Published in final edited form as:

Neurotoxicology. 2008 May ; 29(3): 421–427.

CIGARETTE SMOKE, NICOTINE AND COTININE PROTECT AGAINST 6-HYDROXYDOPAMINE-INDUCED TOXICITY IN SH-SY5Y CELLS

Karen Riveles, Luping Z. Huang, and Maryka Quik*

The Parkinson's Institute, Sunnyvale, CA 94085

Abstract

Epidemiological studies consistently demonstrate a reduced incidence of Parkinson's disease in smokers. As an approach to evaluate whether nicotine in tobacco may be involved in this apparent protective effect, we compared the effect of mainstream 1R4F cigarette smoke solutions, which contain chemicals inhaled by active smokers, and nicotine against 6-hydroxydopamine (6-OHDA)-induced toxicity in an *in vitro* cell culture system. For this purpose we used terminally differentiated SH-SY5Y neuroblastoma cells that exhibit a catecholaminergic phenotype and express nicotinic receptors. Cells were pre-incubated for 24 h in mainstream-cigarette smoke solutions (0.06, 0.2, or 0.6 cigarette puffs/ml) made from University of Kentucky 1R4F research brand cigarettes, followed by the addition of 6-OHDA for another 24–48 h. The 0.2, but not 0.06, puffs/ml dose, significantly protected against 6-OHDA-induced toxicity in SH-SY5Y cells. This dose yielded final nicotine concentrations of $\sim 5 \times 10^{-7}$ M, which is similar to plasma smoking levels. Although the 0.6 puffs/ml dose caused significant toxicity on its own, it also appeared to protect against 6-OHDA-induced damage. We next tested the effect of nicotine, as well as its metabolite cotinine. These agents protected against the toxic effects of 6-OHDA in SH-SY5Y cells at concentrations ranging from 10^{-7} to 10^{-5} M. These combined results support the idea that nicotine is one of the components in cigarette smoke that has a protective effect against neurotoxic insults. These data suggest that nicotine may be of potential therapeutic value for Parkinson's disease.

Keywords

Neuroprotection; Cigarette smoke; Nicotine; Cotinine; 6-Hydroxydopamine; Parkinson's disease

1. Introduction

Numerous epidemiological studies over the last 50 years have consistently reported that smoking is associated with a reduced incidence of Parkinson's disease, suggesting that some smoke component(s) may have a protective effect against this debilitating neurological movement disorder (Morens et al., 1995; Hernan et al., 2002; Allam et al., 2004; Gorell et al., 2004; Ritz et al., 2007; Thacker et al., 2007). Accumulating studies indicate this appears to be due to a true biological protective effect of smoke because the reduced incidence of Parkinson's

*Corresponding author at: The Parkinson's Institute, 675 Almanor Ave, Sunnyvale, CA 94085. Tel: 408 542 5601; fax: 408 734 8455. E-mail address: mquik@parkinsonsinstitute.org (M. Quik).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

disease is correlated with increased smoking duration and intensity, is more pronounced in current than former smokers, is observed in large prospective cohort studies and is observed in monozygotic twins discordant for Parkinson's disease. A critical question is the molecular basis for this apparent protection because identification of the active component(s) may provide a novel therapeutic approach to Parkinson's disease.

One major tobacco constituent for which there is compelling experimental data is nicotine, with support derived from both *in vitro* and *in vivo* models. For instance, nicotine reduces toxicity induced by glutamate, ischemia damage, ethanol, and growth factor deprivation in a variety of different cultured cell systems (Belluardo et al., 2000; Jeyarasasingam et al., 2002; O'Neill et al., 2002; Copeland et al., 2005; Tizabi et al., 2005; Copeland et al., 2007; Quik et al., 2007). Of more immediate relevance to the current study, nicotine pretreatment reduces toxin-induced dopaminergic loss in animal models of nigrostriatal damage. Positive effects are observed across species including rats (Janson et al., 1988; Costa et al., 2001; Ryan et al., 2001; Soto-Otero et al., 2002; Visanji et al., 2006), nonhuman primates (Quik et al., 2006a; Quik et al., 2006b), and mice, although results in this latter model appear somewhat less consistent (O'Neill et al., 2002; Quik et al., 2007).

In vivo, nicotine is rapidly degraded by cytochrome P450 to cotinine (Benowitz and Jacob, 1994; Hukkanen et al., 2005; Matta et al., 2007). This metabolite is of interest because it has a relatively long half-life (~17 h) compared to nicotine (1–2 h), most likely because of its slow metabolism. This results in plasma cotinine levels 5–10 fold greater than those of nicotine. Work to investigate the potential biological significance of this nicotine metabolite has shown that exposure of a pheochromocytoma cell line (PC12 cells) to cotinine led to a significant enhancement in cell viability (~80%), at concentrations in the high nM range (Buccafusco and Terry, 2003). Thus, the protective effects of smoke against toxicity may potentially be due to nicotine and/or its long-lasting metabolite cotinine.

