The neuroprotective effect of nicotine in Parkinson's disease models is associated with inhibiting PARP-1 and caspase-3 cleavage

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Clinical evidence points to neuroprotective effects of smoking in Parkinson's disease (PD), but the molecular mechanisms remain unclear. We investigated the pharmacological pathways involved in these neuroprotective effects, which could provide novel ideas for developing targeted neuroprotective treatments for PD. We used the ETC complex I inhibitor methylpyridinium ion (MPP$^+$) to induce cell death in SH-SY5Y cells as a cellular model for PD and found that nicotine inhibits cell death. Using choline as a nicotinic acetylcholine receptor (nAChR) agonist, we found that nAChR stimulation was sufficient to protect SH-SY5Y cells against cell death from MPP$^+$. Blocking α7 nAChR with methyllycaconitine (MLA) prevented the protective effects of nicotine, demonstrating that these receptors are necessary for the neuroprotective effects of nicotine. The neuroprotective effect of nicotine involves other pathways relevant to PD. Cleaved Poly (ADP-ribose) polymerase-1 (PARP-1) and cleaved caspase-3 were decreased by nicotine in 6-hydroxydopamine (6-OHDA) lesioned mice and in MPP$^+$-treated SH-SY5Y cells. In conclusion, our data indicate that nicotine likely exerts neuroprotective effects in PD through the α7 nAChR and downstream pathways including PARP-1 and caspase-3. This knowledge could be pursued in future research to develop neuroprotective treatments for PD.

1 **The neuroprotective effect of nicotine in Parkinson's disease models is**

2 **associated with inhibiting PARP-1 and caspase-3 cleavage**

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27   **Abstract**

28   Clinical evidence points to neuroprotective effects of smoking in Parkinson's disease (PD), but

29   the molecular mechanisms remain unclear. We investigated the pharmacological pathways

30   involved in these neuroprotective effects, which could provide novel ideas for developing

31   targeted neuroprotective treatments for PD. We used the ETC complex I inhibitor

32   methylpyridinium ion (MPP$^+$) to induce cell death in SH-SY5Y cells as a cellular model for PD

33   and found that nicotine inhibits cell death. Using choline as a nicotinic acetylcholine receptor

34   (nAChR) agonist, we found that nAChR stimulation was sufficient to protect SH-SY5Y cells

35   against cell death from MPP$^+$. Blocking α7 nAChR with methyllycaconitine (MLA) prevented

36   the protective effects of nicotine, demonstrating that these receptors are necessary for the

37   neuroprotective effects of nicotine. The neuroprotective effect of nicotine involves other

38   pathways relevant to PD. Cleaved Poly (ADP-ribose) polymerase-1 (PARP-1) and cleaved

39   caspase-3 were decreased by nicotine in 6-hydroxydopamine (6-OHDA) lesioned mice and in

40   MPP$^+$-treated SH-SY5Y cells. In conclusion, our data indicate that nicotine likely exerts

41   neuroprotective effects in PD through the α7 nAChR and downstream pathways including

42   PARP-1 and caspase-3. This knowledge could be pursued in future research to develop

43   neuroprotective treatments for PD.

Introduction

45

46   Parkinson's disease (PD) is a neurodegenerative disorder affecting the nigrostriatal

47   dopamine tract that regulates the initiation and fluency of voluntary movement. Patients present

48   with a characteristic set of neurological symptoms that include tremor, muscle rigidity,

49   bradykinesia, stooped posture, shuffling gait and a lack of facial expression (Magrinelli et al.

50   2016). In the advanced stages, patients may also develop a subcortical dementia and a variety of

51   neuropsychiatric symptoms (Sveinbjornsdottir 2016). Symptoms can be alleviated temporarily

52   with L-dopa and carbidopa but this does not alter the progression of the illness or the death of

53   nigrostriatal neurons (Connolly & Lang 2014).

54   Although smoking cigarettes has well documented adverse health effects including lung

55   cancer, and cardiovascular disease, smokers are less likely to develop PD (Ascherio &

56   Schwarzschild 2016; Baron 1996; Breckenridge et al. 2016; Hernan et al. 2002; Polito et al. 2016;

57   Ritz et al. 2007). This is also true for passive exposure to second-hand smoke (Searles Nielsen et

58   al. 2012) or chewing tobacco (O'Reilly et al. 2005), and appears to be dose-dependent (Thacker

59   et al. 2007). The mechanisms underlying the potential neuroprotective effects of tobacco

60   exposure remain unclear, but hypotheses include: (1) interactions between the dopamine and

61   acetylcholine neurotransmitter systems, (2) reduction of oxidative stress, (3) modulation of

62   neuroinflammation, and (4) non-specific cognitive enhancing effects (Barreto et al. 2014).

63   Although nicotine is the most well-known component of tobacco, cotinine and other metabolites

64   may also play a role (Barreto et al. 2014).

65   Animal studies have provided useful insights into potential mechanisms for the protective

66   effects of tobacco in PD. Lesioning cholinergic neurons in the pedunculopontine nucleus resulted

67   in loss of substantia nigra dopaminergic neurons (Bensaid et al. 2016), showing that

68   physiological levels of acetylcholine are required for survival of nigrostriatal dopaminergic

69   neurons. In rats with 6-hydroxydopamine (6-OHDA) lesions of the medial forebrain bundle,

70   nicotine or the α7 nAChR agonist ABT-107 improved neurological functioning in conjunction

71   with restoring dopamine transporter levels and dopamine release (Bordia et al. 2015). Treatment

72   with a different α7 nAChR agonist 3-[(2,4-dimethoxy)benzylidene]-anabaseine dihydrochloride

73   (DMXBA) or nicotine also protects dopamine neurons in rats injected with 6-OHDA (Costa et al.

74   2001; Suzuki et al. 2013). Blocking nAChR with chlorisondamine prevents the protective effects

75   of nicotine *in vivo*. Another common animal model for PD relies on 1-methyl-4-phenyl-1,2,3,6-

76   tetrahydropyridine (MPTP) that is toxic to nigrostriatal dopamine neurons. Both cigarette smoke

77   and nicotine increased the survival of these neurons in MPTP mice (Parain et al. 2003). Similar

78   results have been reported in non-human primates exposed to MPTP (Quik et al. 2006), and in

79   mice models of PD using methamphetamine to induce dopamine neuron toxicity (Maggio et al.

80   1997). Nicotine increases the levels of fibroblast growth factor-2 (FGF-2) and the brain-derived

81   neurotrophic factor in rat striatum in these models, which could be one mechanism for

82   neuroprotection (Mudo et al. 2007).

83   Cellular model systems have also been used to investigate specific pathways through

84   which nicotine and other tobacco constituents could protect neurons in PD. Using cultured

85   mouse ventral midbrain neurons that included dopaminergic neurons, one group used

86   tunicamycin as an endoplasmic reticulum stressor and found that nicotine, at levels comparable

87   to those achieved through smoking cigarettes, attenuated the unfolded protein response

88   (Srinivasan et al. 2016). There is also evidence that dopamine release can be regulated by

89   presynaptic nAChR in rat brain slices (Giorguieff-Chesselet et al. 1979), and mouse striatal

90   synaptosomes (Grady et al. 1992; Rapier et al. 1990).

