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Nicotine-mediated improvement in L-dopa-induced dyskinesias in MPTP-lesioned monkeys is dependent on dopamine nerve terminal function

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ABSTRACT

L-Dopa-induced dyskinesias (LIDs) are abnormal involuntary movements that develop with long term L-dopa therapy for Parkinson's disease. Studies show that nicotine administration reduced LIDs in several parkinsonian animal models. The present work was done to understand the factors that regulate the nicotine-mediated reduction in LIDs in MPTP-lesioned nonhuman primates. To approach this, we used two groups of monkeys, one with mild-moderate and the other with more severe parkinsonism rendered dyskinetic using L-dopa. In mild-moderately parkinsonian monkeys, nicotine pretreatment (300 μg/ml via drinking water) prevented the development of LIDs by ~75%. This improvement was maintained when the nicotine dose was lowered to 50 μg/ml but was lost with nicotine removal. Nicotine re-exposure again decreased LIDs. By contrast, nicotine treatment did not reduce LIDs in monkeys with more severe parkinsonism. We next determined how nicotine's ability to reduce LIDs correlated with lesion-induced changes in the striatal dopamine transporter and ^{3}H-dopamine release in these two groups of monkeys. The striatal dopamine transporter was reduced to 54% and 28% of control in mild-moderately and more severely parkinsonian monkeys, respectively. However, basal, K^{+}, $\alpha4\beta2^{*}$ and $\alpha6\beta2^{*}$ nAChR-evoked ^{3}H-dopamine release were near control levels in striatum of mild-moderately parkinsonian monkeys. By contrast, these same release measures were reduced to a significantly greater extent in striatum of more severely parkinsonian monkeys. Thus, nicotine best improves LIDs in lesioned monkeys in which striatal dopamine transmission is still relatively intact. These data suggest that nicotine treatment would most effectively reduce LIDs in patients with mild to moderate Parkinson's disease.

Introduction

The dopamine precursor L-dopa is the primary treatment for the motor symptoms associated with Parkinson's disease (PD). This debilitating neurodegenerative disorder is characterized by tremor, rigidity, bradykinesia and postural instability, which are thought to be due to a loss of nigrostriatal dopaminergic neurons (Halliday et al., 2011; Meissner et al., 2011; Obeso et al., 2010; Rascol et al., 2011; Schapira, 2009; Schapira and Jenner, 2011; Wichmann et al., 2011). L-dopa treatment ameliorates motor deficits because of its ability to elevate nigrostriatal dopamine levels. However, in addition to improving parkinsonian features, L-dopa is also associated with the emergence of side effects including abnormal involuntary movements or L-dopa-induced dyskinesias (LIDs). These develop in a significant proportion of Parkinson's disease patients receiving L-dopa treatment and represent a substantial problem with long term therapy. Current treatment strategies are limited and include a decrease in L-dopa dosing which has the disadvantage that there is a return of parkinsonian features, adjunct therapy with amantadine which has only limited effectiveness or surgical intervention (Brotchie and Jenner, 2011; Iravani and Jenner, 2011; Sankar and Lozano, 2011).

There is therefore a critical need for therapeutic approaches to treat this debilitating side effect of L-dopa administration for PD. Recent studies show that nicotine treatment attenuates LIDs in parkinsonian nonhuman primates, when given either before the onset of dyskinesias or when they are established (Quik et al., 2007). Nicotine also reduced L-dopa-induced abnormal involuntary movements in rodents, when given via several modes of administration, including drinking water, minipumps or injection (Bordia et al., 2008, 2010; Huang et al., 2011a, 2011b).

An important question is the mechanism whereby nicotine alleviates LIDs as this may help in developing optimal therapeutic interventions. The effect of nicotine in the CNS is generally mediated via nicotinic acetylcholine receptors (nAChRs). These are ion channels composed of five subunits, with the predominant subtypes in the striatum being the $\alpha4\beta2^{*}$ and $\alpha6\beta2^{*}$ receptor populations (the asterisk indicates

Abbreviations: LIDs, L-Dopa-induced dyskinesias; nAChRs, nicotinic acetylcholine receptors; PD, Parkinson's disease.

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the possible presence of other subunits in the receptor complex) (Albuquerque et al., 2009; Baddick and Marks, 2011; Gotti et al., 2009; Millar and Gotti, 2009; Quik et al., 2011). Support for the suggestion that nicotine improves LIDs via an interaction at nAChRs is derived from studies showing that nAChR agonists mimic the effect of nicotine (Huang et al., 2011b). In addition, nicotine does not reduce LIDs in β2 subunit knockout mice which do not express α4β2* and α6β2* nAChRs, nor does it reduce LIDs in α6 nAChR subunit knockout mice suggesting that the α6β2* or α6α4β2* subtype may specifically be involved (Huang et al., 2011a; Quik et al., 2012).

The present studies were done to understand the factors that influence the ability of nicotine to reduce LIDs. Since nicotine probably acts at nAChRs on dopamine nerve terminals, presynaptic dopamine function may be important for an optimal antidyskinetic effect (Grady et al., 2007; Quik and Wonnacott, 2011; Threlfell and Cragg, 2011; Wonnacott et al., 2005). To test this possibility, we initiated experiments in which we measured basal, K^+-stimulated and nAChR-stimulated dopamine release in striatum of MPTP-lesioned nonhuman primates with mild-moderate and more severe nigrostriatal damage. The data suggest that nicotine more effectively reduces LIDs when dopamine nerve terminal function is relatively intact.

Materials and methods

Animals

Adult male and female squirrel (*Saimiri sciureus*) monkeys weighing 0.6–1.0 kg were purchased from World Wide Primates (Miami, FL) and quarantined for one month, as mandated by California state regulations. All studies were performed according to the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at SRI. Animals were housed separately in a room maintained at 27 ± 3 °C, with a 12:12-h light/dark cycle. Monkey chow, fruits, and vegetables were provided over the course of the day, with water freely available.

MPTP lesions and parkinsonian ratings

After quarantine, the monkeys were trained to perform various motor tasks for future assessment of parkinsonism (Quik et al., 2007). They were then injected with saline or 2.0 mg/kg 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine sc (MPTP; Sigma-Aldrich, St. Louis, MO) dissolved in saline, as described (Quik et al., 2007). Parkinsonism was rated 3 to 4 wk later using a scale from 0 (normal) to 4 (severely parkinsonian), with a maximum possible score of 28 based on seven parameters. These include spatial hypokinesia (use of available cage space), body bradykinesia (slowness in body movement), manual dexterity in both left and right hands, balance (ability to hold on to the cage bars), freezing, and action tremor. MPTP injections were repeated up to 4 times (1.9 mg/kg sc). Parkinsonism was rated once weekly throughout the study.

Nicotine treatment

The monkeys were next given commercially available orange Gatorade diluted 50% in the drinking water. Gatorade was used to mask the taste of nicotine (Quik et al., 2007). After one wk on Gatorade to habituate the monkeys to its taste, the animals were randomly assigned to Gatorade only (vehicle-treated) or Gatorade plus nicotine (free base; Sigma-Aldrich, St. Louis, MO). Nicotine was started at a dose of 50 μg/ml for 1 wk, increased to 150 μg/ml for 1 wk and then to a final concentration of 300 μg/ml (Fig. 1). A 25 ml aliquot of Gatorade containing nicotine or Gatorade only was also added to the monkey chow. The animals were maintained on the 300 μg/ml dose for 6 wk before starting L-dopa treatment. There were no effects of nicotine on fluid intake, body weight or general behavior.

L-Dopa-treatment and dyskinesia ratings

The monkeys were subsequently orally gavaged with L-dopa (7.5 or 10 mg/kg) plus carbidopa (1.875 or 2.5 mg/kg), twice daily 3.5 h apart 5 d per wk (Fig. 1), as described (Quik et al., 2007). While treated with L-dopa, monkeys were given only fruits and vegetables in the morning to enhance L-dopa absorption. Monkey chow, as well as more fruits and vegetables, were given 4 h after the second dose of L-dopa. The monkeys were taped from 8:00 to 9:00 AM, before the first dose of L-dopa (baseline period) and from 12:30 to 4:30 PM, following the afternoon L-dopa dose. Dyskinesias were rated from the video recordings for a 1 min period every 30 min. Ratings were on a scale of 0 (no dyskinesias) to 4 (severely dyskinetic) with: 1 = subtle dyskinesias that were not sustained (<3 trunk movements in a row); 2 = sustained dyskinesias (≥3 trunk movements in a row); 3 = moderate dyskinesias that impaired the ability to remain stationary; and 4 = severe dyskinesias that were generalized and incapacitating. Scores shown were averaged over several days (Tan et al., 2002).

