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COVALENT PROTEIN BINDING OF A METABOLITE OF 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE TO MOUSE AND MONKEY BRAIN *IN VITRO* AND *IN VIVO**

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Abstract—We have recently reported that a reactive metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is formed in rat brain *in vitro* by type B monoamine oxidase (MAO). In the present study, we further characterize the irreversible binding *in vitro* using tissues from mice and monkeys, two species more sensitive than rats to MPTP neurotoxicity. We also report the occurrence of irreversible binding of radioactivity after administration of tritiated MPTP in the same species *in vivo*. Tissue homogenates were incubated at 37° with 1-[methyl-^{3}H]MPTP in *in vitro* experiments. Animals were injected with labeled MPTP and sacrificed at different times in *in vivo* experiments. The perchloric acid precipitates of tissue homogenates from either procedure were washed exhaustively with organic solvents and counted for radioactivity. The amount of recovered radioactivity in *in vitro* experiments was similar using brain homogenates from mice and monkeys, whereas a considerably lower amount was found in mouse liver. MAO-B inhibitors decreased the covalent binding. However, the combined MAO-B/MAO-A inhibitor pargyline had no effect if added after 2 hr of incubation. Sulfhydryl-containing compounds decreased the covalent binding in a concentration-related manner. GSH reduced the rate of the reaction throughout the incubation. The covalent binding slowly increased in time *in vivo* in mouse brain, not in liver. There was a two-fold variation of covalently bound radioactivity in different brain areas of $^3H_2$-MPTP-treated monkey. This reactive metabolite may play a role in MPTP neurotoxicity.

Parenteral administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)§ produces a parkinsonian syndrome in several species, including humans [1, 2], rhesus [3] and squirrel monkeys [4], selectively destroying dopaminergic neurons in the A9 area of the substantia nigra. Rats are less susceptible to such an irreversible neurotoxic effect of MPTP [5], while mice are generally considered a suitable animal model. In mice, large parenteral doses of MPTP can cause nigral cell loss, even though nerve terminals appear predominantly affected according to neurochemical and histochemical evidence [6, 7]. Although the mechanism of toxicity remains unexplained, there is evidence that reactive intermediates in the oxidative metabolism of MPTP may play a role in the neurotoxic process.

Monoamine oxidase (MAO) metabolizes MPTP *in vitro* [8] and *in vivo* [9]; such biotransformation is essential for the toxicity since pretreatments with inhibitors of MAO, particularly of type B, can prevent the neurological damage in monkeys [10, 11] and mice [12]. We have recently reported that a reactive metabolite of MPTP is formed in rat brain *in vitro* by type B MAO [13]. In the present report, we have further characterized the process leading to the covalent binding *in vitro* using tissues from mice and monkeys, two species more sensitive than rats to MPTP neurotoxicity. Furthermore, we have tested the occurrence of the covalent binding in the same species *in vivo*.

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§ Abbreviations used: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MAO, monoamine oxidase; GSH, glutathione; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridine; MPP^+, 1-methyl-4-phenylpyridine.

MATERIALS AND METHODS

Male C57BL mice (Charles River Laboratories, Wilmington, MA) weighing 18–22 g, two male Squirrel monkeys (*Saimiri sciureus*), and a Rhesus monkey (*Macaca mulatta*) were used in these experiments.

Assay in vitro. Brains and livers from mice and squirrel monkeys were homogenized in 10 vol. (w/v) of ice-cold 0.01 M Tris HCl buffer (pH 7.4) using a Teflon smooth-glass homogenizer (clearance 0.025 cm). The covalent binding assay *in vitro* was performed at 37°, using an incubation volume of 1 ml containing 2–2.5 mg of tissue proteins in 0.01 M Tris HCl buffer (pH 7.4). After preincubation at 37° for 5 min, the reaction was started by the addition of 100 μl of a solution containing ^{3}H-MPTP (0.2 μCi). In the routine assay, the final MPTP concentration was 10^{-5} M. The reaction was stopped at different times by adding 1 ml of 7% perchloric acid, and the precipitates were used as samples.