91   Poly (ADP-ribose) polymerase-1 (PARP-1) and caspase have both been implicated in the

92   pathophysiology or etiology of PD. PARP-1 is a DNA-damage sensor that is activated in some

93   PD models such as the MPTP mouse (Wang et al. 2003), and inhibiting PARP-1 reduced

94   dopamine neuron death from MPTP (Iwashita et al. 2004), alpha synuclein and MPP+ (Outeiro

95   et al. 2007). PARP-1 also mediates dopamine neuron degeneration in the 6-OHDA mouse PD

96   model (Kim et al. 2013). Mutations in PARP-1 protect against mitochondrial dysfunction and

97   neurodegeneration in mouse models of PD with mutations in the Parkin gene (Lehmann et al.

98   2016), and in human clinical populations (Infante et al. 2007).

99   Caspase-3 has been implicated in cleavage of a proapoptotic kinase protein kinase C delta

100   (PKCdelta) that mediates neuron death in both MPP+ and 6-OHDA cellular PD models (da

101   Costa et al. 2003; Kanthasamy et al. 2006; Shimoke & Chiba 2001). There is also evidence that

102   caspase-1 activation is the final step in apoptotic cell death in PD (Hartmann et al. 2000; Tatton

103   2000). Acteoside binding to caspase-3 is neuroprotective in the rotenone rat PD model (Yuan et

104   al. 2016), and caspase-3 activation has been observed to be important in a number of pathways

105   related to PD (Shukla et al. 2014; Zawada et al. 2015). Genetic disruption of caspase-3 is also

106   protective against the effects of MPTP (Yamada et al. 2010). To our knowledge, there have not

107   been attempts to investigate whether the neuroprotective effects of nicotine involve PARP-1 or

108   caspase.

109   In summary, there is evidence that nicotinic cholinergic drugs may delay progression of

110   PD (Perez 2015), and thus, α7 nAChR have been proposed as a target for new medications to

111   treat PD (Quik et al. 2015). However, since the mechanisms underlying the neuroprotective

112   effects remain unclear, we sought to further investigate the role of the α7 nicotinic acetylcholine

113   receptor (α7 nAChR) in mediating the protective effects of nicotine in PD. We used the ETC

114   complex I inhibitor methylpyridinium ion (MPP$^+$) to induce cell death in SH-SY5Y cells as a

115   cellular model for PD and used 6-hydroxydopamine (6-OHDA) lesions as a mouse model for PD.

116   We investigated the potential involvement of PD-related molecules PARP-1 and caspase in both

117   of these model systems.

118

119   **Materials and Methods**

120   **Cell culture and treatment**

121   SH-SY5Y cells are derived from a human neuroblastoma and are often used as a cellular model

122   for PD because the express tyrosine hydroxylase, dopamine-beta-hydroxylase, and the dopamine

123   transporter.(Xie et al. 2010) SH-SY5Y cells (American Type Culture Collection (ATCC),

124   Manassas, VA) were maintained as a monolayer in Dulbecco's Modified Eagle Medium

125   (DMEM) (Gibco, ON, Canada) with 10% fetal bovine serum (Gibco, ON, Canada), 100 U/ml

126   penicillin (Sigma-Aldrich, Oakville, ON, Canada), and 100 U/ml streptomycin (Sigma-Aldrich,

127   Oakville, ON, Canada). Cells were cultured in a humidified atmosphere of 5% CO_2, at 37°C. All

128   cells were cultured in 100-mm (diameter) cell culture plates (BD Biosciences, ON, Canada) until

129   ~80% confluence and then seeded into 24-well plates (BD Bioscience, ON, Canada) to achieve

130   ~90% confluence 24-28 hours prior to treatment. The medium was replaced by DMEM without

131   fetal bovine serum 12 hours before treatments.

132   **Drugs**

133   MPP^+ (methylpyridinium ion) was purchased as MPP^+ iodide from Sigma-Aldrich, dissolved in

134   water to a stock concentration of 500 mM, and wrapped with foil to protect from light. Choline,

135   nicotine and methyllycaconitine (MLA) were purchased from Tocris Bioscience. Nicotine was

136   used at a concentration of 2 mM for *in vitro* experiments based on previous reports (Ke et al.

137   1998; Wang et al. 2011). We used MLA at a concentration of 20 μM based on a previous report

138   that MLA at 5 μM and 10 μM could alleviate amyloid-β peptide-induced cytotoxicity in SH-

139   SY5Y cells, without affecting cell viability (Zheng et al. 2014). At 20 μM, MLA could

140   theoretically interact with α4β2 and α6β2 receptors, but no α4 and α6 receptor subunit mRNA

141   was detected in SH-SY5Y cells (Gould et al. 1992; Lukas et al. 1993). The α7 acetylcholine

142   receptor subunit has good expression levels in SH-SY5Y cells (Peng et al. 1994).

143   **Propidium iodide (PI) and Hoechst33342 staining**

144   Cultured SH-SY5Y cells were gently rinsed with phosphate-buffered saline (PBS) (pre-warmed

145   in 37℃) twice, incubated with 50 μg/ml PI (Invitrogen, Carlsbad, CA) or double labeling of

146   Hoechst 33342 (20 μg/ml) (Invitrogen, Carlsbad, CA) and PI for 30 min, and then rinsed three

147   times with PBS. Fluorescent intensity was measured by a plate reader (Victor 3; Pekin-Elmer,

148   Waltham, MA). The level of cell death was defined as the ration of PI: Hoechst 33342. The

149   fraction of dead cells was normalized to the cell toxicity that occurred in the control group.

150   **Protein extraction**

151   Striatial tissues were dissected from mice in 6-OHDA exposure models. Striata were

152   homogenized in ice cold buffer containing (in mmol/L): 50 Tris-Cl, pH 7.4, 150 NaCl, 2 EDTA,

153   1 PMSF plus 1% Igepal CA-630, 0.5–1% sodium deoxycholate, 1% Triton X-100 and protease

154   inhibitor mixture (5 μL/100 mg of tissue; Sigma-Aldrich, Okaville, ON, Canada) on ice and

155   shaken at 4ºC for 1 hour. Striatal tissues dissolved in the lysis buffer was centrifuged at 12,000 g

156   for 10 minutes at 4ºC to yield the total protein extract in the supernatant. The concentration of

157   protein was measured with the BCA protein assay kit (Pierce Protein Biology, ON, Canada).

