

行政院國家科學委員會專題研究計畫 成果報告

Nicotine 與 Deprenyl 穿透血腦屏障與其抗巴金森氏症機制之探討 研究成果報告(精簡版)

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Introduction

Parkinson's disease (PD) is a progressive disease and is related to the degeneration of nigrostriatal dopaminergic neurons. The cause of PD is still unclear and seems to be multifactorial. To date, several candidate genes, including the parkin gene (Kitada et al., 1998), α -synuclein gene (Polymeropoulos et al., 1997), DJ-1 gene (Bonifati et al., 2003), UCHL1 gene (Wintermeyer et al., 2000), mitochondrial complex I genes (Kösel et al., 1998), mitochondrial t-RNA genes (Grasbon-Frodl et al., 1999), MAOB gene (Kurth et al., 1993), and COMT gene (Kunugi et al., 1997), have been associated with the development of PD. While genes can be causative factors for PD, environmental factors can also be important.

Exposure to the chemical, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), can produce parkinsonian syndrome (Langston et al., 1983). While MPTP has become an important neurotoxin in establishing animal models of PD, neuronal uptake of its toxic metabolite, N-methyl-4-phenylpyridine (MPP^+), or of structural analogs is the major determinant (Javitch et al., 1985). The fact that PD can be caused by MPTP suggests that a similar neurotoxin may cause PD. In this regard, isoquinoline analogs, such as 1,2,3,4-tetrahydroisoquinoline (TIQ) and N-methyl-salsolinol, may be important (Yoshida et al., 1990; Niwa et al., 1993; Naoi et al., 1996). On the other hand, an epidemiological study has revealed an inverse relationship between cigarette smoking and PD (Liou et al., 1997) and nicotine may provide neuroprotection for the substantia nigra.

Nicotine-induced neuroprotection might be due to antioxidative effects (Obata et al., 2002), preservation of mitochondrial function (Cormier et al., 2003), and/or a selective increase in fibroblast growth factor (FGF)-2 mRNA levels in the striatum (Maggio et al., 1998). In addition to its pharmacological activities, nicotine (i.p.) may exert its neuroprotective action by decreasing striatal MPP^+ levels after subcutaneous administration of MPTP (Quik and Di Monte 2001). Given the transfer of nicotine or MPTP/ MPP^+ across the blood-brain barrier can be the prerequisite for these substances to reach brain tissue, the interaction between nicotine and MPTP/ MPP^+ merits an attention. In the present study, transfected immortalized adult rat brain microvascular endothelial cells (ARBEC) were used to study the uptake of MPTP and MPP^+ in the presence of nicotine and a variety of compounds or inhibitors. The mechanisms of the interaction between MPTP or MPP^+ and nicotine were also explored. In addition, the inhibitory effect of nicotine on MPTP transfer to brain extracellular fluid was investigated using an in vivo brain microdialysis technique.

Materials and Methods

Chemicals and reagents

³H-MPP⁺ (73 Ci/mmol) and ³H-nicotine (67 Ci/mmol) were purchased from PerkinElmer Life Sciences Inc. (Boston, MA, USA) and ³H-MPTP (80 Ci/mmol) from American Radiolabeled Chemicals (St. Louis, MO, USA). MPP⁺, MPTP, salsolinol, N-methyl-salsolinol, tetraisoquinoline (TIQ), carnitine, dopamine, nicotine, cotinine, BME vitamin solution, BME amino acid solution, rat tail collagen (Type I), and peptone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Decynium22 (1,1'-diethyl-2,2'-cyanine iodide) was obtained from Acros Organics (Geel, Belgium). Fetal bovine serum, medium 199 (M199), and antibiotic/antimycotic solution were purchased from Gibco (Invitrogen Life Technologies, USA). Other chemicals were obtained from standard sources and were of the highest quality available.

Cultured cells

Clones of SV-40 transfected immortalized ARBEC (Garberg et al., 2005) were kindly provided by National Research Council of Canada, Ottawa. Additionally, ARBEC was characterized by the presence of ZO-1, occludin and claudin-1 (Yeh et al., 2007). The ARBEC were grown routinely at 37 °C under an atmosphere of 5% CO₂/95% air in M199 medium containing 10% fetal bovine serum, 0.05% peptone, 0.9% glucose, BME amino acids, BME vitamins, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of amphotericin B. The culture medium was changed every 2 days and the culture split every 4 days. For subculturing, the cells were removed enzymatically (0.25% trypsin/EDTA), split 1:20, and subcultured in T75 flasks. The cells were used in experiments 5 days after subculturing.

Cellular uptake study

ARBEC (2 x 10⁴ cell/cm²) were grown on collagen-coated 24-well polystyrene Nunclon multidishes (1.9 cm² culture area). The experiments were performed in ECF buffer (122 mM NaCl, 3 mM KCl, 25 mM NaHCO₃, 1.2 mM MgSO₄, 1.4 mM CaCl₂, 10 mM D-glucose, 0.4 mM K₂HPO₄, and 10 mM HEPES, pH 7.4). Initially, the cells were washed three times with 0.5 ml of ECF buffer at 37°C, then pre-incubated in 0.5 ml of ECF buffer for 20 min. The buffer was then aspirated and 0.2 ml of ECF buffer containing ³H-MPTP, ³H-MPP⁺, or ³H-nicotine, with or without inhibitors, was added and incubation continued for 30 sec in MPTP, 10 min in MPP⁺, and 20 sec in nicotine, respectively. The cells were washed three times with 1 ml of ice-cold ECF buffer and solubilized overnight at room temperature in 0.5 ml of 1% Triton X-100 and the released radioactive material measured by liquid scintillation counting using an

external standard method for quench correction. The protein content was determined using the DC protein assay (Bio-Rad) with bovine serum albumin as the standard.