The purpose of this study was to compare the effects of mainstream whole cigarette smoke solutions, nicotine and cotinine to better understand their role in protection against 6-OHDA-induced toxicity. For these studies we used an *in vitro* cell culture system consisting of differentiated SH-SY5Y cells, a cell line of CNS origin expressing both a catecholaminergic phenotype and nicotinic receptors. The results suggest that the nicotine and/or cotinine contribute, at least in part, to the beneficial effects of smoke against dopaminergic cell damage.

2. Materials and Methods

2.1 Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from VWR (West Chester, PA); TrypLE Express, penicillin/streptomycin, and GlutaMax-1 supplement from Invitrogen (Carlsbad, CA); fetal bovine serum from Hyclone (Logan, UT); normal goat serum and Vectastain ABC kit from Vector Laboratories (Burlingame, CA); collagen type 1 from rat tail, Hank's balanced salt solution (HBSS) without calcium or magnesium, nicotine base, cotinine, retinoic acid, phorbol-12-myristate 13-acetate (TPA) and 6-hydroxydopamine chloride (6-OHDA) from Sigma-Aldrich (St. Louis, MO); rabbit polyclonal anti-tyrosine hydroxylase (TH) antibody from Pel-Freez Biologicals (Rogers, AK); and the DAB-metal enhanced substrate kit from Pierce (Milwaukee, WI). All other chemicals were purchased from standard commercial sources. The SH-SY5Y neuroblastoma cells were a gift from Dr. Seung-Jae Lee at The Parkinson's Institute.

2.2. Preparation and treatment of cell cultures

The SH-SY5Y neuroblastoma cell line was grown at 37°C and 5% CO₂ in DMEM-Hi glucose (4,000 mg/L) supplemented with 10% fetal bovine serum, 1% streptomycin/penicillin, and 2 mM Glutamax I supplement. Cells were subcultured every 48 h in 100 mm² dishes. The cell cultures prepared for immunocytochemistry were seeded in 48-well culture plates pre-coated with 8 µg/cm² collagen at a density of 45,000 cells/cm² in DMEM. This density was chosen because individual cells could be distinguished for counting using immunocytochemistry. The SH-SY5Y cells were terminally differentiated in DMEM containing retinoic acid (50 µM) for five days and TPA (50 nM) for the next two days (Joyce et al., 2003; Presgraves et al., 2004a). SH-SY5Y cells were pre-incubated in nicotine (10⁻⁷-10⁻⁵ M), cotinine (10⁻⁶-10⁻⁸ M) or mainstream smoke solution (0.06-0.6 puffs/ml), dissolved in DMEM for 24 h. This was followed by addition of 6-OHDA (60 or 100 µM) for 24–48 h, during which time the nicotine, cotinine and mainstream smoke solution exposure was continued.

2.3. Main stream smoke preparation

The University of Kentucky 1R4F research cigarettes were purchased from the Kentucky Tobacco Research and Development Center at the University of Kentucky (Lexington, KY). Mainstream smoke solutions, which contain chemicals inhaled by active smokers, were prepared in the laboratory of Dr. P. Talbot, University of California Riverside, using a University of Kentucky analytical smoking machine, as previously described (Riveles et al., 2007). The set-up included a puffer box and a peristaltic pump, which pumped the smoke into a 10 ml solution of low glucose DMEM. Mainstream smoke solutions were made from six University of Kentucky 1R4F research cigarettes with 10 standard puffs per cigarette for a total of 60 puffs of smoke pushed through 10 ml of DMEM using a peristaltic pump. This concentration of 60 puffs through 10 ml is equivalent to 6 puffs/ml. The doses of smoke solution used in the culture yielded final concentration of 0.06, 0.2, and 0.6 puffs/ml, with the 0.06 puffs/ml dose providing a nicotine concentration of $\sim 5 \times 10^{-7}$ M, which is similar to plasma smoking levels (Hukkanen et al., 2005; Matta et al., 2007).

2.4. Immunocytochemistry

SH-SY5Y cells were fixed in 48 well culture plates using 4% paraformaldehyde for 40 min at 4°C and subsequently washed in Hank's Balanced Salt Solution (HBSS) supplemented with magnesium. Cells were labeled with a rabbit polyclonal anti-tyrosine hydroxylase antibody as described with minor modifications (Jeyarasasingam et al., 2002). In brief, fixed cultures were incubated in HBSS with 4% normal goat serum, 1% bovine serum albumin, 0.1% Triton X-100 for 1 h at 22°C. This was followed by incubation with a 1:600 dilution of TH antibody in HBSS with 1% bovine serum albumin and 0.1% Triton X-100 overnight at 4°C. The cells were then washed in HBSS and incubated in biotinylated goat anti-rabbit secondary in HBSS for 1 h at 22°C. After washing, the cells were incubated in Vectastain ABC Elite kit for 1 h at 22°C. The cells were then washed and incubated in 0.03% diaminobenzidine with 0.003% H₂O₂ for 5 min for visualization of peroxidase.