158   Equal amounts of samples (50~100 μg) were denatured and subjected to 10% SDS-PAGE and

159   Western blot analyses.

160   **Gel electrophoresis and Western blot analyses**

161   Samples were separated using SDS-PAGE with 10% separating gel and 5% stacking gel, and

162   transferred to a nitrocellulose membrane after gel electrophoresis. After blocking for 1 hour with

163   5% fat-free milk powder in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH7.4), blots

164   were incubated overnight at 4°C with primary antibodies: 1:200 anti-PARP-1 (Santa Cruz

165   Biotechnology, Dallas, Texas), 1:10,000 anti-α-Tubulin (Sigma-Aldrich) and 1:200 anti-caspase-

166   3 (Santa Cruz Biotechnology, Dallas, Texas). After washes, blots were incubated with HRP-

167   conjugated secondary antibodies (Sigma-Aldrich, Okaville, ON, Canada) for 2 hours at room

168   temperature. Immunoactivity was visualized with ECL Western blot detection reagents (GE

169   Healthcare). Data representative of three experimental replicates are shown.

170   **Unilateral 6-OHDA lesions and nicotine administration**

171   The animal studies were approved by the University Animal Care Committee (UACC) at the

172   University of Toronto in accordance with the Canadian Council on Animal Care (CCAC)

173   guidelines (IRB approval number 20010879). Surgeries were performed as previously described

174   (Thiele et al. 2011; Thiele et al. 2012). In brief, 30 minutes prior to surgery, a mixture of

175   desipramine hydrochloride (25 mg/kg, Sigma Aldrich) and pargyline hydrochloride (5 mg/kg,

176   Sigma Aldrich) in 0.9% sterile saline (pH 7.4) was systemically administered intra-peritoneally

177   (i.p.). C57Bl/6J mice (P35, 24-28 g) were anaesthetised (isoflurane (Abbott), 2-3%) and placed

178   in a stereotaxic frame (David Kopf Instruments, USA). 6-hydroxydopamine (6-OHDA) (15

179   μg/μl, 0.02% ascorbic acid, w/v in 0.9% saline) or vehicle was unilaterally injected into the

180   medial forebrain bundle (MFB) at a rate of 0.1 μl/min (total delivery of 3 μg total, as a 0.2 μl

181   bolus) at the following coordinates: AP: -1.2 mm, ML: -1.1 mm, and DV: -5.0 mm (Franklin

182   K.B.J. 2007). This protocol results in a >95% dopamine depletion of the SNc (Thiele et al. 2011;

183   Thiele et al. 2012).

184   Seven days prior to 6-OHDA lesion surgeries, animals were given nicotine or saline

185   control by subcutaneous injection (s.c.) (MP Biomedicals, LLC) twice daily for two weeks. For

186   the first 3 days animals received a dose of 0.4 mg/kg, which was then increased to 0.8 mg/kg for

187   four days prior to surgery. This dose was continued for one week post-surgery until subjects

188   were sacrificed for tissue collection.

189

190   **Statistical analysis**

191   Levene's homogeneity test or F test was used to compare the variances between groups. For

192   equal variances, data were analyzed either by t-test, one-way analysis of variance (ANOVA)

193   followed by Tukey's test, or two-way analysis of variance (ANOVA) followed by Bonferroni or

194   Tukey's post-tests (SPSS Statistics, I.B.M Corporation, USA). For groups with unequal variance,

195   data were analyzed either with a t-test with Welch's correction, a one-way analysis of variance

196   (ANOVA), or two-way ANOVA, followed by Dunnett's *post hoc* test. Data are expressed as

197   mean ± standard error of mean (SEM). The significance levels of $p < 0.05$, $p < 0.01$, or $p < 0.001$

198   were used for all analyses.

199

200   **Results**

201   **Nicotine inhibits MPP$^+$-induced SH-SY5Y cell death.**

202   Tobacco exposure is associated with decreased risk for PD(O'Reilly et al. 2005; Ritz et al.

203   2007; Searles Nielsen et al. 2012) and nicotine is the most prominent psychoactive component of

204   tobacco. Thus, we first investigated if nicotine could protect against cell death in a cellular model

205   of PD: MPP$^+$-induced SH-SY5Y cell death. As shown in Figure 1A, using propidium iodide (PI)

206   staining, MPP$^+$ treatment (500 µM, 24 hours) induced more SH-SY5Y cell death compared to

207   control cells (control: 1.00 ± 0.099; MPP$^+$: 1.40 ± 0.086; t-test $p<0.05$). Pre-treatment with nicotine

208   (2 mM, 30 min) prior to MPP$^+$ treatment, decreased the level of cell death, as compared cells

209   treated with MPP$^+$ alone (MPP$^+$: 1.40 ± 0.086; MPP$^+$ with nicotine: 0.88 ± 0.068; two-way

210   ANOVA followed by Bonferroni post-tests $p<0.05$: figure 1B). These data show that nicotine

211   can inhibit MPP$^+$-induced SH-SY5Y cell death.

212

213   **nAChR is involved in the protective effect of nicotine**

214   To determine whether the protective effects of nicotine are mediated by activation of the

215   nAChR, we investigated if activation of nAChR without using nicotine, inhibits MPP$^+$-induced

216   SH-SY5Y cell death. Nicotine is an nAChR agonist and previous studies have shown that other

217   nAChR agonists can protect against nigrostriatal dopamine neuron damage in PD animal models

218   (Dajas et al. 2001; Janson et al. 1988; Maggio et al. 1998; Mudo et al. 2007). As shown in Figure

219   2, choline (1 mM, 30 min), a nAChR specific agonist, decreased the level of cell death, when

220   administered prior to MPP$^+$ treatment (Control: 1.00 ± 0.099; Choline: 0.98 ± 0.10; MPP+:

221   1.40 ± 0.086; MPP$^+$ with choline: 0.81 ± 0.12; two-way ANOVA followed by Bonferroni post-tests

222   $p<0.05$). These results indicate that activation of nAChR prevents SH-SY5Y cells from MPP$^+$-

223   induced cell death and suggest that nAChR activation is sufficient to protect SH-SY5Y cells against

224   MPP$^+$-induced death.

225   **α7 nAChR mediates the protective effect of nicotine against MPP$^+$-induced SH-SY5Y cell**

226   **death**

227   We hypothesized that the α7 nAChR could be the receptor through which nicotine has

228   neuroprotective effects in PD. Activation of the α7 nAChR has protective effects in other

229   neurodegenerative disorders, and in Alzheimer's disease models (Fan et al. 2015; Hu et al. 2015;

230   Shen & Wu 2015). To confirm if α7 nAChR mediates the effect of nicotine to protect against

231   MPP$^+$-induced cell death, we pre-treated SH-SY5Y cells with methyllycaconitine (MLA) (20

232   μM, 30 min), a α7 nAChR specific antagonist, followed by MPP$^+$ as above and either nicotine (2

233   mM, 30 min) or choline (1 mM, 30 min).. As shown in Figure 3A, MLA treatment increased the

234   level of cell death when administered prior to nicotine and MPP$^+$ treatments, as compared to

235   those treated with nicotine and MPP$^+$ alone (control: 1.00±0.053; MPP$^+$: 1.70±0.119; MPP$^+$ with

236   nicotine: 1.37±0.0351; MPP$^+$ with nicotine and MLA: 1.81±0.0628; one-way ANOVA followed

237   by Tukey's test, $p<0.05$). Similarly, figure 3B shows that choline can reduce the cell death

238   induced by MPP$^+$ and this effect is blocked by MLA (Control: 1.00±0.053; MPP$^+$: 1.70±0.119;

239   MPP$^+$ with choline: 1.34±0.0197; MPP$^+$ with choline and MLA: 1.85±0.0796; one-way ANOVA

240   followed by Tukey's test, $p<0.05$). These data indicate that α7 nAChR signaling is necessary for

241   the neuroprotective effect of nicotine.

