British Journal of Pharmacology (2001) 132, 1650–1656

www.nature.com/bjp

Dose-related neuroprotective effects of chronic nicotine in 6-hydroxydopamine treated rats, and loss of neuroprotection in α4 nicotinic receptor subunit knockout mice

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1 The present study examined the effect of a range of doses of chronic nicotine (0.75, 1.5, 3.0 and 30.0 mg kg^{-1} day^{-1}, s.c., 14 days) upon striatal dopaminergic nerve terminal survival following 6-hydroxydopamine (6-OHDA; 10 μg intrastriatal unilaterally) in rats; and the effects of acute nicotine (1 mg kg^{-1}, s.c.) pretreatment upon striatal neurodegeneration induced by methamphetamine (5 mg kg^{-1}, i.p., three doses at 2 h intervals) in wild-type and α4 nicotinic receptor (nAChR) subunit knockout mice.

2 In both models of Parkinsonian-like damage, loss of striatal dopaminergic nerve terminals was assessed by [^{3}H]-mazindol autoradiography.

3 In rats, chronic nicotine infusion delivered by osmotic minipump implanted subcutaneously 7 days prior to intrastriatal 6-OHDA injection produced significant and dose-related protection against 6-OHDA-induced neurodegeneration. Low (0.75 and 1.5 mg kg^{-1} day^{-1}) but not high (3.0 and 30.0 mg kg^{-1} day^{-1}) nicotine doses significantly inhibited 6-OHDA-induced degeneration.

4 In wild-type mice, acute nicotine treatment produced significant inhibition of methamphetamine-induced neurodegeneration. In α4 nAChR subunit knockout mice, acute nicotine treatment failed to inhibit methamphetamine-induced neurodegeneration.

5 Nicotine is capable of protecting dopaminergic neurons against Parkinsonian-like neurodegeneration *in vivo*. In rats, this neuroprotective effect is critically dependent upon nicotine dose and is consistent with the activation of nAChRs, as high, desensitizing doses of nicotine fail to be neuroprotective. Further, neuroprotection is absent in α4 nAChR subunit knockout mice. The current results therefore suggest that activation of α4 subunit containing nAChRs constitutes a major component of the neuroprotective effect of nicotine upon Parkinsonian-like damage *in vivo*. *British Journal of Pharmacology* (2001) **132,** 1650–1656

Keywords: Nicotine; nicotinic receptor; Parkinson's disease; α4 nicotinic receptor subunit; neurodegeneration; knockout; 6-hydroxydopamine; methamphetamine

Abbreviations: DMI, desmethylimipramine; MPTP, 1-methyl-4-phenyl-2,3,6-tetrahydropyridine; nAChR, neuronal nicotinic receptor; PD, Parkinson's disease; 6-OHDA, 6-hydroxydopamine; SNpc, substantia nigra pars compacta

Introduction

Parkinson's disease (PD) is an age-related neurodegenerative disorder associated with the progressive and persistent degeneration of the dopaminergic nigrostriatal pathway (Levy *et al*., 1997; Olanow & Tatton, 1999). Recent epidemiological studies have documented an approximate halving of the incidence of PD in cigarette smokers relative to non-smokers (Baron, 1986; Morens *et al*., 1995). This neuroprotective effect is likely to be due to nicotine, as subsequent *in vitro* studies demonstrated that pretreatment with nicotine and other nicotinic receptor (nAChR) agonists dose-dependently attenuated dexamethasone-potentiated kainic acid neurotoxicity in primary hippocampal cultures (Semba *et al*., 1996), and glutamate receptor-mediated excitotoxicity in both primary cortical (Akaike *et al*., 1994; Kaneko *et al*., 1997) and striatal cultures (Donnelly-Roberts *et al*., 1996). Furthermore, the neuroprotective effects of nAChR agonists *in vitro* are attenuated by non-selective nicotinic antagonists such as mecamylamine, demonstrating that nAChR activation is a step essential to the neuroprotective process (Akaike *et al*., 1994; Donnelly-Roberts *et al*., 1996; Semba *et al*., 1996; Kaneko *et al*., 1997; Kihara *et al*., 1997).

