Nicotinic receptor-mediated reduction in L-dopa-induced dyskinesias may occur via desensitization

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Running Title Page

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ABBREVIATIONS: AlMs, abnormal involuntary movements; ANOVA, analysis of variance; α-CtxMII, α-conotoxinMII; 6-OHDA, 6-hydroxydopamine; nAChRs, nicotinic receptors

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Abstract

L-dopa-induced dyskinesias in Parkinson's disease are a significant clinical problem for which few therapies are available. We recently showed that nicotine reduces L-dopa-induced abnormal involuntary movements (AIMs) in parkinsonian animals, suggesting it may be useful for the treatment of L-dopa-induced dyskinesias. The present experiments were performed to understand the mechanisms whereby nicotine reduces L-dopa-induced AIMs. We used a well-established model of dyskinesias, L-dopa-treated unilateral 6-hydroxydopamine-lesioned rats. Dose ranging studies showed that injection of 0.1 mg/kg nicotine once or twice daily for 4 or 10 days most effectively reduced AIMs, with no worsening of parkinsonism. Importantly, a single nicotine injection did not reduce AIMs indicating that nicotine's effect is due to long-term rather than acute molecular changes. Administration of the metabolite cotinine did not reduce AIMs, suggesting a direct effect of nicotine. Experiments with the nicotinic receptor (nAChR) antagonist mecamylamine were done to determine whether nicotine acted via a receptor-mediated mechanism. Unexpectedly, several days of mecamylamine injection (1.0 mg/kg) alone significantly ameliorated dyskinesias to a comparable extent as nicotine. The decline in AIMs with combined nicotine and mecamylamine treatment was not additive suggesting that nicotine exerts its effects via a nAChR interaction. This latter finding, combined with data showing that mecamylamine reduced AIMs to a similar extent as nicotine, and that nicotine or mecamylamine treatment both decreased α6β2* and increased α4β2* nAChR expression, suggests that the nicotine-mediated improvement in L-dopa-induced AIMs may involve a desensitization block. These data have important implications for the treatment of L-dopa-induced dyskinesias in Parkinson's disease.
Introduction

A major complication of L-dopa treatment for the management of Parkinson’s disease is the development of dyskinesias. These excessive abnormal involuntary movements (AIMs), which occur in the majority of patients with repeated exposure to L-dopa, can be very troublesome and even disabling (Fahn, 2009; Schapira et al., 2009). Few drugs are currently available to attenuate dyskinesias (Fahn, 2009; Schapira et al., 2009). An alternate approach for the management of L-dopa-induced dyskinesias in Parkinson's disease involves deep brain stimulation; however, this is a serious surgical intervention with its associated risks (Benabid et al., 2009). There is therefore a continued search for pharmacological treatments to manage L-dopa-induced dyskinesias in Parkinson’s disease.

Recent studies from our laboratory show that nicotine treatment reduces L-dopa-induced AIMs in several different parkinsonian animal models (Quik et al., 2008). Nicotine administered via the drinking water attenuated L-dopa-dyskinesias by ~50% in parkinsonian monkeys (Quik et al., 2007). A similar decline in L-dopa-induced AIMs was observed in parkinsonian rats (Bordia et al., 2008) and mice (Huang et al., 2009), attesting to the robustness of this effect across species. These combined data suggest that nicotine may represent a novel pharmacological approach for treating the abnormal movements that arise with chronic L-dopa dosing.

Nicotine generally exerts its effects in the CNS via an interaction at nicotinic acetylcholine receptors (nAChRs) (Albuquerque et al., 2009; Gotti et al., 2009). These are pentameric acetylcholine-gated cation channels assembled from different α (α2 – α7) and β (β2 - β4) subunits. The primary nAChR subtypes present in the brain are the α7 and the α4β2* populations (the asterisk signifies the possible presence of other nAChR subunits in the receptor complex). These two subtypes are very widely distributed throughout the brain and have been
implicated in multiple neuronal functions including reward, addiction, attention, cognition, depression, affect and movement (Albuquerque et al., 2009; Gotti et al., 2009). There is also a smaller population of $\alpha_6\beta_2^*$ nAChRs, which is restricted in localization to CNS catecholaminergic neurons including those in the nigrostriatal pathway, which may be involved in reward, addiction and motor activity (Drenan et al., 2008; Pons et al., 2008). All these three nAChR subtypes ($\alpha_4\beta_2^*$, $\alpha_6\beta_2^*$ and $\alpha_7$) have been implicated in striatal function and shown to influence striatal dopamine release (Grady et al., 2007). In addition, studies using cyclic voltammetry indicate that this regulation of dopamine release most likely represents a complex interplay between nicotinic receptor activation and desensitization (Exley et al., 2008; Meyer et al., 2008; Perez et al., 2008), with the latter resulting in an effective channel block similar to that observed with nAChR antagonists (Corringer et al., 2006; Picciotto et al., 2008; Buccafusco et al., 2009).

The objective of the present study was to gain insight into the mechanism(s) of the nicotine-mediated reduction in L-dopa-induced AIMs. To achieve this, we did a series of behavioral studies to investigate the effect of different nicotine administration regimens and to determine the effect of the nicotinic receptor antagonist mecamylamine on the occurrence of L-dopa-induced AIMs. We also evaluated changes in both $\alpha_4\beta_2^*$ and $\alpha_6\beta_2^*$ nAChR expression under different treatment regimens. Altogether the data suggest that nicotine may attenuate L-dopa-induced dyskinesias via nAChR receptor desensitization or block. Such results are in agreement with an emerging literature that desensitization plays a critical role in nAChR-mediated function.
Methods

6-Hydroxydopamine (6-OHDA) lesions

Male Sprague-Dawley rats (190 to 225 g on arrival; Charles River Laboratories, Gilroy, CA) were housed, two per cage in a temperature- and humidity-controlled environment subject to a 12 h light/dark cycle with free access to food and water. Two to three days after acclimatization, rats were unilaterally lesioned with 6-OHDA (free base; Sigma-Aldrich Co., St. Louis, MO) as previously described (Cenci and Lundblad, 2007; Bordia et al., 2008). They were first anesthetized with isofluorane (5% for induction and 2% for maintenance during the surgical procedure) and subsequently placed in a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, CA). Two injections of 2 μl each of 6-OHDA (3 μg free base/μl dissolved in 0.02% ascorbic acid, 0.9% saline) were made into the right medial forebrain bundle at coordinates: (1) AP, -4.4; ML, 1.2; DV, 7.8; tooth bar at –2.4; (2) AP, – 4.0; ML, 0.75; DV, 8.0; tooth bar at +3.4 relative to the bregma. Delivery of 6-OHDA into the target area was done over 2 min. The needle was left at the site of injection for a further 2 min before withdrawal.

