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26. We thank R. Espinosa, III for technical assistance, D. Gifford for help in the preparation of the manuscript, and E. Lanzl for editorial assistance. Supported in part by U.S. Department of Energy contract No. DE-AC02-80EV10360, by USPHS grants CA 16910 and CA 23954 (J.D.R.), CA 30388 and CA 32738 (D.W.G. and J.C.G.), CA 40163 (J.C.G.), CA 38187 (C.J.S.), by the University of Chicago Cancer Research Foundation (J.D.R. and M.O.D.), by the American Cancer Society grants IN-41-Y (M.M.L. and C.A.W.) and JFRA-134 (J.C.G.), by American Cancer Society–Illinois Division grant 85-7 (M.O.D.), and by the American Lebanese Syrian Associated Charities of St. Jude Children's Research Hospital. M.M.L. is a Special Fellow of the Leukemia Society of America. C.A.W. is the Stratton-Jaffé Fellow of the American Society of Hematology.

12 August 1985; accepted 2 December 1985

Selectivity of the Parkinsonian Neurotoxin MPTP: Toxic Metabolite MPP$^+$ Binds to Neuromelanin

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Methylphenyltetrahydropyridine (MPTP) selectively destroys neuronal cell bodies in the melanin-containing substantia nigra of humans and other primates. We show that methylphenylpyridine (MPP$^+$), an active metabolite of MPTP which is accumulated intraneuronally by the catecholamine uptake system, binds with high affinity to melanin and neuromelanin. MPP$^+$ bound intracellularly to neuromelanin may be released gradually, resulting in subsequent damage to the neurons of the substantia nigra.

METHYLPHENYLTETRAHYDROPYRIDINE (MPTP), a by-product in the synthesis of certain illicit opiates, selectively destroys dopamine neurons of the substantia nigra and produces parkinsonism in human subjects (*1, 2*) and parkinsonian symptoms in animals (*3, 4*). The selective destruction of these neurons by MPTP appears to depend on several factors. MPTP initially binds with high affinity to the enzyme monoamine oxidase (type B) (*5–9*). The conversion by monoamine oxidase of MPTP to methylphenylpyridine (MPP$^+$) is required for neurotoxicity, since prior treatment of animals with monoamine oxidase inhibitors prevents MPTP neurotoxicity (*10, 11*). The initial selectivity of MPP$^+$ for catecholamine neurons appears attributable to the high affinity of MPP$^+$, but not MPTP, for the catecholamine uptake system. Accordingly, catecholamine neurons can concentrate MPP$^+$ to levels many times greater than the surrounding extracellular fluid (*12*).

Several features of MPTP toxicity remain difficult to explain. There are marked species differences in susceptibility to MPTP neurotoxicity. In humans and monkeys, there is an extensive loss of neurons in the substantia nigra at very low doses of MPTP, while in rodents, even at higher doses, there are no permanent deficits. In mice depletion of striatal dopamine can be produced by high doses of MPTP, but neuronal cell bodies are not destroyed (*13, 14*). Monkeys and humans have a high content of neuromelanin in the substantia nigra while little or no neuromelanin exists in the substantia nigra of rodents. Furthermore, the susceptibility of monkeys to MPTP appears to increase with age (*15*) corresponding to the age-related increase of neuromelanin in their substantia nigra. Lyden *et al.* (*16*) described binding of MPTP to synthetic melanin, but only low affinity interactions were observed. We now report high affinity binding of MPP$^+$, the active metabolite of MPTP, to synthetic melanin and neuromelanin which can explain important aspects of MPTP neurotoxicity.

