lack of cotinine-insensitive $\alpha 3/\alpha 6\beta 2^*$ nAChRs on dopaminergic terminals in the lateral caudate may somehow contribute to the increased susceptibility of these nigrostriatal projections to degenerative processes, relative to the medial caudate.

Combined, these data provide further evidence for the presence of a cotinine-insensitive $\alpha 3/\alpha 6\beta 2^*$ nAChR in the medial caudate. The possibility also exists that cotinine is acting as a partial agonist at nicotinic receptors. However, if this were the case, a decline in nicotine- and cotinine-evoked $\alpha 3/\alpha 6\beta 2^*$ nAChR-mediated [3 H]DA release might be expected after nigrostriatal damage. Instead, only nicotine-stimulated release is reduced, with the level of release decreased to that of cotinine-evoked release in the control condition. A further argument against a partial agonist action stems from our results showing that cotinine is a full agonist in the lateral caudate, with cotinine and nicotine eliciting the same amount of [3 H]DA release.

Cotinine also stimulates [3 H]DA release via activation of $\alpha 4\beta 2^*$ nAChRs. These results are in agreement with the receptor competition studies presented here and elsewhere showing that cotinine inhibits binding to both $\alpha 3/\alpha 6\beta 2^*$ and $\alpha 4\beta 2^*$ nAChR sites (Sloan et al., 1984; Anderson and Arneric, 1994; Vainio and Tuominen, 2001). In the control condition, cotinine-evoked $\alpha 4\beta 2^*$ nAChR-mediated release was similar to nicotine-mediated release in each of the caudate regions, indicating that cotinine does not discriminate between striatal $\alpha 4\beta 2^*$ nAChR subpopulations. Nigrostriatal damage significantly decreased $\alpha 4\beta 2^*$ nAChR-mediated DA release in the lateral but not medial caudate, consistent with previous studies showing an enhanced susceptibility of the dorsolateral striatum to MPTP treatment (McCallum et al., 2005, 2006). However, whereas nicotine-evoked release was reduced by approximately half, cotinine-stimulated release was completely abolished. One possible explanation for this discrepancy could be an MPTP-induced alteration in receptor stoichiometry, for instance, $(\alpha 4)_2(\beta 2)_3$ versus $(\alpha 4)_3(\beta 2)_2$. Recent work has shown that alternate stoichiometric arrangements of the $\alpha 4\beta 2$ nAChR yield high- and low-affinity subforms that possess significantly different functional and pharmacological properties (Briggs et al., 2006; Moroni and Bermudez, 2006). Thus, a change in the ratio of $\alpha 4$ to $\beta 2$ subunits with nigrostriatal damage may result in an $\alpha 4\beta 2^*$ nAChR that is cotinine insensitive. Alternatively, the $\alpha 4\beta 2^*$ receptors may have a different composition before and after lesioning, for instance, $\alpha 4\beta 2$ versus $\alpha 4\alpha 2\beta 2$, which both seem to be present in monkey striatum (Quik et al., 2005).

A further question that arises is the composition of the cotinine-sensitive and -insensitive $\alpha 3/\alpha 6\beta 2^*$ nAChR subpopulations. At least three different $\alpha 3/\alpha 6\beta 2^*$ nAChR populations have been identified by radioligand binding in monkey striatum, including $\alpha 6\alpha 4\beta 2\beta 3$, $\alpha 6\beta 2\beta 3$, and $\alpha 3\beta 2^*$ nAChRs, with the $\alpha 6\alpha 4\beta 2\beta 3$ subtype particularly susceptible to nigrostriatal damage in all striatal subregions (Quik et al., 2005; Bordia et al., 2007). However, the present results show differential effects of cotinine on receptor-mediated function in the medial and lateral caudate. One interpretation of these seemingly discrepant results is that the $\alpha 6\alpha 4\beta 2\beta 3$ subtype is insensitive to cotinine in the medial, but it is sensitive in the lateral caudate. This may arise because of differential post-translational modifications and/or stoichiometric conformations of the receptors in the two regions. However, it may not be

possible to correlate the present results derived from release assays with those from radioligand binding studies because the conditions for binding favor a desensitized state and the ligand may bind to both intracellular and extracellular receptors, whereas release assays involve only functional membrane-bound receptors. Further studies with more selective $\alpha 3/\alpha 6\beta 2^*$ nAChR agonists and antagonists are required to understand the specific subtypes involved. On the other hand, the differential experimental conditions of the binding and functional assays may point to an explanation for the greater differences in nicotine and cotinine potencies observed in the binding assays (10,000-fold differences in potency) compared with the functional assays (100–1000-fold). Nicotine may bind particularly well to desensitized receptors of one or more subtypes compared with cotinine. Alternatively, or as well, nicotine and cotinine may possess differential capacities for desensitization of the receptors and thus different affinities for the receptors.