2.5. TH⁺ Cell Counts

SH-SY5Y cell were counted with a light microscope at 200x magnification using a micrometer grid in the ocular lens. The grid was placed as close to the center of the well as possible. The number of TH positive cells inside the micrometer grid was counted in every 4th field along the diameter of the well for a total of 5 fields per measurement. The average value per field was multiplied by 20 to obtain the total number of cells per grid. Each well was counted four times and the average value used.

2.6. [³H]Epibatidine binding in culture

SH-SY5Y cells were plated at 300,000 cells/cm² and terminally differentiated with retinoic acid (RA) and phorbol-12-myristate 13-acetate (TPA), as detailed above. Binding of [³H] Epibatidine to the cultures was done using a saturating concentration of the radioligand (2.0 nM), as previously described (Jeyarasasingam et al., 2002). Nicotine (10⁻⁴ M) was used to define nonspecific binding.

2.7. Cell Viability Assays

After terminal differentiation and exposure to 6-OHDA, the CellTiter96 Aqueous One Solution Cell Proliferation Assay (MTS) kit from Promega (Madison, WI) was used to determine cell viability. For the MTS assay, 20 µl of the CellTiter 96 Aqueous One Solution Reagent was added to each well with 100 µl of culture medium in a 96-well plate. The plate was then incubated for 3 hours at 37°C in a humidified, 5% CO₂ atmosphere. Absorbance was measured at 490nm using a 96-well plate reader. Background absorbance was subtracted using at least six control wells (without cells) containing the same volumes of culture medium and reagent.

2.8. Statistical Analysis

The data are expressed as mean ± S.E.M. They were statistically compared using analysis of variance (ANOVA) followed by Dunnett's multiple comparison test or a Bonferroni *post hoc* test, with $p \leq 0.05$ considered significant.

3. Results

3.1. Phenotypic characteristics of terminally differentiated SH-SY5Y

Our results indicated that SH-SY5Y neuroblastoma cells stained positively for TH, indicating they possessed catecholaminergic characteristics. We next determined whether differentiated SH-SY5Y cells expressed nicotinic receptors since we were interested in investigating effects of nicotine. Cells were plated at a density of 300,000 cells/cm², differentiated, and binding of [³H]epibatidine, a radioligand that interacts with $\alpha 2$ to $\alpha 6$ subunit-containing nicotinic receptors was determined. [³H]epibatidine binding was done at saturating concentrations of the radioligand. Specific binding was 7.11 ± 0.87 fmol/well ($n = 12$), indicating that differentiated cells possess nicotinic receptors.

3.2. Decreased SH-SY5Y cell viability and number of TH⁺ cells after exposure to 6-OHDA

Experiments were subsequently performed to determine the effect of 6-OHDA on SH-SY5Y cells in culture. Cells were terminally differentiated for 7 d followed by a 48-h exposure period to varying concentrations of 6-OHDA (0, 20, 60, or 100 µM). The results in Fig. 1A show that the toxin decreased SH-SY5Y cell viability in a dose-dependent manner as assessed using the CyQuant cell viability assay. There was a main effect of 6-OHDA using one-way ANOVA ($F(3, 40) = 55.37$, $p < 0.001$) (Fig. 2A), with significant declines in cell viability at both the 60 and 100 µM dose using Dunnett's post hoc test ($p < 0.01$).

The effect of 6-OHDA was next determined on the number of TH⁺ cells using immunocytochemistry. There was a similar effect of the toxin ($F(3, 87) = 41.30$, $p < 0.0001$) using this approach (Fig. 1B), with significant declines in the number of immunoreactive cells at 60 and 100 µM 6-OHDA ($p < 0.01$). Because evaluation of the number of TH⁺ SH-SY5Y cells provided a direct measure of the effect of the toxin on the cellular population with catecholaminergic characteristics, all subsequent work was done by counting TH⁺ SH-SY5Y cells.

Since 6-OHDA induced significant declines only at 60 and 100 μ M, studies to evaluate the neuroprotective effect of nicotine against toxicity were done only at these two concentrations.