Melanin can arise by the autoxidation and polymerization of tyrosine, dihydroxyphenylalanine (dopa), dopamine, or norepinephrine. In skin, the synthesis of melanin is catalyzed by the enzyme tyrosinase, while in the brain it is not clear whether the formation of neuromelanin is enzymatic or nonenzymatic. Das *et al.* (*17*) showed that neuromelanin of the substantia nigra is similar to the type formed by dopamine autoxidation. Initially we examined ligand binding to synthetic melanin that was synthesized from dopamine or norepinephrine by autoxidation to model neuromelanin of the substantia nigra or locus coeruleus, respectively (*18*). [^{3}H]MPP$^+$ bound with high affinity to dopamine melanin (Fig. 1). Scatchard analysis of equilibrium-saturation data re-

Table 1. Equilibrium constants for MPP$^+$ and MPTP binding to melanin. Binding constants were calculated by computer fit (*31*). Saturation analysis was performed for [^{3}H]MPP$^+$ and [^{3}H]MPTP binding to various melanin preparations using 14 concentrations of ligand as described in (*32*). Values reported are the means ± standard error of four independent determinations. DA, dopamine; NE, norepinephrine.

	K_D (nM)	B_{max} (nmol/mg melanin)
[^{3}H]MPP$^+$		
DA Melanin	28.0 ± 4.0	1.00 ± 0.17
NE Melanin	32.0 ± 3.1	0.49 ± 0.08*
[^{3}H]MPTP		
DA Melanin	39.0 ± 5.3	0.27 ± 0.06
NE Melanin	37.0 ± 3.2	0.03 ± 0.001†

*Differs from DA melanin value, $P < 0.05$. †Differs from DA melanin value, $P < 0.001$. A modified Student's t-test which accounts for unequal variance between groups was used.

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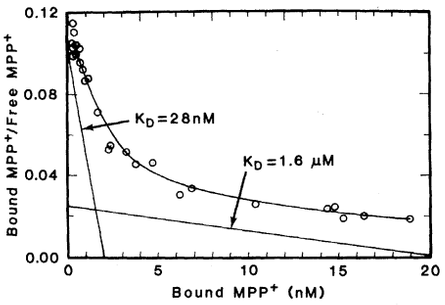

Fig. 1. Scatchard plot of the saturation of specific [^{3}H]MPP$^+$ binding to dopamine melanin. Specific binding was calculated by subtracting nonspecific binding measured in the presence of 10 μM unlabeled MPP$^+$. Data were analyzed by computer-assisted iterative curve fitting (*31*). The x axis represents the concentration of bound MPP$^+$ (nM) in 0.25 ml with 0.5 μg of dopamine melanin. The corresponding B_{max} is thus 1 nmol per milligram of melanin. Data presented are the results of a typical experiment which was replicated four times as described in Table 1.

vealed two distinct populations of binding sites. The high affinity site had a dissociation constant (K_D) of 28.0 ± 4.0 (SEM) nM and a maximal number of binding sites (B_{max}) of 1.00 ± 0.17 (SEM) nmol per milligram of melanin (Table 1). By contrast, the low affinity site had a K_D of 1.6 ± 0.4 μM and a B_{max} of 15.00 ± 0.40 nmol per milligram of melanin. MPP$^+$ bound with similar affinity to norepinephrine melanin but with a 50 percent lower B_{max}.

MPTP interactions with melanin appeared somewhat different. While [^{3}H]-MPTP bound to dopamine melanin with a similar K_D as [^{3}H]MPP$^+$, it interacted with one-fourth as many sites. Even more striking, [^{3}H]MPTP bound to only 11 percent as many sites on norepinephrine melanin as on dopamine melanin.

The binding sites for [^{3}H]MPP$^+$ on dopamine and norepinephrine melanin were highly selective. MPTP was less than 10 percent as potent as MPP$^+$ in competing for the sites (Table 2). Removal of the methyl group of MPP$^+$ yielded 4-phenylpyridine and abolished affinity for the binding sites. Dimethyl-4-phenyltetrahydropyridine which, like MPP$^+$, is a quaternary pyridine derivative, lacked detectable affinity for the binding sites. Dopamine was also inactive. Although many substances were examined, only the antimalarial quinolines, chloroquine and hydroxychloroquine, which bind to melanin (*19*) are potent inhibitors.

