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Characterization of *N*-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) Binding Sites in C57BL/6 Mouse Brain: Mutual Effects of Monoamine Oxidase Inhibitors and σ Ligands on MPTP and σ Binding Sites

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SUMMARY

N-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces Parkinson-like symptoms in humans, nonhuman primates, and mice. Several studies suggest that MPTP is metabolized by monoamine oxidase (MAO) type B to yield *N*-methyl-4-phenylpyridinium (MPP^+), which is responsible for the neurotoxic effects of the drug. In the present study, the pharmacological properties of [^{3}H]MPTP binding sites in C57BL/6 mouse brain membranes were investigated, and a possible relationship to the σ binding sites was examined. Both equilibrium binding experiments and kinetic assays indicate that [^{3}H]MPTP labels two distinct binding sites in C57BL/6 mouse brain. The high affinity [^{3}H]MPTP binding sites (K_d = 13 nM) are selectively blocked by the MAO type A inhibitor clorgyline, and the residual low affinity [^{3}H]MPTP sites (K_d = 1100 nM) display the pharmacological specificity of MAO-B binding sites. In contrast, the low affinity [^{3}H]MPTP binding sites are blocked by the selective MAO-B inhibitor (−)-deprenyl, and the drug-specificity profile of the remaining high affinity sites is consistent with the properties of MAO-A binding sites. The affinities of several MAO inhibitors tested and of MPTP for the high affinity MPTP/MAO-A binding sites correlate well (r = 0.96) with their affinities for the σ binding sites labeled with (+)-[^{3}H]-3-(3-hydroxyphenyl)-*N*-1-(propyl)piperidine [(+)-[^{3}H]-3-PPP]. The σ receptor ligand (+)-3-PPP displays moderately high affinity for the MPTP/MAO-A binding sites but negligible affinity for MPTP/MAO-B sites. Moreover, (+)-3-PPP alters the dissociation kinetics of MPTP from the high affinity MPTP/MAO-A sites. The finding that [^{3}H]MPTP labels MAO-B sites supports the hypothesis that the drug is a substrate for these enzyme binding sites. However, the finding that the high affinity sites, labeled by [^{3}H]MPTP, are particularly sensitive to MAO-A inhibitors, which also display high affinity for the σ binding sites, may suggest a possible relationship between MAO-A and σ binding sites. In turn, the kinetic experiments imply that σ ligands [i.e., (+)-3-PPP] may allosterically modulate the binding to MAO-A binding sites.

MPTP is a neurotoxin that is responsible for the Parkinson-like symptoms seen in humans using illicit synthetic opiate analogs of meperidine that contained MPTP as a contaminant (1, 2). Several studies have demonstrated that nonhuman primates (3–5) and mice (6–9) are highly susceptible to the neurotoxic effects of MPTP and may be useful animal models for Parkinson's disease. Administration of MPTP to experimental animals results in marked reduction in neostriatal content of dopamine and its metabolites (3, 6) and a significant loss of nerve cells in the substantia nigra (3, 6, 8). Two major steps are proposed in the mechanism of action of MPTP. First, MPTP is oxidized by MAO type B to N-methyl-4-phenyl-2,3-dihydropyridinium, which is then transformed to MPP^+ (10–13). Second, MPP^+ is actively transported into dopaminergic neurons by the dopamine uptake system (14). The involvement of MAO-B and dopamine uptake sites in the neurotoxic effects of MPTP is supported by *in vivo* experiments showing that pretreatment of animals with drugs that inhibit MAO-B activity (i.e., deprenyl) (9, 15–17) or block the dopamine uptake sites (14, 18, 19) prevents the neurotoxic effects of MPTP.

Although a significant number of studies have focused on the biochemical events following the administration of MPTP in animals, very few studies have attempted to characterize the specific binding sites of MPTP in mammalian brain (20, 21).

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ABBREVIATIONS: MPTP, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP^+, *N*-methyl-4-phenylpyridinium; MAO, monoamine oxidase; (+)-3-PPP, (+)-3-(3-hydroxyphenyl)-*N*-1-(propyl)piperidine; SKF 10047, *N*-allylnormetazocine; 5-HT, 5-hydroxytryptamine; PEA, phenylethylamine; GBR-12909, 1-[2-[bis-(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]piperazinne;Ro41-1049,*N*-(2-aminoethyl)-5-(3-fluorophenyl)-4-thiazolecarboxamide;Ro11-1163, *p*-chloro-*N*-(2-morpholinoethyl)benzamine; DTG, 1,3-di-*o*-tolylguanidine.

Moreover, because the C57BL/6 mouse strain is particularly sensitive to the neurotoxic effects of MPTP (22) and is commonly used as an animal model of parkinsonism, the characterization of MPTP binding sites in this mouse strain warrants further investigation. This seems to be an important issue, because it has been speculated that endogenously formed or exogenously acquired MPTP-like substances may play a role in the destruction of dopaminergic neurons in idiopathic parkinsonism (23). The purpose of the present study was, first, to characterize *in vitro* the binding sites for MPTP in C57BL/6 mouse brain and, second, to investigate a possible association between MPTP binding sites and the σ receptor sites in the mouse brain.