3.3. Mainstream cigarette smoke attenuates 6-OHDA-induced neurotoxicity

Next, we tested the effect of 6-OHDA on the number of TH⁺ SH-SY5Y cells exposed to varying concentrations of mainstream cigarette smoke solutions (Fig. 2). Cells were pretreated with 0.06 puffs/ml (Fig. 2A), 0.2 puffs/ml (Fig. 2B), or 0.6 puffs/ml (Fig. 2C) of smoke solution for 24 h. 6-OHDA (to a final concentration of 60 or 100 μ M) was then added to the wells with the smoke solution maintained. The results demonstrate that 0.2 puffs/ml of mainstream cigarette smoke solution (Fig. 2B) conferred significant protection against 100 μ M 6-OHDA-induced neurotoxicity with a trend for a reversal at 60 μ M 6-OHDA. The 0.06 puffs/ml dose was ineffective. The 0.6 puffs/ml dose alone led to a significant decline in the number of TH⁺ cells, similar in magnitude to that induced by the dopaminergic neurotoxin, most likely due to the high concentration of numerous toxic components in smoke (Fig. 2C). There was no further reduction in cell number when the 0.6 puffs/ml dose was combined with either the 60 or 100 μ M dose of 6-OHDA.

3.4. Nicotine attenuates 6-OHDA-induced neurotoxicity

As an approach to evaluate whether nicotine may contribute to the protective action of smoke against 6-OHDA-induced cell damage, SH-SY5Y cells were exposed to 10^{-7} to 10^{-5} M nicotine (Table 1). These concentrations were selected because the concentration of nicotine in the smoke solution fell within this range (5×10^{-7} M final concentration in the culture medium). The results show that nicotine attenuated 6-OHDA-induced toxicity at 10^{-6} and 10^{-7} M, concentrations that did not affect the cells on its own (Fig. 3A, Table 1). At the 10^{-5} M concentrations, nicotine alone decreased the number of TH⁺ cells (Table 1); however, since there was no further reduction with 100 μ M 6-OHDA, this may suggest there is protection.

3.5. Cotinine attenuates 6-OHDA-induced neurotoxicity

Since previous work had shown that cotinine exhibited neuroprotective properties, we also tested whether this nicotine metabolite had beneficial effects against 6-OHDA-induced toxicity. We first tested the effect of different doses of cotinine on control SH-SY5Y cells (10^{-8} to 10^{-6} M). There were no appreciable effects of 10^{-8} and 10^{-7} M cotinine under control conditions, although there was a significant decline with 10^{-6} M cotinine (Table 1, Fig. 3B). In cells treated with 60 μ M 6-OHDA, 10^{-7} and 10^{-6} M cotinine both significantly reduced neurotoxicity (Table 1, Fig. 3B). Cotinine exposure did not attenuate the neurotoxic effects of 100 μ M 6-OHDA.

Discussion

The present results show that whole mainstream smoke solutions protect against 6-OHDA-induced damage in cultured SH-SY5Y cells, a cell line used as a model for dopaminergic neurons (Copeland et al., 2007; Lim et al., 2007; Sung et al., 2007). Protection against toxicity was also obtained with nicotine at doses (10^{-7} - 10^{-6} M) similar to those present in the smoke solution (5×10^{-7} M). This concentration of nicotine falls within the range of nicotine present in the plasma of smokers (Hukkanen et al., 2005; Matta et al., 2007). These combined findings suggest that beneficial effects against 6-OHDA-induced toxicity may be attributed, at least in part, to the presence of nicotine in the smoke solution.

The finding that nicotine is a component in smoke that may be involved in protection against toxic insults is consistent with previous work in numerous experimental models. Exposure of primary mesencephalic cultures to 10^{-7} and 10^{-6} M nicotine partially protected against dopaminergic neuron toxicity induced by MPTP (Jeyarasasingam et al., 2002). Administration

of nicotine via injection, infusion, the drinking water and minipump administration also reduced the effect of nigrostriatal damage in different animal models (O'Neill et al., 2002; Quik et al., 2007).

In addition, our data show that cotinine, the primary metabolite of nicotine (Benowitz and Jacob, 1994; Hukkanen et al., 2005; Matta et al., 2007), protected against 6-OHDA-induced cell damage. The biological significance of this long-lasting nicotine metabolite is currently under investigation. Studies have shown that cotinine affects numerous physiological functions in both the peripheral and central nervous system (Buccafusco and Terry, 2003; Terry et al., 2005; Buccafusco et al., 2007). In addition, cotinine protects against toxic insults in PC12 cells, with improved cell viability at concentrations on the order of 10^{-7} M (Buccafusco and Terry, 2003), similar to those observed in the current study. *In vivo* studies have also been done in which cotinine was administered to mice prior to nigrostriatal damage. However, no beneficial effects were observed possibly due to a suboptimal dosing regimen (Parain et al., 2001). SH-SY5Y cells express cytochrome P450 (Guarneri et al., 2000; Armstrong et al., 2005), the enzyme involved in the conversion of nicotine to cotinine (Hukkanen et al., 2005; Matta et al., 2007). Our results may thus suggest that the protective effect of nicotine against 6-OHDA-induced toxicity is, at least in part, due to conversion of nicotine to cotinine by the SH-SY5Y cells.

