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Chronic Nicotine Treatment Increases nAChRs and Microglial Expression in Monkey Substantia Nigra after Nigrostriatal Damage

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Abstract

Our previous work had shown that long-term nicotine administration improved dopaminergic markers and nicotinic receptors (nAChRs) in the striatum of monkeys with nigrostriatal damage. The present experiments were done to determine whether nicotine treatment also led to changes in the substantia nigra, the region containing dopaminergic cell bodies. Monkeys were chronically treated with nicotine in the drinking water for 6 months after which they were injected with low dose MPTP for a further 6-month period. Nicotine was administered until the monkeys were euthanized 2 months after the last MPTP injection. Nicotine treatment did not affect the dopamine transporter or the number of tyrosine hydroxylase positive cells in the substantia nigra of lesioned monkeys. However, nicotine administration did lead to a greater increase in $\alpha 3/\alpha 6\beta 2^*$ and $\alpha 4\beta 2^*$ nAChRs in lesioned monkeys compared to controls. Nicotine also significantly elevated microglia and reduced the number of extracellular neuromelanin deposits in the substantia nigra of MPTP-lesioned monkeys. These findings indicate that long-term nicotine treatment modulates expression of several molecular measures in monkey substantia nigra that may result in an improvement in nigral integrity and/or function. These observations may have therapeutic implications for Parkinson's disease.

Keywords

Neuromelanin; Nicotine; Nicotinic receptor; Substantia nigra; Parkinson's disease

Introduction

Cigarette smoking is the most robust negative risk factor for Parkinson's disease. The results of epidemiological studies suggest that this represents a true biological consequence of smoking since the reduced incidence of Parkinson's disease is dose and time dependent, is diminished with smoking cessation and is not due to enhanced mortality (Morens et al. 1995; Allam et al. 2004; Ritz et al. 2007). Accumulating work suggests that nicotine, a constituent of cigarette smoke, may play a role in this apparent neuroprotective effect (Quik et al. 2007; Picciotto and Zoli 2008).

Evidence for this possibility stems from experiments showing that treatment with nicotine reduces the neurotoxic effects of nigrostriatal damage. This includes studies in parkinsonian rodents in which nicotine pretreatment results in improved dopamine and tyrosine hydroxylase levels in the striatum (Janson et al. 1988; Carr and Rowell 1990; Costa et al. 2001; Visanji et al. 2006). Similar results have also been obtained in MPTP-treated nonhuman primates, a model that bears many resemblances to Parkinson's disease (Bordia et al. 2006; Quik et al. 2006a; Quik et al. 2006b). In these studies, monkeys were administered nicotine in the drinking water for several months. The nigrostriatal dopaminergic neurons were then lesioned using chronic MPTP administration, with nicotine continued for a further six months at which time the study ended. This nicotine treatment regimen led to a significant reduction in the degree of loss of striatal tyrosine hydroxylase, the dopamine transporter, the vesicular monoamine transporter, $\alpha 4\beta 2^*$ and $\alpha 3/\alpha 6\beta 2^*$ nAChRs, as well as in nAChR-mediated dopamine function (Quik et al. 2006a; Quik et al. 2006b).

The question arose whether nicotine treatment also improved reduced molecular measures after lesioning in the substantia nigra, the region that contains the dopaminergic cell bodies that project to the striatum. Although previous work had shown that nicotine treatment did not prevent neurotoxin-induced dopaminergic cell loss monkey substantia nigra (Quik et al. 2006b), it is possible that nicotine treatment improved other molecular measures in the remaining dopaminergic cell bodies in the substantia nigra to enhance nigral integrity and consequently improve function.

