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Neurotoxicity studies with the monoamine oxidase B substrate 1-methyl-3-phenyl-3-pyrroline

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Abstract

The neurotoxic properties of the parkinsonian inducing agent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are dependent on its metabolic activation in a reaction catalyzed by centrally located monoamine oxidase B (MAO-B). This reaction ultimately leads to the permanently charged 1-methyl-4-phenylpyridinium species MPP^+, a 4-electron oxidation product of MPTP and a potent mitochondrial toxin. The corresponding 5-membered analogue, 1-methyl-3-phenyl-3-pyrroline, is also a selective MAO-B substrate. Unlike MPTP, the MAO-B-catalyzed oxidation of 1-methyl-3-phenyl-3-pyrroline is a 2-electron process that leads to the neutral 1-methyl-3-phenylpyrrole. MPP^+ is thought to exert its toxic effects only after accumulating in the mitochondria, a process driven by the transmembrane electrochemical gradient. Since this energy-dependent accumulation of MPP^+ relies upon its permanent charge, 1-methyl-3-phenyl-3-pyrrolines and their pyrrolyl oxidation products should not be neurotoxic. We have tested this hypothesis by examining the neurotoxic potential of 1-methyl-3-phenyl-3-pyrroline and 1-methyl-3-(4-chlorophenyl)-3-pyrroline in the C57BL/6 mouse model. These pyrrolines did not deplete striatal dopamine while analogous treatment with MPTP resulted in 65–73% depletion. Kinetic studies revealed that both 1-methyl-3-phenyl-3-pyrroline and its pyrrolyl oxidation product were present in the brain in relatively high concentrations. Unlike MPP^+, however, 1-methyl-3-phenylpyrrole was cleared from the brain quickly. These results suggest that the brain MAO-B-catalyzed oxidation of xenobiotic amines is not, in itself, sufficient to account for the neurodegenerative properties of a compound like MPTP. The rapid clearance of 1-methyl-3-phenylpyrroles from the brain may contribute to their lack of neurotoxicity.

Keywords: Monoamine oxidase B; Neurotoxicity; MPTP; 1-Methyl-3-phenyl-3-pyrroline; 1-Methyl-3-phenylpyrrole; Dopamine; Striata

Introduction

The flavoenzyme monoamine oxidase B (MAO-B) has been identified as the principal catalyst responsible for the metabolic activation of the proneurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP (**1**)] in the brains of mammals including humans (Fig. 1) (Chiba et al., 1984; Heikkila et al., 1984b). The molecular mechanisms by which MPTP selectively damages nigrostriatal neurons and induces a parkinsonian syndrome has been the subject of extensive research (Heikkila et al., 1984a; Nicklas et al., 1985; Smeyne and Jackson-Lewis, 2005). Critical to its mode of action is the MAO-B-catalyzed ring α-carbon 2-electron oxidation of the parent compound to yield the corresponding 1-methyl-4-phenyl-2,3-dihydropyridinium species $MPDP^+$ ($\mathbf{2H^+}$). This metabolic intermediate, presumably via the corresponding free base **2**, undergoes a second 2-electron oxidation to generate the 1-methyl-4-phenylpyridinium metabolite MPP^+ ($\mathbf{3^+}$), the ultimate neurotoxin (Chiba et al., 1984; Ramsay et al., 1991; Markey et al., 1984). This process appears to take place mainly in MAO-B rich glial cells (Takada et al., 1990). MPP^+ is thought to accumulate via the plasma membrane dopamine transporter (DAT) (Chiba et al., 1985; Javitch and Snyder, 1984) in nigrostriatal nerve terminals where it localizes within the inner mitochondrial membrane (Sayre et al., 1990). Inhibition of complex I of the mitochondrial respiratory chain by MPP^+ then leads to downstream events such as ATP depletion and

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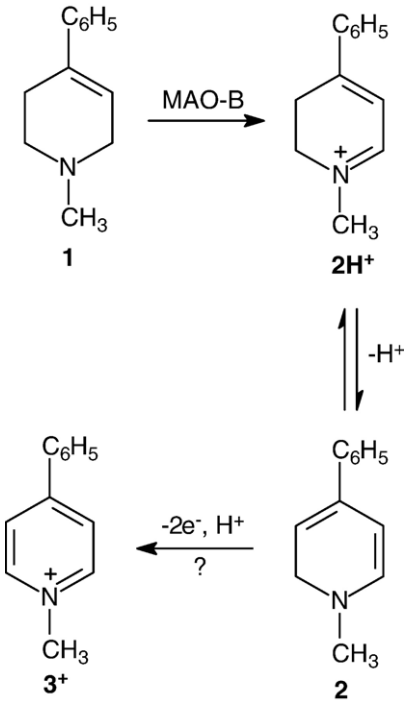

Fig. 1. The MAO-B-catalyzed oxidation of MPTP (**1**) to the corresponding 2,3-dihydropyridinium product $MPDP^+$ ($\mathbf{2H^+}$) and the pyridinium species MPP^+ ($\mathbf{3^+}$).

oxidative stress which eventually result in degeneration of nigrostriatal dopaminergic neurons (Singer et al., 1988). Experimental animals treated with MPTP have become useful models for studying neurodegenerative processes. In a frequently used protocol striatal dopamine concentrations of C57BL/6 mice are measured 7 days after systemic injection (multiple or single doses) of MPTP (Schmidt and Ferger, 2001). The depletion of striatal dopamine is indicative of the permanent loss of nigrostriatal dopaminergic cell bodies in the substantia nigra.

Over the years various tetrahydropyridinyl analogues of MPTP have been found to be MAO-B substrates (Kalgutkar and Castagnoli, 1992). One compound of particular interest is 1-methyl-3-phenyl-3-pyrroline (**4a**), a 5-membered ring analogue that has been shown to be a good and selective substrate of MAO-B (Wang et al., 1998). Unlike MPTP, the MAO-B-generated final metabolite of **4a** is the neutral 1-methyl-3-phenylpyrrole (**5a**). This overall 2-electron oxidation most likely arises via $\mathbf{6H^+}$, the short-lived conjugate acid of the pyrrolyl product **5a** (Fig. 2).