To estimate the kinetic properties of uptake of MPTP, MPP⁺, and nicotine, cells were incubated with increasing concentrations of MPTP (0.02-200 μM), MPP⁺ (0.1-200 μM), or nicotine (0.02-200 μM) prepared using 0.02 μM ³H-MPTP, 0.1 μM ³H-MPP⁺, or 0.02 μM ³H-nicotine adjusted with the unlabeled substance to give the appropriate final concentrations. For inhibition studies, the cells were incubated with 0.02 μM ³H-MPTP, 0.1 μM ³H-MPP⁺, or 0.02 μM ³H-nicotine in the presence of 0.5 mM inhibitors (unlabelled MPTP, MPP⁺, salsolinol, N-methyl-salsolinol, TIQ, carnitine, dopamine, nicotine, or cotinine). In addition, decynium22 (10 μM) was also used to inhibit OCT to investigate its contribution to the uptake of MPTP, MPP⁺, and nicotine.

The mechanism of the effect of nicotine and deprenyl on the uptake of MPTP and MPP⁺ was investigated in the same type of concentration-dependent study except that uptake was measured in the presence of 0.05 mM, 0.3 mM or 0.5 mM nicotine or 0.1 mM and 0.5 mM deprenyl. Each experiment was repeated three times, each in triplicate.

RT-PCR

Total RNA was isolated from rat kidneys and ARBEC (about 5x10⁶ cells) by the acid phenol-guanidinium-chloroform method using TRIzol reagent (Invitrogen). First strand cDNA was synthesized from the total RNA using an oligo(dT)₁₂₋₁₈ primer and the ImProm-II reverse transcription system (Promega) according to the manufacturer's protocol. The primer sequences for the PCR amplification of rat OCT1 (slc22a1), OCT2 (slc22a2), and OCT3 (slc22a3) were: OCT1 primer set (sense, 5'-TGCAGACAGGTTTGGCCGTAA-3'; antisense, 5'-TCGAGGCCGCTATTGGGTAGA), OCT2 primer set (sense, 5'-GCTGGTTAATTGGCTACATCC-3', antisense, 5'-TTCTTGGCCTCTGCATATTC-3'); OCT3 primer set (sense, 5'-CCACCATCGTCAGCCAGTTT-3'; antisense, 5'-ACACGACACCCCTGCCACTA-3') (Friedrich et al. 2001). PCR was performed in a total volume of 25 μl containing Taq polymerase (2.5 U, Fermentas), MgCl₂ (1.5 mM), and dNTPs (200 μM each). The temperature program for OCT1 and OCT3 was denaturation (94°C, 5 min) followed by 30 cycles of 94°C for 60 sec, 54°C for 30 sec, and 72°C for 60 sec, followed by 72°C for 10 min. For OCT2, the annealing temperature was 50°C.

In vivo microdialysis study

Male Wistar rats (230-270 g) were purchased from the Animal Center of the College of Medicine, National Taiwan University and were housed in the animal quarters (air conditioning and a 12-h light/dark cycle). Rats were anesthetized by chloral hydrate (300 mg/kg i.p.) and the femoral vein cannulated with a PE50 tube for MPTP infusion. The rats were fixed on a stereotaxic instrument (Stoelting, Illinois, USA) and the location of the head adjusted as described by Wishaw et al. (1977). The dialysis probes for brain with a dialysis area of 3 mm length were prepared according to the method of Abercrombie and Finlay (1991). The relative recovery of MPTP and MPP⁺ was tested in each probe in vitro prior to use. Only those probes with a recovery greater than 10% were used. The probe was stereotaxically implanted into the right side of the caudate putamen at the following coordinates: A-P + 1.6 mm and M-L -2.5 mm from the bregma and D-V -6 mm below the dura according to the stereotaxic atlas of Paxinos and Watson (2005). The dialysis probes (MAB 7.80.10) for blood were purchased from Microbiotech (Stockholm, Sweden) with a recovery greater than 20%, and were inserted into jugular vein. Each probe was perfused with ECF buffer (for brain microdialysis) or ACD solution (for blood microdialysis) (Change et al., 2005) at a flow rate of 1.5 µl/min using a CMA/102 microdialysis pump.

For the control group, MPTP (10 mg/kg) was given through the femoral vein as a 2-min infusion and 30 min brain dialysates were collected for 150 min. To investigate the effect of nicotine on MPTP uptake into the brain extracellular fluid, nicotine (0.6 mg/kg) were given intraperitoneally 30 min prior to the femoral vein administration of MPTP and brain dialysates were collected every 30 min for 150 min. Also, blood dialysates were collected accordingly. After dialysate collection, 35 µl aliquots were injected onto a HPLC system consisting of a model PU2080 pump (Jasco Co., Tokyo, Japan), a model AS-2055 plus intelligent sampler (Jasco Co.), a model UV-2075 UV-VIS detector (Jasco Co.), and a model FP-1520 fluorescence detector (Jasco Co.). The concentrations of MPTP and MPP⁺ were analyzed as described by Naoi et al. (1987) with modifications. In brief, the mobile phase consisted of 80% acetonitrile in 0.02 M sodium acetate/0.1% triethylamine (final pH 6.8) and the sample was analyzed at a flow rate of 0.8 ml/min through a 250 x 4.6 mm nucleosil C18 column (5 µm, MN, Germany). MPTP was detected by the absorbance at 244 nm and MPP⁺ was detected using an excitation wavelength of 290 nm and an emission wavelength of 370 nm and a fluorescence detector. The retention times for MPTP and MPP⁺ were 10.3 min and 8.7 min, respectively. The lower limits of quantification were 25 ng/ml and 5 ng/ml for MPTP and MPP⁺, respectively. The concentrations of nicotine and cotinine were analyzed according to the methods described by Chang et al. (2005).

