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Parkinsonism-inducing neurotoxin, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: Characterization and localization of receptor binding sites in rat and human brain

(substantia nigra/caudate/locus coeruleus/autoradiography/*N*-methyl-4-phenylpyridine)

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ABSTRACT **N-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produces neuropathologic and clinical abnormalities in humans and animals that closely resemble idiopathic Parkinson disease. [^{3}H]MPTP binds with high affinity (K_d = 28 × 10^{-9} M) to brain membranes. The chemical specificity of the binding sites corresponds to structure–activity requirements for neurotoxicity. Autoradiographic studies in human brain show very high receptor densities in the caudate, substantia nigra, and locus coeruleus, which may explain the neurotoxic and neurochemical sequelae of MPTP administration. In contrast to the human, rat substantia nigra and caudate display only moderate receptor concentrations. This species difference may explain the difficulty in producing selective nigrostriatal degeneration in rats. Sites densely labeled in rat brain include the locus coeruleus, interpeduncular nucleus, arcuate and periventricular hypothalamic nuclei, and the subfornical organ.**

Parkinson disease (PD) produces a constellation of symptoms including rigidity, hypokinesia, and tremor, often accompanied by dementia or depression (1–3). Although the most prominent neuropathologic abnormality in PD is degeneration of the nigrostriatal dopaminergic neurons, other cell groups, including noradrenergic cells of the locus coeruleus, dopaminergic cells of the ventral tegmental area, and cholinergic neurons of the nucleus basalis of Meynert, are affected in many cases (4–7).

Recently, individuals who self-administered an analog of the opiate meperidine have developed apparently irreversible symptoms clinically indistinguishable from those found in PD (8, 9). Analysis of the injected material has revealed an active ingredient, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which also produces a PD syndrome in monkeys when administered in carefully titrated doses (10, 11). In postmortem studies of humans, monkeys, and mice intoxicated with this substance, pathological and neurochemical lesions are concentrated in nigrostriatal dopamine systems (8–13). However, MPTP also affects other neurochemical systems (refs. 10 and 12–14; unpublished observations), and alters motor tone and locomotor activity. In sufficient doses, MPTP is lethal, even in species such as rats, in which nigral cell loss is difficult to demonstrate (15). We now report specific high-affinity binding sites for [^{3}H]MPTP in rat and human brain membranes. The pharmacologic specificity of these binding sites resembles the structure–activity requirements for neurotoxicity. Autoradiographic studies in rat and human reveal discrete localizations of [^{3}H]MPTP binding sites in the substantia nigra, corpus striatum, and other areas such as the locus coeruleus that are also involved pathologically in idiopathic PD.

MATERIALS AND METHODS

[^{3}H]MPTP (85 Ci/mmol; 1 Ci = 37 GBq) was prepared by S. Hurt (New England Nuclear). MPTP·HCl and *N*-methyl-4-phenylpyridine (MPP^+) were kindly supplied by S. Markey (National Institutes of Health, Bethesda, MD). Other analogs of MPTP were obtained from Aldrich. All other reagents were obtained from commercial sources.

For membrane–homogenate binding studies, we used whole brains from male Sprague–Dawley rats (150–250 g) or frozen parietal cortex from a 39-year-old human asthmatic dying acutely of respiratory arrest (postmortem interval, 11 hr). Tissues were homogenized in 40 vol of ice-cold Tris buffer (pH 7.7; 25°C) and centrifuged at 45,000 × *g* for 10 min at 4°C. The pellet was resuspended in fresh buffer and recentrifuged. This procedure was repeated once more, and membranes were resuspended in 40 vol of buffer for use in binding assays. Storage of the homogenate at −70°C for up to 3 months did not significantly alter binding activity. [^{3}H]MPTP (1 nM) was incubated in a final assay volume of 0.25 ml, in the presence or absence of various tested drugs, with 5 mg of original tissue homogenate (wet weight) for 90 min at 4°C. Nonspecific binding was measured in the presence of 10 μM unlabeled MPTP. High-affinity specific binding was defined by subtracting from the total the binding in the presence of 0.3 μM unlabeled MPTP (see below). Incubations were terminated by the addition of 2 ml of ice-cold 10 mM Tris buffer (pH 7.7; 25°C) and filtration under vacuum through glass-fiber filters (Schleicher & Schuell no. 32). Filters were washed with two consecutive 2-ml aliquots of buffer. Radioactivity remaining on the filters was measured by liquid scintillation spectrometry. K_d, B_{max}, and K_i values were calculated from saturation binding data and drug competition data using an iterative curve-fitting program (16).