In addition to nicotine, cigarette smoke also contains numerous other chemicals, some of which have been reported to protect against nigrostriatal damage in mice. This includes components with monoamine oxidase (MAO) inhibitory properties such as 2,3,6-trimethyl-1,4-naphthoquinone and farnesylacetone (Castagnoli et al., 2001; Castagnoli et al., 2003; Khalil et al., 2006). Studies have also shown that smoking results in a decline in MAO activity in human brain (Fowler et al., 1996b; Fowler et al., 1996a). These observations may suggest that a reduction in MAO activity attenuates neurodegeneration by decreasing reactive oxygen species and/or other toxic metabolites implicated in Parkinson's disease. The combined effects of nicotine and MAO inhibitors against nigrostriatal damage remain to be investigated.

SH-SY5Y cells were selected for the present studies because they are derived from human neuroblastoma cells, are of CNS origin and have a catecholaminergic phenotype. Previous studies had shown that differentiated cells express TH, the dopamine transporter, a dopamine uptake system, dopamine receptors, as well as other catecholaminergic markers (Presgraves et al., 2004a; Presgraves et al., 2004b). In addition, they are susceptible to the toxic effects of dopaminergic neurotoxins that damage the nigrostriatal pathway, a major site of pathology in Parkinson's disease (Presgraves et al., 2004a; Presgraves et al., 2004b; Copeland et al., 2007; Lim et al., 2007; Sung et al., 2007). In some studies, nicotine did not prevent toxin-induced degenerative effects (Mazzio et al., 2005); however, this may have been due to the fact that these investigators had used undifferentiated SH-SY5Y cells which are phenotypically distinct from their differentiated counterparts and did not express tyrosine hydroxylase in our hands. These combined findings would suggest that differentiated SH-SY5Y cells represent a suitable model for the current study.

In summary, the present data suggest that nicotine, possibly also via conversion to cotinine, is one component in smoke that protects against neurotoxic insults in SH-SY5Y cells in culture. Since the concentration of nicotine required for neuroprotection corresponds well to the plasma nicotine levels in smokers (Hukkanen et al., 2005; Matta et al., 2007), these combined data suggest that nicotine may be of value as a neuroprotective strategy against Parkinson's disease.

Acknowledgements

This work was supported by NIH Grant # NS47162. We thank Dr. P. Talbot, University of California Riverside, for the use of her equipment to prepare the mainstream smoke solutions.