Data analysis

The uptake data, expressed as the rate of substrate uptake, were analyzed using a nonlinear regression model as follows:

$$V = \frac{V_{\max} \times C}{K_m + C} \text{ with a nonsaturable component, } K \times C,$$

where V represents the uptake rate; V_{\max} , the maximum uptake rate; C , the substrate concentration; K_m , the Michaelis constant; and K , the first order constant. A suitable model for data interpretation was selected on the basis of the goodness of fit evaluated by Model Selection Criteria (MSC), the coefficient of determination, and the standard deviation for the parameter estimated. Nonlinear regression analyses were performed using Scientist v2.01 (MicroMath Scientific Software, Salt Lake City, UT, U.S.A.). For competitive inhibition, the rate of substrate uptake can be expressed as:

$$V = \frac{V_{\max} \times C}{K_m \times (1 + I / K_i) + C}$$

where I is the inhibitor concentration and K_i the inhibition constant. The double reciprocal equation for competitive inhibition can be arranged to that of the Lineweaver-Burk plot,

$$\frac{1}{V} = \frac{K_m \times (1 + I / K_i)}{V_{\max}} \times \frac{1}{C} + \frac{1}{V_{\max}}$$

Statistical analyses were performed using SYSTAT v10 (Systat, Inc., Evanston, IL, U.S.A.). Statistical differences were evaluated by analysis of variance (ANOVA) or Student's t test, with a level of significance of 0.05. Pairwise comparisons between treatment groups were made using the Bonferroni test.

Results

Inhibition of MPTP uptake

^3H -MPTP uptake by ARBEC was investigated in the presence of unlabelled MPTP, MPP^+ , salsolinol, N-methyl-salsolinol or TIQ (MPTP and MPP^+ analogues), carnitine (OCTN2 substrate), dopamine (DAT substrate), nicotine or the nicotine metabolite, cotinine. Also, decynium22, an OCT inhibitor (Martel et al., 1996), was used to investigate the contribution of OCT to MPTP uptake. As shown in Figure 1A, among the compounds tested, the MPTP analogue, TIQ, showed the highest inhibitory activity and reduced MPTP uptake by more than 90%. MPTP and N-methyl-salsolinol caused about 85% and 37% inhibition, respectively. On the other hand, MPP^+ and salsolinol did not show inhibitory effects on MPTP uptake. Nicotine inhibited MPTP uptake by 50%, whereas cotinine caused only 10% inhibition. Carnitine and dopamine had no activity on MPTP uptake. Decynium22 inhibited uptake by about 30%. The

cellular uptake of MPTP was reduced by almost 70% in the presence of both nicotine and decynium22, indicating that nicotine inhibited MPTP uptake through multiple pathways.

MPTP uptake by ARBEC was saturable, with K_m and V_m values (mean \pm SD) of $35.75 \pm 0.85 \mu\text{M}$ and $40.95 \pm 3.56 \text{ pmole/mg-sec}$, respectively (Table 1). Given the V_m/K_m ratio of $1.14 \pm 0.07 \mu\text{l/mg-sec}$, the nonsaturable constant of $0.17 \pm 0.05 \mu\text{l/mg-sec}$ accounts for about 15% of the total uptake under first-order conditions. The inhibitory effect of nicotine on MPTP uptake was competitive, with a K_i of about $328 \mu\text{M}$ (Figure 2).

Inhibition of MPP⁺ uptake

³H-MPP⁺ uptake by ARBEC was also measured in the presence of unlabelled compounds, including MPTP and MPP⁺ analogues, nicotine, cotinine and decynium22. As shown in Figure 1B, MPTP, MPP⁺, N-methyl-salsolinol, and TIQ reduced ³H-MPP⁺ uptake by 80%, salsolinol reduced ³H-MPP⁺ uptake by 68%, nicotine reduced ³H-MPP⁺ uptake by 60%, while its metabolite, cotinine, caused only 25% inhibition, and dopamine caused about 40% inhibition. On the other hand, the OCTN2 substrate, carnitine, did not show inhibitory activity on ³H-MPP⁺ uptake. Decynium22 caused about 70% inhibition of MPP⁺ uptake. In the presence of both nicotine and decynium22, cellular uptake of MPP⁺ was also reduced by almost 70%, indicating that nicotine inhibited MPP⁺ uptake mainly through OCT pathway.

MPP⁺ uptake by ARBEC was saturable, with K_m and V_m values (mean \pm SD) of $10.94 \pm 1.44 \mu\text{M}$ and $0.049 \pm 0.007 \text{ pmole/mg-sec}$, respectively (Table 2). Given the V_m/K_m ratio of $0.0045 \pm 0.0004 \mu\text{l/mg-sec}$, the nonsaturable constant of $0.0014 \pm 0.0001 \mu\text{l/mg-sec}$ accounts for about 30% of the total uptake under first-order conditions. However, in terms of the V_m/K_m or K value, MPP⁺ uptake was much lower than that of MPTP. Nevertheless, inhibition of MPP⁺ uptake by nicotine was competitive, with a K_i value of about $210 \mu\text{M}$ (Figure 3).