The drug specificity for MPTP binding differed from MPP$^+$. MPP$^+$ was only a fifth as potent in competing for [^{3}H]MPTP as for [^{3}H]MPP$^+$ binding. Conversely, MPTP itself was two times more potent than MPP$^+$ in competing for the [^{3}H]MPTP binding sites. Chloroquine and hydroxychloroquine were substantially less potent at [^{3}H]MPTP than [^{3}H]MPP$^+$ binding sites.

In experiments with neuromelanin isolated from monkey substantia nigra and with retinal melanin, [^{3}H]MPP$^+$ bound with similar affinity (30 nM) and pharmacologic specificity as it did to synthetic dopamine melanin (*20*). The high affinity of [^{3}H]MPP$^+$ binding to melanin and neuromelanin implies that such interactions occur in vivo after high intracellular concentrations of MPP$^+$ accumulate via the dopamine uptake system. Between 1 and 72 hours after monkeys receive MPTP, MPP$^+$ concentrations in the substantia nigra almost double, while they fall tenfold in the cerebellum and decline by 50 percent in the corpus striatum (*21*). MPP$^+$ is accumulated by the dopamine uptake process both in the corpus striatum and in the substantia nigra, while neuromelanin occurs only in the neuronal cell bodies. Thus, MPP$^+$ which is bound intracellularly to neuromelanin may form a depot for gradual release and subsequent damage to the neurons. We suggest that these interactions of MPP$^+$ with neuromelanin may be important for MPTP neurotoxicity. The neurotoxicity of other substances such as manganese and chloroquine may also depend on their high affinity for melanin (*19, 22*).

The binding of MPTP to neuromelanin may affect its initial regional distribution. Monkeys pretreated with pargyline, which blocks the formation of MPP$^+$, and then treated with MPTP exhibit striking regional variations in MPTP levels (*21*). MPTP concentration in the substantia nigra is six times as great as any other brain region. Interestingly, levels in the eye are four times as great as the substantia nigra. Thus, binding of MPTP to retinal melanin and neuromelanin may provide an important initial store of MPTP, especially considering the lack of MPTP binding sites on norepinephrine melanin.

In monkeys, MPTP initially depletes both norepinephrine and dopamine. Over a period of months norepinephrine levels return to normal values, and no dramatic neuronal cell loss has been demonstrated in the locus coeruleus, which also contains neuromelanin (*3*). The relative resistance of the locus coeruleus neuronal cell bodies may reflect a lesser exposure to MPP$^+$ compared to the substantia nigra. The locus coeruleus is innervated densely by catecholamine nerve terminal systems. These include dopamine terminals from cell bodies in the ventral tegmentum and the substantia nigra, norepinephrine terminals from cells in the lateral tegmentum, and a moderately dense network of epinephrine terminals (*23*). By contrast, the substantia nigra receives only a few norepinephrine and epinephrine terminals, and it is unclear whether any dopamine terminals innervate the substantia nigra. Catecholamine uptake sites can be detected on both terminals and cell bodies (*24*), but are more concentrated on nerve terminals. These sites can be labeled by [^{3}H]mazindol autoradiography (*25*). [^{3}H]Mazindol binding sites are much denser in the locus coeruleus than the substantia nigra, reflecting the