Several *in vivo* studies that have investigated the neuroprotective effects of nicotine upon nigrostriatal degeneration in animal models of Parkinsonism have reported equivocal results. Chronic nicotine infusion at a dose rate of 3.0 mg kg^{-1} day^{-1}, in combination with acute nicotine injections, significantly inhibited the dopaminergic neuron loss that follows 1-methyl-4-phenyl-2,3,6-tetrahydropyridine (MPTP)-induced lesions of the nigrostriatal system (Janson *et al*., 1988b); as well as the more progressive loss of nigrostriatal neurons that follows partial mesodiencephalic hemitransection (Janson *et al*., 1988a; 1991; 1994; Fuxe *et al*., 1990; Janson & Moller, 1993). Chronic nicotine infusion

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however failed to attenuate 6-hydroxydopamine (6-OHDA)-induced nigrostriatal lesions (Blum *et al*., 1996), and actually enhanced MPTP-induced damage when given chronically either intermittently or *via* infusion (Janson *et al*., 1992; Hadjiconstantinou *et al*., 1994; Ferger *et al*., 1998).

The nAChR is a ligand-gated ion channel composed of five subunits assembled around a central cationic pore. Several nAChR subunits have been identified to date ($\alpha2-\alpha7$ and $\beta2-\beta4$), and while these subunits show a wide and heterogeneous distribution within the mammalian brain (for review, see Wonnacott, 1997; Changeux *et al*., 1998), two major subtypes appear to predominate. The heteromeric $\alpha4/\beta2$ receptor accounts for approximately 90% of high-affinity [^{3}H]-nicotine binding sites centrally, whereas the homomeric $\alpha7$ receptor forms a high-affinity binding site for [^{125}I]-α-bungarotoxin (for review, see Clarke, 1998). The nigrostriatal system however, which is specifically damaged in PD, expresses a diverse array of nAChR subunits (Elliott *et al*., 1998) including, but not restricted to, $\alpha4/\beta2$ and $\alpha7$ nAChR subtypes. Further, nicotinic modulation of striatal dopamine release is consistent with a heterogeneous nAChR population on dopaminergic nigrostriatal neurons, although the nAChR subtypes present have not been identified with certainty (see Reuben *et al*., 2000; Sharples *et al*., 2000).

Attempts to identify the nAChR mediating neuroprotection *in vitro* by blockade with selective nAChR antagonists has implicated both major nAChR subtypes: both dihydro-β-erythroidine and α-bungarotoxin, antagonists selective for $\alpha4/\beta2$ and $\alpha7$ nAChRs respectively, block the neuroprotective effects of nicotine in cortical cultures (Donnelly-Roberts *et al*., 1996; Kaneko *et al*., 1997; Kihara *et al*., 1997). The identity of the nAChR subtype(s) mediating neuroprotection *in vivo* is unknown.

Given the epidemiological data suggesting that nicotine may play an important role in the progression of human PD, coupled with the equivocal data obtained from previous *in vitro* and *in vivo* studies, the aims of the current study were 2 fold. Firstly, we investigated the effect of a range of nicotine doses, chronically administered, upon nigrostriatal system survival against 6-OHDA-induced damage in the rat. Secondly, the current study sought to identify the potential nAChR subtype(s) involved in neuroprotection *in vivo* by examining the effect of deletion of the $\alpha4$ nAChR subunit gene against methamphetamine-induced damage in the mouse. The results of the current study are consistent with activation of $\alpha4$ subunit-containing nAChRs as a necessary and major component of the neuroprotective effect of nicotine upon Parkinsonian-like damage *in vivo*.

Methods

Chronic nicotine treatment experiments

Animals and drug treatment Female Sprague-Dawley rats (180–220 g) were housed under controlled conditions with free access to food and water. All procedures were conducted in accordance with guidelines set out by the Monash University Animal Ethics Committee.

Alzet osmotic minipumps were pre-weighed, filled with saline or (−)-nicotine di-*d*-tartrate in saline and re-weighed to check for complete filling prior to implantation. Solutions were prepared so as to provide final delivery *via* minipump of 0, 0.75, 1.5, 3.0 or 30.0 mg kg^{-1} day^{-1} nicotine in saline for a total treatment period of 14 days. Rats were anaesthetized (1 midazolam, 10 mg kg^{-1} : 1 fentanyl, 1.0 mg kg^{-1} and fluanisone, 20 mg kg^{-1} : 1 sterile water) (i.p.), and an incision made in the back of the neck. A hollow was made beneath the skin in the interscapular space, and the minipump inserted into this hollow. The incision was then closed and the animal allowed to recover.