Inhibition of nicotine uptake

As nicotine competitively inhibited the uptake of both MPP⁺ and MPTP in ARBEC, nicotine uptake was investigated in the presence of several compounds and decynium22. As shown in Figure 4A, of the three MPTP/MPP⁺ analogues tested, TIQ and N-methyl-salsolinol showed substantial inhibitory activity and reduced nicotine uptake by 86% and 70%, respectively, whereas salsolinol had no significant inhibitory effect. Likewise, MPTP inhibited nicotine uptake by 76%, whereas MPP⁺ had no significant inhibitory activity. Dopamine significantly reduced nicotine uptake by 20%. Decynium22 inhibited nicotine uptake by 60%. When the uptake mechanism of

nicotine was further investigated, the nicotine saturable component in ARBEC exhibited K_m and V_m values (mean \pm SD) of $25.29 \pm 6.44 \mu\text{M}$ and $51.15 \pm 14.18 \text{ pmole/mg-sec}$, respectively (Figure 4B). The nonsaturable constant was estimated to be $0.12 \pm 0.02 \mu\text{l/mg-sec}$ and accounted for less than 6% of uptake under first-order conditions.

RT-PCR for OCT1 mRNA in rat brain endothelial cells

RT-PCR showed that OCT1 mRNA was detectable in both rat kidney and ARBEC, whereas OCT2 or OCT3 was only seen in the kidney or brain, respectively (Figure 5).

In vivo microdialysis study

The extracellular levels of MPTP in rat brain after intravenous infusion of MPTP were about $637.9 \pm 30.8 \text{ ng/ml}$ (mean \pm SD) in the first 30-min interval and nicotine pre-treatment significantly reduced this value to $507.4 \pm 8.5 \text{ ng/ml}$ (mean \pm SD) (Table 3). Since MPTP was given 30 min after nicotine treatment, the corresponding (i.e., 30-60 min collection interval) unbound concentrations of nicotine in the blood and brain were 233.1 ± 9.2 and $136.8 \pm 16.6 \text{ ng/ml}$, respectively (Table 4). Although MPP^+ was also detectable throughout the collection period after MPTP administration, its levels in brain extracellular fluid are much lower than that of MPTP. No significant difference in brain extracellular MPP^+ levels between nicotine-treated and control rats.

Discussion

It is known that parkinsonism induced by exposure to the chemical, MPTP, is attributed to its metabolite, MPP^+ . The in vivo study showed that MPTP is rapidly cleared from the circulation and distributed into the brain after arterial bolus injection, whereas blood levels of MPP^+ also decline rapidly after bolus injection, but minimal amounts of MPP^+ are detected in the brain (Riachi et al., 1989). It is reasonably believed that MPTP, but not MPP^+ , passes across the blood-brain barrier and is then converted by intracerebral monoamine oxidase B (MAOB) to MPP^+ . Despite its unfavorable structural characteristics, MPP^+ can be a substrate for a number of membrane transporters, including organic cation transporter (OCT) (Busch et al., 1996; Martel et al., 2001). Previously, OCT1 but not OCT2 and OCT3 was detected in RBE4 cells, another rat brain microvessel endothelial cell line (Friedrich et al., 2001). Similarly, in the present study, the RT-PCR results showed that OCT1 but not OCT2/OCT3 was detected in ARBEC. In addition, while the difference in uptake

capacity (i.e., the V_m) can be attributed to several factors, the K_m for MPP^+ in ARBEC was comparable to that calculated for MPP^+ uptake by rat OCT1 (9.6 μM) (Busch et al., 1996) and by rat hepatocytes (13 μM) (Martel et al., 1996). In addition to MPP^+ uptake, the current results showed that cellular MPTP uptake can be mediated by a saturable pathway. In terms of the V_m/K_m and K values, uptake of MPP^+ across the cell membrane was very unfavorable compared to that of MPTP.

Previously, nicotine-induced reduction in brain striatal MPP^+ levels has been reported in mice (Quik and Di Monte 2001). The results of brain striata can be in consequence of BBB penetration of these substances. The transfer of nicotine from the blood to brain tissue is known to be rapid and has been used as a cerebral blood flow marker in animal studies (Todd and Weeks 1996; Tomiyama et al., 1999; Lockman et al., 2005). Consistently, nicotine was detected in both blood and brain at the first collection interval in the current study. Although nicotine is considered to be passively diffused across the BBB (Hukkanen et al., 2005), studies from cell lines, including Caco-2 cells, LLK-PK1 cells, and JAR cells, showed that nicotine can be transported by a carrier-mediated process, of which the K_m values ranged from 156 μM to 910 μM (Takami et al., 1998; Zevin et al., 1998; Fukada et al., 2002). It was also suggested that nicotine can interact with the renal OCT system in isolated renal tissues (Wong et al., 1992). In the current study, the saturable pathway mediating cellular uptake of nicotine was estimated to have K_m and V_m values comparable to those of MPTP. Both nicotine and MPTP were considered to easily pass across the BBB. Accordingly, at low concentration range (e.g., less than 1 μM), the transport manner appears to be linear despite the contributions of carrier-mediated processes. Our results also indicated the contribution of OCT system on nicotine uptake in ARBEC. However, the partial inhibition (about 60%) of decynium22 on nicotine uptake suggests that nicotine uptake in ARBEC is mediated by multiple pathways. Likewise, cellular uptake of MPTP and MPP^+ were also partly inhibited by decynium22. Given that nicotine competitively inhibited the uptake of both MPTP and MPP^+ , nicotine may in part compete with MPTP and MPP^+ for the OCT in ARBEC. This can be corroborated by the findings on the inhibitory activities of nicotine alone and nicotine coupled with decynium22 on cellular uptake of MPTP/ MPP^+ . Nonetheless, while nicotine inhibited MPP^+ mainly through OCT pathway, nicotine inhibited MPTP uptake by more than one system. In this regard, except the significant difference in uptake capacity, the insignificant inhibition of nicotine or MPTP uptake by MPP^+ may be partly due to the participation of different transport systems. Interestingly, among MPTP/ MPP^+ analogues, salsolinol showed significant inhibition on MPP^+ uptake but not on the uptake of MPTP and nicotine. On the other hand, TIQ and N-methyl salsolinol showed significant inhibition on

MPTP, MPP⁺ and nicotine. Due to structural similarity, BBB transfer characteristics and structure-transport relationship for MPTP/MPP⁺ analogues need to be further elucidated.