The biological significance of cotinine is an important question because this nicotine metabolite has a very long half-life and is present at relatively high levels in plasma compared with nicotine. Behavioral studies have shown that cotinine improves performance on a number of cognitive tasks in rats (Buccafusco and Terry, 2003; Terry et al., 2005). In addition, cotinine protects against toxic insults in PC12 cells, with an ~80% restoration of cell viability and a potency similar to that of nicotine (Buccafusco and Terry, 2003). Our results indicate that cotinine can stimulate striatal dopamine release in an in vitro preparation, suggesting that it may have functional effects in the nigrostriatal pathway. Admittedly, cotinine-mediated functional responses in striatal synaptosomes are observed at higher concentrations than those that are probably present in the brains of smokers based on plasma levels and the brain levels observed in rats (micromolar versus nanomolar concentrations) (Hatsukami et al., 1997; Ghosheh et al., 1999, 2001; Hukkanen et al., 2005). It is possible that cotinine exerts its effects at lower concentrations in intact brain compared with isolated synaptosomes. Alternatively, although brain cotinine concentrations are probably not high enough to activate nAChRs, persistent low levels of agonist are often associated with receptor desensitization (Giniatullin et al., 2005). Thus, at levels found in brains of smokers, cotinine may influence striatal function by chronically desensitizing nAChRs, thereby inhibiting acetylcholine-induced effects. This phenomenon has been demonstrated in a number of experimental systems including rats and bovine chromaffin cells (Vainio et al., 1998a, 2000; Koh et al., 2003; Buccafusco et al., 2007). Because excitotoxicity may be involved in neuronal loss in Parkinson's disease (PD), cotinine inhibition of nAChRs through desensitization may protect against nigrostriatal degeneration by decreasing dopaminergic terminal excitability (Egea et al., 2006). Therefore, cotinine may play a role in the neuroprotection against PD that has been observed in tobacco users, because smokers would presumably have sustained brain levels of cotinine. If cotinine does protect dopaminergic neurons by nAChR desensitization, the cotinine-insensitive receptors in the medial caudate may be more vulnerable to excitotoxic insult, thereby explaining their increased susceptibility to nigrostriatal damage. Therefore, the discrimination of cotinine between $\alpha 3/\alpha 6\beta 2^*$ nAChR subtypes may prove useful for the future development of PD therapies that are optimally beneficial with minimal side effects by specifically targeting this subtype.

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ABBREVIATIONS

nAChR	nicotinic acetylcholine receptor
DA	dopamine
$\alpha 4\beta 2^*$	nAChR containing the $\alpha 4$ and $\beta 2$ subunits, but not $\alpha 3$ or $\alpha 6$
$\alpha 3/\alpha 6\beta 2^*$	nAChR composed of the $\alpha 3$ or $\alpha 6$ subunits and $\beta 2$
DA	dopamine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
RTI-121	3 β -(4-iodophenyl) tropane-2 β -carboxylic acid isopropyl ester
ANOVA	analysis of variance
A-85380	5-[125 I]iodo-3-[2(<i>S</i>)-azetinylmethoxy]pyridine-2HCl
PD	Parkinson's disease

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Fig. 1.

Molecular structures of nicotine and cotinine. In humans, 70 to 80% of nicotine is converted to cotinine by the cytochrome P450 system (Benowitz and Jacob, 1994).

Fig. 2.

Cotinine completely inhibits ^{125}I - α -conotoxinMII and [^{125}I]A-85380 binding to the medial and lateral caudate. Competition binding assays were performed at the indicated concentrations of cotinine (10^{-10} – 10^{-2} M) using tissue sections from the medial and lateral caudate. Because ^{125}I - α -conotoxinMII (A) selectively binds to $\alpha 3/\alpha 6\beta 2^*$ nAChRs, and [^{125}I]A-85380 (B) binds to both the $\alpha 3/\alpha 6\beta 2^*$ and $\alpha 4\beta 2^*$ nAChR subtypes, these data suggest that cotinine interacts at both of these nAChR populations. Curves were generated using nonlinear regression analysis in GraphPad Prism. Data represent the mean $\pm$ S.E.M. of two to four sections.

Fig. 3.

