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Neuroprotective Effects of Nicotine Against Salsolinol-induced Cytotoxicity: Implications for Parkinson's Disease

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Parkinson's disease is associated with degeneration of dopaminergic cell bodies in the substantia nigra. It has been suggested that salsolinol, an endogenous metabolite of dopamine, may be involved in this process. An inverse relationship between Parkinson's disease and smoking (nicotine intake) has been observed in epidemiological studies. Moreover, neuroprotective effects of nicotine in various experimental models have been observed. In this study we sought to determine whether salsolinol-induced cytotoxicity in SH-SY5Y human neuroblastoma cells, a cloned cell line which expresses dopaminergic activity, could also be prevented by nicotine pretreatment, and if so, which nicotinic receptors may mediate the actions of nicotine. Exposure of SH-SY5Y cells to 0.8 mM salsolinol for 24 hours resulted in approximately 80% cell death as determined by 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Pretreatment of cells with 0.1 mM nicotine resulted in inhibition of salsolinol-induced cytotoxicity. The effects of nicotine were blocked by mecamylamine, a non-selective nicotinic antagonist as well as conotoxins with selective antagonism against alpha3-containing nicotinic receptor subunits. The effects of nicotine were not affected by dihydro-beta-erythroidine or methyllycaconitine, selective antagonists against alpha4-beta2 or alpha7 nicotinic receptors, respectively. It is suggested that selective nicotinic agonists may be of therapeutic potential in at least a subpopulation of Parkinsonian patients.

Keywords: Salsolinol; Nicotine; Nicotinic Receptors; SH-SY5Y; Neuroblastoma; Parkinson's disease; Neurotoxicity; Neuroprotection

Parkinson's disease (PD) is a chronic neurodegenerative disorder associated with loss of dopaminergic neurons in substantia nigra and accumulation of Lewy bodies. Its primary symptoms of bradykinesia, rigidity, tremor, postural instability and gait disturbance can be extremely detrimental to the quality of life of the patient. Current pharmacological treatments by dopaminergic precursors or agonists and muscarinic cholinergic antagonists serve as palliative therapy. Hence, there is an urgent need to develop neuroprotective drugs that might impede or halt the progression of the disease.

Although most PD cases are idiopathic (unknown origin or cause), several factors, including environmental toxins and genetic predisposition have been implicated in its etiology. It was discovered that administration of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) results in Parkinson-like syndrome in human and non-human primates (see Storch *et al.*, 2002; Maruyama *et al.*, 2004). Salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline), an endogenous dopamine metabolite has structural similarity to MPTP and might be especially toxic to dopaminergic neurons (Storch *et al.*, 2002; Maruyama *et al.*, 2004; Naoi *et al.*, 2004). Indeed, high levels of salsolinol have been found in the cerebrospinal fluid of patients with PD as well as those treated with L-Dopa (L-3,4-dihydroxyphenylalanine). These findings have led to the suggestion that salsolinol might be involved in the etiology or loss of dopamine neurons and/or accumulation of Lewy bodies in at least some Parkinson patients (Storch *et al.*, 2002; Maruyama *et al.*, 2004).

A number of pre- and prospective epidemiological studies have suggested that there is an inverse relationship between cigarette smoking and PD (Gorell *et al.*,

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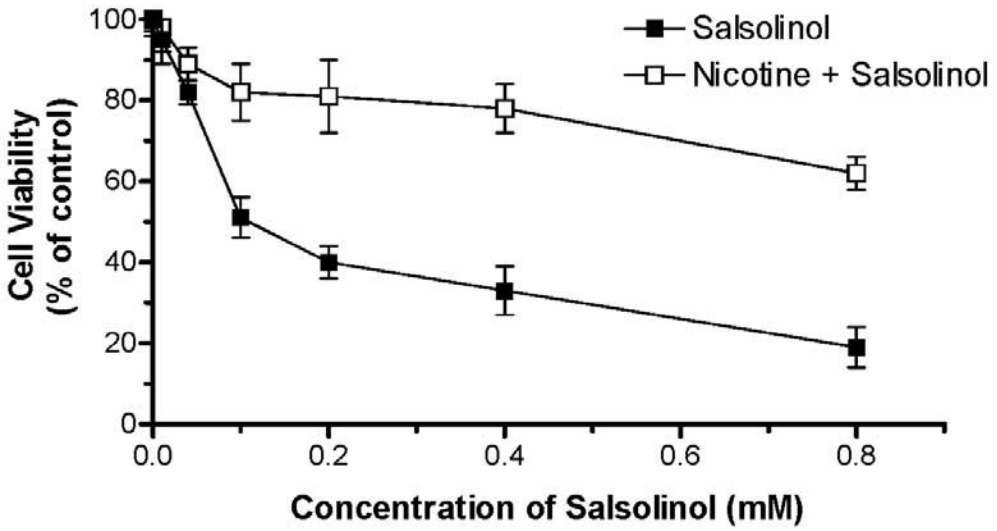