Assay in vivo. Mice were treated either with a single injection of ^{3}H-MPTP (30 mg/kg i.p., at a

specific radioactivity of 100 μCi/mg) or with six injections of ^{3}H-MPTP (30 mg/kg i.p., 12 hr interval, at a specific radioactivity of 16.7 μCi/mg) and killed by cervical dislocation at different times. Whole brains and livers were immediately dissected, homogenized in 10 vol. (w/v) of 0.01 M Tris HCl (pH 7.4), and stored at −20°. Tissue from a Rhesus monkey referred as BZ9 in a previous study by Markey *et al.* [9] was used as well. The animal (6.85 kg) had been treated with a single tracer dose of $^3H_2$-MPTP (9.3 mCi) and sacrificed 24 hr later. Brain areas had been immediately dissected, homogenized in 4 vol. (w/v) of water, and stored at −20°. The homogenates of mice and monkey tissues were subsequently thawed; aliquots were tested for proteins and for radioactivity. Proteins were then precipitated with 150 vol. (wet wt/vol.) of 7% perchloric acid and used as samples.

Washing procedures. In order to remove the non-covalently bound substrate and metabolites, the perchloric acid precipitates from the assays *in vitro* and *in vivo* were washed exhaustively with organic solvents. The samples were centrifuged, the supernatant solution was removed, and the pellet was washed with 500 vol. (initial wt/vol.) of 95% ethanol. The procedure was repeated 10 times. After each washing, the solvent removed by aspiration was tested for radioactivity. At the end of the procedure, the organic wash contained background levels of radioactivity. Protein loss was negligible. The final precipitate was dissolved with 1 ml of 1 N NaOH at 55° for 24 hr and an aliquot was transferred to a counting vial containing 10 ml of scintillation fluid. Radioactivity was measured with a liquid scintillation spectrometer and the values were corrected for quenching by the external standardization method. Similar results were obtained with a different washing procedure. In this case aliquots of the perchloric acid precipitates containing 0.6–0.7 mg of tissue proteins were filtered through Whatman GF/B filters under low vacuum. The filtration was followed by 15 rinses with 10 ml of 95% ethanol. The filters were then suspended in 10 ml of scintillation fluid and radioactivity determined. The assay was performed in triplicate; individual repeats agreed to ±5%. The filtration procedure was used in the routine assay *in vitro*. Proteins were determined according to Lowry *et al.* [14]. Results of the assay *in vitro* are expressed as picomoles of ^{3}H-MPTP covalently bound per mg of protein above the levels bound at zero time. Results of the assay *in vivo* are expressed as dpm/mg of protein above the levels covalently bound to homogenates of tissue from untreated animals added with ^{3}H-MPTP containing the same amount of starting radioactivity.

Drugs and substances. MPTP HCl (Aldrich Chemical Co., Milwaukee, WI); 1-[^{3}H]-MPTP (85 Ci/mmol, New England Nuclear, Boston, MA); pargyline HCl (Sigma Chemical Co., St Louis, MO); deprenyl HCl and clorgyline HCl (Research Biochemicals Inc., Wayland, MA). All other chemicals and solvents were of the purest grade commercially available.

RESULTS

Experiments in vitro

Figure 1 shows the time-course of the irreversible protein binding of ^{3}H-MPTP in mouse brain homogenates. The increase was linear with time up to 4 hr of incubation, when it reached 136.9 ± 0.8 pmol bound/mg of protein. The rate of the reaction decreased significantly in the next 5 hr. The possibility of covalently bound intermediates formed through the non-enzymatic oxidation of MPTP was ruled out by experiments at different incubation conditions, including 0° temperature, use of preboiled tissue or addition of tissue at the end of the incubation (Table 1). Similar results were obtained

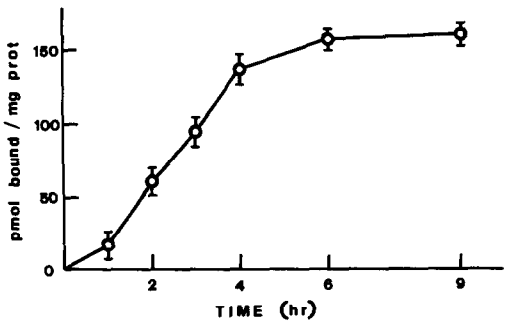

Fig. 1. Time course of irreversible protein binding of MPTP in mouse brain homogenates. Incubation volume of 1 ml containing 0.6 mg of tissue proteins in 0.01 M Tris–HCl buffer (pH 7.4), 1-[methyl-^{3}H]MPTP at a final concentration of 10^{-5} M/0.2 μCi. Incubations were stopped with 1 ml of 7% perchloric acid and the precipitates were washed exhaustively with 95% ethanol. Values are averages of six determinations ± SE.