After recovery, the rats received buprenorphine (0.02 mg/kg sc) as an analgesic treatment. All procedures conformed to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Rotational behavior; amphetamine challenge

Two wks post-surgery rats were behaviorally assessed for the extent of 6-OHDA lesioning by testing for amphetamine-induced rotation (ROTOMAX, AccuScan Instruments Inc. Columbus, Ohio, USA) (Cenci and Lundblad, 2007; Bordia et al., 2008). Each rat was first placed in a cylindrical chamber for 15 min for habituation, followed by a 30 min baseline
recording after which rats were injected ip with 4 mg/kg amphetamine (Sigma-Aldrich Co., St. Louis, MO). Ipsilateral full rotations (360°) were recorded for 90 min. Rats turning > 4 complete turns/ min were used in the study.

**L-dopa-induced abnormal involuntary movement (AIM) ratings**

Three wks after daily injection of 8 mg/kg L-dopa methyl ester and 15 mg/kg benserazide sc (Sigma-Aldrich Co., St. Louis, MO), the rats were evaluated for AIMs, as previously described (Cenci and Lundblad, 2007; Bordia et al., 2008). This included three different AIM components; (1) axial AIMs, contralateral flexion of the neck and upper body that resembles dystonia-like axial torsion; (2) orolingual AIMs, which include stereotyped jaw movements, twitching of facial muscle and contralateral rapid and jerky tongue protrusion, manifested either individually or together; (3) forelimb AIMs, jerky repetitive movement of the forelimb contralateral to the lesion side, which could be fast and irregular or continuous. The above mentioned AIM components were scored on a scale from 0 to 4 based on the duration and persistence of the abnormal behavior as follows; 0 = not present; 1 = occasional; 2 = frequent; 3 = present for the entire observation time and interrupted by a loud stimulus; and 4 = present for entire observation time but not interrupted by a loud stimulus. The rats were rated at 9 time points, for 2 min each, every 20 min. The highest possible score that could be achieved in a session by each animal was 108 (highest total score per time point = 12; number of time points over 3 h = 9). All animals were habituated for a 15 min baseline before rating for L-dopa-induced AIMs.

**NACHR drug treatments**

One set of twenty rats was used for all the behavioral experiments described in this study.
Three days or more washout periods were allotted between the different treatments, with L-dopa treatment continued during the washout. Control studies showed that a 3 d time interval was sufficient for L-dopa-induced AIM scores to return to control values (Bordia et al., 2008). It is possible that L-dopa-induced AIMs may be influenced by previous dosing of agonists and antagonists; however, repeat experiments with the same doses of a specific drug yielded a similar decline in L-dopa-induced AIMs. In one series of experiments, mecamylamine (0.5 to 2 mg/kg; Sigma-Aldrich Co., St. Louis, MO) and/or nicotine (0.01 to 0.75 mg/kg; freebase, Sigma-Aldrich Co., St. Louis, MO) were injected 30 and 10 min before L-dopa/benserazide treatment, respectively, similar to previous studies (Dekundy et al., 2007; Matta et al., 2007). In another set of experiments, nicotine (0.1 to 2 mg/kg/day; freebase, Sigma-Aldrich Co., St. Louis, MO) or cotinine (2.5 and 5 mg/kg/day; Sigma-Aldrich Co., St. Louis, MO) was administered via osmotic minipump (Alzet model 2004, delivery rate, 0.25 μl/h; Durect Corporation, Cupertino, CA) as previously detailed (Bordia et al., 2008).

**Limb use asymmetry test**

We used the forelimb asymmetry test as an index of motor function after nigrostriatal denervation. Exploratory behavior was analyzed as previously described in our laboratory (Bordia et al., 2008) and that of others (Schallert et al., 2000). Rats were placed in a transparent cage and evaluated for contralateral forelimb use 1 h after L-dopa injection, a time at which its antiparkinsonian effects are maximal. Values are expressed as a percent of total limb use before and 1 h after L-dopa treatment. The rats were assessed for their exploratory behavior for 5 min as previously described (Bordia et al., 2008).
**Cotinine measurements**

Plasma levels of cotinine, a primary metabolite of nicotine with a relatively long half-life (Matta et al., 2007) were determined using an EIA kit (Orasure Technologies, Bethlehem, PA). Blood samples were collected 3-4 h after nicotine injection or 2 wks after initiation of nicotine or cotinine treatment via osmotic minipump. Blood was drawn under isofluorane anesthesia from the lateral saphenous vein in heparin-coated tubes. Blood was centrifuged at 1200 g for 15 min at 4°C, and the plasma stored at -80°C until analyzed.

**Measurement of nAChR binding sites**

Another set of unilateral 6-OHDA lesioned rats were used for the binding studies. Rats were administered mecamylamine (1 mg/kg sc) or nicotine (0.2 mg/kg sc) 30 and 10 min before L-dopa/benserazide treatment, respectively, daily for 14 d. On the last day, they were killed 1 h after L-dopa treatment when its effects were maximal. The brains were quickly removed, rinsed and frozen in isopentane on dry ice. Eight μm sections were cut at -15°C in a cryostat, thaw mounted onto poly-L-lysine coated slides, dried, and stored at -80°C until use.