The σ receptors are postulated to be involved in the psychotomimetic effects of certain synthetic opiates (i.e., SKF 10047 and pentazocine) and in the antipsychotic effects of some neuroleptics, such as haloperidol (for review see Refs. 24 and 25). We have recently reported that certain MAO inhibitors display high affinity for the σ receptors in C57BL/6 mouse brain membranes (26). In addition, our preliminary data indicated that MPTP, which is considered an MAO substrate, also displays high affinity for the σ sites in the mouse brain. In light of these findings, we have investigated a possible relationship between MPTP-sensitive/MAO binding sites and the σ receptor sites. The present study provides evidence that indicates the labeling of multiple MPTP-sensitive/MAO binding sites. The high affinity sites display the pharmacological specificity of MAO-A binding sites and may be modulated by σ receptor ligands, whereas the low affinity [^{3}H]MPTP binding sites correspond to the MAO-B binding sites.

Experimental Procedures

Materials. [^{3}H]MPTP (82 Ci/mmol), (+)-[^{3}H]3-PPP (99 Ci/mmol), 5-[2-^{14}C]HT (59 mCi/mmol), and β-[2-^{14}C]PEA (56 mCi/mmol) were purchased from New England Nuclear (Boston, MA). The following unlabeled drugs were purchased from Research Biochemicals Incorporated (Natick, MA): MPTP, MPP^+, nomifensine, GBR-12909, clorgyline, harmaline, (−)- and (+)-deprenyl, pargyline, tranylcypromine, Ro41-1049, (+)-3-PPP, and (+)-SKF 10047. Moclobemide (Ro11-1163), a selective MAO-A inhibitor (27), was a gift from Hoffmann-La Roche (Basel, Switzerland).

***In vivo* treatments.** Male C57BL/6 mice (7–8 weeks old; 22–25 g; Taconic Farms, Germantown, NY) were treated with a single intraperitoneal injection of either (−)-deprenyl (3 mg/kg), clorgyline (2 mg/kg), or saline. One hour after the injection, the mice were sacrificed, the brains were removed, and membranes were prepared as described below.

Preparation of mouse brain membranes. The following procedure was used for both naive mice and those treated with MAO inhibitors. Whole brains were homogenized in 15 volumes of ice-cold Tris·HCl buffer (50 mM; pH 7.8) and centrifuged at 45,000 × *g* for 15 min at 4°. Pellets from untreated mice were resuspended in 6 volumes of sucrose (0.32 M), and aliquots were frozen at −80° until needed for further experiments. Pellets from MAO inhibitor-treated mice were resuspended in 30 volumes of Tris·HCl buffer and centrifuged as described above. This washing procedure was repeated three times, to ensure maximal removal of unbound drug. Pellets were then resuspended in sucrose and frozen as described above.

***In vitro* treatments of mouse brain membranes.** In order to block the MAO-A or MAO-B binding sites, frozen membrane preparations derived from naive mice were diluted (1:10) in 50 mM Tris·HCl buffer (pH 7.8) and incubated with either clorgyline (0.5 μM) (to block MAO-A sites) or (−)-deprenyl (1 μM) (to block MAO-B sites), for 60 min at 25°. Control tissue was incubated under the same condition with vehicle (buffer). The suspension was then diluted 1:1 with Tris·HCl buffer and centrifuged (45,000 × *g*, 15 min, 4°). The supernatant was discarded and the washing procedure was repeated three times, to ensure maximal removal of unbound drugs. Finally, pellets were resuspended in 0.32 M sucrose (1:6) and frozen as described above. All subsequent radioligand binding experiments and enzyme studies were carried out in the various tissue preparations, which were subjected to the same number of washes.

Binding experiments. Binding assays were performed essentially as previously described (28). Membrane preparations were diluted in 20 volumes of Tris·HCl buffer (5 mM; pH 7.8) to yield 0.4–0.5 mg of protein/ml. For competition binding experiments, the reaction was initiated by addition of 1 ml of membrane suspension to test tubes containing various concentrations of unlabeled drug and [^{3}H]MPTP (1.5–2.1 nM). The reaction mixture was incubated at 25° for 60 min, a time period that allowed equilibrium (see kinetic experiments in Results section). Nonspecific binding was determined in the presence of either unlabeled MPTP (50 μM), (−)-deprenyl (50 μM), or clorgyline (20 μM), all of which yielded very similar levels of nonspecific binding (20–25% of total binding). In routine experiments, 50 μM MPTP was used to assess the nonspecific binding. Reaction was stopped by rapid vacuum filtration through Whatman GF/B filters that were presoaked in 0.05% polyethylenimine. Filters were washed (2 × 4 ml) with the buffer, and radioactivity was determined by scintillation counting. Under the assay conditions employed, binding of [^{3}H]MPTP (1.5–2.2 nM) to blank GF/B filters represented 8–11% of total binding and was not inhibited by either 50 μM MPTP, 50 μM (−)-deprenyl, or 20 μM clorgyline.

σ Receptor binding assays were performed using (+)-[^{3}H]3-PPP, which is considered a selective ligand for the σ binding sites (28–30). Competition binding experiments were conducted as we recently described (28), using 2 nM radiolabeled ligand and 10 μM pentazocine to define the nonspecific binding.