Autoradiographic studies were carried out using slide-mounted rat or human brain sections prepared as described (17). Brain sections (8 μM) were preincubated for 5 min in 50 mM Tris buffer (pH 7.7; 25°C) at 4°C and then incubated for 60 min in the presence of 3 nM [^{3}H]MPTP. Adjacent sections were incubated with 10 μM unlabeled MPTP to measure nonspecific binding. After two consecutive 1-min washes in buffer, slides were rinsed in distilled water and dried under a stream of cold dry air. Autoradiograms were generated by apposition of slides to ^{3}H-sensitive film ([^{3}H]Ultrofilm, LKB) for 2–3 weeks at 4°C (18).

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Abbreviations: MPTP, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP^+, *N*-methyl-4-phenylpyridine; PD, Parkinson disease; MAO, monoamine oxidase.

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RESULTS

Characteristics of [^{3}H]MPTP Binding. [^{3}H]MPTP binds saturably and with high affinity to rat brain membranes. In typical experiments using 1 nM [^{3}H]MPTP, total binding is 4000 cpm, while nonspecific binding measured in the presence of 10 μM unlabeled MPTP is 600 cpm, and binding measured in the presence of 0.3 μM unlabeled MPTP is $\approx$1500 cpm (see below). At 4°C, binding of [^{3}H]MPTP reaches equilibrium after 60–90 min. Specific binding is linear with tissue concentration between 1 and 8 mg of tissue (wet weight) per assay volume and is absent in boiled tissue. Treatment of membranes with trypsin or α-chymotrypsin (1 mg/ml; 37°C for 30 min) decreases specific binding to negligible levels.

Scatchard analysis of saturation data indicates the presence of a high-affinity site with a dissociation constant (K_d) of 28 $\pm$ 5 $\times$ 10^{-9} M (7) and maximal number of binding sites (B_{max}) of 225 $\pm$ 55 pmol per g of tissue (7), as well as a low-affinity site with K_d of 3.5 $\pm$ 0.7 $\times$ 10^{-6} M (5) and B_{max} of 8 $\pm$ 4 nmol per g of tissue (5) (Fig. 1). [^{3}H]MPTP binds saturably to filters in the absence of tissue with K_d and B_{max} values similar to the low-affinity component of [^{3}H]MPTP binding. Thus, the low-affinity binding site may largely reflect saturable interactions with the filters. Accordingly, in routine experiments, we use 0.3 μM unlabeled MPTP to define high-affinity specific binding.

Specific binding is temperature dependent, with slightly lower levels at 25°C than at 4°C and with an 80% decrease at 37°C. Routine experiments use 4°C incubations to minimize metabolism. HPLC analysis reveals no evidence of metabolic products in the incubation medium under standard incubation conditions.

Ionic strength influences [^{3}H]MPTP binding. The monovalent cations sodium, potassium, and lithium lower specific binding, with a 50% decrease apparent at $\approx$100 mM concentration. Divalent cations also decrease binding, with a 50% decrease at $\approx$1 mM copper or 10 mM calcium, magnesium, manganese, or cobalt. The cationic inhibition of binding is reversible with washing.

In human cerebral cortical membranes, Scatchard analysis of saturation data reveals a pattern similar to that seen in rat brain membranes. The low-affinity site appears identical, while the high-affinity site has a similar K_d of 24 $\pm$ 5 $\times$ 10^{-9} M (3) and a significantly larger B_{max} of 400 $\pm$ 65 pmol per g of tissue (3).