Plasma cotinine levels

Cotinine, a nicotine metabolite, was used as a measure of nicotine intake because of its long half-life (18 h) compared to nicotine (1 h) (Matta et al., 2007). Blood (1–2 ml) was drawn from the femoral vein under ketamine anesthesia (15–20 mg/kg im). The plasma was collected and stored at −80 °C for cotinine measurements using an enzyme immunoassay (Orasure Technologies, Bethlehem, PA).

Tissue preparation

Monkeys were euthanized according to the recommendations of the Panel of Euthanasia of the American Veterinary Medical Association. They were first injected with 1.5 ml Euthasol (390 mg sodium pentobarbital and 50 mg phenytoin sodium/ml ip; Butler Schein, Chicago, IL) for sedation and then 1.5 ml Euthasol iv for euthanasia. The brains were rapidly removed, rinsed in cold phosphate-buffered saline and cut into 2 mm-thick blocks using a squirrel monkey brain mold. The medial caudate, lateral caudate, dorsal putamen and ventral putamen were dissected from one half of the striatal block at level A15.0-13.0 as per a squirrel monkey brain atlas (Emmers and Akert, 1963) for measurement of synaptosomal ^{3}H-dopamine release (McCallum et al., 2005). The other half was quick frozen in isopentane on dry ice for autoradiography. Ten μm sections were prepared using a cryostat (Leica Microsystems, Inc., Deerfield, IL) cooled to −20 °C. Frozen sections were mounted onto poly-L-lysine coated slides (Superfrost Plus, Fisher Scientific Co., Pittsburgh, PA), dried, and stored at −80 °C.

Dopamine transporter autoradiography

Dopamine transporter levels were measured using ^{125}I-RTI-121 (specific activity, 2200 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) (Quik et al., 2001). Thawed sections were pre-incubated 2× for 15 min each at room temperature in buffer (50 mM Tris–HCl, 120 mM NaCl, 5.0 mM KCl, pH 7.4). They were then exposed to the same buffer containing 0.025% bovine serum albumin, 1.0 μM fluoxetine and 25 pM ^{125}I-RTI-121 for 2 h. Sections were washed in ice cold buffer 4× for 15 min each and 1× for 10 s in ice cold de-ionized H_2O. Slides were exposed to Kodak MR film (Easterman Kodak Co., Rochester, NY, USA) along with ^{3}H-microscale standards (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) for 2–7 days, depending on the decay of the ^{125}I-labelled RTI-121. Background binding was evaluated in the presence of the dopamine uptake inhibitor nomifensine (100 μM), and was subtracted from the total binding. The ImageQuant program from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK) was used to obtain optical density values from autoradiographic images, which were converted to fmol/mg tissue using standard

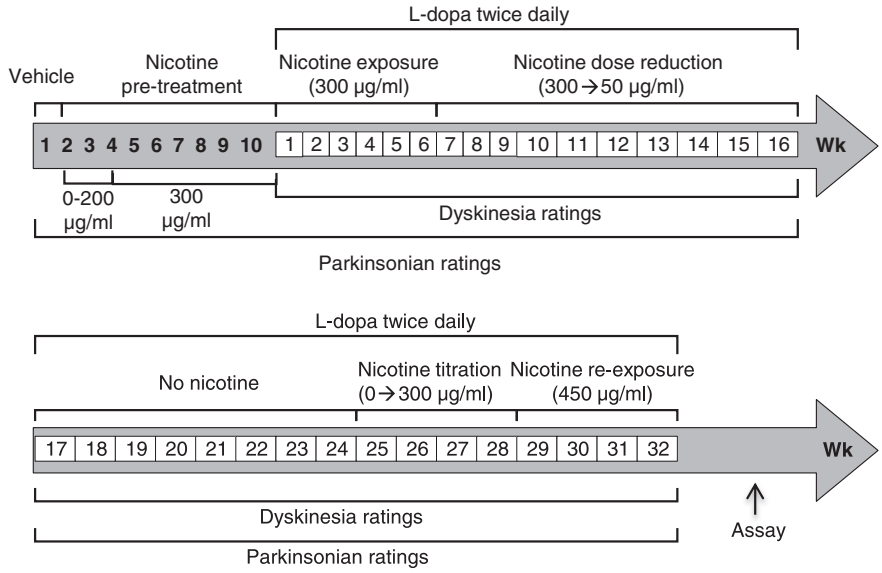


Fig. 1. Treatment timeline to determine the effect of nicotine administration on L-dopa-induced dyskinesias (LIDs) in MPTP-lesioned monkeys. Monkeys were lesioned with MPTP as described in Materials and methods. They were then given 50% diluted Gatorade in the drinking water (vehicle). The vehicle-treated group ($n=4$) was continued on diluted Gatorade throughout the study. Gatorade was used to mask the taste of nicotine, which was added to the drinking water of the nicotine-treated group ($n=4$). The initial dose of nicotine in the diluted Gatorade drinking solution was 50 μg/ml; this was gradually increased to 300 μg/ml over a 2 week period, with the animals maintained on the final dose of nicotine for a further 6 weeks. This first phase of treatment is designated as the nicotine pre-treatment phase. L-Dopa treatment was then initiated, as designated by the boxed numbering in the timeline. Monkeys were gavaged with L-dopa (7.5–10 mg/kg) twice daily at a 3.5 h interval 5 d per week for the remainder of the study. Nicotine treatment was as indicated in the timeline. Dyskinesia and parkinsonian ratings were evaluated throughout the study.

curves generated from ^{3}H-microscale standards. These were calibrated for ^{125}I autoradiography as described (Artymyshyn et al., 1990). Sample optical density readings were within the linear range of the standards.

Nicotine-evoked ^{3}H-dopamine release

Nicotine-evoked ^{3}H-dopamine release from striatal synaptosome preparations was measured as described (McCallum et al., 2005). Striatal tissue (~15 mg wet weight) was homogenized in 2 ml of ice-cold 0.32 M sucrose buffered with 5.0 mM HEPES (pH 7.5). After centrifugation at 12,000 g for 15 min, the pellets were resuspended and incubated for 10 min in 0.8 ml of 37 °C uptake buffer (128 mM NaCl, 2.4 mM KCl, 1.2 mM KH_2PO_4, 3.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM HEPES, pH 7.5, 10 mM glucose, 1 mM ascorbic acid and 0.01 mM pargyline). ^{3}H-Dopamine (100 nM or, specific activity, 30–60 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) was then added to the synaptosomes, followed by a 5 min incubation period. Synaptosomal aliquots are prepared such that each contains ~250,000 cpm of ^{3}H-dopamine; the amount of ^{3}H-dopamine incorporation into the synaptosomes varied with the extent of lesioning, with a lower incorporation in more severely lesioned animals. Synaptosomal aliquots (80 μl) were pipetted onto glass-fiber filters mounted on polypropylene platforms and perfused with perfusion buffer (uptake buffer plus 0.1% bovine serum albumin and 10 μM nomifensine) for 10 min prior to nicotine stimulation. Since the synaptosomes are on the surface of moistened filters, with buffer dripping during the release process, they have ready access to ambient air and are not oxygenated. Our release data is similar to that of others in which buffers were oxygenated (Wonnacott et al., 2000). ^{3}H-Dopamine release was evoked by an 18 s exposure to 20 mM K^{+}. Release was also measured in response to varying concentrations of nicotine. To assess the α4β2* and α6β2* nAChR-mediated components of nicotine evoked-release, one set of filters was perfused with buffer only and another set was perfused with buffer containing 50 nM α-CtxMII for 3 min prior to nicotine stimulation. The toxin α-CtxMII inhibits only α6β2* nAChRs. Thus, nicotine-evoked release in the presence of α-CtxMII would represent release mediated by α4β2* nAChRs. α6β2* nAChR-mediated release is obtained by subtracting nicotine-evoked release minus α4β2* nAChR-mediated release. Twelve 18 s fractions, which included basal release before and after the stimulated release, were collected. Radioactivity was determined using liquid scintillation counting. Data from the ^{3}H-dopamine release studies were plotted as counts per minute (cpm) versus fraction number in the curve-fitting algorithm of SigmaPlot® version 5.0 (Systat Software Inc, San Jose, CA). The basal release was determined by selecting the release fractions before and after the peak and plotting them as a single exponential decay function. To calculate net release (cpm/mg tissue), basal release was subtracted from each fraction, with release normalized to the wet weight of the tissue.

