Partial Recovery of Striatal Nicotinic Receptors in 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-Lesioned Monkeys with Chronic Oral Nicotine

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Received May 7, 2006; accepted July 10, 2006

ABSTRACT

Recent studies in nonhuman primates show that chronic nicotine treatment protects against nigrostriatal degeneration, with a partial restoration of neurochemical and functional measures in the striatum. The present studies were done to determine whether long-term nicotine treatment also protected against striatal nicotinic receptor (nAChR) losses after nigrostriatal damage. Monkeys were administered nicotine in the drinking water for 6 months and subsequently lesioned with the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) over several months while nicotine was continued. 125I-Epibatidine, [125I]5-[125I]iodo-3(S)-azetidinylmethoxy)pyridine (A85380), and 125I-α-conotoxinMII autoradiography was performed to evaluate changes in α4β2* and α3α6β2* nAChRs, the major striatal subtypes. Nicotine treatment increased α4β2* nAChRs by ≥50% in striatum of both unlesioned and lesioned animals. This increase in α4β2* nAChRs was significantly greater in lesioned compared with unlesioned monkey striatum. Chronic nicotine treatment led to a small decrease in α3α6β2* nAChR subtypes. The decline in α3α6β2* subtypes, defined using α-conotoxinMII-sensitive 125I-epibatidine or [125I]A85380 binding, was significantly smaller in striatum of nicotine-treated lesioned monkeys compared with unlesioned monkeys. This difference was not observed for α3α6β2* nAChRs identified using 125I-α-conotoxinMII. These data suggest that there are at least two striatal α3α6β2* subtypes that are differentially affected by chronic nicotine treatment in lesioned animals. In addition, the results showing an improvement in striatal α4β2* and select α3α6β2* nAChR subtypes, combined with previous work, demonstrate that chronic nicotine treatment restores and/or protects against the loss of multiple molecular markers after nigrostriatal damage. Such findings suggest that nicotine or nicotinic agonists may be of therapeutic value in Parkinson’s disease.

Accumulating evidence suggests that activation of the central nervous system nicotinic cholinergic system may influence Parkinson’s disease. First, compelling and consistent epidemiological literature shows that there is a reduced risk of developing Parkinson’s disease with smoking, which has been attributed, at least in part, to the nicotine in tobacco (Morens et al., 1995; Allam et al., 2004). In addition, smoking and the nicotine patch/gum have been reported to alleviate the symptoms of Parkinson’s disease in some studies (Ishikawa and Miyatake, 1993; Fagerstrom et al., 1994; Ebersbach et al., 1999; Kelton et al., 2000; Vierregge et al., 2001; Lemay et al., 2004). Nicotine is thought to exert its effect in the nigrostriatal pathway by interacting with nicotinic acetylcholine receptors (nAChRs) (Gotti and Clementi, 2004; Quik, 2004). Multiple nAChRs have been identified in both rodent and primate brain. These nAChRs include the α4εβ2β3 receptor that may form the major subtype, as well as smaller populations of subtypes containing some combination of these and other minor subunits (α2, α3, α5, and β4) (Zoli et al., 2002; Salminen et al., 2004b; Gotti et al., 2005; Quik et al., 2005). In addition, homomeric α7 nAChRs are present in the striatum (Jones and Wonnacott, 2004). The

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; ANOVA, two-way analysis of variance; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; A85380, 5-[125I]iodo-3(S)-azetidinylmethoxy)pyridine.
ability of different subunits to form functionally diverse nAChRs provides for a complex regulation of nigrostriatal activity after nAChR stimulation under both physiological and pathological conditions.

nAChRs are altered both in Parkinson’s disease and with nicotine treatment. Numerous studies have shown that nAChRs are decreased in the caudate and putamen in Parkinson’s disease. This includes receptors identified using radioligands, such as epibatidine, that label most nAChR subtypes, A85380 that binds to β2* nAChRs, nicotine that identifies α4β2* subtypes, and α-conotoxinMII that binds to α3/α6β2* receptor sites (Gotti and Clementi, 2004; Quik, 2004). Similar declines in nAChRs have also been observed in rodent models with nigrostriatal damage (O’Neill et al., 2002; Quik, 2004). Moreover, studies with MPTP-lesioned primates show that the α3/α6β2* nAChR subtype is selectively targeted, with α4β2* nAChRs affected only after more severe nigrostriatal damage (Quik et al., 2001; Kulak et al., 2002a).