**125I-epibatidine autoradiography**

125I-epibatidine (specific activity, 2200 Ci/mmol; PerkinElmer Life and Analytical Science, Boston, MA, USA) binding was done as previously reported (Quik et al., 2003). Thawed 8.0 μm thick striatal sections were pre-incubated at room temperature 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 incubated for 40 min with 0.015 nM 125I-epibatidine in the presence or absence of α-conotoxinMII (300 nM) to define α4β2* nAChRs binding sites. The slides were then washed,
dried and exposed to Kodak MR film (Eastman Kodak Co., Rochester, NY, USA) with \(^3\)H-microscale standards (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) for several days.

\(^{125}\text{I-}\alpha\text{-conotoxinMII (}\alpha\text{-CtxMII) autoradiography}\)

Binding of \(^{125}\text{I-}\alpha\text{-CtxMII}\) (specific activity, 2200 Ci/mmol) was done as reported previously (Quik et al., 2003). Eight \(\mu\)m striatal sections were preincubated at room temperature for 15 min in binding buffer (144 mM NaCl, 1.5 mM KCl, 2mM CaCl\(_2\) 1mM MgSO\(_4\), 20mM HEPES and 0.1 % BSA (bovine serum albumin), pH 7.5) plus 1 mM PMSF (phenylmethylsulfonyl fluoride) This was followed by 1-h incubation at room temperature in binding buffer also containing 0.5% bovine serum albumin, 5 mM EDTA, 5 mM EGTA and 10 \(\mu\)g/ml each of aprotinin, leupeptin and pepstatin A plus 0.5 nM \(^{125}\text{I-}\alpha\text{-CtxMII}\). The assay was terminated by washing the slides for 10 min at room temperature, 10 min in ice cold binding buffer, twice for 10 min in 0.1X buffer at \(0^\circ\)C and two final 5-s washes in ice cold deionized water. The striatal sections were air-dried and exposed to Kodak MR film (Eastman Kodak Co., Rochester, NY, USA) for 5-7 together with \(^3\)H-microscale standards (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Nicotine (100 \(\mu\)M) was used to determine nonspecific binding.

\([^{125}\text{I}]\text{RTI-121 binding}\)

The dopamine transporter was measured using \([^{125}\text{I}]\text{RTI-121}\) (specific activity, 2200 Ci/mmol; PerkinElmer Life and Analytical Science, Boston, MA, USA) autoradiography as previously described (Bordia et al., 2008). Brain sections were preincubated 2 x 15 min in buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl) and then incubated for 2 h with 50 pM
[\textsuperscript{125}I]RTI-121 in the same buffer also containing 0.025% BSA and 1 \mu M fluoxetine. Sections were washed 4 x 15 min in ice cold buffer, 1 x 10 s in ice cold doubly-distilled H\textsubscript{2}O, dried and exposed to Kodak MR film (Eastman Kodak Co., Rochester, NY, USA) for 2-3 days along with \textsuperscript{3}H-microscale standards (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Nonspecific binding was determined in the presence of the dopamine uptake inhibitor nomifensine (100 \mu M).

**Data analyses**

The optical density measurements from the autoradiograms were evaluated using ImageQuant (GE Healthcare, Little Chalfont, Buckinghamshire, UK) system and converted to fmol/mg tissue by using standard curves generated from \textsuperscript{3}H standards. All statistical analyses were done using GraphPad Prism\textsuperscript{\textregistered} (GraphPad Software, Inc, San Diego, CA.). Differences in ratings between treatment groups were determined using one-way analysis of variance (ANOVA), followed by a Dunnett’s multiple comparison test. For time-course studies, repeated measures analysis of variance (ANOVA) followed by Bonferoni post hoc test was used. A level of 0.05 was considered significant. Values are the mean \pm S.E.M. of the indicated number of rats, and represent data pooled from one to two separate experiments.

**Results**

**Nicotine-mediated reduction in L-dopa-induced AIMs; dose response**

At the beginning of the study, lesioned rats were randomly divided into two groups such that total L-dopa-induced AIM scores were comparable in the two sets of animals (62.9 \pm 6.6, 60.5 \pm 10.4). The rats were then injected with varying doses of nicotine (0.01 to 0.75 mg/kg) or saline
once daily for four consecutive days 10 min before L-dopa treatment. The results in Fig. 1 show that nicotine (0.01 and 0.05 mg/kg) treatments had no significant effect on L-dopa-induced AIMS. However, 0.1 mg/kg nicotine resulted in a maximal reduction in total, orolingual, and forelimb AIMS with a trend for a decline in axial AIMS. Higher doses of nicotine (0.25, 0.5 and 0.75 mg/kg) also resulted in significant reductions in total, orolingual and forelimb AIMS; however, the declines appeared less pronounced than those observed with the 0.1 mg/kg dose. Values for the saline-treated control rats remained stable over the different treatment regimes with a mean total L-dopa-induced AIM score of 69.1 ± 3.2 (n = 54, which represent the scores from 9 rats over 6 separate experiments). Total AIM values for the control group were also stable over time, with the weekly rating for the control group being 67.5 ± 2.8 (n = 9 weekly experiments). It should be noted that the higher doses were also associated with the development of anxiety-like behaviors such as irritability and nervousness. Since stress (anxiety) is known to increase AIMS in Parkinson's disease patients, it is possible that these nicotine-mediated side effects enhance expression of L-dopa-induced AIMS in the rats at the higher nicotine doses.

Measurement of plasma cotinine levels (Table 1) 3 to 4 h after injection of the 0.1 mg/kg nicotine dose yielded concentrations of 202 ± 26 ng/ml (n = 10). Thus, optimal declines in L-dopa-induced AIMS are obtained with nicotine doses that result in plasma cotinine values at the lower end of those observed in the plasma of smokers (Matta et al., 2007).