Statistical analyses

Statistical comparisons were performed with GraphPad Prism (GraphPad Software, Inc, San Diego, CA) using one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparisons *post hoc* test. They were also done using two-way ANOVA followed by Bonferroni *post hoc* test; the interacting factors were treatment and time in Figs. 2–5, and treatment and nicotine concentration in Figs. 7 and 8. A value of $P \leq 0.05$ was considered statistically significant. Values expressed are the mean ± SEM of the indicated number of monkeys.

Results

Monkey treatment groups

For these studies, we had four groups of monkeys (Table 1). This included a set of unlesioned control animals that received 50% diluted Gatorade in the drinking water but were not gavaged with L-dopa ($n=5$). The other monkeys were lesioned with MPTP as described in Materials and methods. The majority were mild to moderately parkinsonian ($n=10$). These animals exhibited mild tremors, and had problems with balance and manual dexterity. Freezing, bradykinesia and spatial hypokinesia were not observed in these animals. The mild-moderately parkinsonian monkeys were randomly divided into 2 groups, one which received Gatorade only ($n=4$) and the other Gatorade plus nicotine ($n=6$); they were all gavaged with L-dopa. There was no difference

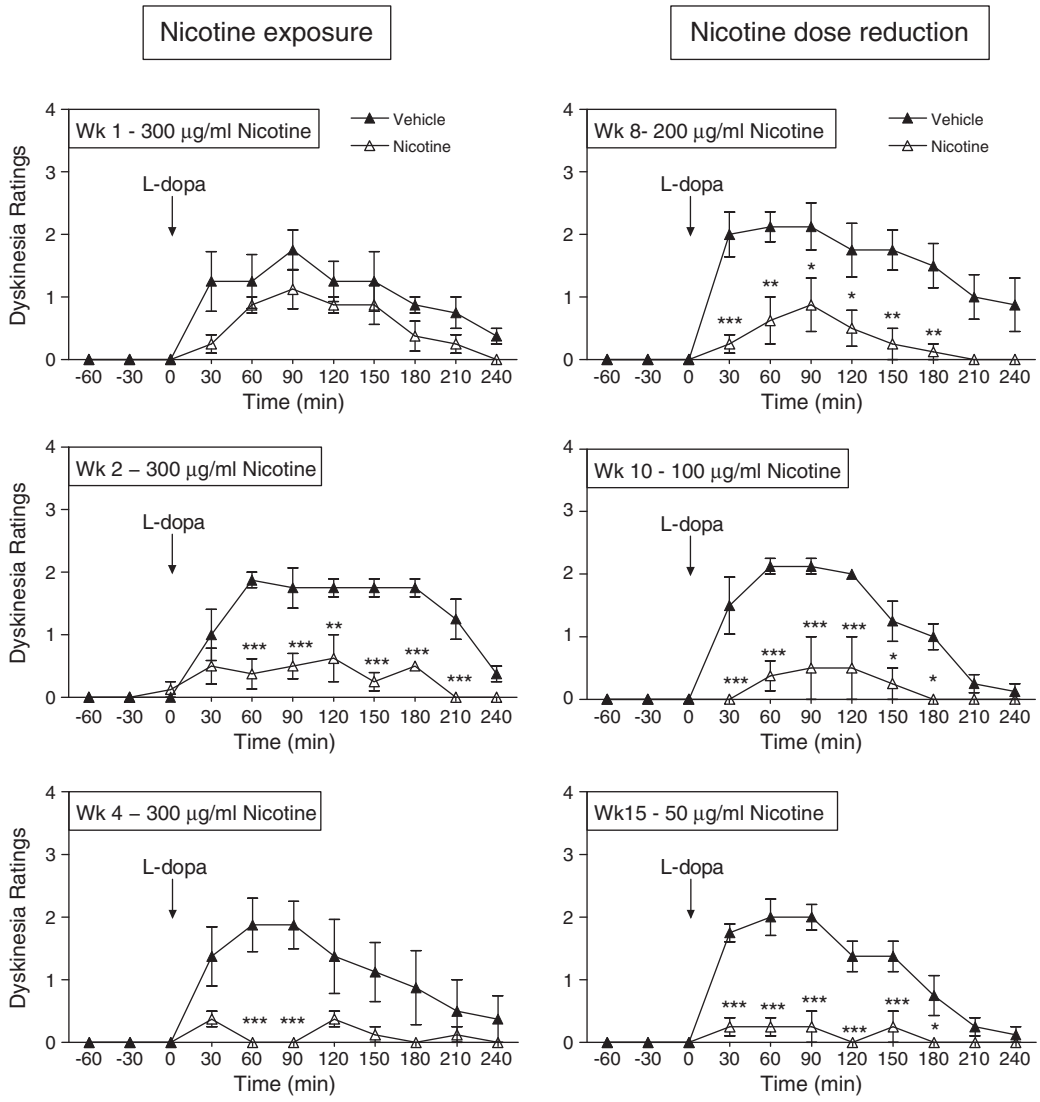


Fig. 2. Daily time course of LID scores with nicotine exposure and dose reduction in mild-moderately parkinsonian monkeys. MPTP-lesioned monkeys were pre-treated with nicotine as detailed in Fig. 1. They were then gavaged with L-dopa twice daily, with nicotine dosing continued. LIDs were assessed for a 1 min period every 30 min, 1 h before the first dose of L-dopa and 4 h following the second dose of L-dopa (afternoon dose). The panels on the left depict the effect of nicotine exposure (300 μg/ml) on LIDs with the first few weeks of L-dopa dosing. LID scores were significantly decreased over the entire 4 h time period in nicotine-treated monkeys as compared to vehicle from wk 2 onwards. The panels on the right (nicotine reduction) depict the effect of decreasing doses of nicotine (from 200 to 50 μg/ml). The reduced LID scores were maintained even when the nicotine dose was lowered to 50 μg/ml. The values represent the mean ± SEM of 4 monkeys. Significance of difference from vehicle, *P<0.05, **P<0.01, ***P<0.001 using two-way repeated ANOVA followed by a Bonferroni *post hoc* test.

in the parkinsonian rating scores with or without nicotine treatment, consistent with previous results (Quik et al., 2007). The small improvement in scores across the different time points was similar in mild-moderately lesioned animals receiving Gatorade only or Gatorade plus nicotine, and suggests there is a slight recovery.

In addition, there was another set of monkeys that developed more severe parkinsonism (n = 3). These animals had more intense tremors and greater difficulty with balance and manual dexterity than the mild-moderately parkinsonian monkeys. They also exhibited freezing and bradykinesia although there was no spatial hypokinesia. These monkeys (n = 3) received Gatorade plus nicotine and were gavaged with L-dopa.

Nicotine treatment reduced LIDs in mild-moderately parkinsonian monkeys

Monkeys were treated with nicotine in the drinking water according to the schedule depicted in Fig. 1, as previously described (Quik et al., 2007). Nicotine was held at a final dose of 300 μg/ml as this yielded plasma cotinine levels of 371 ± 111 ng/ml (n = 4), which correspond to those in the plasma of smokers (Matta et al., 2007). Cotinine levels were not detected in vehicle-treated monkeys. Plasma cotinine was also measured during the course of the nicotine removal phase; the plasma cotinine values at the lowest dose of nicotine (50 μg/ml) were 138 ± 80 ng/ml.

The monkeys were subsequently gavaged with L-dopa (7.5 mg/kg) twice daily. L-Dopa administration resulted in the rapid development of LIDs in vehicle-treated monkeys, which peaked about 1 h after treatment and then gradually declined over the 4 h period (Fig. 2). Nicotine treatment decreased dyskinesias after both the morning and afternoon doses, consistent with previous findings (Quik et al., 2007). Since the data were similar for the morning and afternoon, only the results for the afternoon dose are shown in Fig. 2. The monkeys were kept at 300 μg/ml nicotine for 6 wk after the start of L-dopa treatment, with a significant reduction in LIDs from 2 wk onwards (Fig. 2 left panel). The nicotine-mediated decline in LIDs