242

243   **Nicotine pre-treatment inhibits PARP-1 and caspase-3 cleavage in MPP$^+$-treated SH-SY5Y**

244   **neuroblastoma cells**

245   To investigate potential mechanisms mediating the effect of nicotine in the MPP$^+$ SH-

246   SY5Y cellular model of PD, we examined PARP-1 and caspase-3 cleavage with and without

247   nicotine. As shown in Figure 4, nicotine pre-treatment inhibits the cleavage of caspase-3 (p<0.01,

248   two-way ANOVA; Control: 1.00±0; nicotine: 1.05±0.078; MPP$^+$: 1.27±0.026; MPP$^+$ with

249   nicotine: 0.933±0.073) and PARP-1 (p<0.001, two-way ANOVA; Control: 1.00±0; nicotine:

250   0.919±0.054; MPP$^+$: 1.17±0.022; MPP$^+$ with nicotine: 0.919±0.022), compared to the control

251   cells, using α-Tubulin as the loading control against which the other proteins were normalized.

252   **The neuroprotective effect of nicotine is associated with decreased PARP-1 and caspase-3**

253   **cleavage**

254   To expand on the *in vitro* results above, we performed unilateral 6-hydroxydopamine

255   lesions in mice as an *in vivo* model of PD. We first confirmed that the 6-OHDA lesion was

256   causing the expected death of dopamine neurons and that nicotine had a neuroprotective effect *in*

257   *vivo*, by measuring the amount of tyrosine hydroxylase as a proxy for dopamine neuron survival.

258   Tyrosine hydroxylase is the rate limiting enzyme in the synthesis of catecholamines including

259   dopamine. Figure 5 shows that the lesioned hemisphere of the brain has lost approximately half

260   of the TH-containing neurons, while nicotine treatment protected almost all of these neurons

261   from death (p<0.01, two-way ANOVA, non-lesioned side in 6-OHDA mice: 1.00±0.070; non-

262   lesioned side in 6-OHDA mice with nicotine: 1.08±0.051; lesioned side in 6-OHDA mice:

263   0.49±0.19; lesioned side in 6-OHDA mice with nicotine: 1.06±0.061).

264   To investigate potential mechanisms underlying this neuroprotective effect of nicotine,

265   we measured the expression of Poly [ADP-ribose] polymerase 1(PARP-1) and caspase-3 using

266   Western blots. We analyzed protein from solubilized striatal tissue of mice exposed to 6-

267   hydroxydopamine (6-OHDA) with or without nicotine. Both cleaved PARP-1 (6-OHDA:

268   1.00±0.54; Nicotine+6-OHDA: 0.740±0.022; t-test, p<0.05; figure 6A-C) and cleaved caspase-3

269   (6-OHDA: 1.00± 0.017; Nicotine+6-OHDA: 0.718± 0.053; t=test, p<0.01; figure 6D-F) were

270   decreased by nicotine pre-treatment in 6-OHDA mice pretreated with nicotine. This indicates

271   that nicotine pre-treatment inhibits PARP-1 and caspase-3 cleavage in this PD mouse model. The

272   main cleaved PARP-1 fragment was 89 KDa in size (full length 116 kDa).

273   We also performed a control experiment to examine whether nicotine alone might alter

274   PARP-1 or caspase-3 cleavage, using Western blots to quantify the amount of the intact, full-

275   length protein vs. the cleaved form, in the unlesioned hemisphere of 6-OHDA mice. As shown in

276   figure 7, there is no significant effect of nicotine alone on the cleavage of these two proteins.

277   These data demonstrate that the neuroprotective effect of nicotine for dopamine neurons in PD

278   models is associated with PARP-1 and caspase-3 cleavage pathways.

279

280   **Discussion**

281   The data presented above demonstrate that nicotine inhibits MPP$^+$-induced SH-SY5Y cell

282   death through activating α7 nAChR, and inhibits PARP-1 and caspase-3 cleavage in the 6-

283   OHDA mouse model for PD. We first demonstrated that activation of nAChR with either

284   nicotine or choline is sufficient to protect SH-SY5Y cells from MPP$^+$ toxicity. Nicotine also

285   inhibits PARP-1 and caspase-3 cleavage in MPP$^+$-treated SH-SY5Y cells. We then showed that

286   α7 nAChR activation is necessary for these neuroprotective effects by using the α7 nAChR

287   antagonist methyllycaconitine, which reduces the number of cells rescued by nicotine. Finally,

288   we used the *in vivo* 6-OHDA mouse model for PD to demonstrate that nicotine inhibits PARP-1

289   and caspase-3 cleavage, suggesting a potential downstream molecular mechanism for

290   neuroprotection in PD.

291   This study provides additional knowledge of potential mechanisms to explain the clinical

292   phenomenon of reduced PD incidence in smokers and other people exposed to tobacco. Some

293   have suggested that people who become tobacco users may have an underlying trait that also

294   renders them less susceptible to PD (Barreto et al. 2014), a form of "reverse causation" rather

295   than nicotine actually being neuroprotective. One group found that PD patients are able to quit

296   smoking more easily than matched population controls (Ritz et al. 2014). However, there are two

297   main arguments against this interpretation. The first is that passive exposure to cigarette smoke is

298   also associated with a dose-dependent decreased risk for PD (Searles Nielsen et al. 2012), and

299   the second is the large body of data from experimental animal and cellular models (Barreto et al.

300   2014; Perez 2015).

301   The nAChRs are obvious potential starting points for the mechanism of neuroprotection

302   by nicotine in PD, and these receptors have been investigated in many other studies (Barreto et al.

303   2014; Perez 2015; Quik et al. 2015). α7nAChR agonists are also likely to have significant

304   impacts in PD via the regulation of the immune system and intestinal permeability (Anderson et

305   al. 2016). However, our study is unique in using SH-SY5Y cells exposed to MPP+ as an *in vitro*

306   model to investigate the neuroprotective effects of nicotine and nAChR activation. Also novel is

307   our attempt to investigate the effect of nicotine on PARP-1 and caspase-3 cleavage in the 6-

308   OHDA mouse model for PD. These elements provide insight into molecular mechanisms and

309   potential targets for developing new PD treatments.

310   Our results do not exclude the involvement of other neuroprotective mechanisms. Several

311   signaling pathways that promote cell survival are enhanced by stimulating nAChR, including the

312   Src family-PI3 K-AKT pathway, with subsequent upregulation of Bcl-2 and Bcl-x,

313   JAK2/STAT3 and MEK/ERK (Kawamata & Shimohama 2011). Nicotine can protect SH-SY5Y

314   cells from other types of insults, such as beta-amyloid toxicity, through Erk1/2-p38-JNK-

315   dependent signaling pathways (Xue et al. 2014). However, there are no published studies

316 investigating the role of PARP-1 or caspase in the neuroprotective effects of nicotine in PD

317 disease models.

318   Caspases are a family of proteases that are activated during apopototic cell death

319 (Kroemer & Martin 2005). There are twelve numbered caspases, some of which initiate or

320 execute apoptosis, but these enzymes also regulate inflammation and cell differentiation

321 (Galluzzi et al. 2016). Caspases are initially synthesized as an inactive pro-caspase, which must

322 undergo dimerization or oligomerization and then cleavage to become active (Shi 2004).