References

- Allam MF, Campbell MJ, Hofman A, Del Castillo AS, Fernandez-Crehuet Navajas R. Smoking and Parkinson's disease: systematic review of prospective studies. *Mov Disord* 2004;19:614–621. [PubMed: 15197698]
- Armstrong JL, Ruiz M, Boddy AV, Redfern CP, Pearson AD, Veal GJ. Increasing the intracellular availability of all-trans retinoic acid in neuroblastoma cells. *Br J Cancer* 2005;92:696–704. [PubMed: 15714209]
- Belluardo N, Mudo G, Blum M, Fuxe K. Central nicotinic receptors, neurotrophic factors and neuroprotection. *Behav Brain Res* 2000;113:21–34. [PubMed: 10942029]
- Benowitz NL, Jacob P 3rd. Metabolism of nicotine to cotinine studied by a dual stable isotope method. *Clin Pharmacol Ther* 1994;56:483–493. [PubMed: 7955812]
- Buccafusco JJ, Terry AV Jr. The potential role of cotinine in the cognitive and neuroprotective actions of nicotine. *Life Sci* 2003;72:2931–2942. [PubMed: 12706481]
- Buccafusco JJ, Shuster LC, Terry AV Jr. Disconnection between activation and desensitization of autonomic nicotinic receptors by nicotine and cotinine. *Neurosci Lett* 2007;413:68–71. [PubMed: 17157984]
- Castagnoli K, Petzer JB, Steyn SJ, van der Schyf CJ, Castagnoli N Jr. Inhibition of human MAO-A and MAO-B by a compound isolated from flue-cured tobacco leaves and its neuroprotective properties in the MPTP mouse model of neurodegeneration. *Inflammopharmacology* 2003;11:183–188. [PubMed: 15035820]
- Castagnoli KP, Steyn SJ, Petzer JP, Van der Schyf CJ, Castagnoli N Jr. Neuroprotection in the MPTP Parkinsonian C57BL/6 mouse model by a compound isolated from tobacco. *Chem Res Toxicol* 2001;14:523–527. [PubMed: 11368550]
- Copeland RL Jr, Leggett YA, Kanaan YM, Taylor RE, Tizabi Y. Neuroprotective effects of nicotine against salsolinol-induced cytotoxicity: implications for Parkinson's disease. *Neurotox Res* 2005;8:289–293. [PubMed: 16371323]
- Copeland RL Jr, Das JR, Kanaan YM, Taylor RE, Tizabi Y. Antiapoptotic effects of nicotine in its protection against salsolinol-induced cytotoxicity. *Neurotox Res* 2007;12:61–69. [PubMed: 17513200]
- Costa G, Abin-Carriquiry JA, Dajas F. Nicotine prevents striatal dopamine loss produced by 6-hydroxydopamine lesion in the substantia nigra. *Brain Res* 2001;888:336–342. [PubMed: 11150495]
- Fowler JS, Volkow ND, Wang GJ, Pappas N, Logan J, Shea C, Alexoff D, MacGregor RR, Schlyer DJ, Zezulova I, Wolf AP. Brain monoamine oxidase A inhibition in cigarette smokers. *Proc Natl Acad Sci U S A* 1996a;93:14065–14069. [PubMed: 8943061]
- Fowler JS, Volkow ND, Wang GJ, Pappas N, Logan J, MacGregor R, Alexoff D, Shea C, Schlyer D, Wolf AP, Warner D, Zezulova I, Cilento R. Inhibition of monoamine oxidase B in the brains of smokers. *Nature* 1996b;379:733–736. [PubMed: 8602220]
- Gorell JM, Peterson EL, Rybicki BA, Johnson CC. Multiple risk factors for Parkinson's disease. *J Neurol Sci* 2004;217:169–174. [PubMed: 14706220]
- Guarneri P, Cascio C, Piccoli T, Piccoli F, Guarneri R. Human neuroblastoma SH-SY5Y cell line: neurosteroid-producing cell line relying on cytoskeletal organization. *J Neurosci Res* 2000;60:656–665. [PubMed: 10820437]
- Hernan MA, Takkouche B, Caamano-Isorna F, Gestal-Otero JJ. A meta-analysis of coffee drinking, cigarette smoking, and the risk of Parkinson's disease. *Ann Neurol* 2002;52:276–284. [PubMed: 12205639]
- Hukkanen J, Jacob P 3rd, Benowitz NL. Metabolism and disposition kinetics of nicotine. *Pharmacol Rev* 2005;57:79–115. [PubMed: 15734728]
- Janson AM, Fuxe K, Agnati LF, Kitayama I, Harfstrand A, Andersson K, Goldstein M. Chronic nicotine treatment counteracts the disappearance of tyrosine-hydroxylase-immunoreactive nerve cell bodies, dendrites and terminals in the mesostriatal dopamine system of the male rat after partial hemitransection. *Brain Res* 1988;455:332–345. [PubMed: 2900058]