Stereotaxic injection of 6-OHDA Seven days after the implantation of osmotic minipumps, animals underwent stereotaxic surgery to produce lesions of the nigrostriatal system. Within each experimental group, half of the animals received an intrastriatal injection of 6-OHDA with 0.01% ascorbic acid in saline (lesioned animals) while the remaining animals received an intrastriatal injection of 0.01% ascorbic acid in saline (control animals). The surgical procedure for unilateral intrastriatal injection of 6-OHDA is essentially as described by Przedborski and co-workers (1995). Briefly, rats were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and placed in a Kopf stereotaxic apparatus, where the head was constrained to a tilted skull position (−3.0 mm). An incision was made on the midline of the scalp and a burr hole drilled through the skull at the appropriate coordinates. Through this, an intracerebral injection was delivered into the left striatum using a 30 gauge blunt-tipped cannula. Stereotaxic coordinates for injection were: 0.3 mm anterior and 3.0 mm lateral from Bregma, and 5.2 mm ventral from the cortical surface, according to the atlas of Paxinos & Watson (1986). Lesioned rats received 4 μl of 2.5 μg μl^{-1} 6-OHDA/0.01% (w v^{-1}) ascorbic acid, while control rats received 4 μl of saline/0.01% (w v^{-1}) ascorbic acid. Injections were delivered at a rate of 0.6 μl min^{-1} and the needle left in position for 10 min following injection before being withdrawn slowly. After sealing the skull, the incision was closed and the animals allowed to recover.

Acute nicotine treatment experiments

Preparation of $\alpha4$ subunit knockout mice and drug treatments Knockout mice lacking the $\alpha4$ nAChR subunit were generated by homologous recombination following partial deletion of the coding sequence contained in exon V; full genotypic and phenotypic characterization was carried out, as described by Ross *et al*., (2000).

All procedures were carried out in accordance with guidelines approved by the Monash University Animal Ethics Committee. Mice were housed under controlled conditions with free access to food and water. Knockout mice and wild-type littermates (20–30 g) were treated according to the methods of Maggio *et al*. (1997; 1998). Briefly, mice received a series of three injections of methamphetamine (5 mg kg^{-1}, i.p.) at 2 h intervals. Nicotine treated mice received an injection of nicotine (1 mg kg^{-1}, s.c.) 30 min prior to each injection of methamphetamine. Control mice received no treatment.

Preparation of tissues

Seven days after stereotaxic surgery or acute drug treatment, rats and mice respectively were lightly anaesthetized (CO_2/O_2:

80/20) and decapitated. Brains were rapidly removed and frozen over liquid nitrogen, then stored at −40°C prior to sectioning.

A Reichert Jung cryostat was used to cut consecutive coronal 14 μm sections of striatum at level +0.30 mm from Bregma according to the atlas of Paxinos & Watson (1986). Sections were thaw mounted onto poly-L-lysine coated slides, then stored at −20°C until use.

[^{3}H]-mazindol autoradiography

[^{3}H]-mazindol autoradiography was used to visualize dopaminergic nerve terminals within sections of striatum taken from both rat (chronic treatment) and mouse (acute treatment) brains. All autoradiographic steps were carried out at 4°C to reduce non-specific binding.

Slide mounted sections of striatum were preincubated for 15 min in 50 mM Tris-HCl solution (pH 7.9) containing 120 mM NaCl and 5 mM KCl. Sections were then incubated for 60 min with 4 nM [^{3}H]-mazindol in 50 mM Tris-HCl solution (pH 7.9) containing 300 mM NaCl and 5 mM KCl. Desipramine (DMI; 300 nM) was included in all incubation solutions to prevent non-selective [^{3}H]-mazindol binding at noradrenergic uptake sites. Nomifensine (100 μM), a selective inhibitor of dopamine uptake sites, was used to determine non-specific binding. Sections were washed twice (2 × 3 min) in ice-cold incubation buffer to remove excess [^{3}H]-mazindol and dried under a stream of cold, dry air.

Once dry, radiolabelled sections were apposed to Hyperfilm-^{3}H and exposed for 21 days to allow an image of striatal dopaminergic nerve terminal density to develop on the film. Following the exposure period, films were developed for 5 min in Phenisol X-ray developer, rinsed briefly in a weak solution of stopbath and fixed in Hypam X-ray fixer for 10 min.