These receptor declines after nigrostriatal damage contrast with the effects of nicotine treatment, which typically increases high-affinity α4β2* nAChRs in unlesioned animal models (Marks et al., 1983; Schwartz and Kellar, 1983). Similar increases in central nervous system α4β2* nAChRs have been reported with smoking, which is thought to be an effect of nicotine in tobacco (Benwell et al., 1988; Breese et al., 1997; Perry et al., 1999). Contrary to the nicotine-induced increase in α4β2* nAChRs, nicotine administration decreases α3β2* and/or α6β2* nAChRs in mouse (Salminen et al., 2004a; Lai et al., 2005) and monkey striatum (McCallum et al., 2006), as well as in rat striatum, but not in all studies (Nguyen et al., 2003; Parker et al., 2004; Mugnaini et al., 2006).

In addition to a direct receptor regulation, nicotine treatment also protects against neuronal insults. Studies in rodents show that nicotine partially prevents nigrostriatal degeneration induced by hemisection, 6-hydroxydopamine, or MPTP, although some inconsistencies have been observed (O’Neill et al., 2002; Quik, 2004). In addition, protection against nigrostriatal deficits is observed in MPTP-lesioned nonhuman primates chronically administered nicotine (Quik et al., 2006a,c). Nicotine treatment ameliorated declines in dopaminergic markers and also normalized aberrant dopaminergic activity that developed with MPTP treatment (Quik et al., 2006a,c). These observations, coupled with those in the preceding section, indicate that nicotine administration exerts multiple actions on neuronal systems with the overall response dependent on the integrative effects of combined treatments.

The present experiments were done to determine how nicotine administration affects striatal nAChRs present on dopamine terminals remaining after nigrostriatal damage. To approach this, monkeys were chronically treated with nicotine and the nigrostriatal system subsequently lesioned with the dopaminergic neurotoxin MPTP. To evaluate the effect on receptor expression, we measured both α4β2* and α3/α6β2* nAChRs, the major receptor populations in monkey striatum.

Materials and Methods

Animals and Treatments. Female adult squirrel monkeys (Saimiri sciureus) weighing between 0.5 and 0.7 kg were used, as described previously (Quik et al., 2006a,c). They were purchased from Osage Research Primates (Osage Beach, MO) and from the Primate Research Laboratory (University of South Alabama, Mobile, AL). Monkeys were housed in a room with a 13:11-h light/dark cycle and given food once daily with water ad libitum. They were then quarantined for 1 month according to California State regulations. After quarantine, treatment was conducted as described previously (Quik et al., 2006c) with four groups of monkeys: controls (n = 7), nicotine-treated (n = 6), MPTP-lesioned (n = 7), and nicotine-treated MPTP-lesioned animals (n = 6). All studies were performed 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. After acclimatization (1 month), they received drinking water containing 1% saccharin alone or also containing nicotine (free base), starting at a concentration of 25 μg/ml. The nicotine was gradually increased to 650 μg/ml over a 3-month period (Quik et al., 2006c), a dose at which the animals were maintained for an additional 3 months. They were then lesioned with three doses of MPTP (1.5 mg/kg) administered subcutaneously at 2-month intervals, whereas nicotine was continued. Nicotine was removed 24 h before death, and the monkeys were euthanized according to the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. This was done by injecting 1.5 ml of euthanasia solution intraperitoneally (390 mg of sodium pentobarbital and 50 mg/ml phenytoin sodium), followed by 1.5 ml/kg of the same solution administered intravenously.