- Jeyarasasingam G, Tompkins L, Quik M. Stimulation of non- $\alpha 7$ nicotinic receptors partially protects dopaminergic neurons from 1-methyl-4-phenylpyridinium-induced toxicity in culture. *Neuroscience* 2002;109:275–285. [PubMed: 11801364]
- Joyce JN, Presgraves S, Renish L, Borwege S, Osredkar T, Hagner D, Replogle M, PazSoldan M, Millan MJ. Neuroprotective effects of the novel D3/D2 receptor agonist and antiparkinson agent, S32504, in vitro against 1-methyl-4-phenylpyridinium (MPP+) and in vivo against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): a comparison to ropinirole. *Exp Neurol* 2003;184:393–407. [PubMed: 14637109]
- Khalil AA, Davies B, Castagnoli N Jr. Isolation and characterization of a monoamine oxidase B selective inhibitor from tobacco smoke. *Bioorg Med Chem* 2006;14:3392–3398. [PubMed: 16458520]
- Lim MK, Kawamura T, Ohsawa Y, Ohtsubo M, Asakawa S, Takayanagi A, Shimizu N. Parkin interacts with LIM Kinase 1 and reduces its cofilin-phosphorylation activity via ubiquitination. *Exp Cell Res* 2007;313:2858–2874. [PubMed: 17512523]
- Matta SG, Balfour DJ, Benowitz NL, Boyd RT, Buccafusco JJ, Caggiula AR, Craig CR, Collins AC, Damaj MI, Donny EC, Gardiner PS, Grady SR, Heberlein U, Leonard SS, Levin ED, Lukas RJ, Markou A, Marks MJ, McCallum SE, Parameswaran N, Perkins KA, Picciotto MR, Quik M, Rose JE, Rothenfluh A, Schafer WR, Stolerman IP, Tyndale RF, Wehner JM, Zirger JM. Guidelines on nicotine dose selection for in vivo research. *Psychopharmacology (Berl)* 2007;190:269–319. [PubMed: 16896961]
- Mazzio EA, Kolta MG, Reams RR, Soliman KFA. Inhibitory effects of cigarette smoke on glial inducible nitric oxide synthase and lack of protective properties against oxidative neurotoxins in vitro. *Neurotoxicology* 2005;26:49–62. [PubMed: 15527873]
- Morens DM, Grandinetti A, Reed D, White LR, Ross GW. Cigarette smoking and protection from Parkinson's disease: false association or etiologic clue? *Neurology* 1995;45:1041–1051. [PubMed: 7783862]
- O'Neill MJ, Murray TK, Lakics V, Visanji NP, Duty S. The role of neuronal nicotinic acetylcholine receptors in acute and chronic neurodegeneration. *Curr Drug Target CNS Neurol Disord* 2002;1:399–411.
- Parain K, Marchand V, Dumery B, Hirsch E. Nicotine, but not cotinine, partially protects dopaminergic neurons against MPTP-induced degeneration in mice. *Brain Res* 2001;890:347–350. [PubMed: 11164803]
- Presgraves SP, Ahmed T, Borwege S, Joyce JN. Terminally differentiated SH-SY5Y cells provide a model system for studying neuroprotective effects of dopamine agonists. *Neurotox Res* 2004a;5:579–598. [PubMed: 15111235]
- Presgraves SP, Borwege S, Millan MJ, Joyce JN. Involvement of dopamine D(2)/D(3) receptors and BDNF in the neuroprotective effects of S32504 and pramipexole against 1-methyl-4-phenylpyridinium in terminally differentiated SH-SY5Y cells. *Exp Neurol* 2004b;190:157–170. [PubMed: 15473989]
- Quik M, O'Neill M, Perez XA. Nicotine neuroprotection against nigrostriatal damage: importance of the animal model. *Trends Pharmacol Sci.* 2007
- Quik M, Chen L, Parameswaran N, Xie X, Langston JW, McCallum SE. Chronic oral nicotine normalizes dopaminergic function and synaptic plasticity in 1-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine-lesioned primates. *J Neurosci* 2006a;26:4681–4689. [PubMed: 16641249]
- Quik M, Parameswaran N, McCallum SE, Bordia T, Bao S, McCormack A, Kim A, Tyndale RF, Langston JW, Di Monte DA. Chronic oral nicotine treatment protects against striatal degeneration in MPTP-treated primates. *J Neurochem* 2006b;98:1866–1875. [PubMed: 16882311]
- Ritz B, Ascherio A, Checkoway H, Marder KS, Nelson LM, Rocca WA, Ross GW, Strickland D, Van Den Eeden SK, Gorell J. Pooled analysis of tobacco use and risk of Parkinson disease. *Arch Neurol* 2007;64:990–997. [PubMed: 17620489]
- Riveles K, Tran V, Roza R, Kwan D, Talbot P. Smoke from traditional commercial, harm reduction and research brand cigarettes impairs oviductal functioning in hamsters (*Mesocricetus auratus*) in vitro. *Hum Reprod* 2007;22:346–355. [PubMed: 17071822]

- Ryan RE, Ross SA, Drago J, Loiacono RE. Dose-related neuroprotective effects of chronic nicotine in 6-hydroxydopamine treated rats, and loss of neuroprotection in alpha4 nicotinic receptor subunit knockout mice. *Br J Pharmacol* 2001;132:1650–1656. [PubMed: 11309235]
- Soto-Otero R, Mendez-Alvarez E, Hermida-Ameijeiras A, Lopez-Real AM, Labandeira-Garcia JL. Effects of (–)-nicotine and (–)-cotinine on 6-hydroxydopamine-induced oxidative stress and neurotoxicity: relevance for Parkinson's disease. *Biochem Pharmacol* 2002;64:125–135. [PubMed: 12106613]
- Sung JY, Lee HJ, Jeong EI, Oh Y, Park J, Kang KS, Chung KC. Alpha-synuclein overexpression reduces gap junctional intercellular communication in dopaminergic neuroblastoma cells. *Neurosci Lett* 2007;416:289–293. [PubMed: 17337120]
- Terry AV Jr, Hernandez CM, Hohnadel EJ, Bouchard KP, Buccafusco JJ. Cotinine, a neuroactive metabolite of nicotine: potential for treating disorders of impaired cognition. *CNS Drug Rev* 2005;11:229–252. [PubMed: 16389292]
- Thacker EL, O'Reilly EJ, Weisskopf MG, Chen H, Schwarzschild MA, McCullough ML, Calle EE, Thun MJ, Ascherio A. Temporal relationship between cigarette smoking and risk of Parkinson disease. *Neurology* 2007;68:764–768. [PubMed: 17339584]
- Tizabi Y, Manaye KF, Taylor RE. Nicotine blocks ethanol-induced apoptosis in primary cultures of rat cerebral cortical and cerebellar granule cells. *Neurotox Res* 2005;7:319–322. [PubMed: 16237857]
- Visanji NP, O'Neill MJ, Duty S. Nicotine, but neither the alpha4beta2 ligand RJR2403 nor an alpha7 nAChR subtype selective agonist, protects against a partial 6- hydroxydopamine lesion of the rat median forebrain bundle. *Neuropharmacology* 2006;51:506–516. [PubMed: 16814329]

Fig. 1.