MPP^+ is thought to exert its toxic actions only after accumulating in the inner mitochondrial membrane of the nigrostriatal nerve terminals, a process driven by the transmembrane electrochemical gradient (Sayre et al., 1990). This energy-dependant accumulation of MPP^+ relies upon its permanent charge. Intrastriatal microdialysis studies have established that only permanently charged MPP^+ analogues exhibit dopaminergic neurotoxic properties (Rollema et al., 1990). Interestingly, the neutral *N*-desmethylated form of MPP^+, 4-phenylpyridine, is a much more potent inhibitor of complex I of the isolated respiratory chain than is MPP^+ but since it is not concentrated inside the mitochondria, this compound is not a physiological inhibitor of mitochondrial respiration. Accordingly, chronic exposure of mice to 4-phenylpyridine and 4-phenyl-1,2,3,6-tetrahydropyridine was found not to cause any reduction in striatal dopamine (Perry et al., 1987).

Based on these observations, 1-methyl-3-phenyl-3-pyrrolines and their MAO-B-catalyzed pyrrolyl oxidation products should not be neurotoxic. In this study we have tested this hypothesis by measuring ex vivo striatal dopamine concentrations in C57BL/6 mice 7 days after administration of 1-methyl-3-phenyl-3-pyrroline (**4a**) and 1-methyl-3-(4-chlorophenyl)-3-pyrroline (**4b**). Compound **4b** is also reported to be a MAO-B substrate (Williams and Lawson, 1998). The findings of this study are discussed with reference to the brain concentrations attained by both 1-methyl-3-phenyl-3-pyrroline and its pyrrolyl oxidation product compared to the brain concentrations attained by MPTP, $MPDP^+$ and MPP^+ following systemic treatment of MPTP.

Materials and methods

Caution: MPTP (**1**) is a known nigrostriatal neurotoxin and should be handled using disposable gloves and protective eyewear. Procedures for the safe handling of MPTP have been described previously (Pitts et al., 1986).

Chemicals and instrumentation

All starting materials not described elsewhere were obtained from Sigma-Aldrich and were used without purification. MPTP·HCl, dopamine·HCl and isoprenaline·HCl were purchased from Sigma-Aldrich. Petroleum ether used in this study had a distillation range of 40–60 °C. Melting points (mp) were determined with a Gallenkamp melting point apparatus and all melting points are uncorrected. Proton and carbon NMR spectra were recorded on a Varian Gemini 300 spectrometer. Proton (^1H) spectra were recorded at a frequency of 300 MHz and carbon

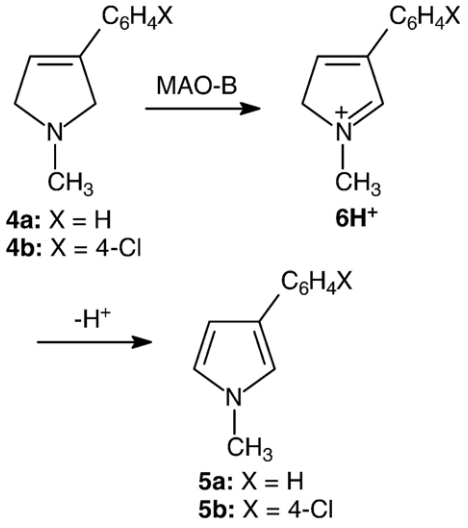

Fig. 2. The MAO-B-catalyzed oxidation of 1-methyl-3-phenyl-3-pyrrolines (**4**) to 1-methyl-3-phenylpyrroles (**5**).

(^{13}C) spectra at 75 MHz. Chemical shifts are reported in parts per million (δ) downfield from the signal of tetramethylsilane added to CD_3OD. Spin multiplicities are given as s (singlet), d (doublet), dd (doublet of doublets), t (triplet) or m (multiplet) and the coupling constants (J) are given in hertz (Hz). Fast atom bombardment mass spectra (FAB-MS) and high resolution mass spectra (HRMS) were obtained on a VG 7070E mass spectrometer. HPLC analyses were performed with an Agilent 1100 HPLC system equipped with an Agilent 1100 series variable wavelength detector. Thin layer chromatography (TLC) was carried out using neutral alumina 60 (Merck) with UV_{254} fluorescent indicator. UV/vis spectral measurements were recorded on a Milton-Roy Spectronic 1201 spectrophotometer.

*Synthesis of 3-phenyl-3-pyrroline hydrochloride (**7**·HCl)*

3-Phenyl-3-pyrroline hydrochloride (**7**·HCl) was prepared from 3-phenyl-3-pyrrolidinol according the method described by the literature (Lee et al., 2002) in a yield of 45%: mp 189–191 °C (ethanol), lit. mp 184 °C (Lee et al., 2002).

*Synthesis of 1-methyl-3-phenyl-3-pyrroline oxalate (**4a**·$C_2H_2O_4$)*

The hydrochloric acid salt of 3-phenyl-3-pyrroline (**7**·HCl) was converted to the corresponding free base with sodium carbonate (2 M). The thick white precipitate that formed was extracted into chloroform and the extract was dried over anhydrous magnesium sulfate. After removal of the chloroform phase, the residue appeared as a light yellow oil which solidified upon cooling to room temperature. The residue (0.55 mmol) was cooled to 0 °C while 98% formic acid (3.3 mmol) was added slowly followed by 40% formaldehyde (3.3 mmol) (Pine and Sanchez, 1971). The reaction mixture was stirred at 80 °C for 3 h and the reaction progress was monitored by TLC on neutral alumina (100% ethyl acetate). During this time, bubbling in the reaction was observed and the reaction turned dark red. Water (5 ml) was added to the reaction followed by an aqueous solution of 5 ml sodium carbonate (2 M). The resulting white precipitate was extracted to diethyl ether and the extract was dried over anhydrous magnesium sulfate. Upon removal of the solvent a light yellow oily residue remained which was converted into its oxalic acid salt by the addition of diethyl ether saturated with oxalic acid. The product was recrystallized from methanol at −20 °C and light yellow colored crystals were obtained in a yield of 30%: mp 152–154 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 7.49–7.35 (m, 5H), 6.28 (d, 1H, J=1.76 Hz), 4.56 (s, 2H), 4.34 (s, 2H), 3.09 (s, 3H). ^{13}C NMR (75 MHz, CD_3OD): δ 166.71, 139.28, 132.78, 130.18, 129.95, 127.03, 18.83, 63.59, 62.53, 42.95. FAB-MS m/z: 160 (MH^+). HRMS calcd. 160.11263, found 160.11260 (MH^+).