FIGURE 1 The effects of various concentrations of salsolinol with and without nicotine (0.1 mM) on cell viability as determined by MTT assay. Cells were exposed for twenty four hours. Nicotine was added 1 hr before salsolinol. Nicotine significantly blocked salsolinol-induced toxicity $p < 0.01$). Values are Mean ± SEM (n=12).

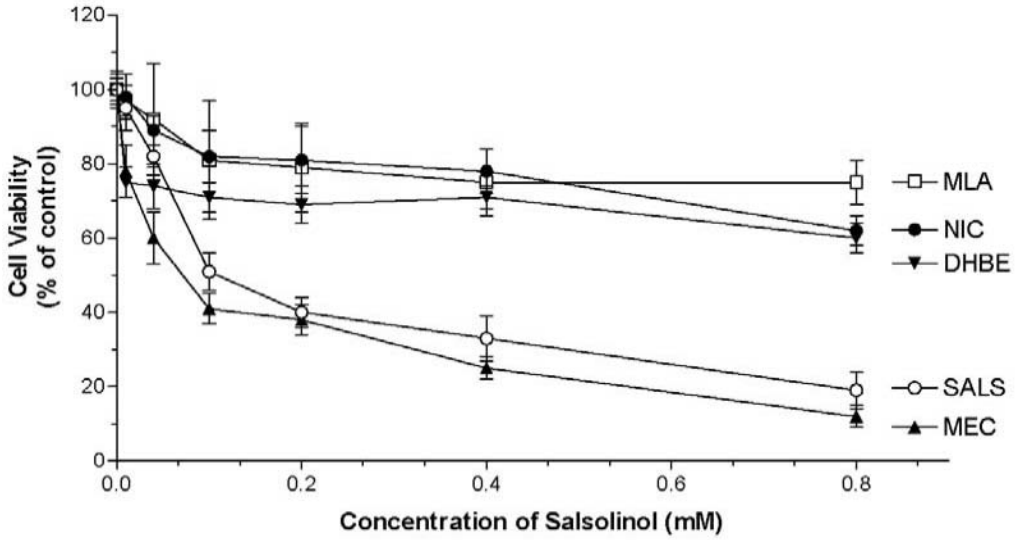

FIGURE 2 The effects of various nicotinic antagonists on nicotine protection against salsolinol-induced toxicity. The antagonists were added one hour before nicotine. Mecamylamine (0.1 mM) completely blocked the effects of nicotine, whereas DHBE (0.1 mM) and MLA (0.1 mM) were ineffective in blocking nicotine's effect. Values are Mean ± SEM (n=4).

1999; Hernan *et al.*, 2002; Wirdefeldt *et al.*, 2005). It has been shown that nicotine, the major psychoactive compound in the cigarette may protect against various neurotoxic insults such as glutamate, hypoxia, beta amyloid or alcohol in various cell cultures (Kihara *et al.*, 1998; Guan *et al.*, 2003; Hejmadi *et al.*, 2003; Stevens *et al.*, 2003; Tizabi *et al.*, 2003, 2004). Moreover, protective effects of nicotine against MPTP-induced toxicity have also been reported (Parain *et al.*, 2003; Liu and Zhao, 2004).

In this study, we sought to determine whether nicotine may protect against salsolinol-induced cytotoxicity in SH-SY5Y cells and if so, which nicotinic receptor subtype may be mediating the action of nicotine. SH-SY5Y cells, derived from human neuroblastoma cells, express nicotinic receptors as well as a high level of dopaminergic activity, and are extensively used as a model to study central dopaminergic neurons (Storch *et al.*, 2002; Maruyama *et al.*, 2004; Naoi *et al.*, 2004). Our findings indicate a protective effect of nicotine, mediated primarily by alpha3 containing subunit against salsolinol-induced toxicity.