**Chronic cotinine treatment does not reduce L-dopa-induced AIMS**

The presence of relatively high plasma cotinine levels with nicotine treatment has led to the idea that cotinine itself may have effects *in vivo*. We tested this possibility by implanting cotinine containing minipumps (2.5 or 5 mg/kg/day) in 6-OHDA-lesioned rats receiving a once daily
injection of L-dopa (8 mg/kg) + benserazide (15 mg/kg). These doses of cotinine were selected because they yielded plasma cotinine levels similar to those in moderate to heavy smokers (Matta et al., 2007). The results in Table 2 show that, despite high plasma cotinine levels, 3 wks of cotinine treatment had no effect on total, axial, orolingual or forelimb AIMS.

**Nicotine treatment does not worsen parkinsonism**

Experiments were next done to evaluate the effect of nicotine on parkinsonism. This is important because treatments that decrease L-dopa-induced dyskinesias have also been reported to worsen motor behavior (Hsu et al., 2004). Two behavior parameters were examined to evaluate the effect of nicotine on L-dopa-induced motor function (Bordia et al., 2008). Fig. 2A demonstrates the effect of nicotine using the forelimb use asymmetry test, a well-characterized model that evaluates contralateral limb use after nigrostriatal damage (Schallert et al., 2000). These experiments were done using the dose of nicotine that optimally reduced L-dopa-induced AIMS, that is, 0.1 mg/kg nicotine. The results show that 4 days of nicotine (0.1 mg/kg) treatment did not affect parkinsonism either ON or OFF L-dopa.

In Fig. 2B, we investigated another measure of parkinsonism, L-dopa-induced contralateral turning (Dekundy et al., 2007). Contralateral turning was similar in rats receiving saline (677 ± 230, n=10) or nicotine (675 ± 166, n = 9), consistent with previous results (Bordia et al., 2008). The observation that nicotine treatment per se does not affect contralateral turning is initially somewhat unexpected, especially in view of recent studies (Gregorio et al., 2009). These authors had shown that acute nicotine, like amphetamine, induces turning behavior in unilateral 6-OHDA treated rats by promoting release of dopamine in the non-lesioned striatum of the rats, although the effects of nicotine were less pronounced than those with amphetamine. This difference
between our study and that of Gregorio et al (2009) is most likely due to the fact that we administered nicotine on a long term basis rather than acutely, with a resultant nAChR desensitization.

The reduction in L-dopa-induced AIMs requires multiple day dosing

We next determined the length of time of nicotine dosing required for an optimal reduction in L-dopa-induced AIMs. The results in Fig. 3 show that a single (acute) injection of nicotine did not attenuate L-dopa-induced AIMs. However, as previously shown, once daily nicotine injection for 4 days significantly \( (p < 0.01) \) reduced total, oral and forelimb AIMs. No further reduction in AIMs was obtained by increasing the length of nicotine treatment time (10 days). We also tested twice daily nicotine injection for 4 days; this treatment resulted in a somewhat greater reduction in total, orolinguial, and forelimb AIMs compared to once daily injection. These combined data suggest that the nicotine-induced decline in L-dopa-induced AIMs most likely involves longer-term molecular changes in the brain.

The time course data in Fig. 4 show that L-dopa-induced AIMs plateau within 60 min after injection and then gradually decline. Multi-day 0.1 mg/kg nicotine treatment (Fig. 4B, C, D) decreased L-dopa-induced AIMs, with a roughly similar pattern in the decline in L-dopa-induced AIMs using the different treatment regimens. Analyses of variance yielded a significant main effect of nicotine treatment with the once \( (p < 0.05) \) and twice daily 4 days \( (D, p < 0.01) \) with a trend for reduction with once daily treatment for 10 days \( (Fig. \ 4 \ C, p < 0.06) \). The single (acute) dose regimen had no effect on L-dopa-induced AIMs (Fig. 4A).

**Chronic mecamylamine treatment reduces L-dopa-induced AIMs**
We next initiated a series of experiments with the general nicotinic receptor blocker, mecamylamine as an approach to evaluate whether the effects of nicotine are receptor-mediated. The results in Fig. 5 show that a single (acute) dose of mecamylamine (2 mg/kg) given 30 min before L-dopa had no significant effect on AIMs. However, 4 days of once daily injection of varying doses of mecamylamine significantly attenuated L-dopa-induced AIMs. Optimal efficacy was observed with the 1.0 mg/kg dose, with a significant reduction in total ($P < 0.05$), orolingual ($P < 0.01$), and forelimb ($P < 0.05$), and a trend for decline in axial AIMs. A 4 day treatment regimen with 0.5 and 2.0 mg/kg mecamylamine also resulted in significant reductions in orolingual ($P < 0.01$) and forelimb ($P < 0.05$) AIMs. It should be noted that mecamylamine treatment was associated with a number of transient adverse effects, including constipation and difficulty in micturation, which subsided several hours after mecamylamine injection. These effects are not unexpected since mecamylamine is a nonspecific nAChR antagonists that blocks ganglionic nicotinic receptors.

**Combined effect of mecamylamine and nicotine on L-dopa-induced AIMs**

We next tested the combined effect of mecamylamine and nicotine on L-dopa-induced AIMs using the multiday injection regimen described in Methods. We injected 0.1 mg/kg nicotine and/or 1.0 mg/kg mecamylamine, doses that resulted in a maximal reduction in L-dopa-induced AIMs when tested alone. Four day mecamylamine or nicotine treatment alone significantly reduced L-dopa-induced AIMs compared to saline treatment (Fig. 6). Interestingly, the effect of combined treatment with both drugs was similar to that observed with nicotine or mecamylamine on their own (Fig. 6). The observation that the effect of nicotine and mecamylamine was not additive, suggests that they act via a similar mechanism. Since mecamylamine is a well-known
nAChR antagonist, these data may suggest that the nicotine-mediated decline in L-dopa-induced AIMs occurs via a desensitization block.