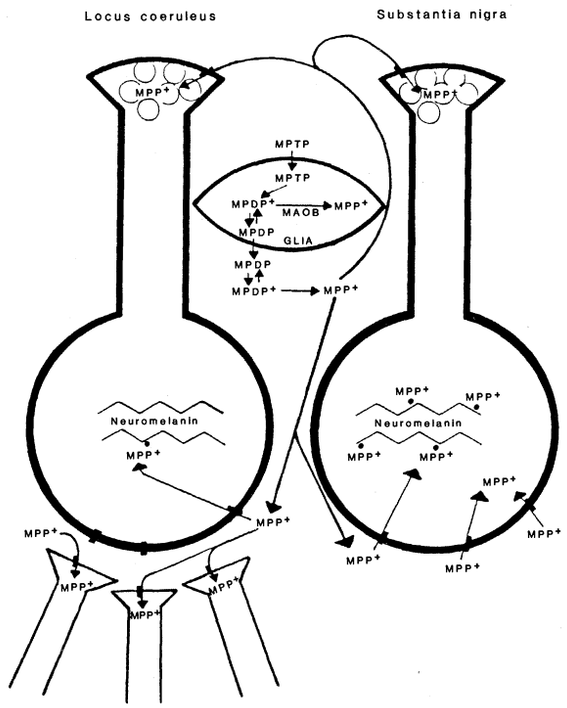

Fig. 2. Model for MPTP neurotoxicity. MPP$^+$ is produced extraneuronally from MPTP by glial monoamine oxidase (type B). The mechanism may involve the diffusion of the intermediate methylphenyldihydropyridine (MPDP$^+$) across the glial membrane and its nonenzymatic conversion to MPP$^+$. Catecholamine uptake sites on terminals and cell bodies accumulate MPP$^+$ resulting in acute neurochemical changes. The amount of MPP$^+$ taken up by cell bodies depends on the density of adjacent catecholamine terminals which can also accumulate MPP$^+$, making less MPP$^+$ available to cell bodies. In the locus coeruleus (left), the dense network of terminals protects the cell bodies from MPP$^+$ accumulation. In the substantia nigra (right) few terminals exist to compete for MPP$^+$ and so it is taken up into the nigral cell bodies. Once it is accumulated, toxic concentrations of MPP$^+$ are maintained in the cells of the substantia nigra by high affinity binding to and slow release from neuromelanin.

greater catecholamine innervation of this region. We propose that MPP^+ formed by monoamine oxidase in glia in the locus coeruleus is accumulated mainly by norepinephrine, dopamine, and epinephrine nerve terminals. Thus, very little MPP^+ is available for accumulation into locus coeruleus neuronal cell bodies (Fig. 2). By contrast, the substantia nigra lacks a dense innervation of catecholamine terminals to remove MPP^+ surrounding the substantia nigra neuronal cell bodies. Interestingly, the ventral tegmental area, which may be partly resistant to MPTP neurotoxicity, also has more [^{3}H]mazindol binding sites than the substantia nigra (*25*).

According to this model, MPTP should destroy catecholamine terminals in the locus coeruleus even though it does not destroy locus coeruleus neuronal cell bodies (*13*). We treated mice with MPTP using a regimen which produces a maximal depletion of dopamine and norepinephrine (*26*). Autoradiographic examination of [^{3}H]mazindol binding to catecholamine uptake sites reveals a 66 ± 2 percent depletion of binding sites in the corpus striatum of MPTP-treated animals relative to controls ($P < 0.001$) reflecting the loss of dopamine terminals in these areas. In the locus coeruleus a 60 ± 5 percent depletion of [^{3}H]mazindol binding sites is also observed (Student's *t*-test, $P < 0.001$). This is consistent with destruction by MPTP of catecholamine terminals innervating the locus coeruleus. The damage to the afferent catecholamine terminals in the locus coeruleus reflects the ability of these terminals to accumulate MPP^+ and thus protect the neuronal cell bodies.

In summary, a number of factors may explain the selective sensitivity of human and monkey substantia nigra to MPTP (Fig. 2). First, the substantia nigra in humans is rich in [^{3}H]MPTP binding sites, reflecting monoamine oxidase in glia and serotonin terminals capable of converting MPTP to MPP^+ (*8, 27*). The mechanism of formation of extracellular MPP^+ may involve the diffusion of the uncharged form of the intermediate methylphenyldihydropyridine ($MPDP^+$) across the glial membrane and its nonenzymatic oxidation (*28*) to MPP^+. Second, the MPP^+ generated is available for uptake into cell bodies. The dense innervation of the locus coeruleus by catecholamine nerve terminals appears to decrease the availability of MPP^+ to locus coeruleus neuronal cell bodies. Finally, although the accumulation of MPP^+ at catecholamine uptake sites results in terminal degeneration, these terminals regenerate in rodents, where the unpigmented substantia nigra neuronal cell bodies remain intact (*25, 29*). Our data imply that neuromelanin in the substantia nigra neuronal cell bodies of primates serves as a depot which gradually releases MPP^+, resulting in cell death. This explains a major component of the exquisite sensitivity of primates to low doses of MPTP and the age-related susceptibility of primates to MPTP.