Receptor Studies. Sections for autoradiography were prepared as described previously (Quik et al., 2006c). In brief, the brains were removed, divided along the midline, and half-placed in a brain mold. One half was then sliced into 6-mm blocks, which were quick-frozen on glass slides in isopentane on dry ice and stored at −80°C. Sections (20 μm) for autoradiography were cut using a cryostat. They were mounted onto Superfrost Plus slides, air-dried, and stored at −80°C. For 125I-epibatidine (2200 Ci/mmol) binding (Kulak et al., 2002b), sections 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. They were then incubated for 40 min with 0.015 nM 125I-epibatidine in the presence or absence of α-conotoxinMII (100 nM). The slides were subsequently washed, dried, and exposed to Kodak MR film (Eastman Kodak Co., Rochester, NY) with 125I standards for several days. Nonspecific binding was assessed in the presence of 100 μM nicotine and was similar to the film blank.

125I(A85380-[5-125I]-iodo-3(2S)-azetidinymethoxy) pyridine, 0.2 nM, 1450 Ci/mmol) binding to brain sections was done at 22°C for 60 min in the same buffer as described for 125I-epibatidine binding (Kulak et al., 2002b), with or without 100 nM α-conotoxinMII. Sections were washed in buffer as described previously, dried and exposed to Kodak MR film with the appropriate 125I standards for several days. Binding in the presence of 100 μM nicotine was defined as the blank binding and was similar to the film background.

125I-α-ConotoxinMII (2200 Ci/mmol) autoradiography was done 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 KC1, 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 nM 125I-α-conotoxinMII in buffer with 0.5% BSA, 5 mM EDTA, 5 mM EGTA, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin A, followed by washing in HEPES buffer. Nonspecific binding was defined as washing 100 μM nicotine or 100 nM epibatidine. After washing and air-drying, slides were exposed to Kodak MR film with the appropriate 125I standards for several days.

Data Analyses. The ImageQuant (GE Healthcare, Little Chalfont, Buckinghamshire, UK) system was used to determine optical density measurements from the autoradiograms. They were converted to nanocuries/milligram of tissue using standard curves generated from 125I standards. The optical density readings were within the linear range of the film. For each radioligand, the receptor binding values (femtomoles/milligram of tissue) for the appropriate
Results

Animal Model. Monkeys were chronically treated with nicotine for 6 months, after which time they were lesioned with multiple doses of MPTP over an additional 6-month period, while the nicotine was continued as described previously (Quik et al., 2006a,c). Such a nicotine treatment regimen yielded plasma nicotine and cotinine levels of 12.6 ± 3.6 and 368.4 ± 47.0 ng/ml (n = 13), respectively, both of which are in the range observed in smokers (Hukkanen et al., 2002b). MPTP-lesioning resulted in significant declines in striatal dopaminergic markers as reported earlier (Quik et al., 2006a,c) and briefly summarized in Table 1.

Effect of Chronic Oral Nicotine and MPTP Treatments on α4β2* or α-ConotoxinMII-Resistant nAChRs in Monkey Striatum. To detect changes in striatal nAChRs after nicotine treatment and nigrostriatal damage, we used 125I-epibatidine, a radioligand that labels both β2* and β4* nAChR subtypes (Davila-Garcia et al., 1997). Because there is little detectable β4 subunit in monkey striatum (Quik et al., 2005), this radioligand most likely targets β2* receptor subtypes. We also used 125I-A85380, an agonist that directly identifies β2* nAChRs (Kulak et al., 2002b). To further define nAChR subtypes, we measured 125I-epibatidine or [125I]A85380 in the presence of α-conotoxinMII (100 nM), which blocks α3/α6β2* receptors. Radioligand binding sites remaining in the presence of α-conotoxinMII were defined as α4β2* nAChRs. Those inhibited by the toxin, i.e., α-conotoxinMII-sensitive binding sites, represent α3/α6β2* nAChRs.