Nicotine administration via minipump yields a similar reduction in L-dopa-induced AIMs as intermittent treatment via injection

In the experiments described thus far, nicotine was given as a bolus once or twice daily for different days via injection 10 min before the assessment of L-dopa-induced AIMs. Our previous work had shown that nicotine also reduced L-dopa-induced AIMs when given via minipump (Bordia et al., 2008). As an approach to compare the effectiveness of these two modes of nicotine administration in reducing L-dopa-induced AIMs, we investigate the dose-response relationship for nicotine administered via minipump. Lesioned rats were surgically implanted with minipumps releasing 0.10 to 2.0 mg/kg/day nicotine or vehicle. Two wks after minipump placement, the rats were rated for L-dopa-induced AIMs as described in Methods. Low dose nicotine (0.10 and 0.25 mg/kg/day) did not significantly alter L-dopa-induced AIMs compared to vehicle-treated rats (Fig. 7). By contrast, significant declines in total, orolingual, and forelimb AIMs were observed with the higher nicotine doses (0.5, 1.0 and 2.0 mg/kg/d), with a trend for a decrease in axial AIMs. The values for the controls remained stable with a mean total L-dopa-induced AIMs score of 62.6 ± 3.8 (n = 27; which represent the scores from 9 rats over 3 separate experiments). The pattern of the dose response curve was very similar to that observed when nicotine was given via injection (see Fig. 1).

Plasma cotinine levels at the dose of nicotine (0.5 mg/kg/day) that resulted in a maximal reduction in L-dopa-induced AIMs were 115 ± 25 ng/ml (n = 10) (Table 1). Again, the greatest reduction in L-dopa-induced AIMs was obtained with a nicotine dose that yielded plasma
cotinine levels at the lower end of those observed in the plasma of smokers.

**Dopamine transporter**

To evaluate the extent of nigrostriatal damage, we measured the dopamine transporter, a marker of the dopaminergic nerve terminal. The results in Table 3 show that there was a 99% decline in the transporter, as anticipated, attesting to the effectiveness of the lesioning paradigm (Cenci and Lundblad, 2007; Bordia et al., 2008).

**Nicotine or mecamylamine treatment resulted in similar changes in nAChR expression in rat striatum**

Rats were treated with nicotine (0.2 mg/kg) or mecamylamine (1 mg/kg) as described in the Methods, together with L-dopa/benserazide (Fig. 8). Both nicotine and mecamylamine treatment led to a significant decrease (p < 0.01) in striatal $^{125}$I-$\alpha$-CtxMII or $\alpha$6$\beta$2$^*$ nAChR binding sites on the unlesioned, but not lesioned side when compared to vehicle-treated rats. These data suggest that the rat striatal $\alpha$6$\beta$2$^*$ nAChR population downregulated by nicotine is lost with nigrostriatal damage.

We also performed $^{125}$I-epibatidine binding in the presence of nonradioactive $\alpha$-CtxMII to study the effects of multiple day nicotine or mecamylamine treatment on $\alpha$4$\beta$2$^*$ nAChRs (Fig 8). Both drugs significantly increased (p < 0.01) $\alpha$4$\beta$2$^*$ binding on the unlesioned but not lesioned side compared to vehicle-treated controls. Thus, the $\alpha$4$\beta$2$^*$ nAChR population upregulated by nicotine appears to be preferentially reduced in striatum of lesioned rats, similar to the results with $\alpha$6$\beta$2$^*$ nAChR described above.
Discussion

The present work is the first to investigate the potential mechanism(s) of the nicotine-mediated decline in L-dopa-induced dyskinesias. The results suggest that nicotine exerts its effects through an interaction at nAChRs, that nAChR desensitization may play an important role in the observed reduction in L-dopa-induced dyskinesias, and that the long-lasting nicotine metabolite cotinine is not involved in these effects. Evidence for these possibilities are discussed below.

The idea that nicotine reduces L-dopa-induced dyskinesias by interacting at nAChRs is based on our experiments with mecamylamine, an antagonist that blocks multiple nAChR subtypes. These data show that the combined effects of mecamylamine and nicotine do not result in an additive decrease in L-dopa-induced AIMs in parkinsonian rats. One interpretation of these observations is that nicotine and mecamylamine attenuate AIMs via a shared mechanism of action, that is, by interacting at nAChRs. This idea is further supported by our studies which show that nAChR subtype agonists decrease L-dopa-induced dyskinesias in rats (Campos et al., 2009; Parameswaran et al., 2009).

Our studies with mecamylamine also showed that an antagonist alone reduced L-dopa-induced dyskinesias to a similar extent as the agonist nicotine. The molecular basis for these seemingly discrepant observations may relate to findings that nicotine initially activates and then subsequently desensitizes or inactivates nAChRs, with a consequent receptor block (Corringer et al., 2006; Picciotto et al., 2008; Buccafusco et al., 2009). These observations suggest that nicotine and the antagonist mecamylamine decrease L-dopa-induced dyskinesias via nAChR blockade.
This possibility is also suggested from the results of the receptor studies. These showed that nicotine or mecamylamine both resulted in declines in α6β2* nAChRs in the unlesioned but not lesioned striatum. The agonist and antagonist also both increased α4β2* nAChRs in the unlesioned, but not lesioned striatum. The similarity in the changes in α6β2* and α4β2* nAChRs with nicotine and mecamylamine treatment with and without nigrostriatal damage suggests a similarity in mechanism of action, that is, that nicotine induces its effects via a receptor desensitization blockade.

Further support for a role for desensitization stems from our experiments showing a similar reduction in L-dopa-induced AIMs whether nicotine was given intermittently via injection or the drinking water (Bordia et al., 2008), or constantly via minipump implantation. This latter mode of administration results in a slow continual release of nicotine that readily promotes receptor desensitization. The idea that a nAChR block either via desensitization or direct antagonism is important would suggest that nicotine reduces L-dopa-induced AIMs by preventing the action of endogenous acetylcholine at CNS nAChRs.