323 Caspases are involved in the pathophysiology of PD through mediating dopaminergic neuron

324 death from MPTP (Furuya et al. 2004; Qiao et al. 2016; Viswanath et al. 2001), promoting

325 synuclein aggregation (Wang et al. 2016), and cleaving Transactivation response DNA-binding

326 protein 43 (TDP-43), which is a primary component of Lewy bodies in PD (Kokoulina & Rohn

327 2010).

328   A number of neuroprotective compounds that have been studied in PD animal models

329 also affect caspases, such as telmisartan (an angiotensin II type 1 receptor blocker) (Tong et al.

330 2016), and nerve growth factor (NGF) (Shimoke & Chiba 2001). Directly blocking a caspase-3

331 cleavage site on the proapoptotic protein kinase C delta has neuroprotective effects in MPP+ and

332 6-OHDA PD models (Kanthasamy et al. 2006). Despite the prominence of caspases in neuronal

333 death in PD, they may not be viable targets for treatment since directly blocking caspase-8

334 resulted in a switch from apoptosis to necrosis (Hartmann et al. 2001), and this may apply to

335 other caspases as well (Kroemer & Martin 2005). Modulating caspase function in PD through the

336 nicotinic receptors may be a better approach for developing new treatments.

337   PARP-1 enzymes are involved in a number of neurodegenerative disorders including

338 Alzheimer's disease and PD (Martire et al. 2015). PARP-1 has DNA binding domains that detect

339 DNA damage and facilitate repair. When PARP-1 levels are too high or when DNA damage is

340 too severe, cell death is initiated (Burkle 2001), and this decision is regulated by NAD+

341 depletion (Alano et al. 2010). During cell death programs, PARP-1 is cleaved into fragments that

342 are specific to different apoptotic pathways (Chaitanya et al. 2010). The 89KDa fragment we

343 detected appears during apoptosis, and could have been generated by the action of caspase-3,

344 caspase-7 (Lazebnik et al. 1994) or the lysosomal proteases cathepsin B or D (Gobeil et al. 2001).

345   Some PARP-1 genetic variants are protective against PD (Infante et al. 2007), and the

346 involvement of PARP-1 in PD pathophysiology includes regulation of alpha-synuclein

347 expression (Chiba-Falek et al. 2005), and modification of p53 in the MPTP model (Mandir et al.

348 2002). Small molecule inhibitors of PARP-1 reduce cell death induced by alpha-synuclein and

349 MPP+ (Outeiro et al. 2007), consistent with our results above. PARP-1 induced depletion of

350 NAD+ could also contribute to decreasing sirtuins and mitochondrial dysfunction in PD

351 (Anderson & Maes 2014).

352

353   In conclusion, we have demonstrated that the neuroprotective effects of nicotine in

354 animal and cellular models of PD is mediated by activation of α7 nAChR and the inhibition of

355 PARP-1 and caspase-3 cleavage. All of these molecules have been previously implicated in the

356 pathophysiology of PD, but until now, have not been linked together. This knowledge could be

357 used to aid development of novel treatments for PD, but further work to delineate the molecular

358 pathway linking α7 nAChR to PARP-1 and caspase-3 is required.

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379   **Figure legends:**

380   **Figure 1: Nicotine protects SH-SY5Y cells against MPP$^+$-induced cell death. A.** MPP$^+$

381   treatment (500 μM, 24 hrs) in SH-SY5Y cells increased the level of cell death, as compared to

382   control cells. *p<0.05 as compared to those in control group, n=5, t-test. **B.** Pretreatment of

383   nicotine (2 mM, 30 min) prior to MPP$^+$ exposure in SH-SY5Y cells decreased the level of cell

384   death as compared to those treated with MPP$^+$ only. *p<0.05 as compared to those of control

385   group, ##p<0.01 as compared to MPP$^+$ group, n=5 for control and MPP$^+$ groups, n=3 for nicotine

386   and MPP$^+$ with nicotine groups, two-way ANOVA followed by Bonferroni post-tests. All data

387   are shown as mean ± SEM. The level of cell death was detected using PI (50 μg/ml) and

388   Hoechst33342 (20 μg/ml) double staining, and was defined as the ratio of fluorescent intensity of

389   PI: Hoechst33342.

390

391   **Figure 2: Choline protects SH-SY5Y cells against MPP$^+$-induced cell death.** Pre-treatment

392   with choline (1 mM, 30 min), a specific nAChR agonist, followed by MPP$^+$ treatment (500 μM,

393   24 hrs) in SH-SY5Y cells decreased the level of cell death as compared to those treated with

394   MPP$^+$ only. *p<0.05 as compared to those of control group, ###p<0.001 as compared to MPP$^+$

395   group, n=5, two-way ANOVA followed by Bonferroni posttests. All data are shown as mean ±

396   SEM. The level of cell death was detected using PI and Hoechst33342 double staining, and was

397   defined as the ratio of fluorescent intensity of PI: Hoechst33342.

398

399   **Figure 3: Blockade of α7 nAChR inhibits the protective effect of nicotine and choline**

400   **against MPP$^+$-induced SH-SY5Y cell death. A.** MLA (20 μM, 30 min), a specific antagonist of

401   α7 nAChR, increased the level of cell death when administered prior to nicotine (2 mM, 30 min)

402   and MPP$^+$ (500 μM, 24 hrs) treatments in SH-SY5Y cells, as compared to those treated with

403   nicotine and MPP$^+$ alone. ***p<0.001 as compared to those of control group, # p<0.05 as

404   compared to MPP$^+$ group, n=4, one-way ANOVA followed by Tukey's test. **B.** MLA (20 μM, 30

405   min), a specific antagonist of α7 nAChR, increased the level of SH-SY5Y cell death when

406   administered prior to choline (1 mM, 30 min) and MPP$^+$ (500 μM, 24 hrs) treatments, as

407   compared to those treated with choline and MPP$^+$ alone. ***p<0.001 compared to the control

408   group, # p<0.05 as compared to the MPP+ group, n=4, one-way ANOVA followed by Tukey's

409   test. All data are shown as mean ± SEM. The level of cell death was detected using PI and

410   Hoechst33342 double staining, and was defined as the ratio of fluorescent intensity of PI:

411   Hoechst33342.

412

413   **Figure 4: Nicotine pre-treatment inhibits PARP-1 and caspase-3 cleavage in MPP⁺-treated**

414   **SH-SY5Y neuroblastoma cells. A.** Western blot analysis showing that cleaved caspase-3

415   decreased in SH-SY5Y cells pre-treated with nicotine before MPP⁺ treatment, as compared to

416   those treated with MPP⁺ only. α-Tubulin was used as a loading control. **B and C.** Densitometric

417   analysis of expression levels of full length (**B**) and cleaved (**C**) caspase-3. The expression level

418   of caspase-3 was defined as the ratio of the intensity of caspase-3: α-Tubulin, and was

419   normalized as percentage of the control group, **p<0.01, n=3, two-way *ANOVA*. **D.** Western

420   blot analysis showing that cleaved PARP-1 was decreased in SH-SY5Y cells pre-treated with

421   nicotine before MPP⁺ treatment, as compared to those treated with MPP⁺ only. α-Tubulin was

422   used as a loading control. **E and F.** Densitometric analysis of the intensity of expression levels of

423   full length (**E**) and cleaved (**F**) PARP-1. The expression level of PARP-1 was defined as the ratio

424   of the intensity of PARP-1: α-Tubulin, and was normalized as percentage of the control group,

425   ***p<0.001, n=3, two-way *ANOVA*. All data are shown as mean ± SEM.