Chronic nicotine treatment increased α4β2* nAChRs or α-conotoxinMII-resistant [125I]-epibatidine binding (Fig. 1), as reported previously (McCallum et al., 2006). There was a significant (p < 0.001) main effect of nicotine (by two-way ANOVA) in all striatal regions, including medial and lateral caudate and ventral and dorsal putamen. In contrast, lesioning decreased α-conotoxinMII-resistant [125I]A85380 binding with a significant (p < 0.01) main effect in all regions but no significant interaction.

The effects of the combined treatments, i.e., nicotine administration and MPTP-lesioning, were then further analyzed on α4β2* binding sites (Fig. 3). Unexpectedly, the percentage increase in binding over the respective control was greater with nicotine administration (p < 0.05 using two-way ANOVA) in the MPTP-lesioned group compared with unle-
date and the dorsal putamen, the two areas with the greatest nigrostriatal damage (Quik et al., 2001; Kulak et al., 2002a). Changes in binding with MPTP-lesioning and nicotine treatment followed the same trend in the medial caudate and ventral putamen.

Similar results were observed using $[^{125}\text{I}]$A85380 (Fig. 5). MPTP treatment led to a significant ($p < 0.001$) decrease in binding sites using two-way ANOVA in all striatal areas. Again, there was no significant interaction between nicotine treatment in the lateral caudate and dorsal putamen, the two regions with the most severe nigrostriatal damage. These data suggest that there is a differential effect of nicotine on $\alpha$-conotoxinMII-sensitive $^{125}\text{I}$-epibatidine and $[^{125}\text{I}]$A85380 binding sites ($\alpha_3/\alpha_6\beta_2^*$), with a decline in binding in striatum of unlesioned monkeys and an increase in lesioned monkeys compared with their respective controls.

**Effect of Chronic Oral Nicotine and MPTP Treatments on Striatal $\alpha_3/\alpha_6\beta_2^*$ nAChRs, Defined Using $^{125}\text{I}$-a-ConotoxinMII.** MPTP-lesioning decreased $^{125}\text{I}$-epibatidine binding (Fig. 6), with a significant ($p < 0.001$) main effect of MPTP treatment using two-way ANOVA. Chronic nicotine treatment increased binding, with a significant ($p < 0.001$) main effect of nicotine treatment (by two-way ANOVA) in all regions. In contrast, MPTP-lesioning led to a significant ($p < 0.01$) decrease in binding sites in all regions, with no significant interaction. Data represent the mean ± S.E.M. of six to seven animals. The symbols indicate significant Bonferroni’s post hoc tests from own control; *, $p < 0.01$.

**Discussion**

The present results are the first to show that long-term nicotine administration partially prevents/restores nAChR declines that occur after nigrostriatal damage, including both
the \( \alpha_4 \beta_2^* \) and select \( \alpha_3/\alpha_6 \beta_2^* \) subtypes. These findings support and extend earlier work showing improved levels of dopaminergic markers in striatum of nicotine-treated MPTP-lesioned monkeys compared with lesioned animals not receiving nicotine. These markers include neurochemical and morphological measures of striatal nerve terminal integrity, such as tyrosine hydroxylase, the dopamine transporter, the vesicular monoamine transporter, and dopamine levels (see Table 1) (Quik et al., 2006c). In addition, previous studies have shown that excessive dopaminergic activity that arises as a result of nigrostriatal damage is normalized in lesioned animals chronically treated with nicotine (Quik et al., 2006a). Moreover, nicotine administration restored synaptic plasticity lost as a result of nigrostriatal damage (Quik et al., 2006a). These results combined with the present data support the hypothesis that chronic nicotine treatment improves dopaminergic nerve terminal integrity and function in lesioned animals.