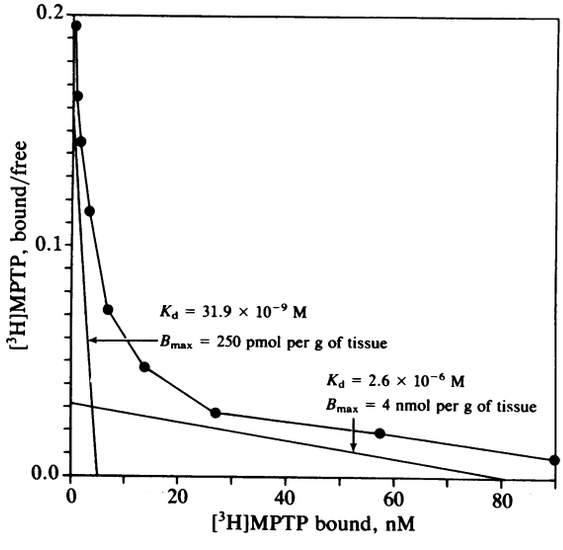

FIG. 1. Scatchard plot of the saturation of specific [^{3}H]MPTP binding to rat brain membranes. Specific binding was calculated by subtracting nonspecific binding in the presence of 10 μM unlabeled MPTP. Analyses of data by computer-assisted iterative curve fitting (16) indicate the presence of two sites. The low-affinity site represents saturable binding to filters (see text). Data represent a typical experiment, which was replicated eight times. Values are the means of triplicate determinations.

Chemical Specificity of [^{3}H]MPTP Binding. Binding sites for [^{3}H]MPTP demonstrate chemical specificity that correlates with structure–activity requirements for neurotoxicity (Table 1). MPP^+, a metabolite of MPTP in which the pyridine ring is fully unsaturated, has a potency similar to MPTP. Demethylation of the pyridine ring eliminates neurotoxicity (13) and also greatly decreases affinity for binding sites. A derivative lacking the phenyl ring also lacks affinity for binding sites and demonstrates no neurotoxicity (13).

The chemical specificity of the binding site is supported by the importance of the double bond in the tetrahydropyridine ring of MPTP. Thus, MPTP derivatives possessing more than one substituent at position 4 are devoid of activity. Numerous pyridine and piperidine structures examined also lack affinity for binding sites. Meperidine, several other opiates, and numerous psychotropic agents are inactive at the binding sites (Table 1). To ascertain whether the MPTP binding site is a receptor for some known neurotransmitter, a series of neurotransmitters were screened against [^{3}H]MPTP binding, but none displayed substantial binding affinity.

Autoradiographic Localization of [^{3}H]MPTP Binding Sites. Autoradiographic analysis indicates selective localization of [^{3}H]MPTP binding sites (Table 2). In human brain (Fig. 2), high densities of autoradiographic grains are apparent in the caudate. The substantia nigra also displays high levels of grain density. In contrast, the adjacent red nucleus has much lower grain density. The locus coeruleus displays high grain density, whereas the adjacent nucleus parabrachialis shows a more modest density and the adjoining cerebellar peduncle is almost devoid of grains. In cerebral cortex, substantial re-

Table 1. Drug inhibitory potencies on [^{3}H]MPTP binding in rat whole brain and human cerebral cortex

Drug	K_i ($\times 10^{-9}$ M) Rat	K_i ($\times 10^{-9}$ M) Human	Neurotoxicity in mice (13)
MPTP	28	24	Yes
MPP^+	41	45	ND
4-Phenyl-1,2,3,6-tetrahydropyridine	315	250	No
N-Methyl-1,2,3,6-tetrahydropyridine	>10,000	>10,000	No
N-Methyl-4-phenylpiperidine	>10,000	>10,000	No