SH-SY5Y cells purchased from American Type Culture Collection (Manassas, VA) were grown in con-

tinuous culture in a 1:1 mixture of Dulbeccos Modified Eagle medium and HAM's F-12 supplemented with 10% fetal calf serum, penicillin/streptomycin (100 IU/ml) and gentamicin (50 µg/ml) at 37°C in 5% CO_2 atmosphere in a humidified incubator. The culture medium was changed every 3-4 days and cells were harvested, once confluent (4-6 days). All buffers and media were of analytical grade.

(±) Salsolinol, (-)-nicotine di-d-tartrate, MTT (4,5 dimethylthiazoyl-2-yl)-2,5 diphenyltetrazolium bromide, mecamylamine, dihydro-β-erythroidine, and methyllycaconitine were purchased from Sigma (St. Louis, MO). α-Conotoxins were generously supplied by Dr. J. Michael McIntosh at University of Utah.

Cell counts were determined using hemacytometer counting procedures. Cell viability was quantitatively measured using 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. SH-SY5Y cells (1.2 x 10^4/well) were cultured in a 96-well culture plate as described by Storch *et al.* (2000). Cells were allowed to attach to the plate for 24 hours. The wells were gently aspirated and fresh media containing 100 µl of various concentrations of ± salsolinol in fresh media (DMEM/Ham's F-12, 5% FBS) was added to each well. Following 24 h, cell viability was determined by MTT assay. Thirty µl of MTT reagent (0.5 mg/ml) in PBS containing 10 mM HEPES was added to each well. The plate was allowed to incubate for 3 hours at 37°C followed by aspiration. The plate was then allowed to dry in the incubator for 1 hour. HCl in isopropanol (100 µl of 0.04 N) was added to each well. The plate was read spectrophotometrically in a multi plate reader at 570 nm. Cell viability was determined by subtracting the test results from background and is expressed as a percentage of control. After establishing the dose-response relationship for salsolinol-induced toxicity, the same procedure was repeated in the presence of nicotine as well as nicotinic antagonists. The antagonists included mecamylamine which blocks all nicotinic receptors, dihydro-β-erythroidine (DHBE, selective for high affinity nicotinic receptors, *e.g.*, alpha4-beta2 subunit), methyllycaconitine (MLA, selective for low affinity nicotinic receptors, *e.g.*, alpha7 subunit) and various α-conotoxins selective for alpha3 and/or alpha6 containing subunits (Nicke *et al.*, 2004). The conotoxins included α-Au1B: selective for alpha 3 containing subunit, α-MII: selective for both alpha3 and alpha6 containing subunits and α-MII-H9A;E11A (E11): selective for alpha6 containing subunit (Luo *et al.*, 1998; Dowell *et al.*, 2003; Quik *et al.*, 2004). In combination studies, nicotine was added one hour before salsolinol and the antagonists were added one hour before nicotine. In all cases the drugs were kept in the wells until cell viability was determined. The doses of nicotine and antagonists were based on studies using similar experimental conditions (Luo *et al.*, 1998; Guan *et al.*, 2003; Stevens *et al.*, 2003; Tizabi *et al.*, 2003; 2004), as well as our preliminary results using various doses of the drugs. Thus, the doses of nicotinic antagonists (mecamylamine, DHBE and MLA) used in this study were sufficient to block specific receptor subtype(s) that might mediate the actions of nicotine. Each experiment was carried out at least four times and each time triplicate samples were used.