Precedence for the idea that nAChRs desensitization makes an important contribution to nicotine’s functional effects is evident from previous work showing that nicotine and nAChR blockers may have comparable pharmacological/physiological effects (Picciotto et al., 2008; Buccafusco et al., 2009). For instance, electrophysiological and neurotransmitter release experiments demonstrate that nicotine and nAChR blockers elicit similar responses in various CNS preparations (Buccafusco et al., 2009). Moreover, it is well established that chronic administration of either nicotine or nAChR antagonists both result in nAChR upregulation (Hulihan-Giblin et al., 1990b; Hulihan-Giblin et al., 1990a; Kuryatov et al., 2005; Picciotto et al., 2008; Buccafusco et al., 2009). Behavioral studies also show that the agonist nicotine and the
antagonist mecamylamine both improve memory-related task performance in rodents and monkeys (Buccafusco and Jackson, 1991; Terry et al., 1999) and exert antidepressant-like effects (Semba et al., 1998; Tizabi et al., 1998; Shytle et al., 2002; Gatto et al., 2004). Reward/reinforcement associated with smoking behavior and effects of nicotine on hormone secretion is also thought to be influenced by both receptor activation and desensitization (Hulihan-Giblin et al., 1990b; Hulihan-Giblin et al., 1990a; Picciotto et al., 2008). Thus, the overall behavioral consequences of nicotine may represent a balance between the integrative effects of nAChR activation and subsequent desensitization.

The mechanisms whereby nAChR-mediated desensitization decreases L-dopa-induced AIMs remain to be investigated. Our data suggest that long-term molecular and cellular adaptations are important since acute nicotine exposure was ineffective. Desensitization most likely involves post-translational receptor changes including receptor phosphorylation (Corringer et al., 2006; Picciotto et al., 2008; Buccafusco et al., 2009). Such modifications and/or others may lead to alterations in receptor maturation and trafficking, possibly via an as yet unknown endogenous substance that modulates receptor expression (Corringer et al., 2006). Alternatively, or as well, nicotine exposure may slowly stabilizes nAChRs in a high-affinity state that is more easily activated, leading eventually to desensitization (Vallejo et al., 2005). Molecular studies such as those described above are a critical next step in understanding how nicotine to improves L-dopa-induced dyskinesias.

The nAChR subtypes responsible for the nicotine-mediated decline in L-dopa-induced dyskinetic-like movements have not yet been elucidated. We anticipate that α4β2* receptors are important since they are widely distributed throughout the brain. In addition, they are present in the striatum, with 80% localized postsynaptically on GABAergic neurons where they modulate
GABA release, and 20% presynaptically on dopaminergic terminals which are involved in regulating dopamine release (Grady et al., 2007; Quik et al., 2008). The present results show that nicotine treatment increased α4β2* nAChRs in the unlesioned but not lesioned striatum. Since nicotine treatment reduces abnormal movements in lesioned rats, these data suggest that remaining nAChRs are involved in the nicotine-mediated decrease in L-dopa-induced AIMs. The nature of this subtype(s) is currently not known. The most likely candidate is the remaining α4β2 nAChR population rather than the α4α5β2 subtype since α4α5β2 nAChRs are localized presynaptically on dopaminergic terminals which are lost with lesioning (Grady et al., 2009).

The α6β2* nAChRs localized to catecholaminergic neurons may also be of relevance for the observed effects of nicotine on L-dopa-induced AIMs (Grady et al., 2007; Quik et al., 2008). The current data show that there is a very marked decline in α6β2* nAChRs with nigrostriatal damage. However, it is possible that a small remaining subset on striatal dopaminergic, and/or possibly serotonergic, neurons may play a crucial role. It is also possible that other subtypes, such as α7 nAChRs, play a role because these receptors are involved in numerous functions throughout the CNS that may be indirectly linked to the development of L-dopa-induced dyskinesias.

The decline in L-dopa-induced AIMs may be due to a direct effect of nicotine at nAChRs; however, it could also be due to a nicotine degradation product. One such compound is cotinine, a primary nicotine metabolite with an extensive half-life (17-18 hours) (Matta et al., 2007). The rather high circulating plasma cotinine levels after nicotine ingestion, coupled with findings that cotinine stimulates nAChRs, has prompted the suggestion that cotinine per se may have biological effects (Dwoskin et al., 1999; O’Leary et al., 2008). Indeed, studies show that cotinine exhibits several nicotine-like properties including effects on acoustic startle, attention and
neuroprotection (Buccafusco and Terry, 2003; Buccafusco et al., 2009). The lack of effect of administered cotinine in the current study may be due to insufficient cotinine entry into the brain such that the CNS levels were too low for efficacy (Lockman et al., 2005). It may also relate to mode of administration. For instance, it is possible that pulsatile cotinine may have a different effect than continuous cotinine. Alternatively, the decline in L-dopa-induced AIMs may be due to a direct effect of nicotine, and/or possibly other metabolites, at the receptor.

The neural substrates that influence the nicotine-mediated reduction in L-dopa-induced dyskinesias most likely involve the nigrostriatal pathway. However, other brain regions are probably also involved since L-dopa-induced AIMs occur via integrative changes in multiple neurotransmitter systems throughout the CNS, including those present in the striatum, globus pallidus, thalamus, cortical areas, subthalamic nucleus, and cerebellum (Carta et al., 2008; Fox et al., 2008). Further support for a contribution of other brain areas in L-dopa-induced AIMs is based on evidence demonstrating beneficial effects of deep brain stimulation of the subthalamic nucleus in reducing dyskinesias (Benabid et al., 2009).