The goal of the present study was to determine whether nicotine treatment enhanced molecular measures in the substantia nigra of MPTP-lesioned nonhuman primates. To approach this, we measured the nigral dopamine transporter, a marker of dopaminergic neuron integrity. In addition, we assessed nAChR expression since both $\alpha 4\beta 2^*$ and $\alpha 3/\alpha 6\beta 2^*$ nAChRs are present in substantia nigra and nicotine exposure influences nigral dopaminergic function (Quik et al. 2002; Gotti et al. 2005; Keath et al. 2007; Nashmi et al. 2007; Pons et al. 2008). Because nicotine also influences inflammatory responses in the CNS (Shytle et al. 2004) and protects dopaminergic neurons from damage via a microglial cell-mediated anti-inflammatory mechanism (Park et al. 2007), we also determined whether nicotine exposure influenced nigral microglial and extracellular neuromelanin levels. The results suggest that nicotine treatment exerts a modulatory influence on the substantia nigra that may be relevant to nicotine-mediated neuroprotection against nigrostriatal damage.

Materials and Methods

Animals and treatment

Monkeys were treated as previously described (Quik et al. 2006a; Quik et al. 2006b). Briefly, female squirrel monkeys (*Saimiri sciureus*) in late adulthood were purchased from Osage Research Primates (Osage Beach, MO, USA) or the University of South Alabama (Mobile, AL, USA). Animals (0.5 to 0.8 kg) were housed separately under a 13/11-h light-

dark cycle, with free access to water. Monkey food chow and fruit/vegetables was provided once daily. All animals were then given a drinking solution containing nicotine plus 1% saccharin to mask nicotine's bitter taste, or saccharin alone. Nicotine in the drinking water was started at 25 µg/ml and gradually increased to 650 µg/ml over a 3 to 4 month period. This final dose yielded plasma nicotine levels of 12.6 ± 1.3 ng/ml ($n = 12$) and cotinine levels of 370 ± 47 ng/ml ($n = 12$), values within the range in the plasma of smokers (Matta et al. 2007). The animals were maintained at this dose for ~3 months. MPTP (1.5 mg/kg sc) was then administered once every 2 months for a total of three doses, and the animals killed 2 months after the last dose; nicotine was continued over the entire period. Animals were euthanized according to the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association by administration of an intraperitoneal injection of solution containing 390 mg/ml sodium pentobarbital and 50 mg/ml phenytoin sodium, followed by 2.2 ml/kg of the same solution intravenously. All procedures used conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Tissue preparation

The brain was quickly removed, cut along the midline and dissected as described (Quik et al. 2006a; Quik et al. 2006b). Six mm-thick coronal blocks were prepared and immediately frozen in isopentane on dry ice. These blocks were cut into 20-µm-thick sections using a cryostat. The sections were thaw-mounted onto poly-L-lysine-coated slides, dried and stored at -80°C until use. The entire substantia nigra was sectioned at 20 µm from level A6.0 to A4.0 to yield ~100 consecutive sections that were used for the autoradiography and immunohistochemistry. All the autoradiographic assays were performed in duplicate sections using sections in the mid portion of the nigra (~A5.0). For the immunohistochemistry, assessment was done using every 15th consecutive section throughout the nigra, as described below.

Dopamine transporter autoradiography

^{125}I -RTI-121 (2200 Ci/mmol; Perkin Elmer Life Sciences, Boston, MA, USA) binding was performed as described (Bordia et al. 2007). Slides were incubated two times for 15 min each at 22°C in the following buffer at pH 7.4; 50 mM Tris-HCl, 120 mM NaCl, and 5 mM KCl. The slides were next incubated for 2 hr in the same buffer also containing 0.025% bovine serum albumin, 1 µM fluoxetine and 50 pM ^{125}I -RTI-121. They were washed, air-dried and exposed for 2 d to Kodak MR film (Perkin Elmer Life and Analytical Science, Boston, MA) with ^{125}I -microscale standards (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Nomifensine (100 µM) was used to evaluate nonspecific binding.