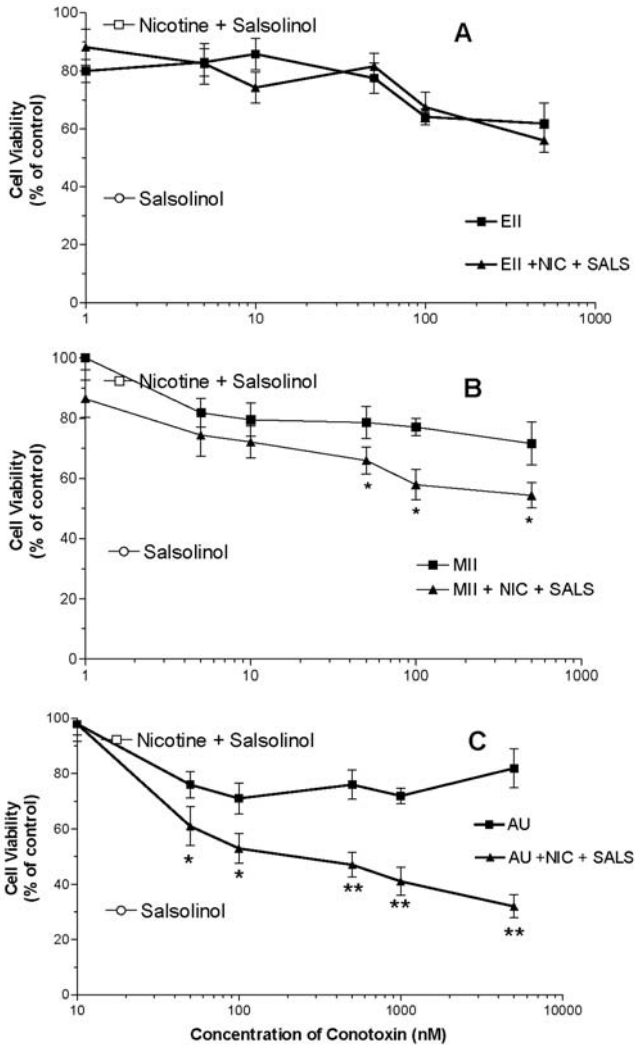

FIGURE 3 The effects of various concentrations of α-conotoxins on nicotine protection against salsolinol-induced toxicity. The conotoxins were added one hour before nicotine. Salsolinol concentration was maintained at 0.4 mM and nicotine concentration at 0.1 mM. **Panel A** depicts the effects α-conotoxin MII-H9A;E11A (E11), selective for α_6 receptors. **Panel B** shows the effects of α-conotoxin MII (MII), selective for α_3 and α_6 receptors. **Panel C** represents the effects of α-conotoxin AuIB (AU), selective for α_3 nicotinic receptors. Values are Mean ± SEM (n=4). *p <0.5, ** p <0.01 *vs* MII or AU.

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's *post-hoc* test. Spectrophotometer values were calculated using the graphing and statistical program GraphPrism (Version 3.0; GraphPad Software). Differences were regarded significant at $P < 0.05$.

Figure 1 shows the effects of various concentrations of salsolinol with and without nicotine on cell viability as determined by MTT assay. Twenty-four hour exposure to salsolinol caused a concentration-dependent decrease of cell viability in SH-SY5Y cells. Exposure to 0.4 mM salsolinol resulted in approximately 65% reduction in cell viability. Maximal toxic effect was observed with 0.8 mM salsolinol where approximately 80% of cells did not survive. Nicotine (0.1 mM) almost completely blocked the effect of 0.4 mM salsolinol and significantly attenuated the toxicity produced by 0.8 mM salsolinol. Nicotine at this concentration did not have any effect on cell viability by itself (data not shown).

Figure 2 depicts the effects of various nicotinic antagonists on nicotine protection against salsolinol-induced toxicity. Mecamylamine (0.1 mM) completely blocked the effects of nicotine, whereas both DHBE (0.1 mM) and MLA (0.1 mM) were ineffective in blocking nicotine's effect, suggesting involvement of other than alpha4-beta2 or alpha7 nicotinic receptor subtypes. Mecamylamine, DHBE or MLA at the concentrations used did not have any effect of their own or on salsolinol-induced toxicity (data not shown).

Figure 3 represents the effects of various concentrations of α-conotoxins on nicotine protection against salsolinol-induced toxicity. Panel A, depicts the effects of α-conotoxin E11, selective for alpha6 containing subunit. E11 did not affect nicotine protection or salsolinol-induced toxicity. Panel B shows the effects α-conotoxin MII, selective for both alpha3 and alpha6 containing subunits. MII attenuated nicotine effect at concentrations of 50 nM and higher. Maximal block of 60% was achieved with the highest MII concentration (500 nM). At these concentrations MII did not have any significant effect of its own or on salsolinol-induced toxicity. Panel C depicts the effects of α-conotoxin AuIB, selective for the alpha3 containing subunit. AuIB significantly attenuated nicotine protection at 50 nM and higher. Complete block (100%) was achieved with 5 μM of α-conotoxin AuIB. AuIB did not have any significant effect of its own or on salsolinol-induced toxicity.

This study demonstrates that salsolinol-induced toxicity in SH-SY5Y dopaminergic neuroblastoma cells can be prevented by nicotine and that the effects of nicotine are most likely mediated by the alpha3 containing nicotinic receptor subtype. Since salsolinol may act as an endogenous neurotoxin to the nigral dopaminergic cells, these findings suggest that nicotine or selective nicotinic agonists may have therapeutic potential in some Parkinson patients.