*Synthesis of 1-methyl-3-(4-chlorophenyl)-3-pyrroline oxalate (**4b**·$C_2H_2O_4$)*

Compound **4b** was synthesized from 1-methyl-3-(4-chlorophenyl)pyrrole (**5b**) according to the procedure reported in the literature (Williams and Lawson, 1998, 1999) with the following modifications. The oily product obtained from the diethyl ether extract solidified at 0 °C. This solid was placed in a vacuum oven at 40 °C for 24 h. The material was converted into its oxalic acid salt in diethyl ether. Following recrystallization from methanol a colorless solid was obtained in 26% yield: mp 150–152 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 7.48–7.34 (m, 4H), 6.32 (t, 1H. J=2.19 Hz), 4.55 (d, 2H, J=1.65 Hz), 4.35 (d, 2H, J=1.51 Hz), 3.09 (s, 3H). ^{13}C NMR (75 MHz, CD_3OD): δ 166.63, 137.14, 135.91, 131.50, 130.06, 128.60, 119.85, 63.60, 62.38, 42.97. FAB-MS m/z: 194 (MH^+). HRMS calcd. 194.07365, found 194.07371 (MH^+).

*Synthesis of 1,1-dimethyl-3-phenyl-3-pyrrolinium iodide (**8**·I)*

Compound **8**·I served as internal standard for the HPLC analysis of **4a**. The hydrochloric acid salt of 3-phenyl-3-pyrroline (**7**·HCl) was converted to the corresponding free base as described above for the synthesis of **4a**. This free base (1.43 mmol) in 10 ml tetrahydrofurane (THF) was treated with 3 equivalents of iodomethane (4.3 mmol). The reaction mixture was stirred for 1 h at room temperature at which time another 10 ml THF was added followed by an additional 3 equivalents of iodomethane. The suspension was heated under reflux for 30 min, cooled to 0 °C and filtered to collect the light yellow precipitate in a yield of 88%. This product was recrystallized from methanol to give the pure product in a yield of 29%: mp 224–226 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 7.52–7.38 (m, 5H), 6.36 (m, 1H), 4.82 (dd, 2H, J=2.06, 2.03 Hz), 4.58 (dd, 2H, J=2.20, 2.09 Hz), 3.43 (s, 6H). ^{13}C NMR (75 MHz, CD_3OD): δ 137.97, 132.43, 130.47, 130.03, 127.11, 118.27, 74.11 (t, J=3.4–3.2 Hz), 73.03 (t, J=3.8–3.7 Hz), 54.61 (t, J=4.0–3.9 Hz). FAB-MS m/z: 174 (M^+), 175 (MH^+). HRMS calcd. 175.13610, found 175.13610 (MH^+).

*Synthesis of 2-aryl-3-(dimethylamino)allylidene(dimethyl)ammonium perchlorates (**9a**–**c**)*

Compounds **9a**–**c** were prepared in relatively high yields from the corresponding phenylacetic acid derivatives, DMF and phosphoryl chloride according to the method described in the literature (Jutz et al., 1969). Compound **9c** served as starting material for the preparation of the HPLC internal standard **5c** (see below). The melting points of compounds were as follows: **9a** mp 203–205 °C (from ethanol), lit. mp 193–194 °C (Jutz et al., 1969); **9b** mp 149–151 °C (from ethanol), lit. mp 142–144 °C (Jutz et al., 1969); **9c** mp 134–136 °C (from ethanol), lit. mp 130–131 °C (Jutz et al., 1969).

*Synthesis of 1-methyl-3-phenylpyrroles (**5a**–**c**)*

1-Methyl-3-phenylpyrroles (**5a**–**c**) were synthesized from compounds **9a**–**c** according to the method described in the literature (Gallagher et al., 1990). The crude products obtained were dissolved in a minimal amount of ethyl acetate and purified on a short column of neutral alumina (Fluka 507C) with 100% petroleum ether (**5a**, **b**) or petroleum ether/ethyl acetate, 90:10 (**5c**) as mobile phase. The fractions containing the product were

recrystallized from an appropriate solvent as indicated below. Compound **5c** served as internal standard for the HPLC analysis of **5a**. The melting points were as follows: **5a** mp 44–46 °C (from petroleum ether), lit. mp 46–47 °C (Gallagher et al., 1990); **5b** mp 112–114 °C (from methanol), lit. mp 117.5–119.5 °C (Gallagher et al., 1990); **5c** mp 120–122 °C (from methanol), lit. mp 126–128 °C (Gallagher et al., 1990).

Steady-state MAO-B activity measurements with **4a** *and* **4b** *as substrates*

Mitochondria were isolated from the tissues of beef liver, baboon liver, mouse liver and mouse brain as described in the literature (Salach and Weyler, 1987) and stored at −70 °C. The mitochondrial isolates were suspended in sodium phosphate buffer (100 mM, pH 7.4, containing 50% glycerol, w/v) and the protein concentrations were determined by the method of Bradford (1976). Since beef liver, baboon liver and mouse liver mitochondria are devoid of MAO-A activity, inactivation of MAO-A was unnecessary where the steady-state kinetic parameters of the oxidation of 1-methyl-3-phenyl-3-pyrrolines by MAO-B were measured (Inoue et al., 1999). The mitochondrial fraction obtained from mouse brain tissue is reported to contain 15% MAO-A activity (Inoue et al., 1999) but since the 1-methyl-3-phenyl-3-pyrrolines (**4a** and **4b**) examined in this study are reported to be MAO-B selective substrates (Wang et al., 1998) inactivation of MAO-A was again not deemed necessary. In order to estimate the K_m and V_{max} values for the oxidation of the 1-methyl-3-phenyl-3-pyrrolines by MAO-B, initial rates were measured at eight substrate concentrations spanning at least two orders of magnitude (6.25–2000 μM). The reactions were carried out in a final volume of 500 μl (in 100 mM sodium phosphate buffer, pH 7.4) and the enzyme concentration used was 0.15 mg mitochondrial protein/ml. All reactions were incubated at 37 °C for 10 min. For this time period the MAO-B-catalyzed production of the 1-methyl-3-phenylpyrrole products was found to be linear. The reactions were terminated by the addition of 70% perchloric acid (10 μl) and the samples were centrifuged at 16,000 *g* for 10 min. The supernatant fractions were removed and the concentrations of the MAO-B-generated 1-methyl-3-phenylpyrrolyl products were measured by HPLC analysis with UV detection (see Chemicals and instrumentation). A Phenomenex Luna C18 column (4.60×250 mm, 5 μm) was used and the mobile phase consisted of 25% distilled water [containing 0.6% (v/v) glacial acetic acid and 1% (v/v) triethylamine] and 75% acetonitrile at a flow rate of 1 ml/min. A volume of 50 μl of the supernatant was injected into the HPLC system and the elution of 1-methyl-3-phenylpyrrole (**5a**) or 1-methyl-3-(4-chlorophenyl)pyrrole (**5b**) was monitored at wavelengths of 270 and 280 nm, respectively. Quantitative estimations of **5a** and **5b** were made by means of linear calibration curves ranging from 1.5 to 25 μM and the initial rates were expressed as nmol of product formed per mg mitochondrial protein per min. The steady-state kinetic data (initial rates as a function of substrate concentration) were fitted to the Michaelis–Menten equation using the nonlinear least-squares fitting routine incorporated into the SigmaPlot software package (Systat Software Inc.). This determination was carried out in triplicate and the K_m and V_{max} values were expressed as means±standard error of the mean (S.E.M.).