^{125}I -α-ConotoxinMII (^{125}I -α-CtxMII) autoradiography

^{125}I -α-CtxMII (2200 Ci/mmol) was synthesized and radiolabeled as described (Whiteaker et al. 2000), and ^{125}I -α-CtxMII binding done as reported (Bordia et al. 2007). Slides were incubated at 22°C for 15 min in binding buffer at pH 7.5; 144 mM NaCl, 1.5 mM KCl, 2 mM CaCl_2 1 mM MgSO_4 , 20mM HEPES and 0.1 % bovine serum albumin, plus 1 mM phenylmethylsulfonyl fluoride. A 60 min incubation was then performed at 22°C in the same buffer also containing 0.5% bovine serum albumin, 5 mM EDTA, 5 mM EGTA and 10 µg/ml each of aprotinin, leupeptin and pepstatin A plus 2.0 nM ^{125}I -α-CtxMII. The slides were then washed, air-dried and exposed to Kodak MR for 2 to 5 days together with ^{125}I -microscale standards. Nicotine (100 µM) was used to evaluate blank binding.

¹²⁵I-Epipatidine autoradiography

¹²⁵I-Epipatidine binding (2200 Ci/mmol, from H. Fan, Dept. Radiology, Johns Hopkins University School of Medicine) was done as described (Quik et al. 2006b). Slides were incubated for 15 min at 22°C in the following buffer at pH 7.0; 50 mM Tris, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, and 1.0 mM MgCl₂. After a 40 min incubation period with 1.0 nM ¹²⁵I-epibatidine in the presence or absence of α-CtxMII (300 nM), the slides were washed and air-dried. They were exposed to Kodak MR film with ¹²⁵I-microscale standards for several days. Nonspecific binding, done in the presence of 100 μM nicotine, was as the film blank.

¹²⁵I-A-85380 autoradiography

Binding of ¹²⁵I-A-85380 (960 Ci/mmol, from H. Fan, Dept. Radiology, Johns Hopkins University School of Medicine) was performed according to previous procedures (Bordia et al. 2006). Slides were incubated for 15 min at 22°C in the following buffer at pH 7.0; 50 mM Tris, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂ and 1 mM MgCl₂. This was followed by a 60 min incubation at 22°C in the same buffer also containing 0.3 to 0.8 nM ¹²⁵I-A-85380, with or without α-CtxMII (300 nM). Slides were washed, air-dried and exposed to Kodak MR film with ¹²⁵I standards for several days. Nonspecific binding was determined using 100 μM nicotine and was similar to the film blank.

Immunocytochemistry

For assessment of HLA-DR⁺ microglia, slides were first fixed in 4% paraformaldehyde for 10 min and quenched with 0.6% peroxide for 15 min. The sections were blocked with 10% normal goat serum for 1 hour at 22°C, followed by exposure to Avidin D and Biotin for 15 min each using an Avidin/Biotin Blocking Kit (Vector, Burlingame, CA). They were subsequently incubated overnight at 4°C with a 1/100 dilution of a mouse monoclonal antibody to HLA-DR (Caltag Labs, Burlingame, CA) diluted in 0.1M phosphate buffered saline (pH 7.4) containing 1% bovine serum albumin and 0.1% Triton X-100. The sections were incubated with a biotinylated goat anti-mouse secondary antibody at 1:50 dilution for 1 hour, and then with preformed Avidin-Biotin complex at 1:700 for 1 hour. Staining was visualized using the avidin-biotin immunoperoxidase reaction with the Vector SG kit (Vector, Burlingame, CA) after a 4 min exposure period. The slides were then dehydrated and coverslipped.

The substantia nigra was delineated using a 10x objective according to a squirrel monkey brain atlas (Emmers and Akert 1963) using tyrosine hydroxylase staining as a guide. The results of the tyrosine hydroxylase immunostaining for these same monkeys have previously been reported (Quik et al., 2006b). Total and activated microglia were counted in every 15th consecutive 20 μm coronal section throughout the nigra using a 20x objective. Activated and resting microglia were defined by the intensity of HLA-DR⁺ staining and cellular morphology as follows. Amoeboid cells with a clearly defined round or ovoid cell body with two or more processes at least twice the length of the cell body were defined as activated microglia. Ramified cells with multiple branching processes without a clearly defined cell body were defined as resting microglia. All data collection was carried out using the Stereo Investigator software (MicroBrightfield, Williston, VT, USA). Cells were counted by one or two researchers blinded to treatment conditions. There was excellent reliability in counting between different researchers, with a Pearson correlation coefficient of $R = 0.84$ ($p < 0.004$).