Analysis of autoradiograms

Computer-assisted densitometry (MCID system, St. Catherine's, Ontario, Canada) was used to quantify the optical density of film images. The system was calibrated using [^{3}H]-standards, so that optical density measurements were made in nCi mm^{-2}. Specific binding was determined by subtracting the non-specific binding image from that of total binding, and was measured in the entire striatum.

Data analysis

The mean optical density and standard error of the mean were determined from independent measurements taken in at least three consecutive coronal sections of striatum for each animal. For chronic treatment studies, six to eighteen animals were contained within each treatment group; for acute experiments, three to eight animals were contained within each treatment group. One-way analysis of variance with a Dunnett's *post-hoc* test, was used for comparisons within treatment groups. In all cases, probability levels of $P<0.05$ were considered statistically significant.

Materials

Alzet osmotic minipumps (model 2002, Alza Corp., U.S.A.); 6-OHDA (RBI); (−)-nicotine di-*d*-tartrate (RBI); nomifensine maleate (RBI); desipramine (RBI); [^{3}H]-mazindol (24 Ci mmol^{-1}; DuPont); Phenisol X-ray developer and Hypam X-ray fixer (Ilford, Australia); Hyperfilm-^{3}H and [^{3}H]-standards (Amersham, Australia).

Results

Chronic treatment experiments

Effect of 6-OHDA lesion upon nigrostriatal dopaminergic nerve terminals Intrastriatal 6-OHDA injection caused a marked and significant loss of dopaminergic nerve terminals, as assessed by specific [^{3}H]-mazindol autoradiography, within the 6-OHDA-injected striatum relative to vehicle-injected controls. This loss accounted for approximately 65% of dopaminergic nerve terminals (see Figure 1). No significant loss of striatal dopaminergic terminals was detected following an intrastriatal injection of vehicle (data not shown). Although a loss of approximately 25% of [^{3}H]-mazindol binding was detected contralateral to the 6-OHDA lesion (specific [^{3}H]-mazindol binding = 1.20 ± 0.07 nCi mm^{-2}) when compared with the contralateral striatum of vehicle-injected controls (specific [^{3}H]-mazindol binding = 0.91 ± 0.12 nCi mm^{-2}), this difference was not statistically significant (unpaired *t*-test, n.s.). Regardless, all comparisons in the neuroprotection studies are only made between ipsilateral injected striata.

Effect of chronic nicotine upon 6-OHDA-induced loss of dopaminergic nerve terminals Chronic nicotine treatment caused a dose-related, neuroprotective effect upon 6-OHDA-induced loss of striatal dopaminergic nerve terminals, as assessed by [^{3}H]-mazindol autoradiography (see Figure 1). In animals treated with either of the lower doses of nicotine (0.75 and 1.5 mg kg^{-1} day^{-1}) and subsequently injected with

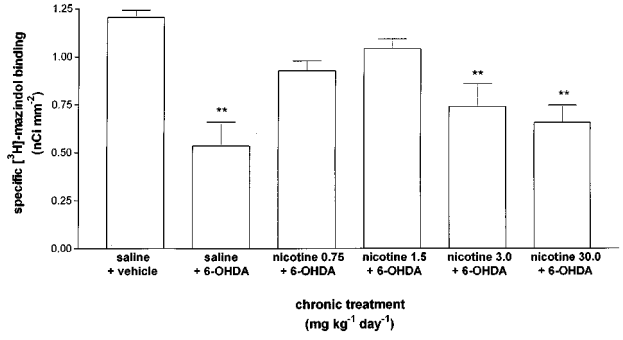

Figure 1 Levels of dopaminergic nerve terminals, measured as density of specific [^{3}H]-mazindol binding (nCi mm^{-2}) within the striatum of chronically treated rats. Each column represents the mean and s.e.mean of averaged triplicates or quadruplicates of the density of specific [^{3}H]-mazindol binding measured in the injected striatum of 3–9 rats. Differences in dopaminergic nerve terminal survival were assessed in striata taken from animals treated chronically with saline and injected with vehicle (saline + vehicle), *versus* those taken from animals treated chronically with saline or nicotine 0.75, 1.5, 3.0 and 30.0 mg kg^{-1} day^{-1}, and injected with 6-OHDA. One-way ANOVA with a Dunnett's *post-hoc* test for multiple comparisons with a control (saline + vehicle) was used to statistically assess differences in the survival of striatal dopaminergic nerve terminals, where $P<0.05$ was considered statistically significant. *($P<0.05$), **($P<0.01$).