MPTP analogs that do not potently inhibit specific [^{3}H]MPTP binding to rat brain membranes (K_i > 10 $\times$ 10^{-6} M) include piperidine, 4-cyano-4-phenylpiperidine, 4-hydroxy-4-phenylpiperidine, 4-acetylpyridine, 2-acetylpyridine, 2-aminopyridine, and meperidine. Other drugs that are weak at [^{3}H]MPTP binding sites include harmaline, harmine, pargyline, phenelzine, and tranylcypromine (K_i > 1 $\times$ 10^{-6} M), and dopamine, apomorphine, haloperidol, domperidone, γ-aminobutyric acid, muscimol, bicuculline, baclofen, picrotoxin, mazindol, imipramine, desipramine, doxepin, citalopram, iprindole, cocaine, norepinephrine, phenoxybenzamine, propranolol, rauwolscine, phentolamine, reserpine, serotonin, lysergic acid diethylamide, naloxone, [D-Ala^2-D-Leu^5]enkephalin, dextromethorphan, fentanyl, bremazocine, α-neoendorphin, β-neoendorphin, hexamethonium, decamethonium, nicotine, atropine, scopolamine, buspirone, verapamil, strychnine, loperamide, nicotine, tetrodotoxin, phencyclidine, cyproheptadine, captopril, L-phenylisopropyladenosine, 2-chloroadenosine, substance P, bradykinin, neurotensin, cholecystokinin (CCK-4, CCK-8, CCK-33), and angiotensin II (K_i > 10 $\times$ 10^{-6} M). K_i values were obtained by computer-assisted curve fitting (16). Results are from experiments using 1 nM [^{3}H]MPTP and 5 mg of tissue (wet weight) in 0.25 ml incubated for 90 min at 4°C. Nonspecific binding was measured in the presence of 0.3 μM unlabeled MPTP. ND, not determined.

Table 2. Distribution of [^{3}H]MPTP binding sites in rat and human brain

Site	Grain density	Site	Grain density
	Human Brain Regions		
Caudate	++++	N. parabrachialis	+ to ++
Substantia nigra	+++	Red n.	Trace
Locus coeruleus	+++	Cerebellar peduncle	Trace
Cerebral cortex grey matter	++ to +++	Subcortical white matter	Trace
	Rat Brain Regions		
Interpeduncular n.	++++	Medial habenula	++
Locus coeruleus	+++	Lateral septum	++
Hypothalamus		Cerebral cortex	+ to ++
Arcuate n.	+++	Hippocampus	+ to ++
Periventricular n.	+++	Cerebellum	+ to ++
Subfornical organ	+++	N. accumbens	+ to ++
Ventricular ependyma	+++	Globus pallidus	+
Superior colliculus	++	Lateral habenula	+
Periaqueductal grey	++	Medial septum	+
Substantia nigra	++	Other hypothalamic n.	+
Corpus striatum	++	Fornix	Trace
Amygdala	++	Corpus callosum	Trace
Bed n. of stria terminalis	++	Internal capsule	Trace

Semiquantitative evaluation of receptor autoradiographic grain densities in rat and human brain structures. (Averaged estimations by two independent observers.) ++++, Very dense; +++, dense; ++, moderate density; +, modest density; trace, slightly greater than background; n., nucleus.

ceptor densities are apparent in all layers of grey matter, with negligible grains in the white matter.

In contrast to the human, rat substantia nigra and caudate display only moderate receptor concentrations (Fig. 3). The highest densities of receptors in rat brain are found in the interpeduncular nucleus, arcuate and periventricular nuclei of the hypothalamus, and the subfornical organ. Ependyma also displays high binding densities in several regions. The rat resembles the human in the high and selective density of grains in the locus coeruleus. In contrast to the very high densities of binding sites in the arcuate and periventricular nuclei of the hypothalamus, other hypothalamic regions have relatively lower grain density, as is the case for the thalamus. Among extrapyramidal structures the highest levels of grains are apparent in the corpus striatum, with similar levels in the nucleus accumbens but substantially lower grain density in the globus pallidus. The interpeduncular nucleus displays high grain densities, while the habenula has moderate levels of receptors. Among limbic structures, moderate grain densities occur in the hippocampus. A moderate density can be seen in the bed nucleus of the stria terminalis, as well as in the amygdala.

DISCUSSION

The radiolabeled neurotoxin [^{3}H]MPTP labels specific binding sites in both rat and human brain with high affinity, appropriate pharmacologic profile, and a discrete pattern of regional distribution. Several lines of evidence indicate that these sites may account for the varied sequelae of MPTP administration. The high binding-site affinity should lead to substantial receptor occupancy at neurotoxic doses. The chemical requirements for binding-site affinity and induction of the Parkinsonian syndrome are similar. Thus, several drugs closely related to MPTP in structure lack MPTP-like neurotoxicity and are much less potent at the MPTP binding site. Recent evidence suggests that an MPTP metabolite, MPP^+, is selectively accumulated in monkey brain, where levels are several times those of MPTP itself (S. Markey, personal communication). The high binding potency of MPP^+ is in accord with a role for this substance in MPTP toxicity.