Steady-state MAO-B activity measurements with MPTP as substrate

In order to estimate K_m and V_{max} values for the oxidation of MPTP by MAO-B, the same incubation procedures were followed as that described for the 1-methyl-3-phenyl-3-pyrroline substrates with the exception that the incubation time chosen was 12.5 min. The rates of oxidation of MPTP by MAO-B present in the mitochondrial fractions of beef liver, baboon liver, mouse liver and mouse brain were found to remain linear over this time period (results not shown). The concentrations of $MPDP^+$ in the incubations were measured spectrophotometrically at a wavelength of 343 nm (ε=16,000 M^{-1}) (Castagnoli et al., 1997). During the time needed to complete a typical experiment it was found, by HPLC analysis of incubations of 30–90 μM of MPTP with mouse brain mitochondria, that the concentrations of MPP^+ present in the incubations ranged from approximately 4.5% to 10.5% of the corresponding $MPDP^+$ concentrations. These HPLC analyses were carried out as described previously (Petzer et al., 2003).

Animal studies

Animal trials were conducted with retired breeder male C57BL/6 mice (30–35 g, 9–11 months of age) which were housed 5 animals per cage in a temperature (21±0.5 °C) and humidity (50±5% relative humidity) controlled room on a 12 h light–12 h dark cycle with free access to food and water. The animals were provided by the Laboratory animal center of the Potchefstroom campus and protocols for all animal experiments were reviewed and approved by the Research Ethics Committee of the North-West University. All injections were intraperitoneal (i.p.) in a volume of 0.2 ml per 30 g mouse. Sterile saline was used as vehicle for all of the test compounds. Mice were sacrificed by rapid cervical dislocation.

Striatal dopamine measurements

The concentrations of dopamine in dissected mouse striata were determined as described previously, (Harvey et al., 2006) and were expressed as means±S.E.M.

Brain MPTP, $MPDP^+$ and MPP^+ measurements

Immediately after sacrifice, the mouse brains were removed, placed in microcentrifuge tubes and frozen in liquid nitrogen. A portion (~30 mg) of the brain tissue of each mouse was weighed and the concentrations of MPTP, $MPDP^+$ and MPP^+ were determined via HPLC analysis as described previously (Castagnoli et al., 1997). The only modification made to the published procedure was that the internal standard used was changed to 1-methyl-4-(1-methylpyrrol-2-yl)pyridinium iodide (MMP^+) at a final concentration of 1.33 μM. MPTP, $MPDP^+$

and MPP^+ concentrations were expressed as pmol/mg wet weight of tissue (mean ± S.E.M.).

Brain measurements of **4a** *and* **5a**

Four groups (n = 5/group) of mice were treated with 238 μmol/kg 1-methyl-3-phenyl-3-pyrroline oxalate (**4a**·$C_2H_2O_4$) (see Animal studies above) and sacrificed at 10, 20, 40 and 60 min post-treatment, respectively. Their brains were removed and placed in microcentrifuge tubes. The brain tissues were frozen in liquid nitrogen until further sample preparation. For the measurement of 1-methyl-3-phenylpyrrole (**5a**) concentrations, a portion (~30 mg) of the brain tissue of each mouse was weighed and 10 μl/mg of the homogenizing solution (an aqueous solution of 50% acetonitrile, 0.305 M perchloric acid and 12.5 μM of compound **5c** as internal standard) was added. Following sonication (2 × 14 s, 14 μm) the samples were placed on ice for 60 min, centrifuged at 16,000 *g* for 15 min and 50 μl of the resulting supernatants were analyzed for 1-methyl-3-phenylpyrrole content by reverse phase HPLC equipped with a UV detection and a Phenomenex Luna C18 analytical column (see Chemicals and instrumentation). The mobile phase consisted of 35% aqueous phase (0.6% glacial acetic acid and 1% triethylamine in distilled water, pH 4.7) and 65% acetonitrile; the solvent was delivered at a flow rate of 1 ml/min. The elutions of **5a** (4.93 min) and internal standard **5c** (4.41 min) were monitored at a wavelength of 270 nm. A linear standard curve was constructed using four calibration standards (3.125–25 μM) prepared in the homogenizing buffer. In order to measure brain concentrations of 1-methyl-3-phenyl-3-pyrroline (**4a**), a portion of brain tissue (~30 mg) of each mouse was weighed and 10 μl/mg of the homogenizing solution (an aqueous solution of 10% acetonitrile, 0.305 M perchloric acid and 12.5 μM of compound **8**·I as internal standard) was added. Following sonication (2 × 14 s, 14 μm) the samples were placed on ice for 60 min, centrifuged at 16,000 *g* for 15 min and 50 μl of the resulting supernatants were analyzed for 1-methyl-3-phenyl-3-pyrroline content by reverse phase HPLC as described for 1-methyl-3-phenylpyrrole above. The mobile phase consisted of 90% aqueous phase (0.6% glacial acetic acid and 1% triethylamine in distilled water, pH 4.7) and 10% acetonitrile and solvent was delivered at a flow rate of 1 ml/min. The elutions of **4a** (9.66 min) and internal standard **8** (7.88 min) were monitored at a wavelength of 250 nm. A linear standard curve was constructed using four calibration standards (3.125–25 μM) prepared in the homogenizing buffer. The concentrations of **4a** and **5a** were expressed as pmol/mg wet weight of tissue (mean ± S.E.M.).

Statistical analysis

Striatal dopamine levels were analyzed using an one-way analysis of variance (ANOVA), followed by multiple comparisons using the Dunnett's *t*-test to compare the experimental groups to the control group. The Statistica software package (StatSoft Inc.) was used for all data analysis and data are expressed as the mean ± S.E.M.