426

427   **Figure 5: Tyrosine Hydroxylase expression level decreased in lesioned hemisphere of 6-**

428   **OHDA mouse model of PD. A.** Western blot analysis showing that tyrosine hydroxylase

429   expression level decreased in striatal tissues of lesioned hemisphere from mice exposed to 6-

430   OHDA only, but did not change in mice pretreated with nicotine before 6-OHDA exposure. α-

431   Tubulin was used as a loading control. **B** Densitometric analysis of expression levels of tyrosine

432   hydroxylase. The expression level of tyrosine hydroxylase was defined as the ratio of the

433   intensity of tyrosine hydroxylase: α-Tubulin, and was normalized as percentage of non-lesioned

434   hemisphere exposed to 6-OHDA only, n=3, two-way *ANOVA*. All data are shown as mean ±

435   SEM. *p<0.05 as compared to Non-lesioned hemisphere in 6-OHDA mice

436

437   **Figure 6: Nicotine pre-treatment inhibits PARP-1 and caspase-3 cleavage in striatal tissue**

438   **from 6-OHDA mouse model of PD. A.** Western blot analysis showing that cleaved PARP-1

439   decreased in striatal tissues from mice pre-treated with nicotine before 6-OHDA exposure, as

440   compared to those exposed to 6-OHDA only. α-Tubulin was used as a loading control. **B and C.**

441   Densitometric analysis of expression levels of full length (**B**) and cleaved (**C**) PARP-1. The

442   expression level of PARP-1 was defined as the ratio of the intensity of PARP-1: α-Tubulin, and

443   was normalized as percentage of the 6-OHDA group, *p<0.05 compared to the 6-OHDA group,

444   n=3, t-test. **D.** Western blot analysis showing that cleaved caspase-3 was decreased in striatal

445   tissues from mice pre-treated with nicotine prior to 6-OHDA exposure, as compared to those

446   exposed to 6-OHDA only. α-Tubulin was used as a loading control. **E and F.** Densitometric

447   analysis of the intensity of expression levels of full length (**E**) and cleaved (**F**) caspase-3. The

448   expression level of caspase-3 was defined as the ratio of the intensity of caspase-3: α-Tubulin,

449   and was normalized as percentage of the 6-OHDA group, **p<0.01 compared to the 6-OHDA

450   group, n=3, t-test. All data are shown as mean ± SEM.

451

452   **Figure 7: Nicotine pre-treatment does not change PARP-1 and caspase-3 cleavage in**

453   **striatal tissue from non-lesioned hemisphere of 6-OHDA mouse model of PD. A.** Western

454   blot analysis showing no difference of cleaved caspase-3 in striatal tissues of non-lesioned

455   hemisphere from mice pre-treated with nicotine before 6-OHDA exposure, as compared to those

456   exposed to 6-OHDA only. α-Tubulin was used as a loading control. **B and C.** Densitometric

457 analysis of expression levels of full length (**B**) and cleaved (**C**) caspase-3. The expression level

458 of caspase-3 was defined as the ratio of the intensity of caspase-3: α-Tubulin, and was

459 normalized as percentage of the 6-OHDA group, n=3, t-test. **D.** Western blot analysis showing

460 no difference of cleaved PARP-1 was decreased in striatal tissues of the non-lesioned

461 hemisphere from mice pre-treated with nicotine prior to 6-OHDA exposure, as compared to

462 those exposed to 6-OHDA only. α-Tubulin was used as a loading control. **E and F.**

463 Densitometric analysis of the intensity of expression levels of full length (**E**) and cleaved (**F**)

464 PARP-1. The expression level of PARP-1 was defined as the ratio of the intensity of PARP-1: α-

465 Tubulin, and was normalized as percentage of the 6-OHDA group, n=3, t-test. All data are

466 shown as mean ± SEM.

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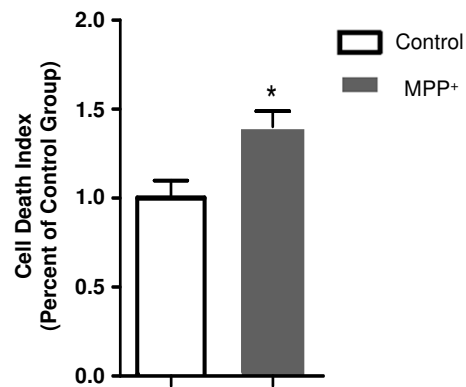
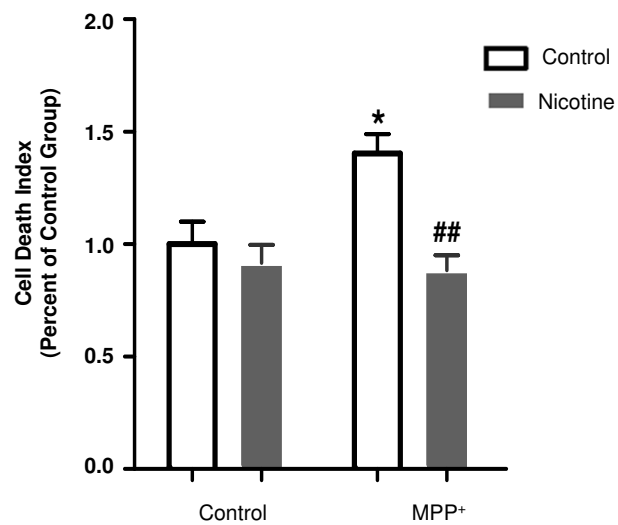
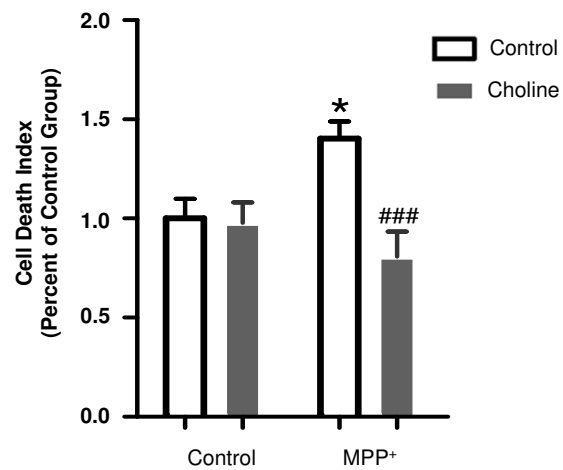
Nicotine protects SH-SY5Y cells against MPP$^+$-induced cell death

A. MPP$^+$ treatment (500 µM, 24 hrs) in SH-SY5Y cells increased the level of cell death, as compared to control cells. *p<0.05 as compared to those in control group, n=5, t-test. **B.** Pretreatment of nicotine (2 mM, 30 min) prior to MPP$^+$ exposure in SH-SY5Y cells decreased the level of cell death as compared to those treated with MPP$^+$ only. *p<0.05 as compared to those of control group, ##p<0.01 as compared to MPP$^+$ group, n=5 for control and MPP$^+$ groups, n=3 for nicotine and MPP$^+$ with nicotine groups, two-way ANOVA followed by Bonferroni post-tests. All data are shown as mean ± SEM. The level of cell death was detected using PI (50 µg/ml) and Hoechst33342 (20 µg/ml) double staining, and was defined as the ratio of fluorescent intensity of PI: Hoechst33342.