Association kinetics of [^{3}H]MPTP binding were conducted in control, (−)-deprenyl-treated, and clorgyline-treated membranes, and pseudo-first-order association rates were calculated (31). After equilibrium was reached (60 min, 25°), dissociation assays were initiated by addition of excess unlabeled MPTP. Rate constants for the dissociation were calculated as we previously described in detail (28).

Binding data were analyzed by using the LIGAND curve-fitting receptor binding analysis program, version 2.3.10 (32, 33). This weighted, nonlinear, model-fitting computer program distinguishes as many as three distinct binding sites. The fits for specific numbers of sites were compared, using an *F* test incorporated into the program and a p of <0.05 to indicate a significantly better fit for the model tested.

Assays for MAO-A and -B. MAO activity was determined in mouse brain membranes prepared as described above and according to the procedure of Wurtman and Axelrod (34) and Da Prada *et al.* (27). In brief, 20-μl aliquots of brain homogenate (3.5 mg of wet tissue/20 μl) were incubated (15 min, 37°) with either the MAO-A substrate 5-[2-^{14}C]HT (0.2 mM) or the MAO-B substrate β-[2-^{14}C]PEA (0.02 mM), in a final volume of 200 μl of potassium phosphate buffer (30 mM; pH 7.4). Reaction was stopped by addition of 200 μl of 2 N HCl, and the deaminated metabolites were extracted by vigorous shaking with 5 ml of diethylether (5-HT extractions) or *n*-heptane (PEA extractions). After centrifugation (1000 × *g*, 1 min), the water phase was frozen in dry ice/actetone and the organic layer was poured into vials containing 8 ml of scintillation fluid. Reaction mixtures as described above but lacking the brain membranes served as blanks. Under the conditions described, 20% and 40% of the substrates 5-HT and PEA, respectively, were metabolized, and radioactivity in blank tubes was less than 0.3% of total substrates added. Protein concentrations were determined by using the method of Lowry *et al.* (35).

Results

Equilibrium binding of [^{3}H]MPTP in control mouse brain membranes. Specific binding of [^{3}H]MPTP (1.5–2.2

nM) to mouse brain membranes increased linearly with protein concentrations of 0.1–1.5 mg of protein/ml (data not shown) and represented 70–80% of total binding (Fig. 1). Because specific [^{3}H]MPTP binding in 50 mM Tris·HCl buffer (pH 7.8) was reduced by 30–35%, compared with that in 5 mM Tris·HCl (pH 7.8) buffer, all subsequent experiments were conducted in 5 mM buffer. In routine binding assays using 2 nM [^{3}H]MPTP and approximately 0.4 mg of protein/ml, total binding represented about 4.5% of total radiolabeled ligand added. Specific [^{3}H]MPTP binding, following equilibrium, was reduced by 85–90% after a single wash of the bound membranes and was completely eliminated beyond the second wash. These finding suggest that [^{3}H]MPTP binding is reversible.

Saturation binding experiments, using a fixed concentration of [^{3}H]MPTP and various concentrations of unlabeled MPTP (1–50,000 nM), resulted in the best fit for a two-site model (p = 0.001; Fig. 1), with apparent K_d values of 11 and 1300 nM. The number of high and low affinity binding sites (B_{max}) was 0.42 and 3.5 pmol/mg of protein, respectively, (Table 1). At concentrations of 1.5–2 nM [^{3}H]MPTP, the proportion of high and low affinity binding sites was approximately 2:1.

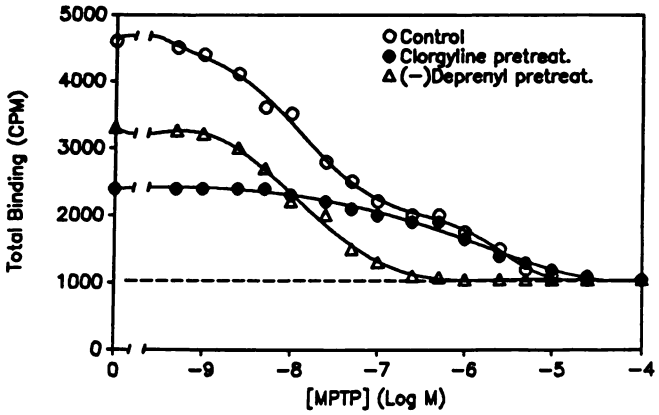

Fig. 1. Homologous competition of unlabeled MPTP for [^{3}H]MPTP (1.6 nM) binding in C57BL/6 mouse brain membranes. Assays were carried out as described in Experimental Procedures, using control tissue and membranes pretreated *in vitro* with either clorgyline (0.5 μM) or (−)-deprenyl (1.0 μM). The LIGAND computer-fitted curve for control membranes resulted in the best fit for a two-site model (F = 18; p = 0.001). For clorgyline- and (−)-deprenyl-treated membranes, the best fit was for a one-site model. *Ordinate*, total binding (in cpm) in the presence of various concentrations of unlabeled MPTP; *dashed line*, the level of nonspecific binding. Results represent the mean of four separate experiments (standard error < 8% of mean values), each carried out in triplicate.

In a series of competition binding experiments, several MAO inhibitors and other drugs were tested for their ability to compete for [^{3}H]MPTP specific binding. As indicated in Fig. 2, the selective MAO-A inhibitors clorgyline and harmaline inhibited [^{3}H]MPTP binding in a biphasic manner. Computer-assisted analysis of the binding data resulted in the best fit for a two-site model (p = 0.002; Table 2). However, the selective MAO-B inhibitor (−)-deprenyl resulted in the best fit for a one-site model. As indicated in Fig. 2 and Table 2, (+)-deprenyl was as potent as (−)-deprenyl in competing for [^{3}H]MPTP binding.