Results

Chemistry

1-Methyl-3-phenyl-3-pyrroline (**4a**) was prepared from 3-phenyl-3-pyrroline (**7**) by the formic acid–formaldehyde methylation procedure (Eschweiler–Clarke reaction) described in the literature (Pine and Sanchez, 1971) (Fig. 3). As expected this method of methylation yielded exclusively the tertiary amine with no trace of the *N*,*N*-dimethylated pyrrolinium species (**8**) as judged by TLC. In contrast, treatment of 3-phenyl-3-pyrroline with one equivalent of iodomethane yielded a mixture of the tertiary and quaternary amines. Treatment with an excess of iodomethane (6 equiv.) yielded exclusively the pyrrolinium iodide salt (**8**·I) which readily separated from the reaction solvent (THF). The starting material 3-phenyl-3-pyrroline (**7**) was prepared from glycine ethyl ester and ethyl chloroformate in a synthetic route involving six steps (Wu et al., 1962; Kuhn and Osswald, 1956; Lee et al., 2002) with an overall yield of less than 3%. For this reason the preparation of 1-methyl-3-(4-chlorophenyl)-3-pyrroline (**4b**) was achieved via an alternative route (Fig. 4) (Williams and Lawson, 1998). The key starting material was 2-(4-chlorophenyl)-3-(dimethylamino)allylidene(dimethyl)ammonium perchlorate (**9b**) which was prepared in high yield from 4-chlorophenylacetic acid and DMF (Jutz et al., 1969). Cyclization of **9b** was achieved by treatment with sodium methoxide in anhydrous pyridine to yield 1-methyl-3-(4-chlorophenyl)pyrrole (**5b**) in fair yield (52.8%) (Gallagher et al., 1990). The preparations of 1-methyl-3-phenylpyrrole (**5a**) and 1-methyl-3-(4-methoxyphenyl)pyrrole (**5c**) were achieved in a similar manner starting from phenylacetic acid and 4-methoxyphenylacetic acid, respectively. The 1-methyl-3-(4-chlorophenyl)pyrrole intermediate was converted to the corresponding 1-methyl-3-(4-chlorophenyl)-3-pyrroline (**4b**) by partial reduction with zinc and hydrochloric acid (Williams and Lawson, 1998; Andrews and McElvain, 1929) in an overall yield of 31.9%. In our experience 3-pyrrolines prepared in this manner are often contaminated with what we believe is the corresponding pyrrolidinyl derivatives (Hudson and Robertson, 1967) in amounts ranging from approximately 10–25% as judged by ^{1}H NMR. We found that in selected instances (for example in the preparation of **4b**, the pyrrolidinyl contaminant may be removed from the 3-pyrrolinyl free base under reduced pressure with slight heating (see

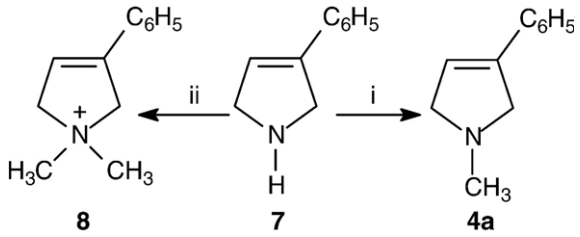

Fig. 3. Synthetic pathways to 1-methyl-3-phenyl-3-pyrroline (**4a**) and 1,1-dimethyl-3-phenyl-3-pyrrolinium iodide (**8**·I). Key: (i) formic acid, formaldehyde, 80 °C; (ii) CH_3I (6 equiv.), K_2CO_3, THF, rt.

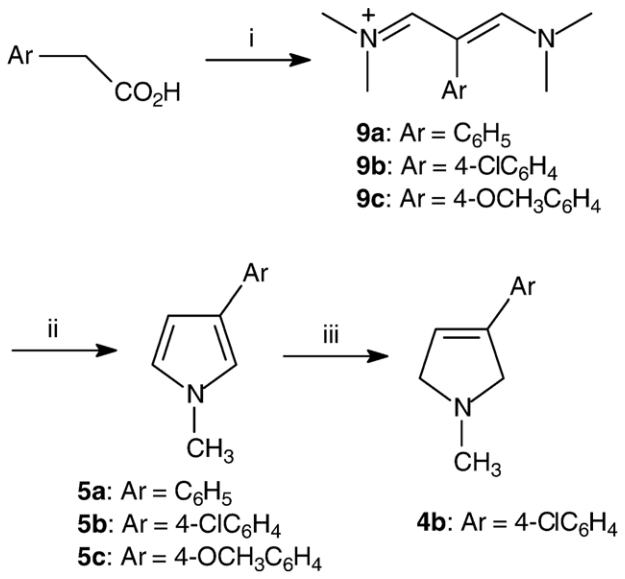

Fig. 4. Synthetic pathway to 1-methyl-3-phenylpyrroles (**5a**–**c**) and 1-methyl-3-(4-chlorophenyl)-3-pyrroline (**4b**). Key: (i) DMF, $POCl_3$, 80 °C; (ii) $NaOCH_3$, pyridine, reflux; (iii) Zn, HCl, 60 °C.

Materials and methods). Alternatively the impurity can also be removed by fractional crystallization of the oxalate salts.