Neurotoxic effect of 6-OHDA on differentiated SH-SY5Y cells. SH-SY5Y cells were plated in a collagen-coated 48-well culture plate. They were differentiated by addition of retinoic acid (50 μ M) for five days, followed by two days in TPA (50 nM). This was followed by a 24–48-h exposure period to varying concentrations of 6-OHDA (20, 60 or 100 μ M). 6-OHDA-induced cell loss was assessed using the MTS viability assay (A) and TH immunocytochemistry (B). Bars represent the mean $\pm$ SEM of 7–32 wells from up to 3 separate experiments. Statistical significance was determined using a one-way ANOVA followed by a Dunnett's multiple comparison test. Significance of difference from no 6-OHDA; ** $p < 0.01$.

Fig. 2.

Effect of different doses of mainstream smoke solution on 6-OHDA-induced neurotoxicity. SH-SY5Y cells were pre-treated with smoke solution at 0.06 puffs/ml (A), 0.2 puffs/ml (B), or 0.6 puffs/ml (C). This was followed 24 h later by addition of either 60 or 100 μ M 6-OHDA. The numbers of TH+ cells were then counted to evaluate the effects of treatment. A beneficial effect against damage was observed with smoke solutions containing 0.2 and 0.6 puffs/ml. The bars represent the mean $\pm$ SEM of 6–24 wells from 2–4 different experiments. Statistical significance was determined using two-way ANOVA, followed by a Bonferroni *post hoc* test. Significance of difference from respective condition, that is, control or smoke solution with

no 6-OHDA; * $p < 0.05$ and *** $p < 0.001$. Significance of difference from own control in the absence of smoke solution; ## $p < 0.01$, ### $p < 0.001$.

Fig. 3.

Neuroprotective effect of nicotine and cotinine against the 6-OHDA-induced loss in SH-SY5Y cells. Cells were pretreated with 10^{-7} M nicotine (A) or 10^{-7} M cotinine (B) for 24 h, followed by co-incubation with 6-OHDA at 60 or 100 μ M for 24–48 h. The cells were then processed for TH immunoreactivity and the number of TH⁺ cells counted. The bars represent the mean $\pm$ SEM of 4–8 wells from 2–3 experiments. Statistical significance was determined using two-way ANOVA, followed by a Bonferroni *post hoc* test. Significance of difference from respective condition with no 6-OHDA; * $p < 0.05$ and *** $p < 0.001$. Significance of difference from own control in the absence of nicotine or cotinine; ### $p < 0.001$.

Table 1

Nicotine and cotinine protect against 6-OHDA-induced damage in SH-SY5Y cells as assessed using TH⁺ immunocytochemistry.

Drug	Concentration (M)	TH ⁺ cells (% control)		
		Control	60 μ M 6-OHDA	100 μ M 6-OHDA
Nicotine	0	100 $\pm$ 6%	44 $\pm$ 3% ^{***}	25 $\pm$ 2% ^{***}
	10 ⁻⁷	113 $\pm$ 2%	75 $\pm$ 2% ^{***###}	52 $\pm$ 8% ^{***, ###}
	10 ⁻⁶	89 $\pm$ 3%	84 $\pm$ 5% ^{###}	68 $\pm$ 0.3% ^{*, ###}
	10 ⁻⁵	63 $\pm$ 3% ^{###}	Not done	54 $\pm$ 7% ^{##}
Cotinine	0	100 $\pm$ 6%	35 $\pm$ 4% ^{***}	16 $\pm$ 2% ^{***}
	10 ⁻⁸	98 $\pm$ 3%	22 $\pm$ 3% ^{***}	16 $\pm$ 2% ^{***}
	10 ⁻⁷	95 $\pm$ 5%	78 $\pm$ 1% ^{####}	10 $\pm$ 3% ^{***}
	10 ⁻⁶	75 $\pm$ 7% ^{###}	82 $\pm$ 3% ^{###}	9 $\pm$ 1% ^{***}

SH-SY5Y cells were plated in a collagen-coated 48-well culture plate. They were differentiated by addition of retinoic acid (50 μ M) for five days, followed by two days in TPA (50 nM). Cultures were exposed to nicotine or cotinine for 24 h, after which 6-OHDA (60 or 100 μ M) was added for a further 24–48 h. The numbers of TH⁺ cells were then counted to evaluate the effects of the different treatments. Each value represents the mean $\pm$ SEM of 4–8 wells from 2–3 separate experiments. Significance of difference from respective condition with no 6-OHDA

* P < 0.05

*** p < 0.001: from own control in the absence of nicotine or cotinine

p < 0.01

p < 0.001. Data were analyzed by two-way ANOVA, followed by a Bonferroni *post hoc* test.