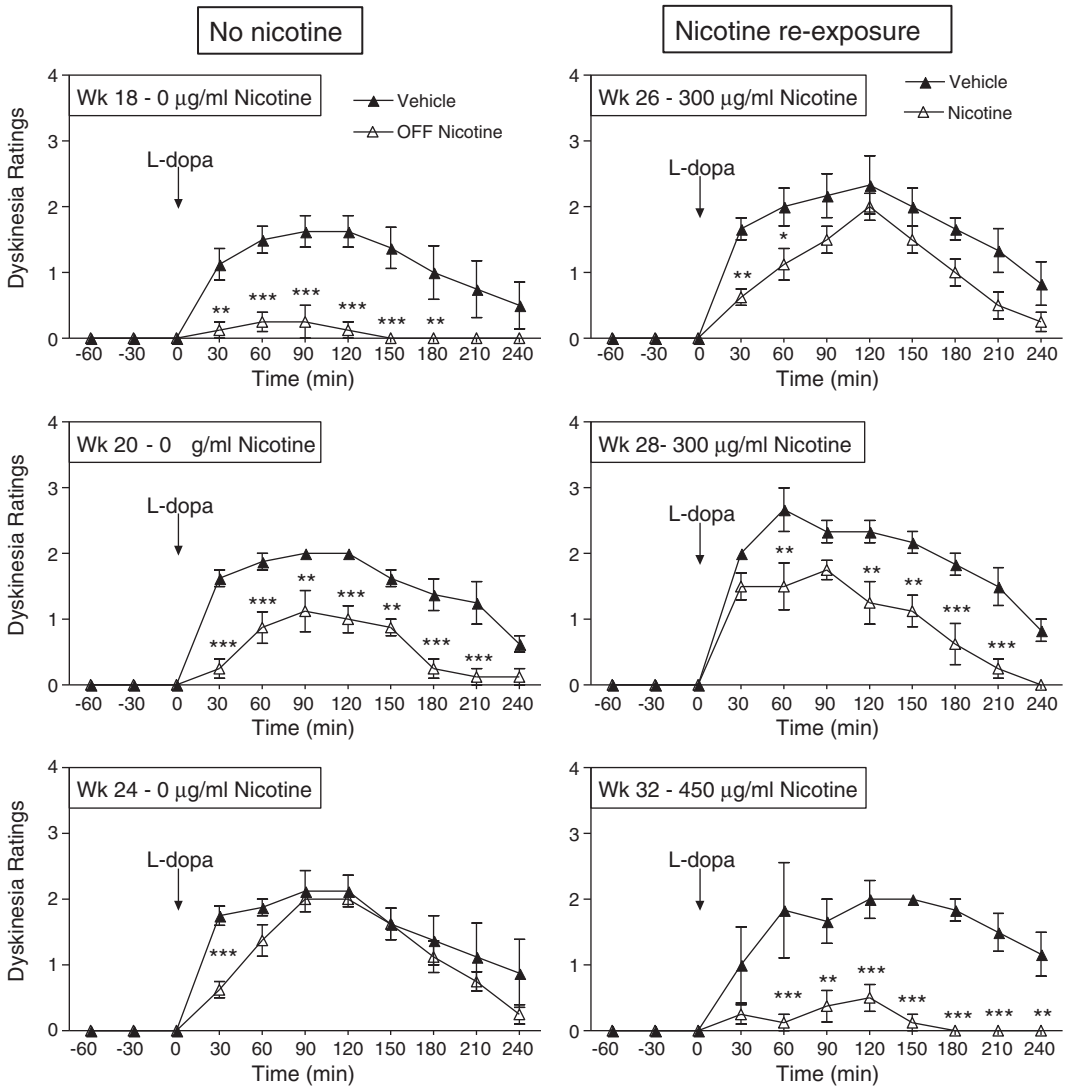


Fig. 3. Daily time course of LID scores with nicotine removal and re-exposure in mild-moderately parkinsonian monkeys. The study in Fig. 2 was continued, with nicotine completely removed (no nicotine) for several weeks (left panels). Twice daily L-dopa treatment was continued. LIDs were assessed for a 1 min period every 30 min, 1 h before the first dose of L-dopa and 4 h following the second dose of L-dopa (afternoon dose). Nicotine removal increased LID scores throughout the 4 h period. By contrast, nicotine re-exposure (right panels) again led to an improvement in LIDs. The values represent the mean ± SEM of 4 monkeys. Significance of difference from vehicle,*P<0.05, **P<0.01, ***P<0.001 using two-way repeated ANOVA followed by a Bonferroni *post hoc* test.

was similar throughout the 4 h time period, that is, nicotine did not modify the onset of LIDs or the decline in LIDs. The total dyskinesia scores are shown in Fig. 4.

The nicotine dose was then gradually reduced from 300 to 200, 100 and finally 50 µg/ml over the next 10 wk. The daily time course studies show that there was no loss in the beneficial effect of nicotine against LIDs over this period (Fig. 2 right), with total dyskinesia scores provided in Fig. 4.

Nicotine removal worsened LIDs while nicotine re-exposure improved LIDs in mild-moderately parkinsonian monkeys

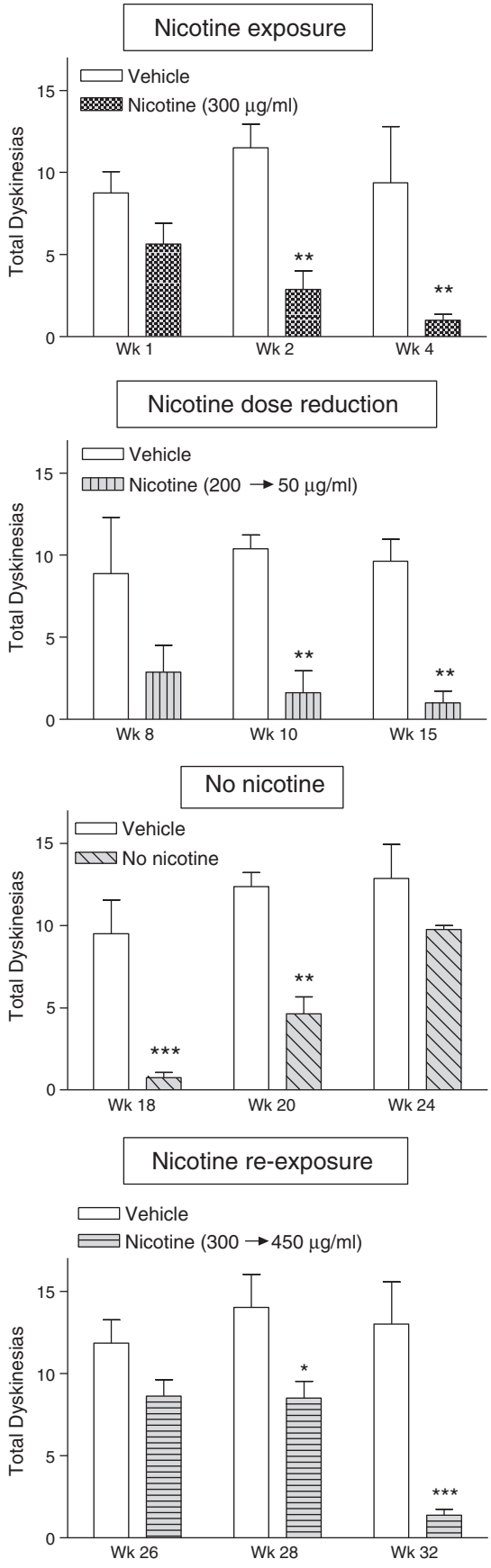
We next tested whether continued nicotine treatment was necessary to maintain the improvement in LIDs. The time course studies depicted in Fig. 3 (left) show that LIDs scores had almost returned to control levels, except for one time point, after 8 wk of nicotine removal. The total LIDs scores were also similar in nicotine- and vehicle-treated monkeys 8 wk after stopping nicotine (Fig. 4).

Lastly, we assessed whether nicotine re-administration would again attenuate LIDs (Figs. 3 right and 4). Monkeys were treated with increasing doses of nicotine to a final dose of 450 µg/ml, which yielded plasma nicotine concentrations (475 ± 72 ng/ml) similar to those with the initial nicotine dosing regimen (wk 10 of nicotine pretreatment phase). The time course studies show that there was a 90% decrease in LIDs four wk after re-initiation of the nicotine treatment (32 wk time point) (Fig. 3), with the total dyskinesia scores provided in Fig. 4.

Nicotine does not reduce LIDs in more severely parkinsonian monkeys

The α4β2* and α6β2* nAChRs that regulate striatal dopaminergic function are located presynaptically on nigrostriatal dopaminergic terminals (Grady et al., 2007; Quik and Wonnacott, 2011; Threlfell and Cragg, 2011; Wonnacott et al., 2005). If these receptors are of relevance to the nicotine-mediated decline in LIDs, one might expect that nicotine would less effectively reduce LIDs with more severe nigrostriatal damage since nAChRs are reduced with lesioning (Quik et al., 2001, 2003). Indeed, after 6 wk of nicotine treatment, there was no decrease in LIDs in a second set of monkeys with more severe parkinsonism compared to animals with mild-moderate

parkinsonism (Fig. 5 top). There was still no improvement in LIDs when the nicotine treatment was extended for an additional 6 wk (Fig. 5 bottom).