6-OHDA, striatal [^{3}H]-mazindol binding levels were not significantly different from those measured in control animals treated with saline and subsequently injected with vehicle. For both of these low nicotine doses, striatal terminal survival was maintained at greater than 75% of control levels. In animals treated with either of the higher nicotine doses (3.0 and 30.0 mg kg^{-1} day^{-1}) however, a significant loss of terminals was still associated with 6-OHDA-injected striata. For all treatment groups, there was no systematic effect of chronic nicotine treatment upon [^{3}H]-mazindol binding levels, as measured in the contralateral striatum of animals injected intrastriatally with vehicle (specific [^{3}H]-mazindol binding (nCi mm^{-2}): saline = 1.20 ± 0.07; nicotine 0.75 mg kg^{-1} day^{-1} = 1.17 ± 0.02; nicotine 1.5 mg kg^{-1} day^{-1} = 1.22 ± 0.11; nicotine 3.0 mg kg^{-1} day^{-1} = 1.07 ± 0.09; nicotine 30.0 mg kg^{-1} day^{-1} = 0.99 ± 0.07; one-way ANOVA, n.s.).

Acute nicotine experiments

Effect of methamphetamine upon nigrostriatal dopaminergic nerve terminals Methamphetamine treatment caused a marked and significant loss of dopaminergic nerve terminals, assessed by specific [^{3}H]-mazindol autoradiography, within the striatum of both wild-type and knockout mice (see Figure 2). This loss of dopaminergic terminals accounted for approximately 50% of terminals in wild-type mice, and approximately 40% of terminals in knockout mice, relative to respective control animals.

Effect of acute nicotine upon methamphetamine-induced loss of dopaminergic nerve terminals Acute nicotine treatment significantly protected against methamphetamine-induced loss of striatal dopaminergic nerve terminals in wild-type mice but not in α4 nAChR subunit knockout mice (see Figure 2). In wild-type mice, nicotine treatment maintained dopaminergic nerve terminal levels at greater than 80% of control levels.

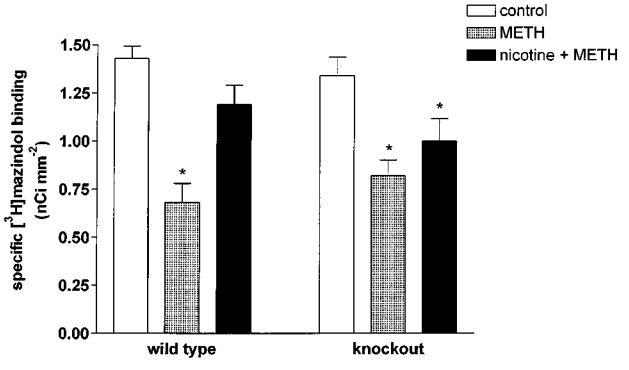

Figure 2 Levels of dopaminergic nerve terminals, measured as density of specific [^{3}H]-mazindol binding (nCi mm^{-2}) within the striatum of wild-type and α4 nAChR subunit knockout mice treated with methamphetamine (5 mg kg^{-1}; three injections i.p.; METH) and nicotine (1 mg kg^{-1}; s.c.) plus methamphetamine (nicotine + METH). Each column represents the mean and s.e.mean of averaged triplicates or quadruplicates of the density of specific [^{3}H]-mazindol binding measured in striata taken from 3–8 mice. Differences in dopaminergic nerve terminal survival were assessed by one-way ANOVA with a Dunnett's *post-hoc* test for multiple comparisons with a control, in wild-type and knockout mice, respectively, where $P < 0.05$ was considered statistically significant. * ($P < 0.05$).

Nicotine treatment, however, failed to significantly attenuate methamphetamine-induced loss of dopaminergic terminals in knockout mice. Although an increase in [^{3}H]-mazindol binding levels (approximately 15% relative to control animals) was detected in striata of knockout mice treated with both nicotine and methamphetamine, this increase was not statistically significant, and levels remained significantly below those measured in control animals.