Table 1. Influence of different incubation conditions on the irreversible protein binding of MPTP in mouse brain homogenates

	Covalently bound material (pmol/mg protein)	Inhibition (%)
Standard conditions (37°)	38.9 ± 0.8	
At 0°	5.8 ± 0.3	85
Preboiled tissue	5.1 ± 0.2	87
Tissue at the end of incubation	3.9 ± 0.2	90

Incubation in ice (0°) with water-boiled tissue (30 min) or addition of tissue at the end of incubation. Incubation time was 120 min. Values are averages of six determinations ± SE.

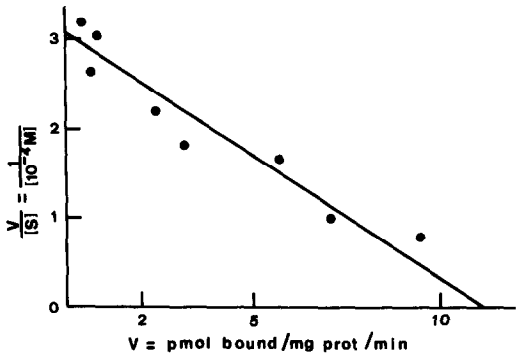

Fig. 2. Scatchard plot of irreversible protein binding of MPTP in mouse brain homogenates. V, velocity of enzyme reaction (pmol bound/mg of protein/min); S, substrate concentration (10^{-4} M). Apparent K_m of $3.7 \pm 0.4 \times 10^{-4}$ M and V_{max} of 11.2 ± 0.6 pmol bound/mg of protein/min as the average of six determinations ± SE. A typical experiment is reported.

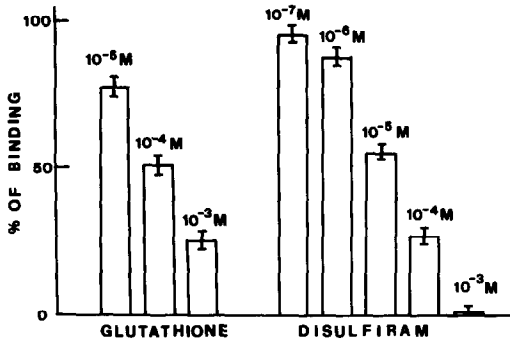

Fig. 4. Inhibition of the irreversible protein binding of MPTP in mouse brain homogenates by SH group containing compounds. Glutathione and disulfiram were added at the concentrations reported in the figure. MPTP was added at 10^{-5} M. Incubation time was 120 min. Values, as percentage of total binding, are averages of six determinations ± SE.

in squirrel monkey brain; in this case, 92.0 ± 0.04 pmol/mg of protein were covalently bound after 3 hr incubation at standard conditions. The amount of covalent binding was considerably lower in liver proteins from mice; the maximum (34 pmol/mg protein) was reached within the first hour and no further radioactivity apparently bound in the next hour.

Kinetic studies, performed incubating mouse brain homogenates with increasing concentrations of ^{3}H-MPTP (10^{-7} M–10^{-3} M), yielded an apparent K_M of $3.7 \pm 0.4 \times 10^{-4}$ M, and a V_{max} of 11.2 ± 0.6 pmol bound/mg of protein per min (Fig. 2). Incubation in the presence of MAO inhibitors significantly decreased the covalent binding in mouse brain (Fig. 3). The selective MAO-A inhibitor clorgyline was about 100 times less potent than the combined MAO-B/MAO-A inhibitor pargyline and less than the selective inhibitor of MAO-B deprenyl. However, if 10^{-4} M pargyline was added 2 hr after starting the reaction with 10^{-5} M ^{3}H-MPTP, no inhibiting effect was observed. Reduced glutathione (GSH) and other sulfhydryl-containing compounds decreased the amount of binding in a concentration-related manner (Fig. 4). In this case, the addition of GSH (10^{-3} M) after 2 hr incubation reduced the rate of the reaction even in the next 4 hr by approximately 50% (0.27 pmol/mg prot/min compared to 0.58 pmol/mg prot/min without GSH).