[^{3}H]MPTP binding and enzyme activity in brain membranes pretreated with MAO inhibitors. Clorgyline and (−)-deprenyl are selective and irreversible blockers of MAO-A and MAO-B, respectively (36). These properties were used to block selectively the MAO-A and MAO-B binding sites in C57BL/6 mouse brain, following *in vivo* or *in vitro* treatments with the drugs, as described in Experimental Procedures. This approach allowed us to further characterize the high and low affinity [^{3}H]MPTP binding sites. Fig. 3 summarizes the results obtained for MAO activity and [^{3}H]MPTP binding following the pretreatments with the MAO inhibitors. As indicated in Fig. 3, the *in vivo* and *in vitro* experiments yielded similar results. MAO-A activity was blocked by 95–98% following pretreatment with clorgyline, but MAO-B activity was reduced by only 2–15%. In contrast, MAO-B activity was completely abolished following (−)-deprenyl pretreatment, whereas MAO-A activity was not significantly different from control levels (Fig. 3). [^{3}H]MPTP binding, however, was reduced by 30–40% following (−)-deprenyl pretreatment, by 60% following *in vitro* clorgyline pretreatment, and by 26% following *in vivo* treatment with clorgyline (Fig. 3). These results further suggest that [^{3}H]MPTP binding is associated with both MAO-B and MAO-A binding sites. The characterization of the high and low affinity [^{3}H]MPTP binding sites in all subsequent experiments was conducted in membrane preparations that were pretreated *in vitro* with clorgyline and (−)-deprenyl. Under these conditions, clorgyline and (−)-deprenyl pretreatments resulted in nearly two thirds and one third inhibition, respectively, of [^{3}H]MPTP (2 nM) binding in mouse brain membranes. This proportion is in accordance with the ratio of high and low affinity binding sites (2:1) labeled with [^{3}H]MPTP (2 nM) in control tissue.

Drug specificity of high and low affinity [^{3}H]MPTP

TABLE 1

Equilibrium and kinetic binding parameters of [^{3}H]MPTP in C57BL/6 mouse brain

Mouse brain membranes were pretreated *in vitro* with either vehicle (control), (−)-deprenyl (1 μM), or clorgyline (0.5 μM) and washed, as described in Experimental Procedures, before binding assays were initiated. Equilibrium and kinetic experiments were carried out with 1.5–2.0 nM [^{3}H]MPTP. Binding data analyzed by the LIGAND program yielded the best fit (p = 0.001) for a two-site model in control tissue only. K_{d_1} and K_{d_2} represent the dissociation constants for the high and low affinity binding sites, respectively, and K_d is the dissociation constant calculated for a single binding site. B_{max_1} and B_{max_2} denote the total number of high and low affinity MPTP binding sites, respectively. K_{-1} denotes the pseudo-first-order association rate constant, and K_{-1} is the first-order dissociation rate constant. Results represent the mean of three or five determinations. Standard error values for kinetic data were ±10–15%.

Tissue	Equilibrium assays				Kinetic assays		
	K_{d_1}	K_{d_2}	B_{max_1}	B_{max_2}	K_{-1}	K_{-1}	K_d
	nM		pmol/mg of protein		$M^{-1}min^{-1}$	min^{-1}	nM
Control	11 ± 1	1300 ± 85	0.42 ± 0.02	3.5 ± 0.3	1.08×10^6	Biexponential[a]	
Deprenyl-treated	16 ± 1		0.51 ± 0.04		6.50×10^6	0.138	21
Clorgyline-treated		850 ± 90		3.2 ± 0.3	5.81×10^5	0.63	1080

[a] The first-order dissociation rate constants (K_{-1}) determined by nonlinear regression are 0.76 ± 0.08 and 0.11 ± 0.01 min^{-1} for the fast and slow dissociation components, respectively.

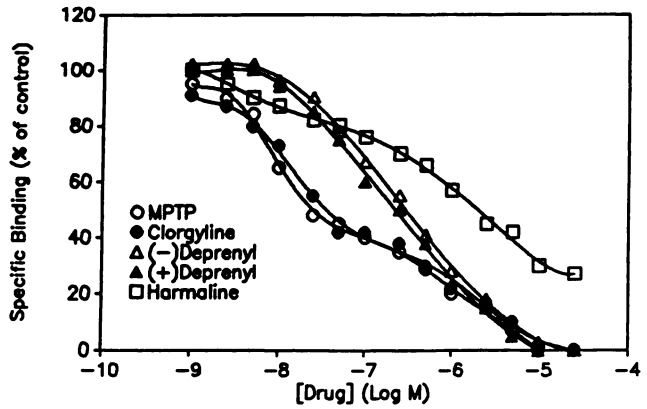

Fig. 2. Inhibition of [^{3}H]MPTP (2.0 nM) binding in C57BL/6 mouse brain membranes by unlabeled MPTP and selected MAO inhibitors. Assays were carried out in control membrane preparations, as described in Experimental Procedures. The LIGAND computer-fitted curves for MPTP (see Fig. 1), clorgyline, and harmaline gave the best fit for a two-site model ($F = 13$; $p = 0.002$). The curves for (−)- and (+)-deprenyl did not yield a better fit for a two-site model than a one-site model. Results are presented as percentage of specific binding and are the mean of three determinations (standard error < 10%).