General enzymology

Both 1-methyl-3-phenyl-3-pyrroline (**4a**) and 1-methyl-3-(4-chlorophenyl)-3-pyrroline (**4b**) have previously been shown to be substrates for MAO-B (Wang et al., 1998; Williams and Lawson, 1998). The 1-methyl-3-phenyl analogue is reported to have a K_m value of 193 μM and a V_{max} value of 397 min^{-1} for oxidation by beef liver MAO-B (Wang et al., 1998) while the K_m and V_{max} values for the oxidation by rat liver MAO-B are reported to be 79 μM and 8.1 nmol/min mg, respectively (Williams and Lawson, 1998). The K_m and V_{max} values for the oxidation of 1-methyl-3-(4-chlorophenyl)-3-pyrroline by rat liver MAO-B are reported to be 67 μM and 10.3 nmol/min mg, respectively (Williams and Lawson, 1998). In the present study we have confirmed both **4a** and **4b** to be good substrates of beef liver, baboon liver, mouse liver and mouse brain MAO-B, and also compared their substrate properties with that of MPTP. In order to compare the substrate properties of **4a**, **4b** and MPTP, it was necessary to determine the steady-state kinetic parameters (K_m and V_{max} values) for all three substrates in a single species since the MAO-B substrate properties often differs between species and even between different tissues in the same species (Inoue et al., 1999). Since the V_{max} values are dependent upon the MAO-B concentration in the mitochondrial preparation used in the assays, and the enzyme concentrations differ from preparation to preparation, cited values could not be used for direct comparison of the substrates. All the substrates were therefore re-examined. In order to measure the steady-state kinetic parameters we chose to measure the extent of oxidation of **4a** and **4b** by HPLC-UV analysis. The concentrations of the MAO-B-catalyzed pyrrole products **5a** and **5b** could be readily measured at wavelengths of 270 and 280 nm, respectively. HPLC-UV was chosen as the analytical technique since background interference in the near-UV wavelength range by the mitochondrial fractions used here as enzyme source was too high to measure the pyrrolyl product concentrations accurately by spectrophotometry. The incubation time of the enzyme-catalyzed reactions were chosen to be 10 min since the oxidation of both substrates (**4a** and **4b**) was found to be linear (results not shown) for at least 12 min at a substrate concentration of 50 μM. Since the mitochondrial fraction obtained from mouse brain tissue is reported to contain 15% MAO-A activity (Inoue et al., 1999) we measured the contribution of MAO-A towards the oxidation of 1-methyl-3-phenyl-3-pyrroline (**4a**) by the mitochondria obtained from this source. The MAO-B isoform was inactivated by pre-incubating the mitochondria with the MAO-B selective inactivator (*R*)-deprenyl (Inoue et al., 1999). Following incubation of **4a** (250 μM) with the MAO-B inactivated mitochondria only trace amounts (0.28±0.03 μM) of the pyrrolyl oxidation product were detected (results not shown). This was less than 5% of the product concentration (8.58±0.31 μM) detected in experiments with mitochondria not previously inactivated. In contrast, when the MAO-A isoform was inactivated by pre-incubating the mitochondria with the MAO-A selective inactivator clorgyline (Inoue et al., 1999) relatively larger amounts (7.18±0.43 μM) of the pyrrolyl product were detected which was approximately 84% of the product concentration detected in experiments with mitochondria not previously inactivated. These results document that MAO-A does not contribute significantly to the oxidation of **4a** by mouse brain mitochondrial isolates and that the steady-state kinetic parameters measured for **4a** are representative of its oxidation by the MAO-B isoform. Since mitochondria obtained from beef liver, baboon liver and mouse liver tissues are devoid of MAO-A activity (Inoue et al., 1999), the oxidation of **4a** and **4b** by these enzyme sources can be exclusively attributed to the action of the MAO-B isoform.

MAO-B substrate properties of **4a** *and* **4b**

As illustrated by example in Fig. 5 the steady-state oxidation of the substrates by MAO-B followed Michaelis–Menten behavior. The K_m and V_{max} values obtained for the oxidation of the two pyrroline substrates and MPTP by beef liver, baboon liver, mouse liver and mouse brain MAO-B are summarized in Table 1. Except for the kinetic data generated with mouse liver mitochondria no significantly large interspecies differences of the K_m values were apparent. The K_m values of **4a**, **4b** and MPTP were significantly larger with mouse liver mitochondria than with the other mitochondrial sources examined here. For example, a K_m value of 461±26.9 μM was observed for the oxidation of **4a** by mouse liver MAO-B while the corresponding value with mouse brain mitochondria was found to be 125±7.04 μM. Similarly, for the oxidation of MPTP, a K_m value of 797±11.5 μM was recorded with mouse liver MAO-B compared to a value of 52.1±3.89 μM observed with mitochondria from mouse brain. These observations are in agreement with the literature which reports a K_m value for the

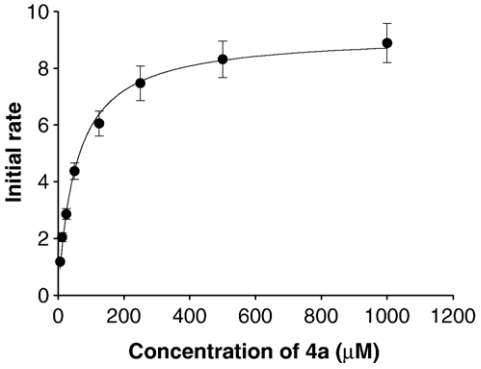

Fig. 5. Determination of the K_m and V_{max} values for the oxidation of **4a** by baboon liver MAO-B. The concentration of **5a** produced was measured by HPLC analysis following a 10 min incubation with 0.15 mg/ml baboon liver mitochondria at 37 °C. The rate data were fitted to the Michaelis–Menten equation using a nonlinear least-squares fitting routine. All measurements were conducted in triplicate and the concentration of **4a** in the incubations ranged from 6.25 to 1000 μM. The initial rates are expressed as nmol/min mg protein of **5a** formed.

oxidation of MPTP by mouse liver MAO-B of 520 μM while a K_m value of 96.8 μM was measured with mouse brain mitochondria (Inoue et al., 1999). The factors contributing to these tissue dependent differences in MAO-B activity remain to be identified. Also of note, the V_{max} values for the oxidation of **4a** and **4b** by mouse brain mitochondrial MAO-B were significantly lower than the corresponding values obtained with beef, baboon and mouse liver mitochondria as enzyme sources. Assuming that there are no large interspecies differences of the turnover numbers (k_{cat}) for the oxidation of **4a** and **4b** by MAO-B, this difference in V_{max} is possibly due to the lower density of MAO-B in brain mitochondrial fractions compared to the liver. The trend has been previously observed in various species where consistently lower V_{max} values have been measured with brain mitochondria as MAO-B source compared to the liver (Inoue et al., 1999). For example baboon liver mitochondria oxidize MPTP with a V_{max} value of 6.3 nmol/min mg protein while with brain mitochondria a V_{max} value of 2.4 nmol/min mg protein was observed. These

Table 1
Steady-state kinetic parameters for the oxidation of **4a**, **4b** and MPTP by MAO-B present in the mitochondrial fractions of beef liver, baboon liver, mouse brain and mouse liver tissue

		K_m (μM)	V_{max}^a	V_{max}/K_m^b
Beef liver	**4a**	56.4±2.82; (193)[c]	9.0±0.5	0.16
	4b	44.8±5.97	9.1±0.8	0.20
	MPTP	138±2.90; (191)[c]	3.1±0.04	0.023
Baboon liver	**4a**	54.6±3.21	9.2±0.7	0.17
	4b	46.1±10.6	4.7±0.4	0.10
	MPTP	173±6.93 (87.5)[d]	6.7±0.1	0.039
Mouse brain	**4a**	125±7.04	0.8±0.02	0.007
	4b	21.0±3.54	0.4±0.02	0.019
	MPTP	52.1±3.89; (40)[e]; (96.8)[d]	0.9±0.1	0.017
Mouse liver	**4a**	461±26.9	5.9±0.2	0.013
	4b	63.5±6.57	2.2±0.3	0.034
	MPTP	797±11.5; (520)[d]	10.6±0.1	0.013