Some localizations of [^{3}H]MPTP binding correlate with the neurotoxic specificity of the drug. The human substantia nigra and caudate possess high and relatively selective receptor densities. However, in the rat only modest concentrations occur in these areas. Conceivably, this species difference may explain the difficulty in producing selective nigrostriatal degeneration in rats with MPTP (15).

Several areas other than the substantia nigra also display moderate to high densities of MPTP binding sites in both human and rat brain. Some of these localizations may account for the behavioral and neurochemical sequelae of MPTP administration that are not necessarily accompanied by cell

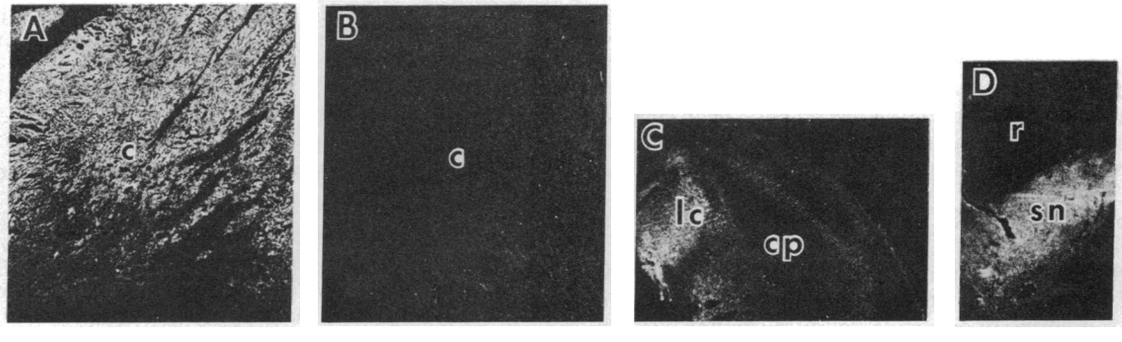

FIG 2. Autoradiogram of specific [^{3}H]MPTP binding sites in neurologically normal human brain. (Bright areas contain increased receptor densities.) (*A*) Caudate (c); (*B*) adjacent section to *A* (10 μM unlabeled MPTP was added to define nonspecific binding); (*C*) locus coeruleus (lc) and cerebellar peduncle (cp); (*D*) red nucleus (r) and substantia nigra (sn). (×8.)