Extracellular neuromelanin assessment

Extracellular neuromelanin deposits were also evaluated in every 15th consecutive section throughout the substantia nigra. These were defined as deposits ≥ 5 μm that were not

associated with neuronal cell bodies, as determined by Nissl staining. Data collection was carried out using Stereo Investigator software (MicroBrightfield, Williston, VT, USA) by an investigator blinded to treatment conditions.

Data analysis

The ImageQuant system (GE Healthcare, Little Chalfont, Buckinghamshire, UK) was used to determine the optical density measurements from the autoradiograms. These were converted to nCi/mg tissue using standard curves generated from ^{125}I -standards, with optimal density readings within the linear range of the film.

Results are expressed as mean $\pm$ SEM for the indicated number of animals. Statistical analyses were done with GraphPad Prism (GraphPad, San Diego, CA, USA) using two-way ANOVA followed by Bonferroni *post hoc* test. $P \leq 0.05$ was considered significant.

Results

Effect of nicotine treatment on the nigral dopamine transporter

The results in Table 1 show that nigrostriatal damage decreased the nigral dopamine transporter with an overall main effect of MPTP treatment ($p < 0.01$). The ~25% decline in the transporter is consistent with ~20% decrease in the number of tyrosine hydroxylase positive cells previously reported in the substantia nigra (Quik et al. 2006b). Nicotine treatment did not affect dopamine transporter levels in the nigra of either control or lesioned monkeys.

Long-term nicotine treatment enhances $\alpha 3/\alpha 6\beta 2^*$ nAChR expression in substantia nigra of MPTP-lesioned monkeys

The levels of $\alpha 3/\alpha 6\beta 2^*$ nAChRs in the substantia nigra were next measured using several different radioligand binding assays. This included binding of ^{125}I - α -CtXMII, which directly measures $\alpha 3/\alpha 6\beta 2^*$ nAChRs. We also assessed α -CtXMII-sensitive ^{125}I -epibatidine binding sites and α -CtXMII-sensitive ^{125}I -A85380 binding sites, with the $\alpha 3/\alpha 6\beta 2^*$ nAChR component determined by subtracting binding in the presence of α -CtXMII from total binding (Fig. 1). MPTP-treatment alone decreased $\alpha 3/\alpha 6\beta 2^*$ nAChR levels suggesting that this subtype is present, at least in part, on dopaminergic cell bodies in the nigra. Nicotine treatment alone had no significant effect on $\alpha 3/\alpha 6\beta 2^*$ nAChRs, in contrast to previous results in the striatum where a decrease was obtained (Nguyen et al. 2003; Lai et al. 2005; Bordia et al. 2006; Mugnaini et al. 2006; Perry et al. 2007). However, nicotine treatment did elevate $\alpha 3/\alpha 6\beta 2^*$ nAChRs in the nigra as compared to MPTP-lesioned monkeys treated with saccharin, as it had in monkey striatum (Bordia et al. 2006). Similar results were obtained using the different binding assays suggesting that the three radioligands bound to similar populations of $\alpha 3/\alpha 6\beta 2^*$ nAChRs. The finding that there is a preferential increase in $\alpha 3/\alpha 6\beta 2^*$ nAChRs with nicotine treatment after nigrostriatal damage suggests that nicotine treatment may ameliorate nigral function via nAChR-mediated mechanisms.

Long-term nicotine treatment enhances $\alpha 4\beta 2^*$ nAChR expression in substantia nigra of saline-treated and MPTP-lesioned monkeys