The values are expressed in [a]nmol/min mg mitochondria and [b](min mg protein)$^{-1}$. [c]Wang et al. (1998). [d]Inoue et al. (1999). [e]Castagnoli et al. (1997).

two compounds (**4a** and **4b**) were approximately equally good substrates for MAO-B as judged by the similarity of their V_{max}/K_m values. These V_{max}/K_m values were found to be consistently higher than for MPTP which was confirmed to be also a relatively good substrate of MAO-B. These results are in accordance with literature reports that MPTP and 1-methyl-3-phenyl-3-pyrroline act as good substrates of beef liver (Wang et al., 1998) as well as rat liver (Williams and Lawson, 1998) MAO-B. We have previously shown that the well-known MAO-B substrate benzylamine is oxidized by baboon liver MAO-B with K_m and V_{max} values of 616±23 μM and 63±2.2 nmol/min mg, respectively (Vlok et al., 2006). This yields a V_{max}/K_m value of 0.10 (min mg protein)$^{-1}$ which compares with the corresponding values obtained for the pyrroline substrates **4a** and **4b** [0.17 and 0.10 (min mg protein)$^{-1}$]. Therefore, consistent with expectation, it can be concluded that **4a** and **4b** are good substrates of MAO-B isolated from a variety of species and therefore these compounds should be oxidized efficiently in vivo to the corresponding pyrrolyl products (**5a** and **5b**).

Neurotoxicity studies

It is well documented that a single intraperitoneal injection of MPTP (95–238 μmol/kg) causes depletion of striatal dopamine in aged C57BL/6 mice while still being sub-lethal (Di Monte et al., 1997; Castagnoli et al., 2001). This depletion measured 7–10 days following treatment is frequently used as marker for the nigrostriatal degeneration resulting from the neurotoxic action of MPP^+. In this study we investigated whether similar

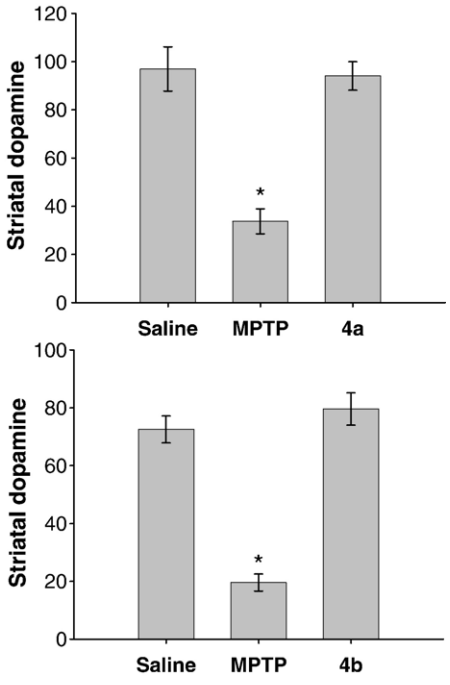

Fig. 6. Striatal dopamine levels of C57BL/6 male mice (n=10 mice/group) treated i.p. with saline, MPTP·HCl (167 μmol/kg), **4a**·$C_2H_2O_4$ (238 μmol/kg) (top) or **4b**·$C_2H_2O_4$ (238 μmol/kg) (bottom). Dopamine levels were measured 7 days after treatment and are expressed as pmol/mg tissue. ∗Significantly different (p<0.01) from the saline treated group.

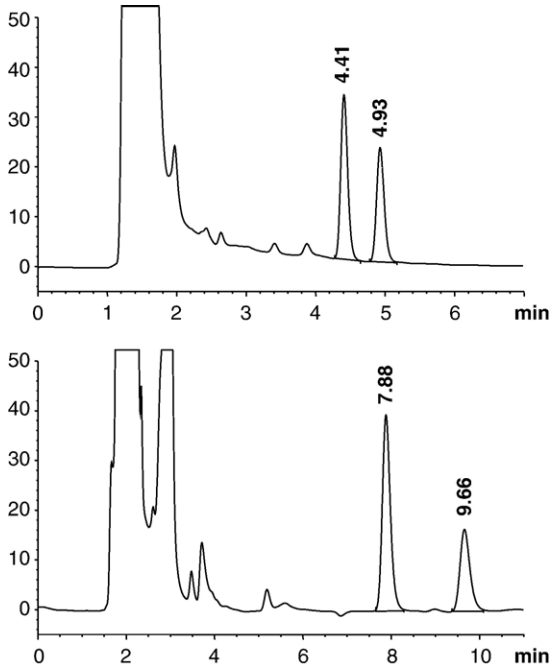

Fig. 7. HPLC-UV tracings showing the presence of 1-methyl-3-phenylpyrrole (**5a**) (top; 4.93 min; λ=270 nm) and 1-methyl-3-phenyl-3-pyrroline (**4a**) (bottom; 9.66 min; λ=250 nm) in the brain tissue of mice sacrificed 10 min following treatment with 238 μmol/kg 1-methyl-3-phenyl-3-pyrroline oxalate (**4a**·$C_2H_2O_4$). The internal standards used were 1-methyl-3-(4-methoxyphenyl) pyrrole (**5c**) (top; 4.41 min) and 1,1-dimethyl-3-phenyl-3-pyrrolinium iodide (**8**·I) (bottom; 7.88 min).

treatment of mice with **4a** and **4b** also results in a loss of dopamine in the striatum and, by association, nigrostriatal injury. Two studies were carried out each containing a control group (n=10 mice) which received saline (0.20 ml/30 g mouse) and a MPTP group (n=10 mice) which received a relatively lower dose of MPTP·HCl (167 μmol/kg). The third group (n=10 mice) received **4a**·$C_2H_2O_4$ or **4b**·$C_2H_2O_4$ at a relatively high dose of 238 μmol/kg. The animals were sacrificed 7 days later, the striata were dissected and the dopamine concentrations were determined by HPLC-ECD analysis as described in the Materials and methods. The results (Fig. 6) show that neither **4a** nor **4b** induced depletion of dopamine with the measured dopamine concentrations at 97% (Fig. 6, top) and 110% (Fig. 6, bottom) of the control values, respectively. In contrast, MPTP treatment significantly (p<0.001) reduced the dopamine levels to 35% (Fig. 6, top) and 27% (Fig. 6, bottom) of the control values.