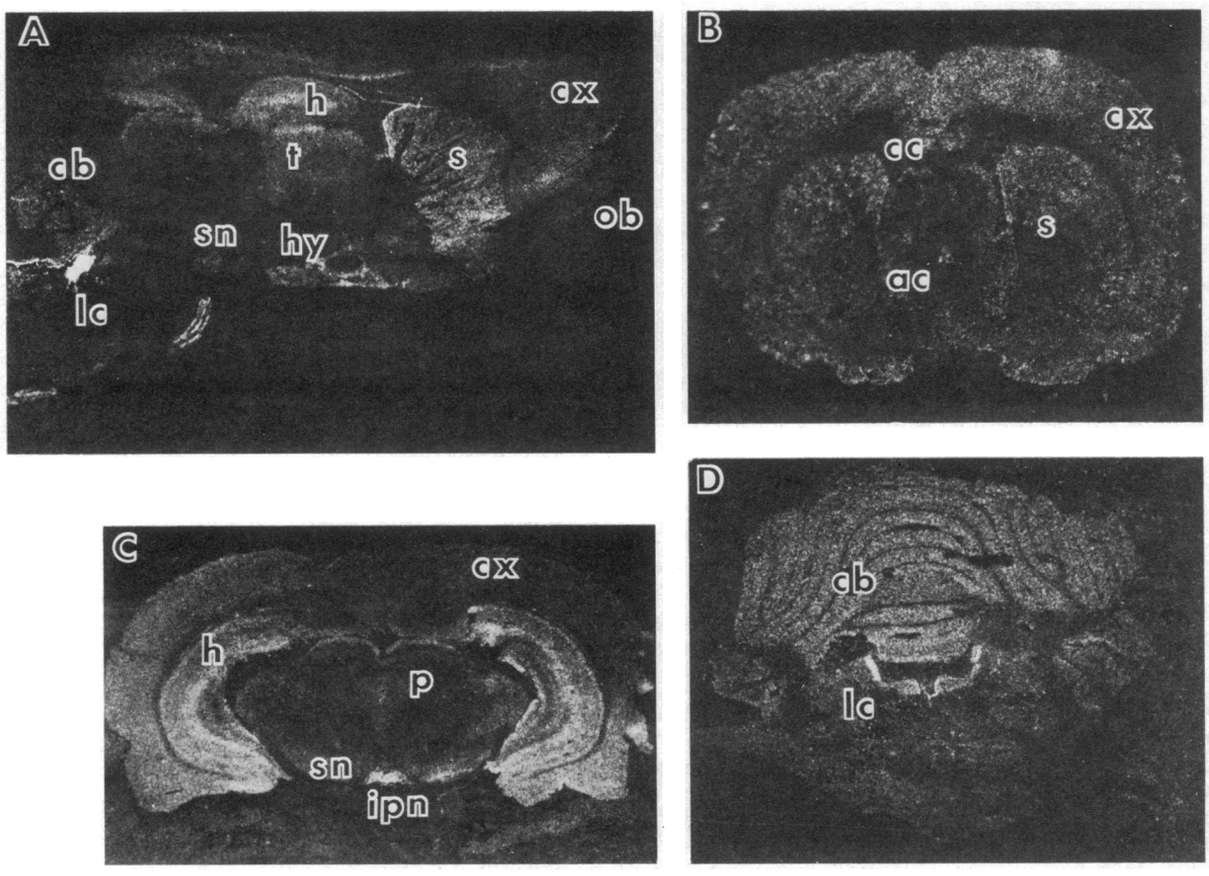

FIG. 3. Autoradiograms of specific [^{3}H]MPTP binding sites in rat brain. (Bright areas contain increased receptor density.) (*A*) Parasagittal section; (*B*) level of the anterior commissure; (*C*) level of the interpeduncular nucleus; (*D*) level of the locus coeruleus. ac, Anterior commissure; cb, cerebellum; cc, corpus callosum; cx, cerebral cortex; h, hippocampus; hy, hypothalamus; ipn, interpeduncular nucleus; lc, locus coeruleus; ob, olfactory bulb; p, periaqueductal grey; s, corpus striatum; sn, substantia nigra; t, thalamus. (×5.)

loss. For example, receptors in the locus coeruleus may explain the depletion of 3-methoxy-4-hydroxyphenylethylene glycol and norepinephrine levels in monkey and rat, respectively (10, 14).

Other binding-site localizations could be responsible for certain MPTP actions. MPTP receptors in the arcuate nucleus of the hypothalamus, as well as in the periventricular zones, suggest the possibility of endocrine effects, although endocrine markers have not been extensively evaluated after MPTP intoxication. Cerebral cortical MPTP sites could allow influences on cognitive function. The acute alterations in tone and locomotion after MPTP administration could arise from interaction with nigrostriatal or non-nigrostriatal binding sites.

Some areas that lose cells in PD, such as the locus coeruleus, contain high densities of MPTP binding sites and are targets for MPTP-induced neurochemical change. Dysfunction of morphologically intact cells in these areas could be responsible for the close parallels between MPTP intoxication and idiopathic PD. Indeed, it has recently been suggested (19) that an insult resembling MPTP is involved in the pathophysiology of idiopathic PD. Therefore, administration of MPTP may provide a better model for PD than selective destruction of the nigrostriatal pathway with 6-hydroxydopamine.