The present results show that diverse conditions, including nicotine administration and nigrostriatal damage, result in varying effects on nAChR subtypes in monkey striatum. 1) Chronic oral nicotine treatment up-regulated \( \alpha_4 \beta_2^* \) receptors and, in addition, partially protected against and/or restored MPTP-induced receptor losses. 2) By contrast, nicotine administration down-regulated \( \alpha_3/\alpha_6 \beta_2^* \) nAChRs (McCallum et al., 2006), although there was a similar protection/restoration of the \( \alpha_3/\alpha_6 \beta_2^* \) nAChRs defined using \( \alpha \)-conotoxinMII-sensitive \( ^{125}\)I-epibatidine and \( ^{125}\)I-A85380 binding sites in lesioned animals treated with nicotine compared with lesioned-only ani-
Fig. 5. Effect of chronic oral nicotine and MPTP treatments on striatal α3/α6β2* nAChRs, defined using α-conotoxinMII-sensitive [125I]A85380 binding. Monkeys were administered nicotine and MPTP as described under Materials and Methods. α3/α6β2* nAChR binding sites were determined by measuring the difference between total [125I]A85380 binding and binding in the presence of α-conotoxinMII (100 nM). MPTP-lesioning led to a significant (p < 0.001) decrease in binding sites (by two-way ANOVA) in all regions. There was no significant main effect of chronic nicotine treatment on binding. However, the data in the inset show that there was a significant interaction (p < 0.05) between nicotine treatment and MPTP lesioning, indicating that there was a differential effect of nicotine in unlesioned and lesioned monkeys. Values represent the mean ± S.E.M. of six to seven animals. The symbols indicate significant Bonferroni’s post hoc tests from the respective saline-treated group, +, p < 0.05; ++, p < 0.01.

Fig. 6. Effect of chronic oral nicotine and MPTP treatments on [125I]-α-conotoxinMII binding sites in striatum. Monkeys were administered nicotine and lesioned with MPTP as described under Materials and Methods. MPTP-lesioning decreased binding of the α3/α6β2*-selective nAChR radioligand, with a significant (p < 0.001) main effect of MPTP treatment (by two-way ANOVA). In contrast, there was no significant main effect of nicotine treatment and no interaction between nicotine and MPTP treatments. Data represent the mean ± S.E.M. of six to seven animals. The symbols indicate significant Bonferroni’s post hoc tests from the respective saline-treated group, +, p < 0.001.

3) On the other hand, α3/α6β2* nAChRs measured using [125I]-α-conotoxinMII were also decreased with nicotine treatment (Quik et al., 2001; McCallum et al., 2006) but they did not seem to be protected and/or restored by nicotine treatment in striatum of lesioned animals. These differential effects of nicotine treatment on α4β2* and α3/α6β2* nAChR subtypes are consistent with previous results (McCallum et al., 2006). In addition, we had previously reported a differential regulation of α3/α6β2* subtypes after l-DOPA administration (Quik et al., 2003). The subunit composition of these different α3/α6β2* receptor subtypes is currently not known, although they may represent α3β2* and/or α6β2* nAChR subtypes also expressing α2, α4, and/or β subunits, all of which are present in monkey striatum (Quik et al., 2005).

The α3/α6β2* nAChRs, which are thought to be confined primarily to dopaminergic terminals (Quik et al., 2001), were decreased ~70%, a value that corresponds to the ~80% declines in other dopaminergic markers (see Table 1). By contrast, the decrease in α4β2* binding sites after MPTP lesioning was ~30% (Kulak et al., 2002a,b). This apparent discrepancy relates to the fact that this latter nAChR subtype is not only localized to dopaminergic terminals but is also present on other striatal cells unaffected by MPTP treatment.