The striatal dopamine transporter is decreased after MPTP treatment

^{125}I-RTI-121 autoradiography was done to determine the extent of the MPTP-induced decline in the striatal dopamine transporter, a marker of striatal dopaminergic integrity. There were 25–35% decreases in the transporter in the medial caudate and ventral putamen in monkeys with mild-moderate parkinsonism and 50–65% declines in the lateral caudate and dorsal putamen (Fig. 6). These data are consistent with previous findings showing there is more severe damage in striatal regions distal from the midline (Quik et al., 2002).

The more severely parkinsonian monkeys exhibited significantly greater reductions in the dopamine transporter in all striatal areas (Fig. 6), with 60–65% reductions in the medial caudate and ventral putamen in monkeys and 75–85% declines in the lateral caudate and dorsal putamen.

Basal and evoked ^{3}H-dopamine release are similar to control in striatum of mild-moderately but not more severely parkinsonian monkeys

To understand whether alterations in cellular dopaminergic mechanisms may be associated with the nicotine-mediated decline in dyskinesias, we measured basal ^{3}H-dopamine release levels and K^{+}-evoked release (20 mM). We also measured both the α4β2* and α6β2* nAChR-mediated components of nicotine-evoked release in synaptosomes prepared from different striatal subregions. For these studies there were no significant differences in basal or stimulated striatal ^{3}H-dopamine release in mild-moderately parkinsonian monkeys treated with and without nicotine. Therefore, the data in Tables 2 to 5 are for the nicotine-treated mild-moderately parkinsonian monkeys only. The results for basal release are shown in Table 2. In mild-moderately parkinsonian monkeys, basal ^{3}H-dopamine release was decreased only in lateral caudate as compared to unlesioned monkey group (Table 2). By contrast, basal release was decreased in all striatal subregions of more severely parkinsonian animals as compared to the unlesioned animals (Table 2).

Stimulus evoked ^{3}H-dopamine release was then assayed in response to 20 mM K^{+} (Table 3). In mild-moderately parkinsonian monkeys, K^{+}-evoked ^{3}H-dopamine release was decreased only in dorsal putamen. By contrast, release was decreased in medial caudate, ventral putamen and dorsal putamen of more severely parkinsonian animals, with a trend for a decline in the lateral caudate (22%).

We also measured nicotine-evoked ^{3}H-dopamine release in the different striatal subregions using a range of nicotine concentrations from 30 nM to 30 μM. Extensive studies have shown that there are two major populations of nAChRs in striatum, the α4β2* and α6β2* receptors (Champtiaux et al., 2003; Luetje, 2004; Quik and Wonnacott, 2011; Salminen et al., 2004). Nicotine-evoked dopamine release occurs in response to stimulation of both these subtypes. The α6β2* selective toxin (α-conotoxinMII) blocks all release mediated via α6β2* nAChRs. Thus release done in the presence of this toxin provides a measure of α4β2* nAChR-mediated release. Subtraction of α4β2* nAChR-mediated release from total nicotine-evoked release defines the component of release occurring via α6β2* nAChRs (Champtiaux et al., 2003; Luetje, 2004; Quik and Wonnacott, 2011; Salminen et al., 2004). ^{3}H-Dopamine release was thus measured in

Fig. 4. Total LID scores with nicotine treatment, removal and re-exposure in mild-moderately parkinsonian monkeys. MPTP-lesioned monkeys were administered nicotine as detailed in the timeline in Fig. 1 and gavaged with L-dopa twice daily. Total dyskinesias are shown during the 4 h period following the second dose of L-dopa (afternoon dose), with LIDs assessed every 30 min for a 1 min period for 4 h. Nicotine exposure reduced total LID scores up to ~90% compared to vehicle-treated monkeys. Improvement was maintained even when the nicotine dose was lowered to 50 μg/ml. LIDs partially returned to control levels with nicotine removal, but again decreased with nicotine re-exposure. The values represent the mean ± SEM of 4 monkeys. Significance of difference from vehicle, *$P<0.05$, **$P<0.01$, ***$P<0.001$ using two-way repeated ANOVA followed by a Bonferroni *post hoc* test.

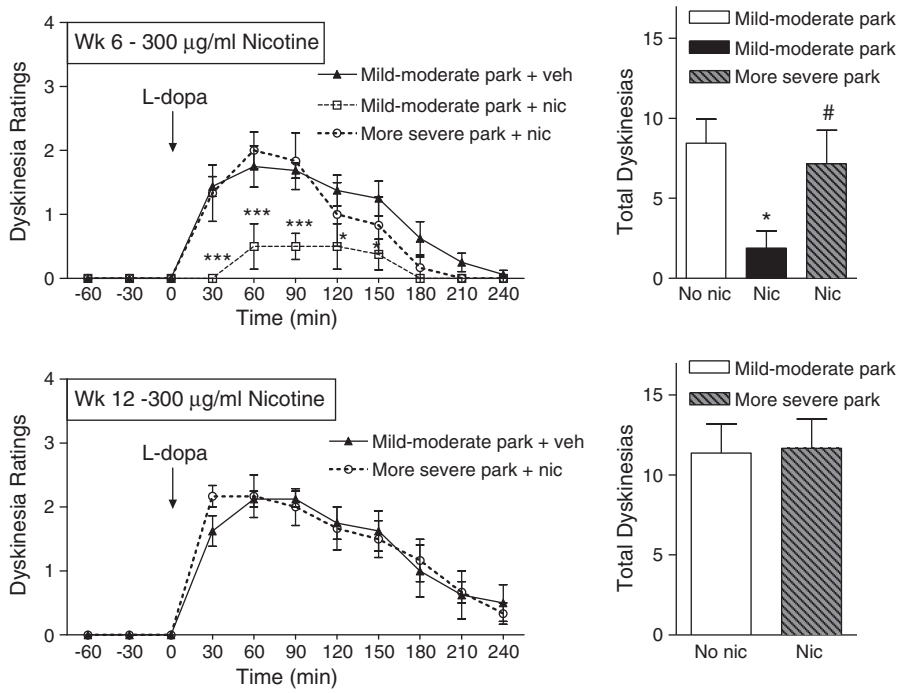


Fig. 5. Nicotine treatment does not reduce LIDs in more severely parkinsonian monkeys. The effect of nicotine was subsequently tested in a separate group of monkeys with more severe parkinsonism (park). The nicotine pre-treatment timeline for the more severely parkinsonian monkeys was similar to that for the mild-moderately parkinsonian animals depicted in Fig. 1. After the initial nicotine pre-exposure period, the monkeys were gavaged with L-dopa twice daily with the nicotine dosing maintained at 300 µg/ml. LIDs were assessed every 30 min for a 1 min period during 1 h of baseline and 4 h following the second dose of L-dopa at 6 wk (top panel) and 12 wk (bottom panel). The daily time course is depicted in the panels on the left and the total LID scores on the right. Data for mild-moderately parkinsonian monkeys is provided for comparison at the 6 wk time point. The values represent the mean ± SEM of 3–6 monkeys. Significance of difference from vehicle, *P<0.05, ***P<0.001; from mild-moderate parkinsonism, #P<0.05 using one or two-way repeated ANOVA followed by a Bonferroni *post hoc* test.

the absence and presence of α-conotoxinMII (50 nM). There was no decrease in α4β2* nAChR-mediated release in any striatal region with mild-moderate parkinsonism (Fig. 7, Table 4). This contrasts with the results in more severely parkinsonian animals in which there is a decline in ^{3}H-dopamine release in all striatal regions except the medial caudate (Fig. 7, Table 4), with a significant main effect of MPTP-treatment using two-way ANOVA.

α6β2* nAChR-mediated ^{3}H-dopamine release was also less affected in striatum of mild-moderately parkinsonian monkeys as compared to more severely parkinsonian animals (Fig. 8, Table 5). α6β2* nAChR-mediated release was decreased only in the lateral caudate and dorsal putamen of mild-moderately parkinsonian monkeys, while release was decreased in all striatal regions in more severely parkinsonian monkeys (Fig. 8, Table 5).

These combined data indicate that measures of release were generally less impaired in striatum of mild-moderately parkinsonian monkeys, the group that also showed improvement in dyskinesias with nicotine treatment. These data suggest that relatively intact presynaptic dopaminergic function is necessary for an optimal antidyskinetic effect of nicotine.