Neuroblastoma cells may represent nigral dopaminergic cells which are particularly vulnerable in Parkinsonism. SHSY5Y cells, derived from human neuroblastoma cells express high level of dopaminergic activity and are used extensively as a model to study central dopaminergic neurons (Storch *et al.*, 2002; Maruyama *et al.*, 2004; Naoi *et al.*, 2004). In addition, these cells express various nicotinic receptor subtypes containing alpha3, alpha5, alpha7, beta2 and beta4 subunits (Lukas *et al.*, 1993). It is noteworthy that the nigral terminals in the striatum are also rich in nicotinic receptors which have a stimulatory effect on dopamine release. A decrease in α-conotoxin MII sensitive nicotinic receptors has been recently observed in the striatum of Parkinson patients (Quik *et al.*, 2004). Moreover, it appears that these receptors are preferentially vulnerable to the toxic damage by MPTP which has a close structural similarity to salsolinol. Because α-conotoxin MII interacts with alpha3 and/or alpha6 containing nicotinic receptor subtype (Dowell *et al.*, 2003; Quik 2004), it may be suggested that agonists of these receptors, acting at both the soma and terminal regions, would be of particular benefit in Parkinson disease. This therapy could prove especially beneficial in those patients whose symptoms may be precipitated or exacerbated by high level of salsolinol or genetic susceptibility to this compound.

Although involvement of other nicotinic receptors in protective effects of nicotine may be suspected, the results of this study do not implicate the alpha7 containing subunit. Thus, the expression of this receptor subtype in SHSY5Y cells may be associated with other cellular functions (Dajas-Bailador *et al.*, 2002) and hence a minimal role in neuroprotective effects of nicotine. The lack of DHBE in attenuating nicotine effect was not surprising as these cells do not express alpha4-beta2 receptor subtype.

Salsolinol-induced toxicity in SH-SY5Y cells appears to be mediated by an apoptotic mechanism (Maruyama *et al.*, 2004; Naoi *et al.*, 2004). Hence, nicotine's attenuation of salsolinol-induced toxicity is likely to be mediated by inhibition of apoptosis. In this regard, antiapoptotic effects of nicotine against several insults including hypoxia-, beta-amyloid- and alcohol-induced apoptosis in various cell cultures have been reported (Hejmadi *et al.*, 2003; Liu and Zhao, 2004; Tizabi *et al.*, 2005). Collectively, these findings suggest a protective effect of nicotine or nicotinic agonists in

Parkinson's disease as well as other neurodegenerative disorders involving apoptotic mechanisms.

In summary, salsolinol-induced toxicity in SH-SY5Y cells can be blocked by nicotine via activation of the alpha3 nicotinic receptor subtype, suggesting therapeutic potential of selective nicotinic agonists in Parkinson's disease.

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References

Dajas-Bailador FA, L Soliakov and S Wonnacott (2002) Nicotine activates the extracellular signal-regulated kinase I/2 via the α_7 nicotinic acetylcholine receptor and protein kinase A, in SH-SY5Y cells and hippocampal neurones. *J. Neurochem.* **80**, 520-530.

Dowell C, BM Olivera, JE Garrett, ST Staheli, M Watkins, A Kuryatov, D Yoshikami, JM Lindstrom and JM McIntosh (2003) α-Conotoxin PIA is selective for α_6 subunit-containing nicotinic acetylcholine receptors. *J. Neurosci.* **23**, 8445-8562.

Gorell JM, BA Rybicki, CC Johnson and EL Peterson (1999) Smoking and Parkinson's disease: a dose-response relationship. *Neurology* **52**, 115-119.

Guan ZZ, WF Yu and A Nordberg (2003) Dual effects of nicotine on oxidative stress and neuroprotection in PC12 cells. *Neurochem. Intl.* **43**, 243-249.

Hejmadi MV, F Dajas-Bailador, SM Barns, B Jones and S Wonnacott (2003) Neuroprotection by nicotine against hypoxia-induced apoptosis in cortical cultures involves activation of multiple nicotinic acetylcholine receptor subtypes. *Mol. Cell. Neurosci.* **24**, 779-786.

Hernan MA, B Takkouche, F Caamano-Isorna and JJ Gestal-Otero (2002) A meta-analysis of coffee drinking, cigarette smoking, and the risk of Parkinson's disease. *Ann. Neurol.* **52**, 276-284.