The high affinity, chemical specificity, and selective localization of MPTP binding sites suggests that they may be receptors for a normally occurring neurotransmitter-like substance. Of numerous neurotransmitters examined, none demonstrates high affinity for the [^{3}H]MPTP binding site. Nevertheless, this does not rule out the possible existence of an "endogenous MPTP." The [^{3}H]MPTP binding site is not a biogenic amine uptake site, because potent inhibitors of dopamine, norepinephrine, and serotonin uptake display negligible potency and [^{3}H]MPTP is not accumulated into rat cerebral cortical synaptosomes (unpublished observations). Interactions of MPTP with neuronal pigments have been demonstrated (20). However, the very low affinity of this interaction and the ability to produce loss of virtually unpigmented nigral cells in species such as mice argue against a central role for neuronal pigments in either high-affinity [^{3}H]MPTP binding or MPTP neurotoxicity. Membrane-associated enzymes can act as "receptor" binding sites (17). Monoamine oxidase (MAO) inhibitors prevent MPTP-induced nigrostriatal damage in mice (R. Heikkila, personal communication). It is possible that membrane-bound MAO may act as an MPTP receptor. Although potent inhibitors of MAO-A, such as harmaline and harmine, only weakly inhibit [^{3}H]MPTP binding, less selective MAO inhibitors, such as pargyline, protect against MPTP-induced neurotoxicity and compete for [^{3}H]MPTP binding at concentrations that may be pharmacologically relevant. However, MPTP is a very weak inhibitor of MAO activity in rat brain preparations ($IC_{50} > 10\ \mu M$; unpublished observations).

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1. Calne, D. B. (1977) *Ann. Neurol.* **1,** 111–119.
2. Mayeux, R., Stern, Y., Rosen, J. & Leventhal, J. (1981) *Neurology* **31,** 645–650.
3. Pirozzolo, F. J., Hansch, E. C., Mortimer, J. A., Webster, D. D. & Kuskowski, M. A. (1982) *Brain Cogn.* **1,** 71–83.
4. Alvord, E. C., Jr., Forno, L. S., Kusske, J. A., Kauffman, R. J., Rhodes, J. S. & Goetowski, C. R. (1974) in *Second Canadian–American Conference on Parkinson's Disease: Advances in Neurology*, eds. McDowell, F. H. & Barbeau, A. (Raven, New York), Vol. 5, pp. 175–193.
5. Greenfield, J. G. & Bosanquet, F. D. (1953) *J. Neurol. Neurosurg. Psychiatry* **16,** 213–226.
6. Whitehouse, P. J., Hedreen, J. C., Whilte, C. L., III, & Price, D. L. (1983) *Ann. Neurol.* **13,** 243–248.
7. Uhl, G. R., Hedreen J. C. & Price, D. L. (1983) *Neurology* **33,** 131 (abstr.).
8. Davis, G. C., Williams, A. C., Markey, S. P., Ebert, M. H., Caine, E. D., Reichert, C. M. & Kopin, I. J. (1979) *Psychiatry Res.* **1,** 249–254.
9. Langston, J. W., Ballard, P., Tetrud, J. W. & Irwin, I. (1983) *Science* **219,** 979–980.
10. Burns, R. S., Chieuh, C. C., Markey, S. P., Ebert, M. H., Jacobowitz, D. M. & Kopin, I. U. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 4546–4550.
11. Langston, J. W., Forno, L. S., Rebert, C. S. & Irwin, I. (1983) *Brain Res.* **292,** 390–394.
12. Hallman, H., Olson, L. & Jonsson, G. (1984) *Eur. J. Pharmacol.* **97,** 133–136.
13. Heikkila, R. E., Cabbat, F. S., Manzino, L. & Duvoisin, R. C. (1984) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43,** 743 (abstr.).
14. Steranka, L. S., Polite, L. N., Perry, K. W. & Fuller, R. W. (1984) *Res. Commun. Subs. Abuse* **4,** 315–323.
15. Chiueh, C. C., Markey, S. P., Burns, R. S., Johannessen, J., Jacobowitz, D. M. & Kopin, I. J. (1983) *Pharmacologist* **25,** 131 (abstr.).
16. McPherson, G. A. (1983) *Comput. Programs Biomed.* **17,** 107–114.
17. Strittmatter, S. M., Lo, M. M. S., Javitch, J. A. & Snyder, S. H. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1599–1603.
18. Unnerstall, J. R., Niehoff, D. L., Kuhar, M. J. & Palacios, J. M. (1982) *J. Neurosci. Methods* **6,** 59–73.
19. Calne, D. B. & Langston, J. W. (1983) *Lancet* **ii,** 1457–1459.
20. Lyden, A., Bordesson, V., Larsson, B. S. & Lindquist, N. G. (1983) *Acta Pharmacol. Toxicol.* **53,** 429–432.