We also measured $\alpha 4\beta 2^*$ nAChR levels in the substantia nigra of control and lesioned monkeys treated with and without nicotine (Fig. 2). To approach this, we performed ^{125}I -epibatidine or ^{125}I -A85380 autoradiography in the presence of α -CtXMII, which defines the $\alpha 4\beta 2^*$ nAChR population (McIntosh et al. 2004). Nigrostriatal damage alone decreased $\alpha 4\beta 2^*$ nAChRs, as previously shown in both monkeys and rodents (Kulak et al. 2002a; Kulak et al. 2002b; Zoli et al. 2002). Nicotine treatment alone increased nigral $\alpha 4\beta 2^*$ nAChR expression in saline-treated monkeys, in agreement with previous studies across

species (Marks et al. 1992; McCallum et al. 2006; Perry et al. 2007). Nicotine exposure also increased $\alpha 4\beta 2^*$ nAChRs in the substantia nigra of MPTP-lesioned monkeys. Interestingly, there was a trend for a greater percent increase in $\alpha 4\beta 2^*$ nAChR in the nigra of the lesioned monkeys with nicotine treatment compared to those only receiving saccharin, although this did not reach statistical significance. For the ^{125}I -epibatidine binding studies, nicotine treatment increased $\alpha 4\beta 2^*$ nAChR levels $100 \pm 10\%$ ($n = 5$) over control in the lesioned group and $60 \pm 14\%$ ($n = 6$) over control in the saline group. Similarly, for ^{125}I -A85380 binding, there was a trend for a greater increase in $\alpha 4\beta 2^*$ nAChRs with nicotine treatment in the lesioned group ($71 \pm 14\%$, $n = 6$) as compared to the control group ($50 \pm 12.8\%$, $n = 6$). The similarity in treatment-induced receptor changes with the two radioligands suggests they bind to similar $\alpha 4\beta 2^*$ nAChR subtypes.

Nicotine treatment increases microglial cell numbers in substantia nigra

Previous work had shown that nicotine treatment results in immune activation, including an increase in microglial cells (de Jonge and Ulloa 2007). Experiments were therefore performed to determine microglial number in the substantia nigra using antibodies against HLA-DR (Fig. 3). Two-way ANOVA showed there was a main effect of nicotine treatment ($p < 0.05$) on the total number of microglia. As well, there was a significant increase using a Bonferroni *post hoc* test in total nigral microglia in the nicotine-treated MPTP-lesioned group compared to lesioned only monkeys ($p < 0.05$). Experiments were next done to count the number of activated microglia, which are distinguished from resting microglia by an amoeboid as compared to a ramified cell morphology. Nicotine treatment increased activated microglia, with a significant main effect using two-way ANOVA ($p < 0.05$). The number of resting HLA-DR⁺ microglial cells, determined by subtraction of activated microglia from the total number of microglia, were also increased with a significant main effect using two-way ANOVA ($p < 0.05$). Thus, nicotine treatment increases both resting and activated microglia, with a somewhat greater increase with nigrostriatal damage.

Nicotine treatment reduces the number of extracellular neuromelanin deposits in the substantia nigra after MPTP treatment

Dopaminergic neurons in primate substantia nigra are well known to contain intracellular neuromelanin inclusions, which present as darkened punctate deposits within the cell body. In addition, extracellular neuromelanin deposits arise with nigrostriatal damage (Fig. 4A). These are distinct from intraneuronal neuromelanin as they are larger in size, located extracellularly and most likely reflect degraded dopaminergic neurons. Since the number of extracellular neuromelanin deposits generally correlates with the extent of nigrostriatal damage (Gibb 1992; Kastner et al. 1992), we assessed the number of these deposits throughout the nigra of saline and MPTP-lesioned animals treated with and without nicotine (Fig. 4B). Only isolated extracellular neuromelanin deposits were detected in non-lesioned monkeys treated with and without nicotine. By contrast, MPTP treatment dramatically elevated the number of extracellular neuromelanin deposits. This increased number of neuromelanin deposits with nigrostriatal damage was reduced with nicotine treatment ($p < 0.05$). Thus, chronic nicotine treatment is associated with fewer extracellular neuromelanin deposits in the substantia nigra of monkeys with nigrostriatal damage.

Discussion

The present results are the first to show that long-term nicotine treatment improves molecular measures in the substantia nigra of monkeys with nigrostriatal damage. Nicotine administration lead to greater increases in $\alpha 3/\alpha 6\beta 2^*$ and $\alpha 4\beta 2^*$ nAChRs in the nigra of lesioned compared to unlesioned monkeys. Nicotine treatment also significantly elevated microglia in the substantia nigra of MPTP-lesioned monkeys, and reduced the number of

nigral extracellular neuromelanin deposits. These findings suggest that nicotine has the potential to improve select aspects of nigral integrity and/or function. These observations may be relevant to nicotine-mediated neuroprotection against nigrostriatal damage, and thus be of therapeutic importance for Parkinson's disease.