Table 1
Monkey groups. Unlesioned control monkeys were not lesioned with MPTP; they received 50% diluted Gatorade in the drinking water and were not gavaged with L-dopa (n = 5). The remainder of the animals were lesioned with MPTP as described in Materials and methods. The majority were mild to moderately parkinsonian (n = 10). These were randomly divided into 2 groups, one which received Gatorade (n = 4) and the other Gatorade plus nicotine (n = 6); they were all gavaged with L-dopa. There was also a more severely parkinsonian group that received Gatorade plus nicotine and were gavaged with L-dopa (n = 3). Parkinsonism was rated throughout the study. Significance of difference from unlesioned controls, *P<0.05, **P<0.01, ***P<0.001; from more severely parkinsonian monkeys, ###P<0.001. Data were analyzed by one-way ANOVA followed by a Newman-Keuls *post hoc* test.

Parkinsonism	Nicotine	*n*	Parkinsonian score		
			Vehicle period (Week 1)	Pre-treatment period (Week 8)	Exposure period (Week 6)
Unlesioned	No	5	0.4 ± 0.2	0.5 ± 0.3	0.8 ± 0.3
Mild-moderate	No	4	3.9 ± 0.2***###	2.5 ± 0.6*###	2.3 ± 0.3*###
Mild-moderate	Yes	6	3.8 ± 0.6***###	3.3 ± 0.5**###	3.2 ± 0.4**###
More severe	Yes	3	9.4 ± 0.5***	6.7 ± 0.9***	7.3 ± 0.9***

Discussion

L-dopa treatment is currently the most successful approach to manage motor symptoms in Parkinson's disease; however, the emergence of LIDs with continued use is a significant problem (Ahlskog and Muenter, 2001; Brotchie and Jenner, 2011; Cenci and Lindgren, 2007; Iravani and Jenner, 2011; Lang and Lozano, 1998a; Lang and Lozano, 1998b; Obeso et al., 2010; Olanow and Tatton, 1999). Our previous studies showed that nicotine administration reduced LIDs in several parkinsonian animal models, including rats, mice and nonhuman primates (Bordia et al., 2006, 2008; Huang et al., 2011a; Quik et al., 2007). This reduction in LIDs across species provides strong support for the idea that nicotine may also reduce LIDs in Parkinson's disease. In fact, a small double-blinded clinical trial showed that nicotine was generally safe and well-tolerated in Parkinson's disease patients given L-dopa; moreover, nicotine treatment led to significant improvements in some dyskinesia components (http://www.neuraltus.com/pages/news_rel12_03_10.html). Altogether these data suggest that nicotine may be useful for attenuating LIDs in Parkinson's disease.

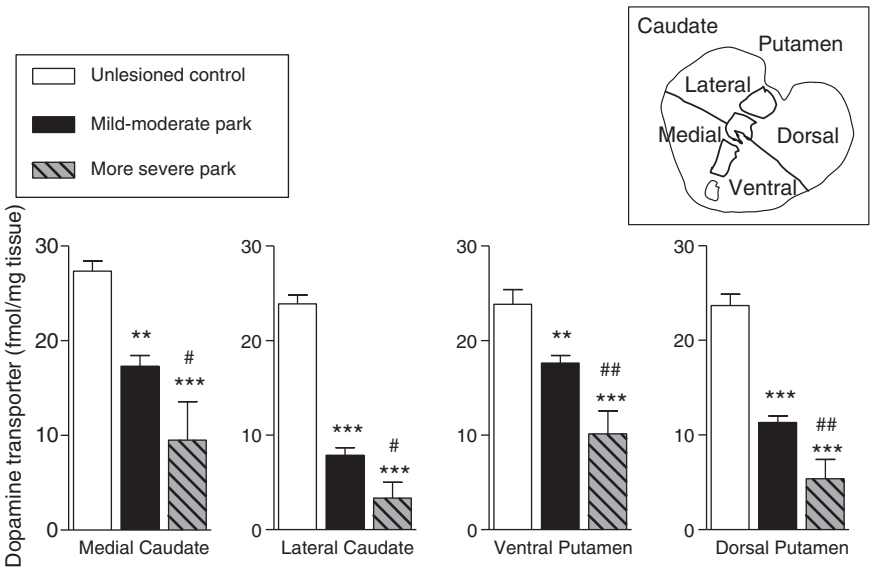


Fig. 6. Dopamine transporter levels in striatum of mild-moderately and more severely parkinsonian monkeys. MPTP-lesioned monkeys were administered nicotine as detailed in the timeline in Fig. 1 and gavaged with L-dopa twice daily. At the end of the study they were euthanized, the brains removed and the striatal dopamine transporter assayed using ^{125}I-RTI-121 autoradiography. Levels were significantly lower in more severe compared to mild-moderately parkinsonian monkeys in all striatal regions. Values from unlesioned control monkeys are shown for comparison. The values represent the mean ± SEM of 3–6 monkeys. Significance of difference from unlesioned control, ** P<0.01, *** P<0.001. Significance of difference from mild-moderate lesion, #P<0.05, ##P<0.01. Data were analyzed by one-way ANOVA followed by a Newman–Keuls *post hoc* test.

If nicotine is to be used successfully in the clinic, it is important to understand the conditions under which it most effectively reduces LIDs. To study this, we used parkinsonian nonhuman primates, an animal model that bears many resemblances to Parkinson's disease. Nicotine was given in the drinking water as this provides a nonstressful, oral mode of treatment that may be analogous to pill administration in Parkinson's disease patients. The dose of nicotine selected for these studies yielded nicotine metabolite levels in the range of those in

Table 2
Basal ^{3}H-dopamine release from monkey striatal regions. Synaptosomes were prepared from freshly dissected monkey striatal subregions and ^{3}H-dopamine release was determined as described in Materials and Methods. Basal release was defined as release occurring in the absence of a stimulus. Basal ^{3}H-dopamine release was decreased only in lateral caudate of mild-moderately lesioned monkeys, but all striatal regions of more severely lesioned animals. Values represent the mean ± SEM of 3–6 monkeys. Significance of difference from unlesioned controls, ***P<0.001; from mild-moderate lesion, ##P<0.01, ###P<0.001 using one-way ANOVA followed by a Newman-Keuls *post hoc* test.

Region	Basal ^{3}H-dopamine release					
	Unlesioned control		Mild–moderate lesion + nicotine		More severe lesion + nicotine	
	cpm/mg tis	% control	cpm/mg tis	% control	cpm/mg tis	% control
Medial caudate	1099 ± 23.0	100	1174 ± 23.4	106	888 ± 44.0*** ###	81
Lateral caudate	1092 ± 22.44	100	908 ± 35.2***	83	727 ± 13.2*** ##	67
Ventral putamen	1167 ± 30.4	100	1190 ± 43.4	102	811 ± 21.8*** ###	69
Dorsal putamen	1060 ± 58.9	100	993 ± 37.3	94	581 ± 18.9*** ###	55

Table 3
K^{+}-evoked ^{3}H-dopamine release from monkey striatal regions. Synaptosomes were prepared from freshly dissected monkey striatal subregions as described in Materials and Methods. Evoked-^{3}H-dopamine release was then assayed in the presence of 20 mM K^{+}. K^{+}-evoked ^{3}H-dopamine release is decreased only in dorsal putamen of mild-moderately lesioned monkeys, but in three of the four striatal regions of more severely lesioned animals. Values represent the mean ± SEM of 4–7 monkeys. Significance of difference from unlesioned controls, **P<0.01, ***P<0.001; from mild-moderate lesion, #P<0.05, using one-way ANOVA followed by a Newman–Keuls *post hoc* test.

Region	K^{+}-evoked ^{3}H-dopamine release					
	Unlesioned control		Mild–moderate lesion + nicotine		More severe lesion + nicotine	
	cpm/mg tis	% control	cpm/mg tis	% control	cpm/mg tis	% control
Medial caudate	17,022 ± 1736	100	23,448 ± 2081	138	12,763 ± 1832#	75
Lateral caudate	13,491 ± 1248	100	11,765 ± 1518	87	10,532 ± 1493	78
Ventral putamen	16,462 ± 1703	100	18,174 ± 659	110	12,507 ± 1880#	76
Dorsal putamen	15,979 ± 1155	100	10,773 ± 561**	67	65,15 ± 1533***#	41

Table 4
Maximal α4β2* nAChR-evoked ^{3}H-dopamine release from monkey striatal regions. Synaptosomes were prepared from freshly dissected monkey striatal subregions as described in Materials and Methods. α4β2* nAChR-mediated ^{3}H-dopamine release was determined by measuring release in the presence of the α6β2* nAChR blocker α-CtxMII. In mild-moderately lesioned monkeys, α4β2* nAChR-mediated release was not significantly different from control in any striatal region. By contrast, there was a trend for a decrease in all striatal subregions except medial caudate in more severely lesioned monkeys. Values are the mean ± SEM of 3–6 monkeys. There was a significant main effect of lesioning using two-way ANOVA ($^{\delta}P<0.05$).