binding sites. Equilibrium saturation binding experiments with [^{3}H]MPTP in deprenyl-treated membranes indicated the labeling of a single high affinity binding site (Fig. 1, Table 1), with an apparent K_d of 16 nM and B_{max} of 0.51 pmol/mg of protein. In contrast, binding of [^{3}H]MPTP in clorgyline-treated membranes revealed the labeling of a single low affinity binding site (K_d = 850 nM, B_{max} = 3.2 pmol/mg of protein; Fig. 1, Table 1). To further establish that the high affinity [^{3}H]MPTP binding site has the properties of MAO-A sites and the low affinity site corresponds to MAO-B sites, competition binding experiments were carried out in each tissue preparation, using various MAO inhibitors. As indicated in Fig. 4, clorgyline inhibited [^{3}H]MPTP binding in deprenyl-treated tissue in a manner manifesting the labeling of a single high affinity site. The pseudo K_i value calculated was 12 nM. The affinities of the various MAO inhibitors for [^{3}H]MPTP sites in deprenyl- and clorgyline-pretreated tissues are summarized in Table 2. As indicated, all drugs considered to be MAO-A inhibitors displayed relatively higher affinity for [^{3}H]MPTP binding sites in the deprenyl-treated tissue. However, (−)-deprenyl, pargyline, and tranylcypromine displayed higher affinity for [^{3}H]MPTP binding sites in the clorgyline-treated tissue, suggesting their preferential affinity for MAO-B sites. Interestingly, the isomers of deprenyl did not display differential affinity for [^{3}H]MPTP binding in control tissue (Fig. 2). However, (−)-deprenyl was about 10-fold more potent than (+)-deprenyl in tissue where MAO-A binding sites were blocked, whereas (+)-deprenyl was about 12-fold more potent in membrane preparations where MAO-B sites were blocked (Table 2).

In order to examine whether, under the assays conditions employed, [^{3}H]MPTP is not metabolized, the following assays were performed. Mouse brain membranes were incubated with 2 nM [^{3}H]MPTP (60 min, 25°) and centrifuged (45,000 × *g*, 15 min), and supernatant aliquots were applied to a silica gel thin layer chromatography plate, which was developed in acetone/1 N HCl (30:1). Radioactivity determined by a Bioscan System 200 imaging scanner indicated a major peak, which represented 92% of total radioactivity, that corresponded to authentic [^{3}H]MPTP (95% purity) or unlabeled MPTP. To further assess the issue of a possible conversion of [^{3}H]MPTP to [^{3}H]MPP$^+$,

TABLE 2

Affinities of various compounds for [^{3}H]MPTP binding sites in C57BL/6 mouse brain

Mouse brain membrane preparations were pretreated *in vitro* with either vehicle (control), (−)-deprenyl (1 μM), or clorgyline (0.5 μM) and washed, as described in Experimental Procedures, before the binding assays were initiated. Assays were carried out using 1.5–2.0 nM [^{3}H]MPTP. The equilibrium binding parameters of [^{3}H]MPTP under the various conditions were entered into the LIGAND program for calculation of the affinity constant (K_i) of the drugs tested. Data for the irreversible inhibitors clorgyline and deprenyl represent "pseudo-K_i" values. Assays with unlabeled MPTP, clorgyline, and harmaline resulted in the best fit for a two-site model (p = 0.001–0.002; pseudo-Hill coefficient values are 0.54–0.67), presented as high and low affinity constants. For all other drugs tested, the Hill coefficient values are between 0.78 and 1.1 and the best fit observed was for a one-site model. Results represent the mean ± standard error of three or four separate experiments.

Drug	K_i			
	Control		Deprenyl-treated	Clorgyline treated
	High affinity	Low affinity		
			μM	
MPTP and MAO inhibitors				
MPTP	0.011 ± 0.001	1.3 ± 0.2	0.016 ± 0.001	0.85 ± 0.08
Clorgyline	0.010 ± 0.001	4.1 ± 0.3	0.012 ± 0.001	3.8 ± 0.3
Harmaline	0.085 ± 0.005	7.3 ± 0.5	0.094 ± 0.002	>100
(+)-Deprenyl	0.42 ± 0.02		0.075 ± 0.006	0.85 ± 0.04
(−)-Deprenyl	0.51 ± 0.03		0.855 ± 0.07	0.08 ± 0.005
Pargyline	0.98 ± 0.07		4.10 ± 0.32	0.07 ± 0.006
Tranylcypromine	1.20 ± 0.06		5.20 ± 0.38	0.11 ± 0.01
Ro41-1049	4.50 ± 0.40		2.10 ± 0.28	8.20 ± 0.60
Ro11-1163	35.00 ± 4.00		1.71 ± 0.60	>100
Dopamine uptake inhibitors				
MPP$^+$	0.4 ± 0.05		0.5 ± 0.03	0.3 ± 0.03
GBR-12909	3.8 ± 0.2		4.5 ± 0.5	2.8 ± 0.1
Nomifensine	11.5 ± 0.8		10.3 ± 1.5	13.0 ± 1.1
σ Ligands				
(+)-3-PPP	6.1 ± 0.4		0.75 ± 0.03	13.5 ± 0.2
(±)-Pentazocine	7.2 ± 0.5		0.51 ± 0.05	5.2 ± 0.4
(+)-SKF 10047	31.0 ± 4.1		6.5 ± 0.50	48.0 ± 3.0
(−)-3-PPP	46.5 ± 7.0		28.4 ± 4.0	22.3 ± 3.1
(−)-SKF 10047	71.0 ± 8.0		78.0 ± 6.0	109.0 ± 9.0