Our previous studies had shown that nicotine treatment improved MPTP-induced declines in several dopaminergic measures in the striatum, including the dopamine transporter (Quik et al. 2006b). These findings contrast with the current results, which show no improvement in nigral dopamine transporter levels with nicotine treatment after nigrostriatal damage. However, they are consistent with our previous studies showing no improvement in the number of dopaminergic neurons in the nigra of nicotine-treated lesioned versus unlesioned monkeys (Quik et al. 2006b), similar to findings by others in rodents with nigrostriatal damage (Visanji et al. 2006). By contrast, we did identify a significantly larger increase in $\alpha 3/\alpha 6\beta 2^*$ nAChRs in the substantia nigra of lesioned monkeys treated with nicotine, as well as a trend for a greater increase in nigral $\alpha 4\beta 2^*$ nAChRs, as we had found in striatum (Bordia et al. 2006). These data add further support to the idea that long-term nicotine treatment may ameliorate nAChR-mediated deficits that arise with nigrostriatal damage.

Nicotine treatment also had significant effects in the nigra of unlesioned monkeys. Exposure to nicotine increased $\alpha 4\beta 2^*$ nAChRs, similar to its effects in other brain regions in rodents and monkeys (Marks et al. 1992; McCallum et al. 2006). However, nicotine treatment did not decrease $\alpha 3/\alpha 6\beta 2^*$ nAChRs in the nigra of control monkeys as it had in the striatum (Nguyen et al. 2003; Lai et al. 2005; Bordia et al. 2006; Mugnaini et al. 2006). These differential effects of nicotine treatment on the $\alpha 3/\alpha 6\beta 2^*$ nAChR subtype in controls suggests that α -CtxMII-sensitive nAChRs in the nigra are distinct from those in the striatum. In fact, previous observations in rodents indicate there are differences in the $\alpha 6\beta 2^*$ subtypes in nigral dopaminergic cell bodies and terminals (Gotti et al. 2005). The precise subunit composition of ^{125}I - α -CtxMII binding sites or $\alpha 3/\alpha 6\beta 2^*$ receptor subtypes in the monkey nigrostriatal system is still not known with certainty. They may contain $\alpha 3$ and/or $\alpha 6$ nAChR subunits and also express $\alpha 2$, $\alpha 4$, $\beta 2$ and/or $\beta 3$ subunits, which have all been identified in the monkey nigrostriatal system (Quik et al. 2005). With respect to localization, the finding that both $\alpha 3/\alpha 6\beta 2^*$ and $\alpha 4\beta 2^*$ were significantly decreased in the nigra with MPTP treatment suggests that these subtypes are present on dopaminergic cell bodies in the nigra. Whether they are also present on other neuronal or non-neuronal elements in the nigra awaits further study.

Accumulating evidence suggests that the nicotinic cholinergic system can regulate immune function through a nicotinic anti-inflammatory pathway (de Jonge and Ulloa 2007). In the peripheral nervous system, vagal nerve stimulation attenuates the production of proinflammatory cytokines and inhibits the inflammatory process in experimental models, most likely via activation of $\alpha 7$ nAChRs on microglia. In the brain, nAChR activation also reduces microglial activation that arises after exposure to lipopolysaccharide or the dopaminergic neurotoxin MPTP (Shytle et al. 2004; Park et al. 2007). These findings have led to the hypothesis that effects of nicotine against cellular damage are mediated, at least in part, through an immune mechanism whereby nicotine dampens microglia activation. In the nigrostriatal system, this most likely occurs via activation of $\alpha 4\beta 2^*$ and/or $\alpha 3/\alpha 6\beta 2^*$ as nicotine-mediated protection is not observed in $\alpha 4\beta 2^*$ nAChR null mutant mice and protection is prevented by treatment with nAChR antagonists that target $\alpha 4\beta 2^*$ nAChRs (Costa et al. 2001; Ryan et al. 2001). The $\alpha 3/\alpha 6\beta 2^*$ receptor population may also be involved since changes in expression of $\alpha 3/\alpha 6\beta 2^*$ receptor subtypes correlate with protection against paraquat-induced toxicity (Khwaja et al. 2007).