Region	Maximal α4β2* nAChR-mediated ^{3}H-dopamine release						
	Unlesioned control		Mild–moderate lesion + nicotine		More severe lesion + nicotine		
	cpm/mg tis	% control	cpm/mg tis	% control	cpm/mg tis	% control	
Medial caudate	3454 ± 438	100	4378 ± 537	126	4442 ± 1428	128	δ
Lateral caudate	1726 ± 290	100	1730 ± 287	100	780 ± 161	45	
Ventral putamen	3420 ± 606	100	3728 ± 688	109	1451 ± 196	43	
Dorsal putamen	1911 ± 189	100	2066 ± 425	108	772 ± 168	38	

smokers, suggesting that it could be tolerated on a long term basis. Such a treatment regimen led to a sustained reduction in dyskinesias. This decrease in LIDs was maintained when the nicotine dose was incrementally decreased from 300 to 50 μg/ml over an 8 wk period. Thus the effect of nicotine persists even at relatively low doses of nicotine. However, constant nicotine dosing appears to be required for the reduction in LIDs, as they reverted back to control levels several wk after nicotine removal. These data indicate that the effect of nicotine is reversible. The subsequent re-introduction of nicotine and the resultant decline in LIDs indicate that the mechanisms through which nicotine exerts its antidyskinetic effect are still functional even after its removal and re-administration.

For the above studies, we used monkeys with mild to moderate parkinsonism, which may be likened to early stage Parkinson's disease. Striatal dopamine transporter values ranged from 63 to 74% of control in ventromedial striatum, and 32–48% of control in the dorsolateral striatum, a region generally more sensitive to the effects of MPTP. We also tested the effect of nicotine in animals with more severe parkinsonism since it is well known that the extent of nigrostriatal damage influences drug effectiveness. Striatal dopamine transporter values in these animals ranged from 35 to 42% of control in ventromedial striatum, and 14–23% of control in the dorsolateral striatum. Three months of nicotine administration to the more severely parkinsonian animals, at a dose similar to those used in the monkeys with mild to moderate parkinsonism, did not significantly reduce LIDs. Thus nicotine decreased LIDs in monkeys in which striatal dopamine transporter levels average ~50% of control, but was no longer effective when the transporter was reduced to ~25%.

The finding that ~25% higher dopamine transporter level could underlie the nicotine-mediated improvement in dyskinesias in mild-moderately as compared to the more severely parkinsonian monkeys was initially somewhat unexpected. However, subsequent measurement of ^{3}H-dopamine release, an index of dopaminergic function, yielded a possible explanation. These data showed that the various release components (basal, K^+-evoked, α6β2* and α4β2* nAChR-evoked) were at control levels or only somewhat decreased primarily in the dorsolateral striatal areas in monkeys with mild-moderate parkinsonism as compared to unlesioned animals. By contrast, these same release measures were decreased in all or most striatal regions in more severely parkinsonian animals as compared to unlesioned monkeys. Thus, nicotine most effectively improves LIDs in parkinsonian monkeys in which striatal dopamine function is at or near control levels, that is, in which dopamine transmission is maintained.

The observation that striatal dopamine release is still relatively normal with mild-moderate parkinsonism as compared to unlesioned controls despite considerable declines in the dopamine transporter is probably due to functional compensation. Support for compensation stems from studies by Hornykiewicz and coworkers who showed an increase in the ratio of dopamine metabolites to dopamine in rat striatum with moderate nigrostriatal damage (Hornykiewicz, 1975, 1998; Zigmond et al., 1990). Subsequent experiments showed that extracellular dopamine was maintained at normal levels despite dopamine terminal loss, suggesting enhanced dopamine release by remaining terminals and/or decreased reuptake (Robinson and Whishaw, 1988; Snyder et al., 1990; Stachowiak et al., 1987; Zigmond et al., 1984). Data in nonhuman primates also show that dopamine release remains at or near control levels in monkey ventromedial striatum despite ~50% declines in striatal dopamine (McCallum et al., 2006). These observations suggest that dopamine terminal integrity is critical for the action of nicotine.

Our findings that nicotine exerts an antidyskinetic effect but also induces presynaptic dopamine release initially appears somewhat paradoxical, since LIDs are thought to arise as a result of excessive dopaminergic activity after bolus L-dopa administration (Bezard et al., 2001; Cenci and Lindgren, 2007; Cenci, 2007; Cenci and Konradi, 2010; Jenner, 2008; Obeso et al., 2000). However, although acute nicotine application to an *in vitro* synaptosomal preparation stimulates dopamine release, long term nicotine administration may lead to very different results *in vivo*. Extensive evidence now indicates that chronic nicotine dosing leads to nAChR desensitization with consequent declines in nAChR-mediated function (Buccafusco et al., 2009; Picciotto et al., 2008). In addition, CNS imaging studies have shown that the majority of nAChRs are occupied after smoking, that is, after nicotine exposure, and thus possibly desensitized (Brody et al., 2006). Long term receptor desensitization or inactivation may attenuate the aberrant dopamine tone that arises with exogenous L-dopa administration. As well, nAChR desensitization may lead to additional downstream changes in signaling mechanisms that improve LIDs. This interpretation is consistent with the finding that a partially intact dopaminergic system is required for the antidyskinetic effect of nicotine.

Table 5
Maximal α6β2* nAChR-evoked ^{3}H-dopamine release from monkey striatal regions. Synaptosomes were prepared from freshly dissected monkey striatal subregions as described in Materials and Methods. α6β2* nAChR-mediated ^{3}H-dopamine release was assessed by subtracting total nicotine-evoked release from release determined in the presence of the α6β2* nAChR blocker α-CtxMII. Striatal α6β2* nAChR-mediated release was decreased only in the lateral caudate and dorsal putamen of mild-moderately-lesioned monkeys. By contrast, release was decreased to a much greater extent in all striatal regions of more severely-lesioned monkeys. Values are the mean ± SEM of 3–6 monkeys. Significance of difference from unlesioned control, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; from mild-moderate lesion, #P<0.05, ##P<0.01, using one-way ANOVA followed by a Newman–Keuls *post hoc* test.

Region	Maximal α6β2* nAChR-mediated ^{3}H-dopamine release					
	Unlesioned control		Mild–moderate lesion + nicotine		More severe lesion + nicotine	
	cpm/mg tis	% control	cpm/mg tis	% control	cpm/mg tis	% control
Medial caudate	8150 ± 776	100	7673 ± 821	94	2037 ± 556**##	25
Lateral caudate	4459 ± 335	100	3088 ± 300*	69	2569 ± 410*	59
Ventral putamen	5023 ± 616	100	4531 ± 604	90	1990 ± 277*#	44
Dorsal putamen	4583 ± 393	100	2766 ± 285**	60	1361 ± 277***#	29