In the *in vivo* microdialysis study, nicotine reduced brain extracellular MPTP levels, but not MPP⁺ levels. Due to the negligible transfer of MPP⁺ and nicotine inhibition, brain extracellular MPP⁺ level can be attributed to MPTP metabolism and brain uptake. Once in the brain, MPTP is converted to MPP⁺ within nondopaminergic cells and MPP⁺ is released by unknown mechanism into the extracellular space, which is then taken by brain cells (Dauer and Przedborski 2003). In this regard, an inhibition of brain uptake of MPP⁺ may result in lower striatal MPP⁺ levels, which can be corroborated by the findings of Quik and Di Monte (2001). The compensatory elevation of extracellular MPP⁺ levels can then minimize the consequence of MPTP reduction caused by nicotine inhibition, as it was observed in the current study. Nicotine plasma concentrations can be 80-100 ng/ml in humans smoking one pack of cigarettes per day (Murrin et al., 1987; Lockman et al., 2005). In the present microdialysis study, blood concentrations of nicotine were estimated to be about 100-250 ng/ml after intraperitoneal administration of 0.6 mg/kg (about 1 μ mole) nicotine. While nicotine concentrations were higher than physiological levels, MPTP was given intravenously at a higher molar dose of 10 mg/kg (about 14 μ mole). To further elucidate the interaction between nicotine and MPTP or MPTP analogs, effects of dose dependency and dosing duration may merit an attention. In addition, once it reaches the systemic circulation, nicotine is mainly metabolized to cotinine by CYP2A6 in humans (Malaiyandi et al., 2005) and by CYP2B and CYP2C11 in rats (Nakayama et al., 1993). Cotinine is also transferred across the blood-brain barrier in rats (Riah et al., 1998; Lockman et al., 2005), as it was observed in the current study. Although its inhibitory activity was weaker than that of nicotine, it should be noted that the plasma half-life of cotinine is more than 8 times longer than that of nicotine (Rosenberg et al., 1980) and a long-term effect of cotinine cannot be neglected. In conclusion, ARBEC uptake of MPTP, MPP⁺ and nicotine can be partly mediated by a system inhibited by decynium22, a specific inhibitor of OCT. Nicotine inhibited both *in vitro* cellular uptake and *in vivo* brain extracellular levels of MPTP.

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Figure Legends:

Figure 1. Uptake of (A) ^3H -MPTP (0.02 μM) or (B) ^3H -MPP⁺ (0.1 μM) by ARBEC in the presence of various compounds (0.5 mM) and a selective inhibitor of the OCT (decynium22, 10 μM). An asterisk denotes a statistically significant difference ($p < 0.05$) compared to the control. The data are presented as the mean \pm SEM for three experiments, each in triplicate.

Figure 2. Competitive inhibition of MPTP (0.02-200 μM) uptake by ARBEC in the presence of 0 mM (black circles), 0.05 mM (black triangles) or 0.5 mM (white circles) nicotine. (A) Nonlinear regression analysis of MPTP uptake. (B) Lineweaver-Burk plot of MPTP uptake; the insert indicates a value of 328 μM for the inhibition constant (K_i) for nicotine. The data are presented as the mean \pm SEM for three experiments, each in triplicate. Each plot was generated from the mean value of the individually fitted parameters.

Figure 3. Competitive inhibition of MPP⁺ (0.1-200 μM) uptake by ARBEC in the presence of 0 mM (black circles), 0.3 mM (white circles), or 0.5 mM (black triangles) nicotine. (A) Nonlinear regression analysis of MPP⁺ uptake. (B) Lineweaver-Burk plot of MPP⁺ uptake; the insert indicates a value of 210 μM for the inhibition constant (K_i) for nicotine. The data are presented as the mean \pm SEM for three experiments, each in triplicate. Each plot was generated from the mean value of the individually fitted parameters.

Figure 4. (A) Uptake of ^3H -nicotine (0.02 μM) by ARBEC in the presence of various compounds (0.5 mM) or a selective inhibitor of the OCT (decynium22, 10 μM). An asterisk indicates a statistically significant difference ($p < 0.05$) compared to the control. (B) Concentration-dependent uptake of nicotine in the range 0.02 to 200 μM . Uptake is presented as total uptake (black circles) with a saturable component (white circles) and a linear component (black triangles). Each plot was generated from the mean value of the individually fitted parameters. The data are presented as the mean \pm SEM for three experiments, each in triplicate.

Figure 5. Competitive inhibition of MPTP (0.02-200 μM) uptake by ARBEC in the presence of 0 mM (black circles), 0.1 mM (open circles) or 0.5 mM (black triangle) deprenyl. (A) Nonlinear regression analysis of MPTP uptake. (B) Lineweaver-Burk plot of MPTP uptake; the insert indicates a value of 246 μM for the inhibition constant (K_i) for nicotine. The data are presented as the mean \pm SEM for three

experiments, each in triplicate. Each plot was generated from the mean value of the individually fitted parameters.

Figure 6. RT-PCR results for the mRNA of rOCT1, rOCT2 and rOCT3 in rat kidney, brain and ARBEC.