The current results show that chronic nicotine treatment increased the number of resting and activated microglia in both control and MPTP-lesioned monkeys, with a somewhat greater enhancement with nigrostriatal damage. These results are distinct from previous work, which had shown that nicotine inhibited microglial activation (Shytle et al. 2004; Park et al. 2007). However, in these latter studies the effects of nicotine occurred via activation of $\alpha 7$ nAChRs. This subtype is present at only very low levels in the nigrostriatal system compared to the $\alpha 4\beta 2^*$ and $\alpha 3/\alpha 6\beta 2^*$ subtypes (Quik et al. 2000; Quik et al. 2001). These latter subtypes may modulate immune function via alternate inflammatory mechanisms compared to those linked to $\alpha 7$ nAChR activation. There is precedence for this possibility from other systems. For instance, acetylcholine is predominantly pro-inflammatory in lymphocytes and epithelial cells, anti-inflammatory for mast cells and macrophages, both pro- and anti-inflammatory for monocytes, with variable effects in neutrophils and eosinophils (Gwilt et al. 2007).

Previous studies using brain tissue from Parkinson's disease cases and from parkinsonian animal models have shown that nigrostriatal damage alone is generally associated with enhanced inflammatory activity, including changes in cytokines and the presence of activated microglial cells (Nagatsu and Sawada 2006; Sawada et al. 2006; Wersinger and Sidhu 2006). However, we observed no increase in microglial cell numbers with MPTP treatment alone. This difference may be due to the fact that nigrostriatal damage was chronic and fairly mild in the present study, with only a 20% loss of nigral dopaminergic neurons (Quik et al. 2006b). This smaller decline in cell number may have been insufficient to maintain an ongoing inflammatory response for 2 months after the last toxic insult, at which time the animals were euthanized.

The present results show that chronic nicotine administration decreased the number of extracellular neuromelanin deposits in the substantia nigra of lesioned monkeys. Extracellular neuromelanin is a breakdown product of dopaminergic cell degeneration (McGeer et al. 1988). Neuromelanin, a large polymer of indole subunits formed as a result of dopamine auto-oxidation, is normally present in membrane-enclosed granules in the neuronal cytoplasm (Zecca et al. 2002; Fasano et al. 2006; Rao et al. 2006). Its function is unclear and it has been implicated in both neuroprotection by acting as a buffer reservoir for toxic reagents, but also with detrimental effects as dying nigrostriatal neurons release neuromelanin to result in the formation of extracellular deposits that most likely disrupt cellular homeostasis. The observed decline in extracellular neuromelanin deposits with nicotine exposure may be due to an accelerated breakdown as a result of the nicotine-induced increase in activated microglia. This may result in an enhanced phagocytosis or scavenging of extracellular neuromelanin deposits, with a resultant decline in their number. This decrease in extracellular neuromelanin may improve the overall integrity of the substantia nigra with a consequent amelioration of striatal dopaminergic function, as was observed in our previous studies (Bordia et al. 2006; Quik et al. 2006a; Quik et al. 2006b).

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Figure 1.