As mentioned earlier, [125I]A85380 interacts selectively with β2* nAChRs (Kulak et al., 2002b), and [125I]-epibatidine is thought to bind to β2* nAChRs in monkey striatum because there is no appreciable β4 subunit in this region (Quik et al., 2005). Therefore, we anticipated that the receptors labeled by these two radioligands in the presence of α-conotoxinMII represented similar populations of α4β2* nAChRs. Indeed, an increase was observed in both these measures in striatum after nicotine treatment. However, this increase in α4β2* nAChR was substantially greater using [125I]A85380 than [125I]-epibatidine. Such findings were also obtained in rodent striatum following nicotine treatment (Lai et al., 2005). This differential increase in binding may suggest that the two radioligands identify unique α4β2* nAChR subtypes. Monkey striatum also expresses the α2 and α3 subunits (Quik et al., 2005), whereas rodent striatum contains the α5 and β4 subunits (Zoli et al., 2002). These data may suggest that [125I]A85380 recognizes additional α4β2* subtypes containing one or more of these subunits, which are preferentially increased by nicotine treatment. This hypothesis that [125I]-epibatidine and [125I]A85380 identify distinct striatal nAChR subtypes is further supported by the observation that there were also differential effects of MPTP...
on α4β2* nAChR subtypes, with a greater decline in receptors labeled by [125I]A85380 than 121I-epibatidine (see Table 1).

Because striatal nAChRs stimulation results in dopamine release, improved levels of α4β2* and select α3/α6β2* nAChR subtypes in the nicotine-treated lesioned group compared with lesioned-only animals not receiving nicotine may be important for maintaining normal striatal function. As indicated earlier, the primary nAChR populations in monkey striatum are the α4β2* and α3/α6β2* subtypes (Quik and McIntosh, 2006b). The α4β2* nAChRs involved in dopamine release are located presynaptically on nigrostriatal dopaminergic terminals and are responsible for ~30% of evoked release. The α3/α6β2* subtype is predominantly present on striatal dopamine terminals and mediates ~70% nicotine-stimulated dopamine release (McCallum et al., 2005). The present data show that the α4β2* and select α3/α6β2* subtypes are improved to a greater extent in striatum of nicotine-treated lesioned monkeys compared with lesioned-only animals, suggesting they are present on dopamine nerve terminals that are partially restored and/or protected by chronic nicotine treatment.

These studies demonstrate a protective effect of nicotine in a nonhuman primate model, supporting previous data in other experimental model systems. An extensive literature has demonstrated a protective effect of nicotine against toxicity in neuronal cells in culture (O’Neill et al., 2002; Quik, 2004). Furthermore, such studies have provided insight into the molecular mechanisms, which may involve initial changes in intracellular calcium, followed by activation of diverse downstream pathways and processes, including alterations in caspases, kinases, cAMP-response element-binding protein, apoptotic signaling, the nitric oxide/cGMP pathway, and others (Dajas-Bailador and Wonnacott, 2004; Wonnacott et al., 2005). Protective effects of nicotine have also been shown in vivo using rodent models of nigrostriatal damage. A consistent improvement in nigrostriatal mark-ers has been observed in unilaterally lesioned rats administered nicotine, although studies indicate that the nicotine-dosing regimen and nature of the lesion are important variables that influence the degree of neuroprotection observed in rodents (O’Neill et al., 2002; Quik, 2004). The data in mouse models of nigrostriatal damage are somewhat more variable compared with nicotine-induced protection observed in some studies but not others (O’Neill et al., 2002; Quik, 2004). These discrepancies most probably relate to differences in the proportion and subtype of nAChRs present in striatum, mechanism(s) of induction of nigrostriatal damage, and pharmacokinetics/metabolism of nicotine. The present data using a chronic nicotine regimen, coupled with long-term induction of nigrostriatal damage in a nonhuman primate model, would lend support to the idea that nicotine protects against and/or restores dopaminergic measures in striatum and suggest a potential beneficial role for nicotine in Parkinson’s disease therapy.

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