Experiments in vivo

Figure 5 shows the amount of radioactivity recovered after exhaustive extraction in tissue proteins of mice sacrificed at different times after either a single injection of ^{3}H-MPTP (30 mg/kg i.p., at a specific radioactivity of 100 μCi/mg) or six injections of ^{3}H-MPTP (30 mg/kg i.p., 12-hr interval, at a specific radioactivity of 16.7 μCi/mg). There was a slow increase of covalent binding in time in brain, not in liver.

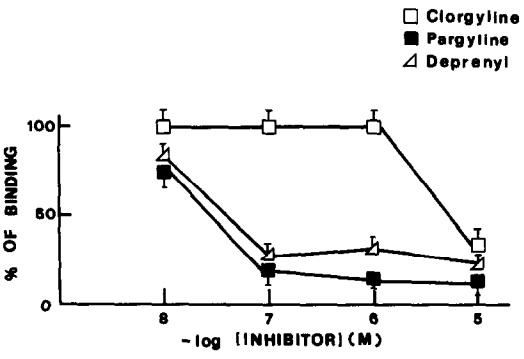

Fig. 3. Inhibition of the irreversible protein binding of MPTP in mouse brain homogenates by different MAO inhibitors. Clorgyline, pargyline and deprenyl dissolved in 0.01 M Tris–HCl were added 20 min before (preincubation time) at the concentration reported in the figure. MPTP was added at 10^{-7} M. Incubation time was 120 min. Values, as percentage of total binding, are averages of six determinations ± SE. IC_{50} values: clorgyline, 3.3×10^{-6} M; pargyline, 5.6×10^{-8} M; deprenyl, 5.6×10^{-8} M.

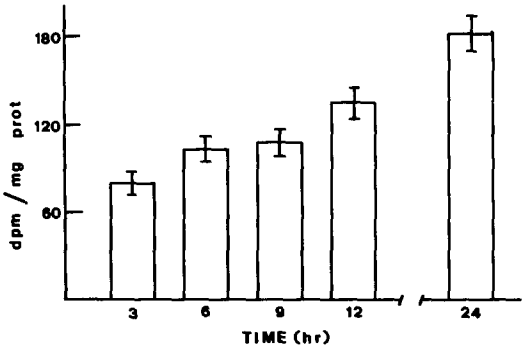

Fig. 5. Time course of the irreversible protein binding of MPTP in brain homogenates from mice treated with 1-[methyl-^{3}H]MPTP. Each animal received approximately 60 μCi of total radioactivity. Four groups (N = 3) were treated with a single injection (30 mg/kg i.p., at a specific radioactivity of 100 μCi/mg) and sacrificed 3 hr, 6 hr, 9 hr and 12 hr later. The fifth group (N = 4) was treated with six doses (30 mg/kg i.p. 12 hr interval, at a specific radioactivity of 16.7 μCi/mg) and sacrificed 24 hr after the last injection. Values are averages ± SE.

Table 2. Irreversible protein binding of MPTP in different monkey brain areas *in vivo*

Brain area	Covalently bound material (dpm/mg protein)
Cerebellum cortex	776
Pons	1057
Midbrain	1084
Hippocampus	1119
Globus pallidus	1162
Medulla oblongata	1178
Occipital cortex	1208
Amygdala	1384
Temporal cortex	1400
Hypothalamus	1430
Parietal cortex	1438
Frontal cortex	1492
Caudate nucleus	1576
Thalamus	1595
Putamen	1730
Parabrachial nuclei	1908

Values are the mean value of two different determinations each performed in duplicate.

Table 2 shows the irreversibly bound radioactivity recovered in 16 brain areas of a rhesus monkey sacrificed 24 hr after a tracer dose of $^3H_2$-MPTP. Cerebellum cortex showed the lowest level, two-fold less than that found in other areas including the neostriatum (caudate nucleus and putamen).

DISCUSSION

The prevention of MPTP-induced damage in mice and monkeys by MAO-B inhibitors [10–12] has raised the hypothesis that reactive intermediates in the oxidative metabolism of MPTP may play a role in the neurotoxic process. An alternative and widely accepted hypothesis is that the final product of oxidation, 1-methyl-4-phenylpyridine (MPP^+), which accounts for about 80% of radioactivity recovered in CNS of tritiated-MPTP-treated monkeys [9], is the toxic metabolite via its accumulation and persistence in the substantia nigra, where it finally destroys the dopaminergic neurons interrupting the mitochondrial electron transport (for a review see Singer *et al.* [15]).