In vivo kinetic studies

The finding that high doses of **4a** and **4b** do not mimic the characteristic striatal dopamine depletion effect of MPTP is in accordance with the idea that the permanent positive charge of MPP^+ is a key structural feature contributing to its neurotoxic action. While the lack of neurotoxicity by **4a** and **4b** may be explained by this hypothesis, it is also possible that the 1-methyl-3-phenyl-3-pyrrolines are not metabolized as efficiently as MPTP by MAO-B in vivo. This could result in relatively lower brain concentrations of the pyrrolyl products compared to a concentration of MPP^+ that leads to neuronal injury. Another explanation for the lack of neurotoxicity of **4a** and **4b** is that the bioavailability of 1-methyl-3-phenyl-3-pyrrolines to the brain may be lower than that of MPTP or that the clearance of 1-methyl-3-phenyl-3-pyrrolines and/or their pyrrolyl oxidation products from the brain may be faster than MPTP or MPP^+. To test these theories we injected mice (n=5 mice/group) with 238 μmol/kg of **4a**·$C_2H_2O_4$ and measured the brain concentrations of **4a** and its MAO-B-catalyzed oxidation product **5a** at various time points (10, 20, 40 and 60 min) following treatment (Fig. 7). In a second experiment, mice (n=6 mice/group) similarly received 167 μmol/kg MPTP·HCl and the concentrations of MPTP, $MPDP^+$ and MPP^+ in the brain tissue were measured at 10 min and 90 min following treatment. It is reported that MPTP concentrations reach a maximum at 10 min following i.p. injection while MPP^+ concentrations reach a maximum at approximately 90 min following MPTP injection (Castagnoli et al., 1997). Results in Tables 2 and 3 show that, like MPTP, **4a** reaches maximum brain concentrations relatively early with a measured concentration of 65.5±11.2 pmol/mg tissue at 10 min following treatment. This concentration is similar to the MPTP concentration in the brain of 80.17±4.99 pmol/mg at 10 min following treatment with a neurotoxic dose of MPTP. The MAO-B-generated pyrrole product **5a** was also found to reach maximal brain concentrations relatively early with a measured concentration of 53.0±9.89 pmol/mg tissue at 10 min following treatment with **4a**. This relatively high concentration of **5a** is in contrast to the MPP^+ concentration in the brain of only 11.17±2.15 pmol/mg tissue at 10 min following MPTP treatment. The pyrrolyl product **5a**, however, was found to clear relatively quickly from the brain; a concentration of only 3.11±2.54 pmol/mg tissue was detected at 60 min post-treatment. This is again in contrast to MPP^+ which was found to be present in the brain in a relatively high concentration of 77.67±4.87 pmol/mg tissue even at 90 min

Table 2
Brain concentrations of **4a** and **5a** in C57BL/6 mice (n=5 mice/group) treated with 238 μmol/kg of **4a**·$C_2H_2O_4$ and sacrificed at the indicated times (min)

Time	Brain concentrations (pmol/mg of wet tissue)	
	4a	**5a**
10	65.5±11.2	53.0±9.89
20	34.1±2.71	37.0±5.29
40	6.92±0.86	6.39±1.60
60	2.20±0.40	3.11±2.54

Table 3
Brain concentrations of MPTP, $MPDP^+$ and MPP^+ in C57BL/6 mice (n=6 mice/group) treated with 167 μmol/kg of MPTP·HCl and sacrificed at the indicated times (min)

Time	Brain concentrations (pmol/mg of wet tissue)		
	MPTP	$MPDP^+$	MPP^+
10	80.17±4.99	25.67±1.23	11.17±2.15
90	4.44±1.60	4.60±1.37	77.67±4.87[a]

[a] MPP^+ concentrations reach a maximum at ~90 min following MPTP injection (Castagnoli et al., 1997).

post-MPTP treatment. The literature reports that MPP^+ concentrations remain relatively high even at a time point of 240 min post-treatment (Di Monte et al., 1997; Castagnoli et al., 1997).

Discussion

In this investigation the nigrostriatal neurotoxic potentials of the 1-methyl-3-phenyl-3-pyrrolines **4a** and **4b** were compared with that of MPTP. It is generally accepted that the neurotoxicity of MPTP is a consequence of its MAO-B-catalyzed metabolism in the brain that ultimately yields the mitochondrial toxin MPP^+ (Chiba et al., 1984; Heikkila et al., 1984b). Evidence suggests that the mitochondrial toxicity and subsequent neurotoxic action of MPP^+ relies on it being permanently charged (Sayre et al., 1990; Rollema et al., 1990). Since the pyrrolyl oxidation products of 1-methyl-3-phenyl-3-pyrrolines are neutral species, **4a** and **4b** are not expected to be neurotoxic.

Here both **4a** and **4b** were shown to act as good substrates for beef liver, baboon liver, mouse liver and mouse brain MAO-B. Judging by the steady-state kinetic parameters, the in vitro MAO-B-catalyzed oxidation of **4a** and **4b** was at least as efficient as that of MPTP and, in some instances, even superior. The results of the neurotoxicity studies, however, show that even at high doses, **4a** and **4b** do not result in depletion of striatal dopamine as observed with MPTP. We conclude, therefore, that these pyrrolines, and probably also other 1-methyl-3-phenyl-3-pyrrolines, are not MPTP-type dopaminergic neurotoxins.

From the in vivo kinetic studies we conclude that **4a** is sufficiently bioavailable to reach brain concentrations similar to those of MPTP following a neurotoxic dose. The pyrrolyl product **5a** also reaches the brain in relative high concentrations which are comparable to the maximum concentration measured for MPP^+. The concentrations measured for **5a** in the brain are probably representative of **5a** generated centrally by the action of MAO-B on **4a** as well as **5a** partitioning from the periphery where it is produced by peripherally located MAO-B and possibly by the cytochrome P450 isozymes. Unlike **5a**, MPP^+ carries a permanent positive charge and is not expected to cross the blood–brain barrier (BBB). Therefore MPP^+ concentrations measured in the brain are representative of centrally generated MPP^+ only. The slow clearance of MPP^+ from the brain may possibly be explained by its low BBB permeability. In contrast pyrrole **5a** is cleared from the brain relatively quickly since it probably crosses the BBB freely. The relatively fast clearance from the brain may be another factor that contributes to the observed lack of neurotoxicity of the 1-methyl-3-phenylpyrroles **5a** and **5b**.

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