Figure 1

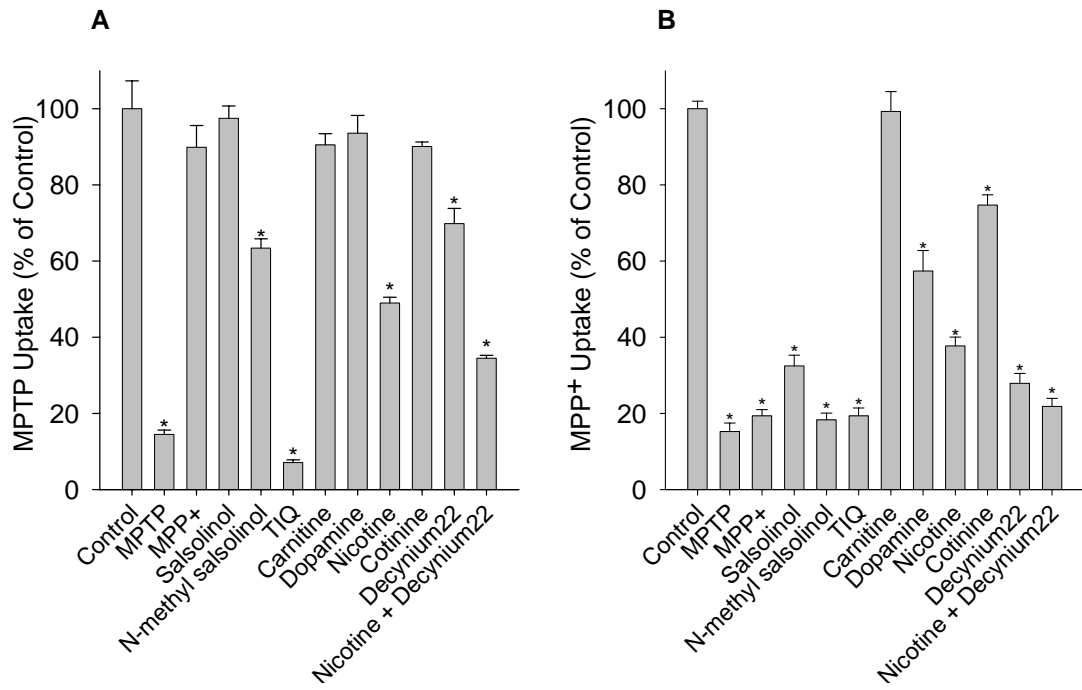


Figure 2

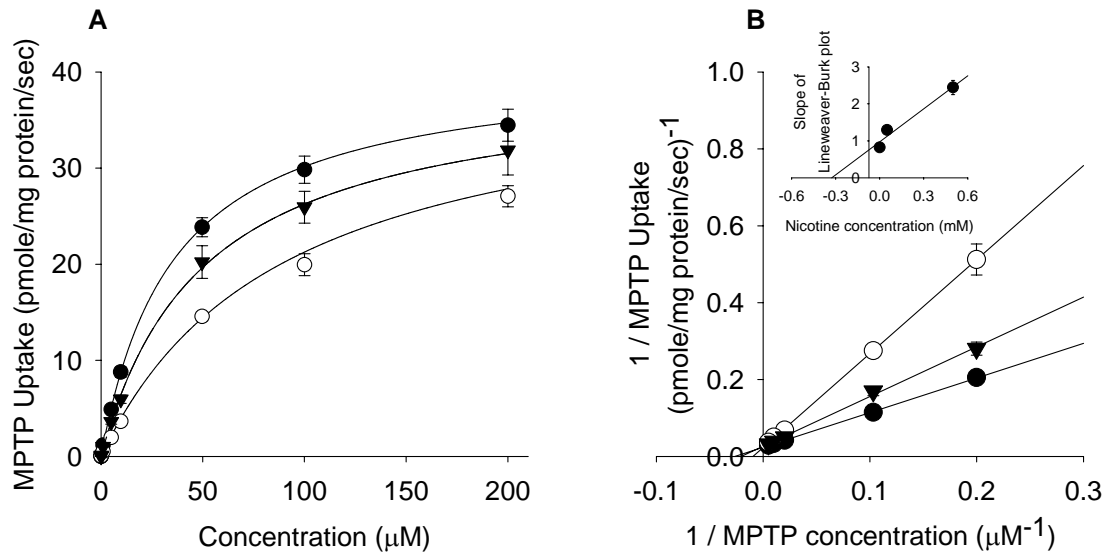


Figure 3

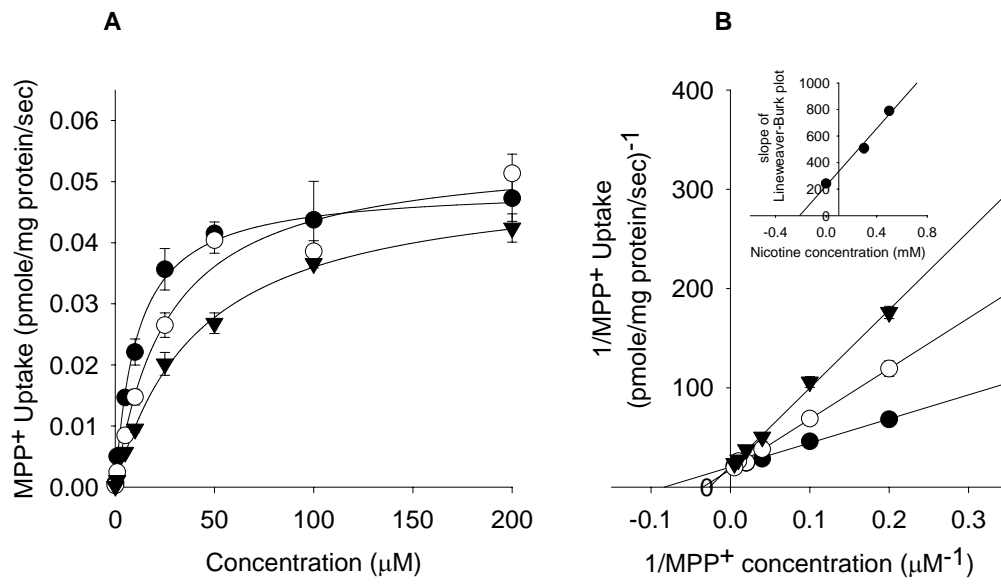


Figure 4

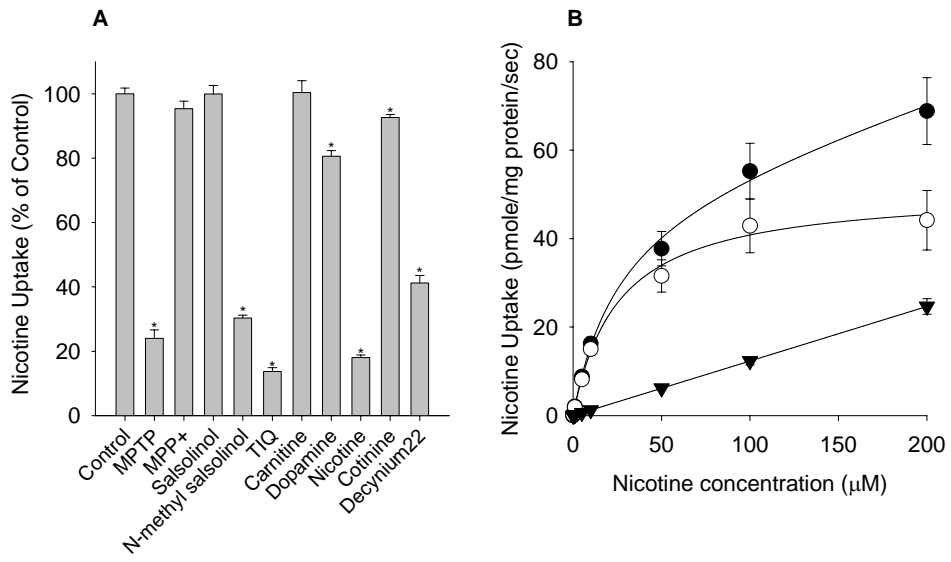


Figure 5

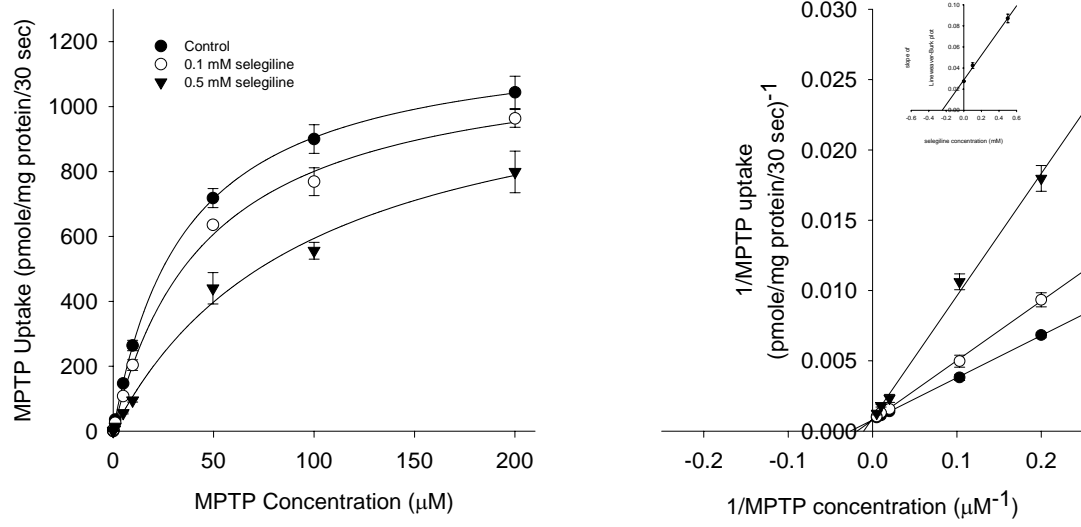


Figure 6

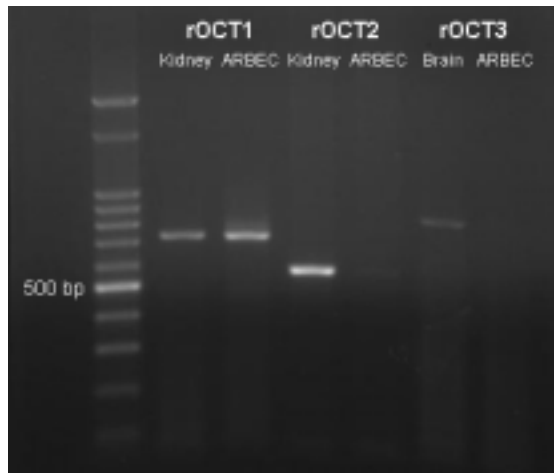


Table 1. Inhibitory effects of nicotine on MPTP uptake in adult rat brain endothelial cells (ARBEC).

	Km μM	Vmax pmole/mg-sec	K μL/mg-sec	Ki μM
Nonlinear regression				
MPTP only	35.75±0.85	40.95±3.56	0.17±0.05	
+ Nicotine, 0.05 mM	50.32±2.50 ^a	39.46±4.95	0.16±0.05	
+ Nicotine, 0.5 mM	89.67±4.13 ^{a,b}	40.32±4.04	0.14±0.02	
Lineweaver-Burk plot				
MPTP only	34.64±5.36	42.10±5.77		
+ Nicotine, 0.05 mM	48.45±2.61 ^a	37.89±5.80		
+ Nicotine, 0.5 mM	105.50±24.73 ^{a,b}	42.80±4.42		
Nicotine Ki ^c				328

The data are presented as the mean ± SD for three experiments, each in triplicate.