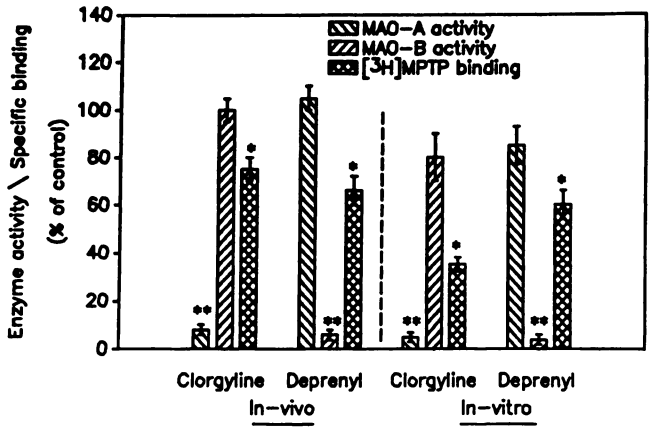

Fig. 3. Effect of clorgyline and (−)-deprenyl treatments on MAO-A and MAO-B activity and [^{3}H]MPTP binding in mouse brain membranes. *Left*, results from *in vivo* treatments; *right*, results from *in vitro* treatments. Enzyme activity is presented as percentage of control values. Control MAO-A and MAO-B activities, determined as described in Experimental Procedures, were 1530 ± 70 and 510 ± 24 pmol/15 min/1 mg of tissue, respectively. Specific [^{3}H]MPTP binding (1.7 nM) is expressed as percentage of control binding (0.135 ± 0.006 pmol/mg of protein). Results are the mean ± standard error of three separate experiments. **, $p < 0.001$; *, $p < 0.01$ as compared with control.

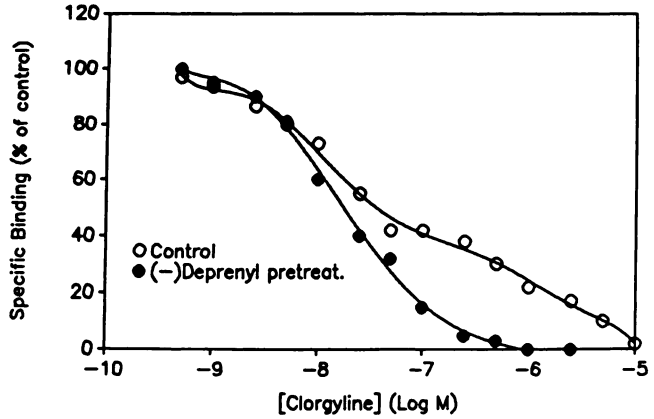

Fig. 4. Inhibition of [^{3}H]MPTP (2.0 nM) binding to control and *in vitro* deprenyl-treated mouse brain membranes by clorgyline. Membranes were prepared as described in Experimental Procedures. The LIGAND computer-fitted curve for experiments in control tissue resulted in the best fit for a two-site mode ($p = 0.002$), whereas assays in deprenyl-treated tissue resulted in the best fit for a one-site model. Results represent the mean of four experiments (standard error < 7%).

which is known to bind to the dopamine carrier (10–14), the affinities of several dopamine uptake inhibitors were examined. As indicated in Table 2, MPP$^+$, nomifensine, and GBR 12909 did not display preferential high affinity for [^{3}H]MPTP binding sites in any of the various membrane preparations tested, suggesting that dopamine uptake sites were not labeled. Thus, it seems unlikely that the high and low affinity [^{3}H]MPTP binding sites detected correspond to the dopamine carrier. Because the concentration of [^{3}H]MPTP used (2 nM) in the binding assays was approximately 100,000-fold lower than its K_m value as a substrate for either MAO-A or -B (37), it would be difficult to detect any metabolites under the assay conditions empoyed.

Because our data indicated that MPTP displays relatively high affinity for the σ binding sites labeled with (+)-[^{3}H]3-PPP (Table 3), we examined the effects of selected σ ligands on [^{3}H]MPTP binding in the various membrane preparations. Results

TABLE 3

Affinities of MPTP and MAO inhibitors for (+)-[^{3}H]3-PPP binding sites in C57BL/6 mouse brain

Competition binding assays were carried out as described in Experimental Procedures; using 2.0 nM (+)-[^{3}H]3-PPP and 10 μM pentazocine to determine the nonspecific binding. The equilibrium binding parameters of (+)-[^{3}H]3-PPP, as determined previously (26), were entered into the LIGAND program to calculate the affinity constant (K_i) of the drugs tested. The analysis resulted in the best fit for a one-site model for all drugs tested. n_H denotes Hill coefficient values. Results represent the mean ± standard error of three to five determinations.