Both the A and B forms of MAO oxidize MPTP rapidly; however, the rate of oxidation by the B enzyme is some 14-fold higher than that by the A enzyme [16]. Moreover, the powerful inhibition of the A form by the products 1-methyl-4-phenyl-2,3-dihydropyridine ($MPDP^+$) and MPP^+ also indicates that only the B form plays a significant role in the bioactivation of MPTP [17].

In a previous work we have reported that MPTP is converted *in vitro* to a reactive metabolite which binds irreversibly to proteins in rat brain [13]. In the present study, we have further characterized the irreversible binding *in vitro* using tissue homogenates from mice and monkeys, in attempts to identify the reactive metabolite(s). Moreover, we have demonstrated the covalent binding *in vivo* in the same species, which is a fundamental point in view of its potential relevance to MPTP neurotoxicity after systemic administration.

The covalent binding reported here is conceivably related to the fact that, besides being substrates and competitive inhibitors of MAO, MPTP and $MPDP^+$ also cause time-dependent, mechanism-based inactivation of both forms of the enzyme [16, 18, 19]. The partition ratio of turnover to irreversible inactivation is high for both forms, so that the inactivation does not prevent the progressive transformation of MPTP to MPP^+ *in vivo* [18, 20]. This indicates that only a very small fraction of the electrophilic product formed reacts with a nucleophile at the active site; most of the product dissociates and can react with other proteins in the cell (or with MAO itself at unspecific sites) or may leave the cell where it is formed by way of an appropriate transport mechanism. This could explain how reactive products from the oxidation of MPTP exert their toxic effects on nigrostriatal dopamine-containing neurons, which contain little or no MAO-B [21]. According to the present experiments with MAO inhibitors, the covalent binding takes place in more than one step. Perhaps the MAO-B-dependent intermediate is formed within the first two hours, but then slowly reacts with tissue. Interestingly, Singer *et al.* [22] have suggested that inactivation of MAO-B by MPTP may be due to the formation of more than one type of adduct with the enzyme, since extensive dialysis partially reverses the inhibition. The adduct dissociated on dialysis may involve the thiol group at the substrate site, as is true of the suicide inhibition of MAO by arylcyclopropylamines. The possibility that the thiol group, rather than a group in the flavin prosthetic group of MAO, is the nucleophile involved in the adduct formation, is also suggested by the absorbance changes which accompany the time-dependent inactivation [22]. The inactivated form contains more than a stoichiometric amount of oxidation product(s) suggesting that these products can bind both specifically and unspecifically to MAO or other tissue proteins [22].

Our experiments with SH-containing compounds, which may react themselves with the metabolite(s), suggest an involvement of thiol groups throughout the course of covalent binding, perhaps both at the active site of MAO and at the binding sites on unspecific proteins. $MPDP^+$ and its subsequent metabolites including MPP^+ can be ruled out as covalently bound electrophiles. In fact, incubation of tritiated MPTP with rat brain homogenates in the presence of excess of unlabeled $MPDP^+$ results in an increased amount of covalently bound radioactivity [13], indicating that the metabolite(s) may be formed either before $MPDP^+$ or in a different but related metabolic pathway.

MPP^+ persists intraneuronally in MPTP-treated monkeys, but is easily extracted into water on homogenization and remains in solution after protein precipitation; it is not covalently bound to intracellular proteins [9]. It has been claimed that MPP^+ is also a time-dependent, irreversible inhibitor of MAO-B [23], but in view of the high concentration required, this may be a nonspecific effect. Moreover, because MPP^+ cannot be further oxidized by the enzyme, it cannot act as a mechanism-based

inhibitor. We cannot exclude that the covalently bound metabolite may correspond to the reactive photosensitive substance which inactivates MAO-B, as reported by Buckman and Eiduson [24].