Two $\alpha 3/\alpha 6\beta 2^*$ nAChR subtypes regulate [^3H]DA release in the medial but not lateral caudate. Stimulation of a cotinine-sensitive $\alpha 3/\alpha 6\beta 2^*$ subpopulation (Cot-sens) elicits equivalent levels of DA release in the medial (A and C) and lateral (B and C) caudate. Nicotine activates an additional, cotinine-insensitive $\alpha 3/\alpha 6\beta 2^*$ subtype (Cot-insens) in the medial caudate (A and C), resulting in higher overall $\alpha 3/\alpha 6\beta 2^*$ -mediated DA release. The $\alpha 3/\alpha 6\beta 2^*$ -specific antagonist α -conotoxinMII was used to identify $\alpha 3/\alpha 6\beta 2^*$ nAChR-evoked release, as described under *Materials and Methods*. R_{max} values and dose-response curves were generated by nonlinear regression analysis using GraphPad Prism. (***, $p < 0.001$; cotinine R_{max} is significantly different compared with nicotine in the given region by two-way ANOVA followed by a Bonferroni post hoc test.) Data represent the mean $\pm$ S.E.M. of five to seven monkeys per group.

$\alpha 4\beta 2^*$ nAChR-mediated release **Fig. 4.**

Cotinine and nicotine elicit a similar level of $\alpha 4\beta 2^*$ nAChR-evoked $[^3H]DA$ release in each caudate region. These results suggest that cotinine does not discriminate between $\alpha 4\beta 2^*$ nAChR subtypes mediating $[^3H]DA$ release in either the medial (A) or lateral (B and C) caudate. The $\alpha 3/\alpha 6\beta 2^*$ -specific antagonist α -conotoxinMII was used to discriminate between $\alpha 3/\alpha 6\beta 2^*$ and $\alpha 4\beta 2^*$ nAChR-evoked release. Release remaining in the presence of α -conotoxinMII was defined as that mediated by $\alpha 4\beta 2^*$ nAChRs. R_{max} values were derived from dose-response curves generated by nonlinear regression analysis using GraphPad Prism. Data represent the mean $\pm$ S.E.M. of five to seven monkeys per group.

$\alpha 3/\alpha 6\beta 2^*$ nAChR-mediated DA release **Fig. 5.**

Nigrostriatal damage selectively decreases cotinine-insensitive $\alpha 3/\alpha 6\beta 2^*$ nAChR-mediated [^3H]DA release in the medial caudate, but in the lateral caudate it reduces nicotine- and cotinine-evoked release to the same extent. MPTP lesioning resulted in a decline in (A) nicotine- but not (B) cotinine-evoked DA release in the medial caudate. The finding that nicotine-stimulated $\alpha 3/\alpha 6\beta 2^*$ nAChR-mediated release was reduced to the level of cotinine-evoked release after MPTP treatment suggests that the $\alpha 3/\alpha 6\beta 2^*$ cotinine-insensitive (Cot-insens) component is selectively abolished by nigrostriatal damage, whereas the cotinine-sensitive (Cot-sens) is retained. In contrast, in the lateral caudate, which seems to lack the cotinine-insensitive $\alpha 3/\alpha 6\beta 2^*$ nAChR subtype, the MPTP lesion reduced both nicotine- and cotinine-evoked $\alpha 3/\alpha 6\beta 2^*$ nAChR-mediated [^3H]DA release to the same extent (C and D, respectively). Dose-response curves were generated by nonlinear regression analysis using GraphPad Prism. Control and MPTP curves in A, C, and D were significantly different ($p < 0.001$) by two-way ANOVA analysis of the experimental values obtained from the release assays. Data represent the mean $\pm$ S.E.M. of four to eight monkeys per group.

Fig. 6.

Nigrostriatal damage leads to a similar decline in $\alpha 3/\alpha 6\beta 2^*$ nAChR binding and nicotine- but not cotinine-evoked [^3H]DA release in the medial caudate. $^{125}\text{I}-\alpha\text{-conotoxinMII}$ binding and nicotine-evoked $\alpha 3/\alpha 6\beta 2^*$ nAChR-mediated [^3H]DA release are both reduced with nigrostriatal damage in the medial caudate (A and B). The lack of change in cotinine-evoked $\alpha 3/\alpha 6\beta 2^*$ nAChR-mediated release suggests that the decrease is due to a loss of cotinine-insensitive $\alpha 3/\alpha 6\beta 2^*$ receptors. This contrasts with results in the lateral caudate, in which $^{125}\text{I}-\alpha\text{-conotoxin-MII}$ binding and nicotine- and cotinine-mediated [^3H]DA release were all reduced in parallel with MPTP lesioning (C and D). $R_{\max}$ values were derived from dose-response curves generated by nonlinear regression analysis using GraphPad Prism. (*, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ compared to respective controls in the given region by two-way ANOVA followed by a Bonferroni post hoc test.) Data represent the mean $\pm$ S.E.M. of five to eight monkeys per group.