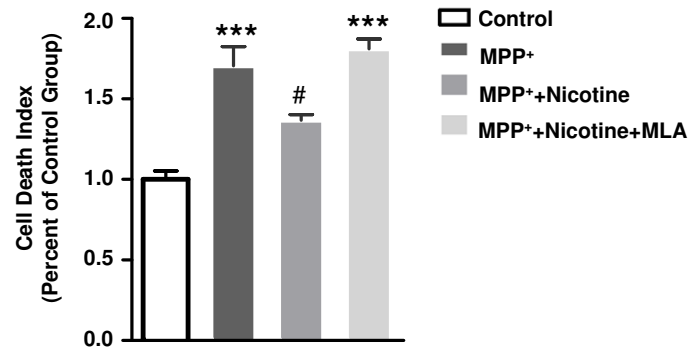
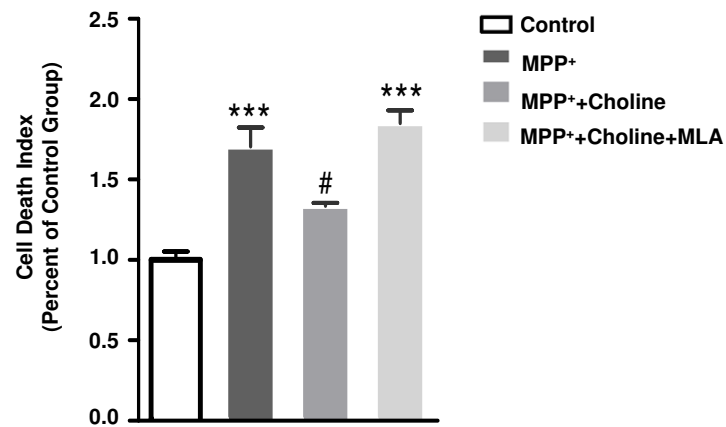
A

B

Choline protects SH-SY5Y cells against MPP⁺-induced cell death.

Pre-treatment with choline (1 mM, 30 min), a specific nAChR agonist, followed by MPP⁺ treatment (500 μM, 24 hrs) in SH-SY5Y cells decreased the level of cell death as compared to those treated with MPP⁺ only. $*p<0.05$ as compared to those of control group, $\#\#\#p<0.001$ as compared to MPP⁺ group, n=5, two-way ANOVA followed by Bonferroni posttests. All data are shown as mean ± SEM. The level of cell death was detected using PI and Hoechst33342 double staining, and was defined as the ratio of fluorescent intensity of PI: Hoechst33342.

Blockade of α7 nAChR inhibits the protective effect of nicotine and choline against MPP$^+$-induced SH-SY5Y cell death.

A. MLA (20 μM, 30 min), a specific antagonist of α7 nAChR, increased the level of cell death when administered prior to nicotine (2 mM, 30 min) and MPP$^+$ (500 μM, 24 hrs) treatments in SH-SY5Y cells, as compared to those treated with nicotine and MPP$^+$ alone. ***$p<0.001$ as compared to those of control group, # $p<0.05$ as compared to MPP$^+$ group, n=4, one-way ANOVA followed by Tukey's test. **B.** MLA (20 μM, 30 min), a specific antagonist of α7 nAChR, increased the level of SH-SY5Y cell death when administered prior to choline (1 mM, 30 min) and MPP$^+$ (500 μM, 24 hrs) treatments, as compared to those treated with choline and MPP$^+$ alone. ***$p<0.001$ compared to the control group, # $p<0.05$ as compared to the MPP+ group, n=4, one-way ANOVA followed by Tukey's test. All data are shown as mean ± SEM. The level of cell death was detected using PI and Hoechst33342 double staining, and was defined as the ratio of fluorescent intensity of PI: Hoechst33342.

A

B

Nicotine pre-treatment inhibits PARP-1 and caspase-3 cleavage in MPP⁺-treated SH-SY5Y neuroblastoma cells

A. Western blot analysis showing that cleaved caspase-3 decreased in SH-SY5Y cells pre-treated with nicotine before MPP⁺ treatment, as compared to those treated with MPP⁺ only. α-Tubulin was used as a loading control. **B and C.** Densitometric analysis of expression levels of full length (**B**) and cleaved (**C**) caspase-3. The expression level of caspase-3 was defined as the ratio of the intensity of caspase-3: α-Tubulin, and was normalized as percentage of the control group, **p<0.01, n=3, two-way *ANOVA*. **D.** Western blot analysis showing that cleaved PARP-1 was decreased in SH-SY5Y cells pre-treated with nicotine before MPP⁺ treatment, as compared to those treated with MPP⁺ only. α-Tubulin was used as a loading control. **E and F.** Densitometric analysis of the intensity of expression levels of full length (**E**) and cleaved (**F**) PARP-1. The expression level of PARP-1 was defined as the ratio of the intensity of PARP-1: α-Tubulin, and was normalized as percentage of the control group, ***p<0.001, n=3, two-way *ANOVA*. All data are shown as mean ± SEM. Western blot analysis showing that cleaved caspase-3 decreased in SH-SY5Y cells pre-treated with nicotine before MPP⁺ treatment, as compared to those treated with MPP⁺ only. α-Tubulin was used as a loading control. **B and C.** Densitometric analysis of expression levels of full length (**B**) and cleaved (**C**) caspase-3. The expression level of caspase-3 was defined as the ratio of the intensity of caspase-3: α-Tubulin, and was normalized as percentage of the control group, **p<0.01, n=3, two-way *ANOVA*. **D.** Western blot analysis showing that cleaved PARP-1 was decreased in SH-SY5Y cells pre-treated with nicotine before MPP⁺ treatment, as compared to those treated with MPP⁺ only. α-Tubulin was used as a loading control. **E and F.** Densitometric analysis of the intensity of expression levels of full length (**E**) and cleaved (**F**) PARP-1. The expression level of PARP-1 was defined as the ratio of the intensity of PARP-1: α-Tubulin, and was normalized as percentage of the control group, ***p<0.001, n=3, two-way *ANOVA*. All data are shown as mean ± SEM.