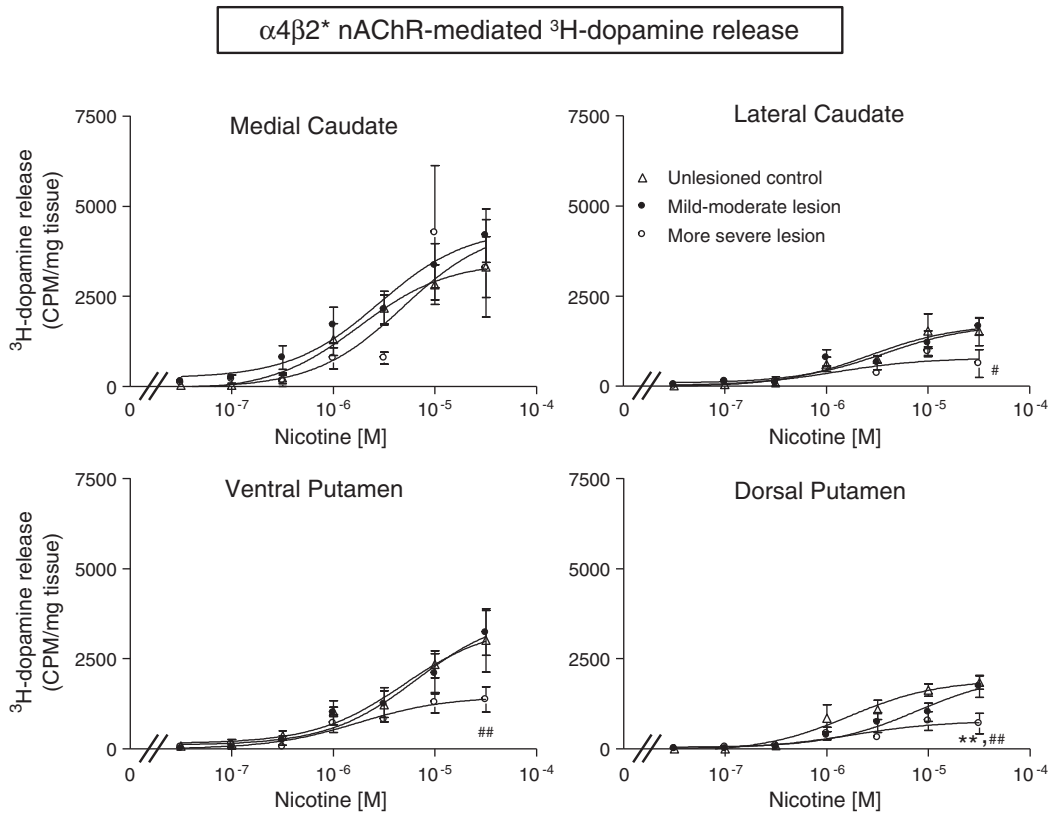


Fig. 7. α4β2* nAChR-mediated ^{3}H-dopamine release in monkey striatum. MPTP-lesioned monkeys were treated as detailed in the timeline depicted in Fig. 1 and gavaged with L-dopa twice daily. At the end of the study, the animals were euthanized, synaptosomes prepared from freshly dissected striata and nicotine-evoked ^{3}H-dopamine released measured in different striatal subregions. The α4β2* nAChR-mediated ^{3}H-dopamine release component was defined as nicotine-evoked release remaining in the presence of the α6β2* nAChR antagonist α-CtxMII. There was no decline in α4β2* nAChR-mediated release in any striatal region with mild-moderate lesioning. By contrast, more severe lesioning reduced release in all striatal regions except the medial caudate. The values represent the mean ± SEM of 3–6 monkeys. Significance of difference from unlesioned control, **P<0.01; from mild-moderate lesion, #P<0.05, ##P<0.01. Data were analyzed by two-way ANOVA followed by a Bonferroni *post hoc* test.

Another possibility is that nicotine may exert its antidyskinetic effect indirectly via nondopaminergic mechanisms. For instance, nicotine may act by modulating serotonergic tone. This system plays a major role in the occurrence of L-dopa-induced dyskinesias because of the non-physiological synthesis and release of dopamine derived from L-dopa in serotonergic and possibly other neurons (Carta and Bezard, 2011; Carta et al., 2008; Eskow et al., 2007, 2009; Huot et al., 2011; Iravani and Jenner, 2011). There is accumulating evidence for a functional relationship between the nicotinic and serotonergic systems, with serotonin receptor blockers reducing nicotine mediated-locomotor responses, as well as modulating nicotine use and dependence (Dao et al., 2011; Fletcher et al., 2008; Levin et al., 2008, 2011; Zaniewska et al., 2009, 2010). Another neuronal population that may be involved in the nicotine-mediated decline in LIDs are striatal cholinergic neurons. Cholinergic interneurons anatomically and functionally overlap with striatal dopaminergic terminals and play a key role in synaptic plasticity and motor learning (Pisani et al., 2007). Furthermore, LIDs are associated with enhanced striatal cholinergic activity (Ding et al., 2011). Nicotine also modulates the glutamatergic system (Timofeeva and Levin, 2011), which plays a major role in the development of LIDs, particularly NMDA and mGluR5 receptors (Blandini and Armentero, 2012; Rylander et al., 2010; Sgambato-Faure and Cenci, 2012). An interaction of nicotine with the noradrenaline, opiate, cannabinoid, adenosine and/or histamine system, which are all implicated in the expression of LIDs, is also a possibility (Iravani and Jenner, 2011). If other CNS systems are involved in the nicotine-mediated decline in LIDs, this may suggest that the loss of the antidyskinetic effect of nicotine in more-severely parkinsonian animals and the impairment of striatal dopamine release are two unique events not casually associated.

In summary, the present work identifies conditions under which nicotine best reduces LIDs in nonhuman primates. These data have therapeutic implications with respect to successful nicotine treatment regimens for Parkinson's disease patients. They also demonstrate that nicotine improves LIDs in lesioned monkeys in which striatal dopamine release mechanisms are still relatively intact, but not in monkeys with more severe parkinsonism. These results suggest that nicotine would most successfully reduce LIDs in other primate and rodent parkinsonian models with a partially intact dopaminergic system. This assumption is consistent with our experiments in parkinsonian rats which show that nicotine and nicotinic agonists most effectively improve L-dopa-induced abnormal involuntary movements with partial nigrostriatal damage (Bordia et al., 2008, 2010; Huang et al., 2011a, 2011b). Overall, these data suggest that nicotine treatment may not attenuate LIDs with severe disease, but would most effectively reduce LIDs in patients with mild to moderate Parkinson's disease.

Relevant conflict of interest

M. Quik is on a patent for the use of nicotine for L-dopa-induced dyskinesias. There are no other conflicts of interest.

Acknowledgments

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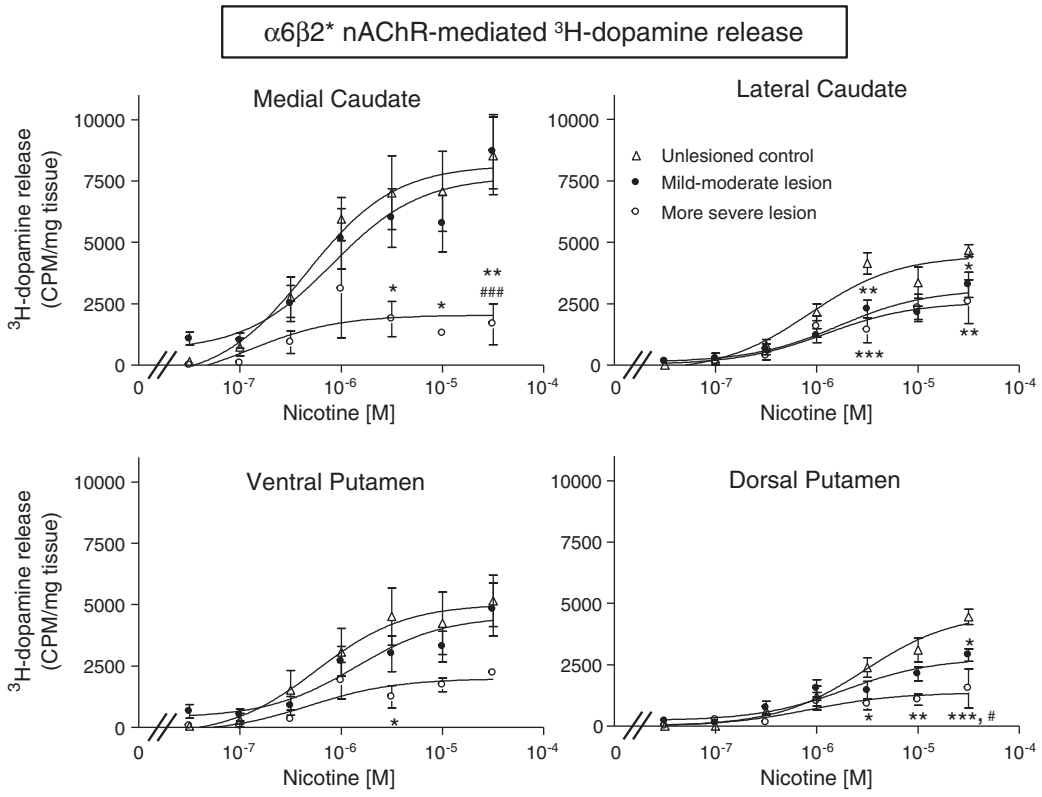

Fig. 8. α6β2* nAChR-mediated ^{3}H-dopamine release in monkey striatum. MPTP-lesioned monkeys were treated as detailed in the timeline depicted in Fig. 1 and gavaged with L-dopa twice daily. At the end of the study, the animals were euthanized, synaptosomes prepared from freshly dissected striata and nicotine-evoked ^{3}H-dopamine released measured in different striatal subregions. α6β2* nAChR-mediated ^{3}H-dopamine release was determined by subtracting nicotine-evoked release in the presence of α-CtxMII from total nicotine-evoked release. In mild-moderately-lesioned monkeys, striatal α6β2* nAChR-mediated release was decreased only in the lateral caudate and dorsal putamen, while release was decreased in all striatal regions in more severely-lesioned monkeys. Values represent the mean ± SEM of 3–6 monkeys. Significance of difference from unlesioned control: *P<0.05; **P<0.01; ***P<0.001. Significance of difference from mild-moderate lesion: #P<0.05; ###P<0.001. Data were analyzed by two-way ANOVA followed by a Bonferroni *post hoc* test.

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