Discussion

Nicotine has been investigated previously as a neuroprotective agent both *in vitro* and *in vivo*. The current study demonstrates that both chronic and acute nicotine treatment protect against 6-OHDA and methamphetamine-induced neurodegeneration *in vivo*, in rats and mice, respectively. The neuroprotective effect of chronic nicotine *in vivo* was critically dependent upon the dose administered, where low (0.75 and 1.5 mg kg^{-1} day^{-1}), but not high (3.0 and 30.0 mg kg^{-1} day^{-1}) doses protected against 6-OHDA-induced nigrostriatal degeneration. These results are consistent with the possibility that it is the activation of nAChRs, rather than receptor desensitization, that mediates the neuroprotective effect of nicotine *in vivo*. An active role for nAChRs in neuroprotection is further supported by the loss of neuroprotection observed in α4 nAChR subunit knockout mice, demonstrating that nAChRs containing the α4 subunit contribute strongly to protection of dopaminergic neurons by nicotine *in vivo*.

Intrastriatally injected 6-OHDA is thought to damage nigrostriatal neurons by promoting the production of hydroxyl radicals: this rapidly destroys the nerve terminal (Gerlach & Riederer, 1996; Lotharius *et al*., 1999), and produces a gradual retrograde loss of cell bodies in the substantia nigra pars compacta (SNpc). Such damage mimics the progressive and incomplete nigrostriatal degeneration that characterizes early human PD (Berger *et al*., 1991; Sauer & Oertel, 1994; Przedborski *et al*., 1995; Lee *et al*., 1996). In comparison, excitatory amino acids have been directly implicated in the mechanism of methamphetamine-induced nigrostriatal toxicity (Sonsalla *et al*., 1989; 1991).

Both 6-OHDA and methamphetamine treatments produced significant nigrostriatal damage, where striatal dopaminergic nerve terminal survival was used as a measure of nigrostriatal damage, and was assessed by [^{3}H]-mazindol autoradiography. This method has been shown previously to be a useful tool for quantifying striatal dopaminergic populations, where DMI-insensitive [^{3}H]-mazindol binding sites represent dopamine uptake sites (Javitch *et al*., 1984; 1985). Further, the loss of [^{3}H]-mazindol binding following intrastriatal 6-OHDA correlates well with cell body loss from the SNpc (Przedborski *et al*., 1995; Lee *et al*., 1996), and with the degree of striatal dopamine depletion following both 6-OHDA (Ryan & Loiacono, unpublished observations) and methamphetamine treatments (Maggio *et al*., 1997; 1998).

The effect of nicotine upon nigrostriatal neuron survival was investigated at four dose levels: 0.75, 1.5, 3.0 and 30.0 mg kg^{-1} day^{-1}. Doses of 0.75, 1.5 and 3.0 mg kg^{-1} day^{-1} are likely to achieve brain nicotine concentrations of 0.2 to at least 0.5 μM (Rowell & Li, 1997). This range approximates the EC_{50} reported for nicotine-evoked dopamine release in striatal

synaptosomes and slices (0.16–0.5 μM) (Grady *et al*., 1992; 1994; Whiteaker *et al*., 1995; Clarke & Reuben, 1996; Wonnacott, 1997), and falls within the range that is maximally protective against neurotoxic damage *in vitro* (approximately 0.1–5 μM) (Donnelly-Roberts *et al*., 1996; Semba *et al*., 1996). The dose of 3.0 mg kg^{-1} day^{-1} was of particular interest, as it is widely cited as a dose that reflects plasma nicotine concentrations in smokers, where smokers demonstrate a diminished incidence of PD (Baron, 1986; Morens *et al*., 1995).

The four nicotine doses used by the current study fall into two ranges; a low range ($\leqslant$1.5 mg kg^{-1} day^{-1}) and a high range ($\geqslant$3.0 mg kg^{-1} day^{-1}). Doses of approximately 2.4 mg kg^{-1} day^{-1} and higher have been shown to upregulate nAChRs centrally (Rowell & Li, 1997): this is more than likely to occur as a result of desensitization block (see Wonnacott, 1990). One recent functional study for example showed that while nicotine infused at a dose of 4.0 mg kg^{-1} day^{-1} desensitized nicotine-stimulated striatal dopamine release, a lower dose of 1.0 mg kg^{-1} day^{-1} showed no such desensitization of function (Benwell & Balfour, 1997). It should be noted however that an enhancement of function has also been associated with nAChR upregulation *in vivo* (see Marshall *et al*., 1997).