Drug	K_i	n_H
	μM	
Clorgyline	0.0035 ± 0.0002	0.92 ± 0.07
MPTP	0.041 ± 0.002	1.08 ± 0.09
(+)-Deprenyl	0.081 ± 0.005	0.95 ± 0.06
Harmaline	0.450 ± 0.050	0.77 ± 0.06
(−)-Deprenyl	0.310 ± 0.040	0.82 ± 0.08
Ro11-1163	1.020 ± 0.075	0.95 ± 0.05
Ro41-1049	1.500 ± 0.080	0.88 ± 0.07
Pargyline	3.120 ± 0.134	1.05 ± 0.08
Tranylcypromine	6.050 ± 0.306	0.85 ± 0.08

summarized in Table 2 indicate that the σ ligands tested displayed preferential moderately high affinity for [^{3}H]MPTP binding sites only in membrane preparations where MAO-B sites were blocked. (+)-3-PPP, (+)-SKF 10047, and pentazocine were 5–12-fold more potent inhibitors in the deprenyl-pretreated tissue, compared with control membranes. Moreover, (−)-3-PPP and (−)-SKF 10047 were significantly less potent displacers (Table 2). These findings suggest that the high affinity [^{3}H]MPTP binding site, which has the characteristics of MAO-A sites, is also sensitive to the σ ligands tested.

Affinities of MAO inhibitors and MPTP for (+)-[^{3}H]3-PPP binding sites. We have previously reported that clorgyline and other MAO inhibitors display relatively high affinity for the σ binding sites (26). In the present study, we examined the affinity of additional MAO inhibitors and MPTP for the σ binding sites labeled with 2 nM (+)-[^{3}H]3-PPP. Results summarized in Table 3 indicate that the rank order of potencies of MAO inhibitors and MPTP for competing for (+)-[^{3}H]-3-PPP binding sites is very similar to that for competing for the high affinity [^{3}H]MPTP binding site (Table 2). Fig. 5 illustrates the high degree of correlation ($r = 0.96$) between the affinities of the MAO inhibitors and MPTP for the high affinity [^{3}H]MPTP/MAO-A sites and (+)-3-PPP/σ sites.

Kinetic experiments. We further investigated the kinetics of [^{3}H]MPTP binding in mouse brain membranes. The association time courses in control, clorgyline-pretreated, and (−)-deprenyl-pretreated membranes are presented in Fig. 6A. The pseudo-first-order association rates are presented in Fig. 6B and summarized in Table 1. The association rate of [^{3}H]MPTP binding to the high affinity site, in deprenyl-treated membranes, was about 10-fold faster than the association rate to the low affinity site, in clorgyline-treated membranes (6.5×10^6 and 5.8×10^5 M^{-1} min^{-1}, respectively). The dissociation of [^{3}H]MPTP binding is presented in Fig. 7. In control tissue the dissociation rate gave the best fit for a biexponential decay, whereas the dissociation time course from the high affinity site (deprenyl-treated tissue) and the low affinity site (clorgyline-treated tissue) gave the best fit for a monophasic dissociation curve (Fig. 7). The first-order dissociation rate constant for the low affinity site was 5- to 7-fold greater than that calculated for the high affinity site (Table 1). The dissociation constants (K_d values) calculated from the kinetic experiments for the high

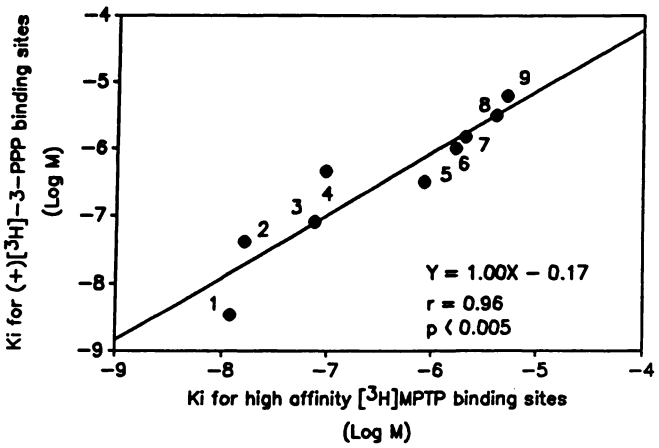

Fig. 5. Correlation between the affinities of MAO inhibitors and MPTP for the σ binding sites labeled with (+)-[^{3}H]-3-PPP and the high affinity [^{3}H]MPTP binding sites. Binding of (+)-[^{3}H]-3-PPP (2 nM) was carried out in control (untreated) mouse brain membranes. The K_i values of the drugs for the σ binding sites are presented in Table 3. Binding of [^{3}H] MPTP (2 nM) to the high affinity sites was assessed in membrane preparations pretreated *in vitro* with (−)-deprenyl to block the MAO-B binding sites, as described in Experimental Procedures. The K_i values of the drugs for the high affinity [^{3}H]MPTP binding sites are summarized in Table 2. The following drugs are presented: **1**, clorgyline; **2**, MPTP; **3**, (+)-deprenyl; **4**, harmaline; **5**, (−)-deprenyl; **6**, Ro11-1163; **7**, Ro41-1049; **8**, pargyline; **9**, tranylcypromine.

and low affinity [^{3}H]MPTP binding sites are in good agreement with the K_d values calculated from equilibrium experiments (Table 1).