A

Anti-Caspase-3

Full-length ➤ ◄ 35 kDa

Cleaved ➤ ◄ 17 kDa

Anti-α-Tubulin ◄ 50 kDa

Control Nicotine MPP⁺ Nicotine +MPP⁺

B

☐ control ■ nicotine

Full length Caspase-3 / α-Tubulin (Percent of Control Group)

Control   MPP⁺

C

☐ control ■ nicotine

Cleaved Caspase-3 / α-Tubulin (Percent of Control Group)

**

Control   MPP⁺

D

Anti-PARP-1

Full-length ➤ ◄ 113 kDa
Cleaved ➤ ◄ 89 kDa

Anti-α-Tubulin ◄ 50 kDa

Control Nicotine MPP⁺ Nicotine +MPP⁺

E

☐ control ■ nicotine

Full length PARP-1 / α-Tubulin (Percent of Control Group)

Control   MPP⁺

F

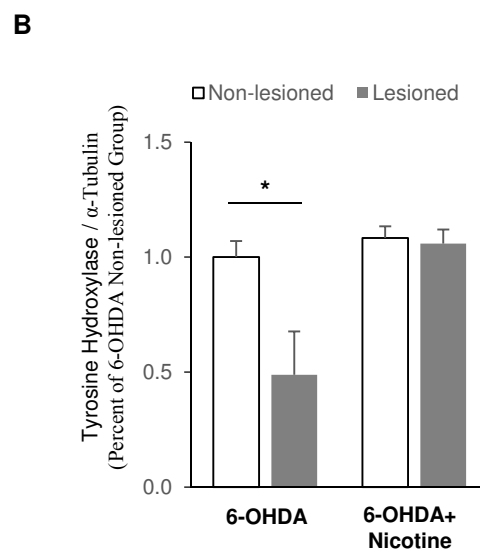
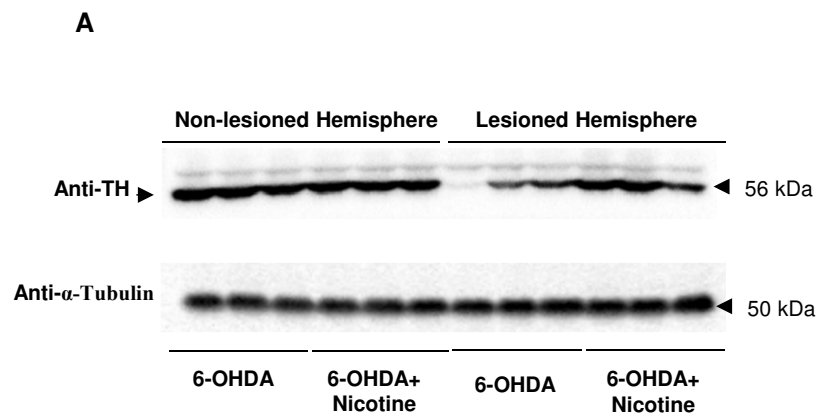
☐ control ■ nicotine

Cleaved PARP-1 / α-Tubulin (Percent of Control Group)

***

Control   MPP⁺

Tyrosine Hydroxylase expression level decreased in lesioned hemisphere of 6-OHDA mouse model of PD

A. Western blot analysis showing that tyrosine hydroxylase expression level decreased in striatal tissues of lesioned hemisphere from mice exposed to 6-OHDA only, but did not change in mice pretreated with nicotine before 6-OHDA exposure. α-Tubulin was used as a loading control. **B** Densitometric analysis of expression levels of tyrosine hydroxylase. The expression level of tyrosine hydroxylase was defined as the ratio of the intensity of tyrosine hydroxylase: α-Tubulin, and was normalized as percentage of non-lesioned hemisphere exposed to 6-OHDA only, n=3, two-way *ANOVA*. All data are shown as mean ± SEM. *$p<0.05$ as compared to Non-lesioned hemisphere in 6-OHDA mice.

A

	Non-lesioned Hemisphere	Lesioned Hemisphere

Anti-TH ▶ ◀ 56 kDa

Anti-α-Tubulin ◀ 50 kDa

6-OHDA   6-OHDA+Nicotine   6-OHDA   6-OHDA+Nicotine

B

☐ Non-lesioned   ■ Lesioned

Nicotine pre-treatment inhibits PARP-1 and caspase-3 cleavage in striatal tissue from 6-OHDA mouse model of PD

A. Western blot analysis showing that cleaved PARP-1 decreased in striatal tissues from mice pre-treated with nicotine before 6-OHDA exposure, as compared to those exposed to 6-OHDA only. α-Tubulin was used as a loading control. **B and C.** Densitometric analysis of expression levels of full length (**B**) and cleaved (**C**) PARP-1. The expression level of PARP-1 was defined as the ratio of the intensity of PARP-1: α-Tubulin, and was normalized as percentage of the 6-OHDA group, *p<0.05 compared to the 6-OHDA group, n=3, t-test. **D.** Western blot analysis showing that cleaved caspase-3 was decreased in striatal tissues from mice pre-treated with nicotine prior to 6-OHDA exposure, as compared to those exposed to 6-OHDA only. α-Tubulin was used as a loading control. **E and F.** Densitometric analysis of the intensity of expression levels of full length (**E**) and cleaved (**F**) caspase-3. The expression level of caspase-3 was defined as the ratio of the intensity of caspase-3: α-Tubulin, and was normalized as percentage of the 6-OHDA group, **p<0.01 compared to the 6-OHDA group, n=3, t-test. All data are shown as mean ± SEM.

A

Anti-PARP-1

Full length → ◄ 113 kDa

Cleaved → ◄ 89 kDa

Anti-α-Tubulin ◄ 50 kDa

6-OHDA   6-OHDA+Nicotine

B

☐ 6-OHDA
■ 6-OHDA+Nicotine

C

☐ 6-OHDA
■ 6-OHDA+Nicotine

*

D

Anti-Caspase-3

Full length → ◄ 35 kDa

Cleaved → ◄ 17 kDa

Anti-α-Tubulin ◄ 50 kDa

6-OHDA   6-OHDA+Nicotine

E

☐ 6-OHDA
■ 6-OHDA+Nicotine

F

☐ 6-OHDA
■ 6-OHDA+Nicotine

**

Nicotine pre-treatment does not change PARP-1 and caspase-3 cleavage in striatal tissue from non-lesioned hemisphere of 6-OHDA mouse model of PD

A. Western blot analysis showing no difference of cleaved caspase-3 in striatal tissues of non-lesioned hemisphere from mice pre-treated with nicotine before 6-OHDA exposure, as compared to those exposed to 6-OHDA only. α-Tubulin was used as a loading control. **B and C.** Densitometric analysis of expression levels of full length (**B**) and cleaved (**C**) caspase-3. The expression level of caspase-3 was defined as the ratio of the intensity of caspase-3: α-Tubulin, and was normalized as percentage of the 6-OHDA group, n=3, t-test. **D.** Western blot analysis showing no difference of cleaved PARP-1 was decreased in striatal tissues of the non-lesioned hemisphere from mice pre-treated with nicotine prior to 6-OHDA exposure, as compared to those exposed to 6-OHDA only. α-Tubulin was used as a loading control. **E and F.** Densitometric analysis of the intensity of expression levels of full length (**E**) and cleaved (**F**) PARP-1. The expression level of PARP-1 was defined as the ratio of the intensity of PARP-1: α-Tubulin, and was normalized as percentage of the 6-OHDA group, n=3, t-test. All data are shown as mean ± SEM.

A

Anti-Caspase-3   **Non-lesioned Hemisphere**

Full-length ➤   ◀ 35 kDa
Cleaved ➤   ◀ 17 kDa

Anti-α-Tubulin   ◀ 50 kDa

6-OHDA   **6-OHDA+ Nicotine**

B

C

D

Anti-PARP-1   **Non-lesioned Hemisphere**

Full-length ➤   ◀ 113 kDa

Cleaved ➤   ◀ 89 kDa

Anti-α-Tubulin   ◀ 50 kDa

6-OHDA   **6-OHDA+ Nicotine**

E

F