$\alpha 4\beta 2^*$ nAChR-mediated release

Fig. 7. MPTP lesioning differentially affects nicotine- and cotinine-evoked $\alpha 4\beta 2^*$ -mediated [3 H]DA release in the medial and lateral caudate. Nigrostriatal damage does not reduce $\alpha 4\beta 2^*$ -evoked nicotine or cotinine-mediated [3 H]DA release in the medial caudate (A and B). In contrast, in the lateral caudate, there was ~50% decrease in nicotine-stimulated $\alpha 4\beta 2^*$ -mediated [3 H]DA release (C) and a complete loss of cotinine-evoked release (D). Control and MPTP curves in C and D were significantly different ($p < 0.001$) by two-way ANOVA analysis of the experimental values obtained from the release assays. Data represent the mean $\pm$ S.E.M. of five to eight monkeys per group.

TABLE 1

EC₅₀ values and 95% confidence intervals (CI) for $\alpha 3/\alpha 6\beta 2^*$ and $\alpha 4\beta 2^*$ nAChR-mediated nicotine- and cotinine-evoked [³H]DA release from the medial and lateral caudate synaptosomes

Region	EC ₅₀ (CI)			
	$\alpha 3/\alpha 6\beta 2^*$ nAChRs		$\alpha 4\beta 2^*$ nAChRs	
	Nicotine	Cotinine	Nicotine	Cotinine
	μM			
Medial caudate	0.9 (0.2–5.8)	234 (39.1–1400)	0.9 (0.3–2.4)	495 (109–2240)
Lateral caudate	1.4 (0.6–3.4)	266 (87.4–808)	0.9 (0.3–2.8)	737 (211–2580)

Values were derived from nonlinear regression analysis using GraphPad Prism. Data are from six to eight animals.

TABLE 2

Decrease in striatal dopamine transporter with MPTP administration

Region	[¹²⁵I]RTI-121 Binding		
	Control	MPTP	% Control
	<i>nCi/mg</i>		
Medial	19.2 ± 1.1	15.7 ± 0.7 *	82
Lateral	15.7 ± 0.6	6.7 ± 0.8 ***	42

MPTP lesioning significantly reduced [¹²⁵I]RTI-121 (50 pM) binding, a measure of dopamine transporter levels, in the medial and lateral caudate. Data represent the mean ± S.E.M. of six to eight animals per group.

*
 $p < 0.05$ and

 $p < 0.001$ compared to respective controls by two-way ANOVA followed by a Bonferroni post hoc test.

TABLE 3

Summary of $\alpha 3/\alpha 6\beta 2^*$ nAChR-mediated [^3H]DA release in the medial and lateral caudate of control and MPTP-lesioned animals

Condition	Region	$\alpha 3/\alpha 6\beta 2^*$ nAChR-Mediated [^3H]DA Release		
		Total	Cotinine-Sensitive	Cotinine-Insensitive
Control	Medial	++++	++	++
	Lateral	++	++	Not present
MPTP	Medial	++	++	0
	Lateral	+	+	Not present

A similar level of cotinine-sensitive $\alpha 3/\alpha 6\beta 2^*$ nAChR-mediated [^3H]DA release is present in the medial and lateral caudate. This release component represents 100% of the release in the lateral caudate. In contrast, it forms only 50% of total $\alpha 3/\alpha 6\beta 2^*$ nAChR-mediated release in the medial caudate, and the remaining 50% release is cotinine-insensitive. Nigrostriatal damage results in a complete loss of this cotinine-insensitive release in the medial caudate, whereas cotinine-sensitive release remains unchanged in the medial caudate, but it is reduced by ~50% in the lateral caudate. The level of total $\alpha 3/\alpha 6\beta 2^*$ nAChR-mediated [^3H]DA release in the medial caudate was set to++++, with ++ and + equal to ~50 and ~25% of this value, respectively.

TABLE 4

Summary of $\alpha 4\beta 2^*$ -mediated [^3H]DA release in the medial and lateral caudate of control and MPTP-lesioned animals

Condition	Region	$\alpha 4\beta 2^*$ nAChR-Mediated [^3H]DA Release		
		Total	Cotinine-Sensitive	Cotinine-Insensitive
Control	Medial	++++	++++	Not present
	Lateral	++	++	Not present
MPTP	Medial	++	++	Not present
	Lateral	+	0	+

Nicotine- and cotinine-evoked $\alpha 4\beta 2^*$ nAChR-mediated [^3H]DA release are equivalent within each region, but release in the lateral caudate is only ~50% of that in the medial caudate. Neither nicotine- or cotinine-evoked release is affected by nigrostriatal damage in the medial caudate. In the lateral caudate, nicotine-evoked release is reduced by ~50%, whereas cotinine-evoked release is completely lost. Unexpectedly, a cotinine-insensitive component was present in the lateral caudate of the MPTP-lesioned group, possibly a compensatory mechanism that arises in response to nigrostriatal damage. Nicotine-evoked $\alpha 4\beta 2^*$ nAChR-mediated [^3H]DA release in the medial caudate was set to++++, with ++ and + equal to ~50 and ~25% of that value, respectively.