Our experiments *in vitro* and *in vivo* have evidenced that the covalent binding is considerably lower in liver than in brain, and the time-course in liver is also much faster. A possible explanation is the presence of different protective systems, considering that depletion of GSH has been found in liver of MPTP-treated mice, perhaps due to the utilization of GSH in detoxification of reactive metabolites of MPTP [25]. It has been also known that MPTP may undergo oxidative pathways other than MAO, especially in peripheral tissues. In liver microsomal preparations from rodents, MPTP is oxidized by cytochrome P-450 to the *N*-demethylated compound, which is not neurotoxic, and by flavin mono-oxygenases to the *N*-oxide [26]. Lactam and pyridone metabolites of MPTP have also been detected in mouse liver incubations via the action of aldehyde oxidases on $MPDP^+$ and MPP^+ [27].

The occurrence of covalent binding *in vivo* was not unexpected in view of the fact that MPTP is also *in vivo* an irreversible inhibitor of MAO-B. Melamed *et al.* [28] have shown, for instance, that striatal MAO-B, but not MAO-A, activity decreases in mice at 2 and 10 days after systemic administration of MPTP at doses which induce persistent striatal dopaminergic depletion. Such an effect is no longer evident at 20 and 30 days post-treatment, possibly due to the synthesis of new enzyme.

It is unlikely that the irreversible inhibition of MAO-B is in itself important in the neurotoxicity of MPTP, since treatment with irreversible MAO inhibitors does not result in nigrostriatal damage. However, the related covalent protein binding may still play a role, considering the concept that many chemically inert xenobiotics are cytotoxic because of their metabolic conversion to reactive intermediates, whose covalent binding to tissue macromolecules is often proportional to the toxicity [29].

The demonstration *in vivo* is a necessary, even if not sufficient, condition for a role of covalent binding in MPTP neurotoxicity. The relatively low amount of covalently bound radioactivity recovered in brain after systemic administration of labeled MPTP may depend on different reasons, besides the possible "first pass" effect due to hepatic metabolism and detoxification. We can speculate that the presence of protective systems (e.g. GSH) and the pattern of MAO activity might affect both the covalent binding and the different MPTP susceptibility of animal species and anatomic regions.

Both lower brain GSH levels [25] and higher brain MAO-B activity [30] have been claimed to explain why black mice are more susceptible to MPTP than albino mice [6, 31]. Moreover, it has been reported that, besides in liver, GSH is utilized in the detoxification of reactive metabolites in brain, since its levels are decreased in the brainstem [32] and substantia nigra [33] of MPTP-treated mice. Interestingly, Perry and Yong [34] have found a significant deficiency of GSH in the substantia nigra, but not in other brain regions of patients with idiopathic Parkinson's disease, suggesting a GSH consumption due to the presence of a possible causative neurotoxin. However, we cannot suggest, at least in our experimental conditions, any correlation between the covalent binding distribution in different brain regions and the hypothetical low GSH levels in primate substantia nigra [35], which may in part account for the specific nigrostriatal toxicity of MPTP. This negative finding may be in part due to the use of a non-toxic tracer dose of MPTP in the monkey. The brain area distribution of covalent binding is conceivably related to the MAO-B activity, since it parallels the distribution of MPP^+ reported in the same animal by Markey *et al.* [9]. Besides the localization to catecholamine-containing areas, covalent binding sites are found in other parts of the brain, including the cerebral cortex, where a low MAO-A/MAO-B ratio, particularly in primates, has been reported by Kalaria *et al.* [36]. These authors have also suggested that the low MAO activity found in the mouse cerebral cortex compared to that in the human cortex, may in part explain the intermediate susceptibility of the mouse to MPTP neurotoxicity [36]. The resistance of the rat to systemic MPTP may instead depend on the very high MAO activity in cerebral microvessels, where MAO may act as an "enzyme barrier" [36]. Whatever the importance of such findings, we must be cautious in generalizing from data obtained in rodents to the actual mechanism of MPTP toxicity in primates, even as for the covalent binding. Accordingly, the possible role of covalent binding in the neurotoxicity cannot be ruled out in primates notwithstanding the recent finding of Markey [37], that the amount of covalently bound radioactivity recovered after the injection of labeled MPTP in mice, is not affected by pretreating the animals with deprenyl or the dopamine-uptake inhibitor mazindol, which are known to prevent the toxicity [12, 38]. In fact, several differences between rodents and primates are still to be explained, including the fact that dopamine uptake blockers fail to protect primates from developing permanent parkinsonism [39], and the blockade of MPP^+ production by MAO-B inhibitors appears to be complete in primates treated with MPTP [10], while not in mice [40].

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