The present results have important implications for the management of L-dopa-induced dyskinesias, one of the major complications of long-term L-dopa therapy for Parkinson’s disease. They show that nicotine given intermittently via multiday injection or constantly via minipump for several days both attenuate L-dopa-induced AIMs in parkinsonian rats, an animal model predictive of the human condition. In addition, our previous data demonstrate that intermittent exposure via the drinking water also effectively reduces AIMs in both rats and monkeys (Quik et al., 2007; Bordia et al., 2008). Thus, nicotine consistently reduced L-dopa-induced AIMs across species, using several different treatment paradigms. This latter observation is of relevance for the treatment of dyskinesias in Parkinson's disease patients. Several different nicotine
formulations are currently available in humans for other indications primarily smoking cessation, that is, to combat addiction. This includes the nicotine patch, gum, lozenge, nasal spray and nasal inhalant (Matta et al., 2007). Our data would suggest that any one of these applications has potential in the treatment of L-dopa-induced dyskinesias. The final test of efficacy will require a clinical trial in subjects with Parkinson's disease patients.
References


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Footnotes

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Figure Legends

Fig. 1. Dose response of the nicotine-mediated decline in L-dopa-induced AIMs. 6-OHDA-lesioned rats exhibiting stable L-dopa-induced dyskinesias were injected with the indicated doses of nicotine or saline for 4 consecutive days, 10 min before L-dopa (8mg/kg) plus benserazide (15 mg/kg). On day 4, the rats were evaluated for axial, oral and forelimb AIMs for 2 min every 20 min over a 3 h period, with the AIM scores on the y-axis representing the sum of these three components. A 3 day washout was allotted between doses. The results show that a 4 day once daily injection of 0.1 mg/kg nicotine resulted in a maximal reduction in total AIMs. The data represent the mean ± S.E.M. of 9-10 rats. Significance of difference from saline control: *p < 0.05; **p < 0.01 using one-way ANOVA followed by Dunnett’s multiple comparison test.

Fig. 2. Nicotine treatment does not interfere with motor function in 6-OHDA-lesioned rats. Parkinsonism was assessed using (A) the asymmetric forelimb use or cylinder test, performed in the absence and presence of L-dopa. Nicotine (0.1 mg/kg) treatment had no effect on impaired forelimb use evaluated before or 1 h after L-dopa treatment. (B) The effect of nicotine treatment was also assessed on L-dopa-induced contralateral rotations in 6-OHDA-lesioned rats. Nicotine treatment had no significant effect on L-dopa-induced contralateral rotations when compared with saline-treated rats. Each value represents the mean ± S.E.M. of 9-10 rats.

Fig. 3 Multiple day nicotine treatment is required for a reduction in L-dopa-induced AIMs. Stably dyskinetic 6-OHDA-lesioned rats were administered 0.1 mg/kg nicotine once (1x) or twice (2x) daily for 4 and/or 10 days 10 min before L-dopa treatment. Rats were rated for axial,
oral and forelimb AIMs on the last day of any given nicotine treatment, with the AIM scores on the y-axis representing the sum of these three components. The results show that a single (acute) injection of nicotine administered 10 min before L-dopa did not diminish L-dopa-induced AIMs. However, a significant reduction in total, oral and forelimb AIMs was observed with multiple daily dosing. Each value represents mean ± S.E.M. of 9-10 rats. Significance of difference from saline control: *p < 0.05; **p < 0.01 using one-way ANOVA followed by Dunnett’s multiple comparison test.

**Fig. 4.** Time course of the reduction in total L-dopa-induced AIMs for different nicotine treatment regimes. 6-OHDA-lesioned rats were administered 0.1 mg/kg nicotine for the indicated number of days, 10 min before L-dopa administration. Rats were rated for axial, oral and forelimb AIMs on the last day of nicotine treatment, with the AIM scores on the y-axis representing the sum of these three components. There was a significant main effect of nicotine on L-dopa-induced AIMs in (B) p < 0.05, and (D) p < 0.01 using two-way repeated measures, ANOVA, with a trend for a decline in (C) (p < 0.063) and no effect with the acute dose in (A) (p < 0.89). Each symbol is the mean ± S.E.M. of 9-10 rats.

**Fig. 5.** nAChR antagonist treatment reduces L-dopa-induced AIMs. 6-OHDA-lesioned rats were injected with saline or varying doses (0.5 to 2.0 mg/kg) of the general nAChR antagonist mecamylamine 30 min before L-dopa for 1 or 4 days. The results show that single (acute) injection of mecamylamine did not modify L-dopa-induced AIMs. However, 4 d mecamylamine treatment led to significant reductions in total, oral and/or forelimb AIMs, with a trend for decline in axial AIMs. Each value represents mean ± S.E.M. of 9-10 rats. *p < 0.05; **p < 0.01
indicates significance of difference from saline control using one-way ANOVA followed by a Dunnett’s multiple comparison test.

**Fig. 6.** Effect of combined nicotine and mecamylamine treatment on L-dopa-induced AIMs. Stably dyskinetic 6-OHDA-lesioned rats were injected with saline, mecamylamine (1 mg/ kg), nicotine (0.1 mg/kg) or mecamylamine (1 mg/kg) + nicotine (0.1 mg/kg), followed 30 min later by L-dopa. Treatment was for 4 consecutive days, after which axial, oral and forelimbs AIMs were assessed. Each value represents the mean ± S.E.M. of 6-8 rats. Significance of difference from saline control using two-way ANOVA followed by a Bonferoni post hoc test, *p < 0.05; **p < 0.01.

**Fig. 7.** Nicotine administration via minipump yields a similar pattern of reduction in L-dopa-induced AIMs as intermittent treatment via injection. Unilateral 6-OHDA-lesioned rats were surgically implanted with minipumps releasing 0.10 to 2.0 mg/kg/day nicotine or vehicle. The animals were scored for L-dopa-induced AIMs 2 wks after minipump placement. Administration of the higher nicotine doses led to significant declines in total, orolingual, and forelimb AIMs, with a trend for a decrease in axial AIMs. The data represent the mean ± S.E.M. of 9-10 rats. Significance of difference from vehicle: *p < 0.05; **p < 0.01 using one-way ANOVA followed by Dunnett’s multiple comparison test.