Long-term nicotine treatment enhances $\alpha 3/\alpha 6\beta 2^*$ nAChR expression in substantia nigra of MPTP-lesioned monkeys. Monkeys were given saccharin (Con) or nicotine (Nic) in the drinking water and subsequently lesioned with MPTP, as described in the Materials and Methods. Nicotine treatment was continued until the animals were euthanized. Nigral $\alpha 3/\alpha 6\beta 2^*$ nAChR levels were measured using ^{125}I - α -CtxMII (A), ^{125}I -epibatidine (B), ^{125}I -A85380 (C) autoradiography. The ^{125}I -epibatidine and ^{125}I -A85380 binding was done in the absence and presence of α -CtxMII, with the difference defined as the $\alpha 3/\alpha 6\beta 2^*$ subtype. MPTP-treatment alone decreased $\alpha 3/\alpha 6\beta 2^*$ nAChR levels, while nicotine treatment alone had no significant effect on $\alpha 3/\alpha 6\beta 2^*$ nAChRs. However, nicotine treatment did elevate nigral $\alpha 3/\alpha 6\beta 2^*$ nAChRs compared to the MPTP-lesioned saccharin-treated monkeys. Significance of difference from Con+MPTP; * $p < 0.05$, ** $p < 0.01$: from Con +Saline; # $p < 0.05$, ## $p < 0.01$. Values represent the mean $\pm$ SEM of 5 to 6 monkeys.

Figure 2.

Long-term nicotine treatment enhances $\alpha 4\beta 2^*$ nAChR expression in substantia nigra of saline-treated and MPTP-lesioned monkeys. Monkeys were given saccharin (Con) or nicotine (Nic) in the drinking water and subsequently lesioned with MPTP, with nicotine treatment continued until the animals were euthanized. Nigral $\alpha 4\beta 2^*$ nAChR expression was determined using ^{125}I -epibatidine (A) and ^{125}I -A85380 (B) autoradiography, both performed in the presence of α -CtxMII to define the $\alpha 4\beta 2^*$ nAChR subtype. MPTP-treatment alone decreased $\alpha 4\beta 2^*$ nAChRs. Nicotine treatment alone increased nigral $\alpha 4\beta 2^*$ nAChR expression in both saline-treated and MPTP-lesioned monkeys. Significance of difference from own control; ** $p < 0.01$, *** $p < 0.001$; from Con+Saline; # $p < 0.05$. Values represent the mean $\pm$ SEM of 5 to 6 monkeys.

Figure 3.

Chronic nicotine treatment increases the numbers of HLA-DR⁺ microglia in monkey substantia nigra. Monkeys were given saccharin (Control) or nicotine in the drinking water and subsequently lesioned with MPTP, with nicotine treatment continued until the animals were euthanized. The number of total and activated microglia were counted throughout the substantia nigra, with resting microglia representing the difference between these two values. Results are the mean $\pm$ SEM of 6 to 7 monkeys. There was a significant main effect of nicotine by two-way ANOVA, $\Psi p < 0.05$. Significance of difference from Control-MPTP using a Bonferroni *post hoc* test, $\delta p < 0.05$.

Figure 4.

Chronic nicotine treatment decreases the number of extracellular neuromelanin deposits after MPTP-induced nigrostriatal damage. (A) Nissl staining of dopaminergic neurons in monkey substantia nigra showing intraneuronal neuromelanin (arrow head) distributed throughout the cytoplasm. The arrow marks an extracellular neuromelanin deposit in the substantia nigra of MPTP-lesioned animals. Scale bar = 25 μ m. (B) Quantitation of the number of extracellular neuromelanin deposits in the substantia nigra. MPTP-lesioning alone significantly increased the number of extracellular neuromelanin deposits. These were significantly reduced with nicotine treatment. Significance of difference from Saline-Control; *** p <0.001; from MPTP-Control; δ p <0.05. Values represent the mean $\pm$ SEM of 6 to 7 monkeys.

Table 1

MPTP treatment decreases dopamine transporter levels in monkey substantia nigra

Group	Treatment	Number of monkeys	Dopamine transporter (fmol/mg tissue)
Control	Saccharin	7	16.4 ± 1.02
	Nicotine	6	17.9 ± 1.85
MPTP-lesioned	Saccharin	7	12.1 ± 1.50
	Nicotine	6	13.3 ± 1.01

Monkeys were given nicotine in the drinking water and subsequently lesioned by subcutaneous injection of MPTP, with the nicotine treatment continued until the animals were euthanized. Nigral dopamine transporter autoradiography was then performed using ^{125}I -RTI-121 as described in Methods. There was a significant main effect of MPTP-lesioning ($p < 0.01$), with no main effect of nicotine treatment using two-way ANOVA.