Kihara T, S Shimohama, M Urushitani, H Sawada, J Kimura, T Kume, T Maeda and A Akaike (1998) Stimulation of alpha4 beta2 nicotinic acetylcholine receptors inhibits beta-amyloid toxicity. *Brain Res.* **792**, 331-334.

Liu Q and B Zhao (2004) Nicotine attenuates beta-amyloid peptide-induced neurotoxicity, free radical and calcium accumulation in hippocampal neuronal cultures. Br. J. Pharmacol. 141, 746-754.

Lukas RJ, SA Norman and L Lucero (1993) Characterization of nicotinic acetylcholine receptors expressed by cells of the SH-SY5Y human neuroblastoma clone line. *Mol. Cell. Neurosci.* **4**, 1-12.

Luo S, JM Kulak, GE Cartier, RB Jacobson, D Yoshikami, BM Olivera and JM McIntosh (1998) α-Conotoxin AuIB selectively blocks $\alpha_3\beta_4$ nicotinic acetylcholine receptors and nicotine-evoked norepinephrine release. *J. Neurosci.* **18**, 8571-8579.

Maruyama W, H Yi, T Takahashi, S Shimazu, H Ohde, F Yoneda, K Iwasa and M Naoi (2004) neuroprotective function of R-(-)-1-(benzufuran-2-yl)-2-propylaminopentane, [R-(-)-BPAP], against apoptosis induced by *N*-methyl(R)salsolinol, an endogenous dopaminergic neurotoxin, in human dopaminergic neuroblastoma SH-SY5Y cells. *Life Sci.* **75**, 107-117.

Naoi M, W Maruyama and GM Nagy (2004) Dopamine-derived salsolinol derivatives as endogenous monoamine oxidase inhibitors: occurrence, metabolism and function in human brains. *Neurotox*. **25**, 193-204.

Nicke A, S Wonnacott and RJ Lewis (2004) α-Conotoxins as tools for the elucidation of structure and function of neuronal nicotinic acetylcholine receptor subtypes. *Eur. J. Biochem.* **271**, 2305-2319.

Parain K, C Hapdey, E Rousselet, V Marchand, B Dumery and EC Hirsch (2003) Cigarette smoke and nicotine protect dopaminergic neurons against the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine Parkinsonian toxin. *Brain Res.* **984**, 224-232.

Quik M, T Bordia and JM McIntosh (2004) Loss of α-conotoxin MII- and A85380-sensitive nicotinic receptors in Parkinson's disease striatum. *J. Neurochem.* **88**, 668-679.

Stevens TR, SR Krueger, RM Fitzsimonds and MR Picciotto (2003) Neuroprotection by nicotine in mouse primary cortical cultures involves activation of calcineurin and l-type calcium channel inactivation. *J. Neurosci.* **23**, 10093-10099.

Storch A, A Kaftan, K Burkhardt and J Schwarz (2000) 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (salsolinol) is toxic to dopaminergic neuroblastoma SH-SY5Y cells via impairment of cellular energy metabolism. *Brain Res.* **855**, 67-75.

Storch A, S Ott, Y Hwang, R Ortmann, A Hein, S Frenzel, K Matsubara, S Ohta, H Wolf and J Schwarz (2002) Selective dopaminergic neurotoxicity of isoquinoline derivatives related to Parkinson's disease: studies using heterologous expression systems of the dopamine transporter. *Biochem. Pharmacol.* **63**, 909-920.

Tizabi Y, M Al-Namaeh, KF Manaye and RE Taylor (2003) Protective effects of nicotine on ethanol-induced toxicity in cultured cerebellar granule cells. *Neurotoxicity Res.* **5**, 315-322.

Tizabi Y, KF Manaye, DT Smoot and RE Taylor (2004) Nicotine inhibits ethanol-induced toxicity in cultured cerebral cortical cells. *Neurotoxicity Res.* **6**, 311-316.

Tizabi Y, KF Manaye and RE Taylor (2005) Nicotine blocks ethanol-induced apoptosis in primary cultures of rat cerebral cortical and cerebellar granule cells. *Neurotoxicity Res.* **7**, 319-322.

Wirdefeldt K, M Gatz, Y Pawitan and NL Pedersen (2005) Risk and protective factors for Parkinson's disease: a study in Swedish twins. *Ann. Neurol.* **57**, 27-33.