Environmental toxins have been implicated in the etiology of idiopathic Parkinson's disease and may act in a fashion analogous to MPTP (*30*). The toxin-binding capacity of neuromelanin in the substantia nigra could account for a component of the selective loss of nigral neurons in idiopathic Parkinson's disease. In normal humans there is an age-related selective loss of dopamine neurons which may also be caused by the accumulation of toxic substances on nigral neuromelanin.

Table 2. Drug inhibition of MPP^+ and MPTP binding to melanin. MPTP analogues and drugs which do not inhibit specific binding of [^{3}H]MPP^+ or [^{3}H]MPTP at 1 μM concentration include methyltetrahydropyridine, 4-phenylpyridine, nortriptyline, atropine, buspirone, fluphenazine, 8-phenyltheophylline, lorazepam, cimetidine, phentolamine, verapamil, arecoline, pargyline, strychnine, 6-hydroxydopamine, phencyclidine, chlorpromazine, and 7-hydroxychlorpromazine. IC_{50} values (concentration producing 50 percent inhibition of specific binding), were calculated by computer fit (*31*). Competition curves were performed with [^{3}H]MPP^+ and [^{3}H]MPTP using 14 concentrations of drug (each in triplicate) as described in (*32*). IC_{50} values are means of three independent IC_{50} determinations whose values varied by less than 30 percent.

Drug	IC_{50} (nM)	
	[^{3}H]MPP^+ binding	[^{3}H]MPTP binding
MPP^+	40	200
Chloroquine	190	570
Hydroxychloroquine	220	620
Paraquat	600	>1000
Quinacrine	750	800
Haloperidol	810	1000
Spiperone	1000	1000
MPTP	1000	100
3-Phenylpyridine	1000	>1000
Propranolol	1000	>1000
4-Phenyltetrahydropyridine	>1000	570
Dimethyl-4-phenyltetrahydropyridine	>1000	>1000
4-Phenylpyridine	>1000	>1000
Dopamine	>1000	>1000
Carbachol	>1000	>1000

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26. Three C57bl mice per group were injected with saline or MPTP (40 mg/kg) for three consecutive days and then sacrificed 1 week later. The autoradiography was done by the method of Javitch *et al.* (*25*) and the optical densities were quantitated by computer-assisted microdensitometry.
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32. The incubation mixture contained 4 nM [^{3}H]MPP^+ or [^{3}H]MPTP (85 Ci/mM) with various concentrations of MPP^+ or MPTP and melanin. For [^{3}H]MPP+ binding, 0.5 μg of DA melanin and 0.7 μg of NE melanin were used. For [^{3}H]MPTP binding, 2.5 μg of DA melanin and 12.5 μg NE melanin were used. The total volume was 0.25 ml of 50 mM tris buffer (*p*H 7.4 at 37°C). After 2 hours 2 ml of cold buffer was added and the mixture was filtered under vacuum through glass-fiber filters (Schleicher & Schuell, No. 32) treated with 0.5 percent polyethylimine. The filters were washed with 4 ml of buffer. Radioactivity remaining on filters was measured by liquid scintillation spectrometry. In typical experiments with dopamine melanin, total [^{3}H]MPP^+ binding was 8000 cpm and nonspecific binding, as measured in the presence of 10 μM unlabeled MPP^+, was 1000 cpm. Total [^{3}H]MPTP binding in parallel experiments was 8000 cpm and nonspecific binding was 1500 cpm in the presence of 10 μM unlabeled MPTP.

33. Supported by USPHS grants MH-18501, NS-16375, MSTP training grant GM-07309 to R.D.'A., Research Scientist Award DA-00074 to S.H.S., and a grant from the Bernard Weinberg Fund.

12 September 1985; accepted 2 December 1985