**Fig. 8.** The agonist nicotine and antagonist mecamylamine resulted in similar alterations in α4β2* and α6β2* nAChRs in unlesioned and lesioned rat striatum. Unilateral 6-OHDA-lesioned rats were treated once daily with nicotine (nic, 0.2 mg/kg sc) or mecamylamine (meca, 1 mg/kg
sc) or saline (con) 10 and 30 min before L-dopa treatment, respectively. They were killed when AIM scores were maximal, that is, 1 hr after L-dopa treatment. $\alpha_6\beta_2^*$ nAChR expression was measured using $^{125}$I-$\alpha$-CtxMII and $\alpha_4\beta_2^*$ nAChRs using $^{125}$I-epibatidine in presence of $\alpha$-CtxMII. Nicot ine and mecamylamine treatment both decreased striatal $\alpha_6\beta_2^*$ nAChRs, and increased striatal $\alpha_4\beta_2^*$ nAChRs, in the unlesioned, but not lesioned striatum. The data represent the mean ± S.E.M. of 6-8 rats. Significance of difference from vehicle: $^*p < 0.05; ^{**}p < 0.01$ using one-way ANOVA followed by Dunnett’s multiple comparison test.
TABLE 1

Plasma cotinine levels in rats treated with nicotine or cotinine

6-OHDA-lesioned L-dopa-treated rats were administered nicotine or cotinine as indicated in the Table. Blood was drawn 3-4 h after a 4-day nicotine injection regimen, or 2 wks after nicotine or cotinine minipump implantation. Plasma cotinine levels were assessed as described in Methods. Both modes of nicotine treatment decreased L-dopa-induced AIMs with plasma cotinine levels comparable to those at the low end in the plasma of smokers. By contrast, cotinine treatment did not reduce L-dopa-induced AIMs, despite high plasma cotinine levels. Values represent the mean ± S.E.M. of 9-10 rats.

<table>
<thead>
<tr>
<th>Regime</th>
<th>Treatment</th>
<th>Dose</th>
<th>Cotinine (ng/ml)</th>
<th>Reduction in L-dopa-induced AIMs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection</td>
<td>Vehicle</td>
<td>--</td>
<td>0 ± 0</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Nicotine</td>
<td>0.1 mg/kg</td>
<td>202 ± 26</td>
<td>Yes</td>
</tr>
<tr>
<td>Minipump</td>
<td>Vehicle</td>
<td>--</td>
<td>0 ± 0</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Nicotine</td>
<td>0.5 mg/kg/day</td>
<td>115 ± 25</td>
<td>Yes</td>
</tr>
<tr>
<td>Minipump</td>
<td>Vehicle</td>
<td>--</td>
<td>0 ± 0</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Cotinine</td>
<td>2.5 mg/kg/day</td>
<td>353 ± 126</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0 mg/kg/day</td>
<td>1202 ± 61</td>
<td>No</td>
</tr>
</tbody>
</table>
TABLE 2
Chronic cotinine treatment does not reduce L-dopa induced AIMs

6-OHDA-lesioned rats were implanted with minipumps containing vehicle or cotinine (2.5 or 5 mg/kg/day). They were injected once daily with L-dopa (8 mg/kg) + benserazide (15 mg/kg) and evaluated for total, axial, orolingual and forelimb 3 wks after cotinine minipump implantation, as detailed in Methods. Cotinine administration did not decrease any AIM component despite high plasma cotinine levels. Values represent the mean ± S.E.M. of the indicated number of animals.

<table>
<thead>
<tr>
<th>Regime</th>
<th>Treatment</th>
<th>Dose (mg/kg/day)</th>
<th>N</th>
<th>L-dopa-induced AIM scores/session</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Minipump</td>
<td>Vehicle</td>
<td>--</td>
<td>10</td>
<td>56 ± 4</td>
</tr>
<tr>
<td></td>
<td>Cotinine</td>
<td>2.5</td>
<td>9</td>
<td>63 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>5</td>
<td>66 ± 3</td>
</tr>
</tbody>
</table>
TABLE 3

Decline in the striatal dopamine transporter with unilateral 6-OHDA lesioning

\(^{125}\text{I}-\text{RTI-121}\) was done as detailed in Methods. Values represent the mean ± S.E.M. of the indicated number of animals.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>(^{125}\text{I}-\text{RTI-121}) binding</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>fmol/mg tissue</td>
<td></td>
</tr>
<tr>
<td>Unlesioned striatum</td>
<td>18</td>
<td>24 ± 0.7</td>
<td>100 ± 2.9</td>
</tr>
<tr>
<td>Lesioned striatum</td>
<td>18</td>
<td>0.2 ± 0.1</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>
Nicotine delivery via injection

Fig 1

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Fig 2

(A) Contralateral forelimb use (% total)

- Saline
- Nicotine

OFF L-dopa  ON L-dopa

(B) L-Dopa-induced contralateral rotations

Saline  Nicotine
Fig 3

![Graphs showing AIM scores (% control) for different conditions.](image-url)
Fig 4

(A) Single dose

(B) Once daily for 4 d

(C) Once daily for 10 d

(D) Twice daily for 4 d
Effect of mecamylamine treatment

- **Total**
- **Axial**
- **Orolingual**
- **Forelimb**
Fig 6

- **Total**
  - AIM scores/session for Control and Mecamylamine groups under Saline and Nicotine conditions.

- **Axial**
  - AIM scores/session for Control and Mecamylamine groups under Saline and Nicotine conditions.

- **Orolingual**
  - AIM scores/session for Control and Mecamylamine groups under Saline and Nicotine conditions.

- **Forelimb**
  - AIM scores/session for Control and Mecamylamine groups under Saline and Nicotine conditions.
Fig 7

Nicotine delivery via osmotic minipump

![Graph showing AIM scores (% control) for Total, Axial, Orolingual, and Forelimb across different log nicotine doses (mg/kg/day)].

- **: p < 0.01
- *: p < 0.05

Log [nicotine] (mg/kg/day) vs. AIM scores (% control)
Fig 8

\[ \alpha 6 \beta 2^* \text{nAChRs} \]

\[ \alpha 4 \beta 2^* \text{nAChRs} \]