^a P < 0.05 compare with MPTP uptake without nicotine.

^b p < 0.05 compare with MPTP uptake with 0.05 mM nicotine

^c The Ki value was estimated by plotting the slope of the Lineweaver-Burk plot vs. inhibitor concentration, when the x intercept is equal to -Ki.

Table 2. Inhibitory effects of nicotine on MPP⁺ uptake in adult rat brain endothelial cells (ARBEC).

	Km μM	Vmax pmole/mg-sec	K μL/mg-sec	Ki μM
Nonlinear regression				
MPP ⁺ only	10.94±1.44	0.049±0.007	0.0014±0.0001	
+ Nicotine, 0.3 mM	26.36±4.19 ^a	0.055±0.006	0.0026±0.0011	
+ Nicotine, 0.5 mM	42.13±4.77 ^{a,b}	0.051±0.003	0.0010±0.0002	
Lineweaver-Burk plot				
MPP ⁺ only	12.38±3.31	0.051±0.011		
+ Nicotine, 0.3 mM	28.62±1.55 ^a	0.057±0.005		
+ Nicotine, 0.5 mM	38.11±3.63 ^{a,b}	0.049±0.006		
Nicotine Ki ^c				210

The data are presented as the mean ± SD for three experiments, each in triplicate.

^a p < 0.01 compare with MPP⁺ uptake without nicotine.

^b p < 0.05 compare with MPP⁺ uptake with 0.3 mM nicotine.

^c The Ki value was estimated by plotting the slope of the Lineweaver-Burk plot vs. inhibitor concentration, when the x intercept is equal to -Ki.

Table 3. Effects of nicotine (0.6 mg/kg i.p.) on brain extracellular levels (ng/ml) of MPTP and MPP⁺ after single intravenous administration of MPTP (10 mg/kg) in male Wistar rats.

Collection Interval (min)	Control		Nicotine Treatment	
	MPTP	MPP ⁺	MPTP	MPP ⁺
0-30	637.9 ± 30.8	54.6±13.6	507.4±8.5*	48.8±12.7
30-60	212.0 ± 43.7	33.2±6.2	201.5±50.2	35.3±10.9
60-90	78.1±26.3	13.5±2.2	90.2±27.4	15.3±3.7
90-120	51.6±22.2	9.2±2.3	50.7±19.0	9.5±2.4
120-150	45.4±12.1	7.9±2.1	41.0±18.1	8.9±1.1

The data are presented as the mean ± SD for five experiments.

* p < 0.001 compare with control group (without nicotine treatment).

Table 4. Unbound concentrations (ng/ml) of nicotine and cotinine in blood and brain after intraperitoneal (i.p.) injection of 0.6 mg/kg nicotine in male Wistar rats.

Collection Interval (min)	Blood		Brain	
	Nicotine	Cotinine	Nicotine	Cotinine
0-30	191.6±18.1	42.0±5.1	131.2±5.7	UD
30-60	233.1±9.2	106.6±4.3	136.8±16.6	46.2±6.2
60-90	163.0±7.2	166.1±19.3	109.4±13.9	62.6±4.4
90-120	115.7±5.4	187.2±15.2	UD	75.9±5.7
120-150	UD	189.2±20.9	UD	76.5±8.0

The data are presented as the mean ± SD for five experiments.

UD represents undetectable.

出席國際學術會議心得報告

計畫編號	NSC 95-2320-B-002-103
計畫名稱	Nicotine 與 deprenyl 穿透血腦屏障與其抗巴金森式症機制之探討
出國人員姓名 服務機關及職稱	臺灣大學醫學院藥學系 許浩睿
會議時間地點	2007/4/22-25 荷蘭 阿姆斯特丹
會議名稱	(中文) 第三屆世界藥學科學會 (英文) 3 th pharmaceutical sciences world congress
發表論文題目	(中文) 尼古丁競爭型抑制MPTP及MPP ⁺ 被大鼠大腦內皮細胞攝取 (英文) Nicotine competitively inhibits MPTP and MPP ⁺ uptake by rat brain endothelial cells

一、參加會議經過

此次會議共有來自世界各國的一千多位藥學研究人員參與，在4/22簡單的開幕式後，會議在4/23正式展開，同時我的壁報論文也在此日張貼，中午需要花一個小時站在壁報論文前講解，期間約有七至八位與會人士提問，大多希望我能簡單的介紹實驗的流程，我也一一為他們解說，雖然因為語言的關係，溝通起來比較花時間，但基本上他們都能得到滿意的答覆，除了壁報展示之外，每天都有六個會議室同時在進行演講或是討論的活動，因此在張貼壁報以外的時間，我聽了一些和我研究上相關或是我自己有興趣的演講，或是觀看其他人張貼的壁報論文，並且和一些壁報論文的作者交換心得，四天下來認識不少來自世界各國的研究生，並且獲益良多。

二、與會心得

雖然之前已經有參加學術研討會的經驗，但這是本人第一次參與國際級的學術會議，自然感到特別的新鮮，在會議的邀請演講中，可以吸收到目前最新在進行的各種研究，在壁報論文的展場，則可看到來自全世界各式各樣不同的研究題材，另外，講解自己的壁報論文或是和其他壁報的作者溝通也是一個特別的經驗，我發現在學術的領域要以英文和其他人討論並不如想像中的困難，反倒是日常對話常常講的吞吞吐吐不知所云。總之，參與這次會議之後，我對於日後繼續出國深造的計畫更具信心，但也體認到持續加強語文能力的重要性。最後我要感謝本人的指導老師林君榮老師給我這個機會，讓我在碩士生涯就能有此難得的經歷。