Evidence from *in vitro* studies strongly implicates nAChR activation as an essential step in neuroprotection, as neuroprotection is blocked by nAChR antagonists (Akaike *et al*., 1994; Donnelly-Roberts *et al*., 1996; Semba *et al*., 1996; Kaneko *et al*., 1997; Kihara *et al*., 1997). Data from the present study is consistent with the possibility that it is the activation of nAChRs, rather than desensitization, that underlies the neuroprotective effect of nicotine *in vivo*. While low nicotine doses ($\leqslant$1.5 mg kg^{-1} day^{-1}) significantly protected striatal dopaminergic terminals, higher nicotine doses ($\geqslant$3.0 mg kg^{-1} day^{-1}) failed to significantly attenuate 6-OHDA-induced damage. Consistent with the current results, a prior study that infused nicotine at a dose of 3.0 mg kg^{-1} day^{-1} failed to find any effect of this treatment upon 6-OHDA-induced neurodegeneration (Blum *et al*., 1996). The results of the current study are in contrast with those of several prior studies examining the effect of nicotine treatment upon Parkinsonian-like damage *in vivo*. Such studies have suggested that it is a desensitization of nAChRs on nigrostriatal neurons that underlies neuroprotection by nicotine, resulting in a decrease in the firing of nigrostriatal neurons (Grenhoff *et al*., 1991), and a decrease in nigrostriatal dopamine (Fuxe *et al*., 1990) and glucose (Owman *et al*., 1989) utilization. Importantly, these prior studies used nicotine at a dose rate of 3.0 mg kg^{-1} day^{-1}: this dose was not significantly protective in the current study. As nAChR regulation and function *in vivo* are affected not only by nicotine dose but also the duration (acute *versus* chronic) and method (continuous *versus* intermittent) of treatment (see Marshall *et al*., 1997; Rowell & Li, 1997), these factors may potentially influence the neuroprotective capacity of nicotine *in vivo*.

We sought to resolve whether it is the activation or desensitization of nAChRs that underlies neuroprotection *in vivo*, by examining the neuroprotective capacity of nicotine in a nAChR knockout mouse model. We chose to examine the α4 nAChR subunit knockout mouse, as the α4/β2 nAChR is the most common within the mammalian brain. While acute nicotine attenuated methamphetamine-induced nigrostriatal damage in wild-type mice, no significant neuroprotection was detected in mice lacking the α4 subunit. A contribution from α4-containing nAChRs therefore appears to be essential if a significant degree of neuroprotection is to be achieved: this implies that the activation of nAChRs, and more specifically, α4-containing nAChRs, contribute significantly to the neuroprotective process *in vivo*. The current results do not rule out the involvement of additional nAChR subtypes in neuroprotection *in vivo*: although failing to reach statistical significance, a trend towards increased dopaminergic neuron survival following nicotine treatment was observed in α4 knockout mice. This effect may be due to the involvement of additional nAChR subtype(s) *in vivo*: the expression of multiple nAChR subunits and modulation of dopaminergic function by a heterogeneous nAChR population within nigrostriatal neurons (see Reuben *et al*., 2000; Sharples *et al*., 2000) adds strength to this possibility. Further, the wide distribution of nAChRs, particularly α4 subunit-containing nAChRs, implies that the effect of systemically administered nicotine need not be restricted to direct actions upon the nigrostriatal system, but may involve nAChRs at several sites within the brain.

Conclusion

The results of the current study are consistent with the view that nAChR activation mediates the neuroprotective effects of nicotine *in vivo*: firstly because low, potentially activating nicotine doses are neuroprotective whereas higher, potentially desensitizing nicotine doses fail to significantly protect nigrostriatal neurons; and secondly, because neuroprotection is abolished in α4 nAChR subunit knockout mice. Taken together, these results suggest that the activation of α4 subunit-containing nAChRs comprises a major component of the neuroprotective effect of nicotine upon Parkinsonian-like damage *in vivo*.

This research was supported by a grant from the Smoking and Health Research Foundation of Australia (R.E. Loiacono), and by a grant from the Australian National Health and Medical Research Council (J. Drago). R. Ryan is supported by an Australian Postgraduate Award. J. Drago is a Logan Research Fellow at Monash University. Part of the work contained in this manuscript was performed in the Department of Pharmacology at the University of Melbourne. The authors are grateful to Professor R.J. Summers for critical reading of the manuscript. This study is not intended to promote cigarette smoking, and has not been supported by any tobacco company.

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(Received June 26, 2000
Revised December 18, 2000
Accepted January 29, 2001)