As indicated in Table 2, the affinity of (+)-3-PPP for the high affinity [^{3}H]MPTP binding site is moderately high and obviously lower than the K_d of (+)-3-PPP for the σ sites in the mouse brain (9 nM) (26). In order to assess a possible "indirect" interaction of (+)-3-PPP with the high affinity [^{3}H]MPTP binding site, the effect of (+)-3-PPP on the dissociation kinetics of [^{3}H]MPTP was examined. As indicated in Figs. 7 and 8, the dissociation of [^{3}H]MPTP from the high affinity site resulted in the best fit for a monophasic decay ($K_{-1} = 0.13 \pm 0.06$ min^{-1}). However, when dissociation of [^{3}H]MPTP was initiated in the presence of both MPTP (2.2 μM) and (+)-3-PPP (10 μM) (Fig. 8), the dissociation rate not only was facilitated but also resulted in the best fit ($p < 0.005$) for a biexponential decay (K_{-1} values are given in Fig. 8). Similar studies carried out with (−)-3-PPP (10 μM) did not affect the dissociation kinetics of [^{3}H]MPTP (data not shown). These findings suggest that (+)-3-PPP may interact with an allosteric site associated with the high affinity MPTP binding site.

Discussion

Three major findings are presented in this study. First, specific MPTP binding to C57BL/6 mouse brain membranes is associated with the labeling of both MAO-A and MAO-B binding sites. Second, MAO inhibitors and MPTP display similar rank orders of potencies in competing for MAO-A binding sites and σ receptor sites. Third, the σ receptor ligand (+)-3-PPP may allosterically modulate the binding to MAO-A sites.

Several lines of evidence suggest that MPTP labels multiple sites in the mouse brain. (i) Saturation binding experiments revealed the labeling of high and low affinity binding sites. (ii) Competition binding assays indicated that some of the selective MAO-A inhibitors (clorgyline and harmaline) inhibit [^{3}H] MPTP binding in a biphasic manner, consistent with the labeling of high and low affinity sites. The finding that (−)-deprenyl and (+)-deprenyl display the same affinity for [^{3}H] MPTP binding sites, in control tissue preparations, argues against a selective labeling of MAO-B binding sites by [^{3}H] MPTP in control membranes, because the stereospecificity of MAO-B is for the (−)-isomer of deprenyl. (iii) Blocking of MAO-B binding sites by either *in vivo* or *in vitro* pretreatments with (−)-deprenyl resulted in only 30–35% inhibition of [^{3}H] MPTP binding, suggesting that the residual binding represents association with additional binding site(s). Subsequently, blocking of MAO-A binding sites by clorgyline resulted in approximately 60–65% inhibition of [^{3}H]MPTP binding. (iv) The dissociation kinetics of [^{3}H]MPTP binding in control untreated membrane preparations are also indicative of the labeling of two binding components, one with a fast dissociation rate and a second with a slow dissociation rate.

The proposal that the high affinity [^{3}H]MPTP binding site has the pharmacological properties of MAO-A sites and the low affinity site corresponds to MAO-B sites is supported by the following findings. (i) Pretreatment of membranes with (−)-deprenyl resulted in the labeling of a single high affinity binding site, whereas treatment with clorgyline yielded the labeling of a single low affinity site (Fig. 1). (ii) Drugs that are considered relatively selective MAO-A inhibitors became significantly more potent displacers of [^{3}H]MPTP binding in membrane preparation where all MAO-B sites were blocked (Table 2) or inhibited [^{3}H]MPTP binding in a manner that is consistent with the labeling of a single high affinity site (Fig. 4). (iii) (−)-Deprenyl but not (+)-deprenyl became a more potent inhibitor of [^{3}H]MPTP binding in membranes where all MAO-A sites were blocked. (iv) The association rate of [^{3}H] MPTP in membranes where MAO-B sites were blocked was about 10-fold faster than in membranes where MAO-A sites were blocked. Consistent with the different kinetics of MPTP association to MAO-A and MAO-B sites is the finding that the dissociation rate of MPTP was about 5-fold slower in membranes where MAO-B sites were blocked than in membranes where MAO-A sites were blocked. Thus, the kinetic experiments further support the concept that the high affinity [^{3}H] MPTP binding site corresponds to MAO-A and the low affinity site corresponds to MAO-B. This conclusion is in accordance with enzyme experiments indicating that the K_m value of MPTP for MAO-A is significantly lower than the K_m value for MAO-B, suggesting that MPTP has higher affinity for MAO-A than MAO-B (37). Previous studies that have also indicated that MPTP labels the MAO enzyme (20, 21) did not fully characterized these sites.

Because (−)-deprenyl, but not clorgyline, can protect against the neurotoxicity of MPTP, the involvement of MAO-B in the metabolism of MPTP was implicated (10–13, 15–17). Our present findings, which strongly suggest the association of MAO-A with the binding of MPTP, are not necessarily in contrast to the hypothesis that MAO-B may be primarily responsible for the neurotoxic products of MPTP. Nevertheless, our present findings and the observations of other investigators (37) suggest that MAO-A may also play an important role in the mechanism of action of MPTP. Interestingly, it has been reported that MAO-A is primarily involved in the metabolism of 1-methyl-

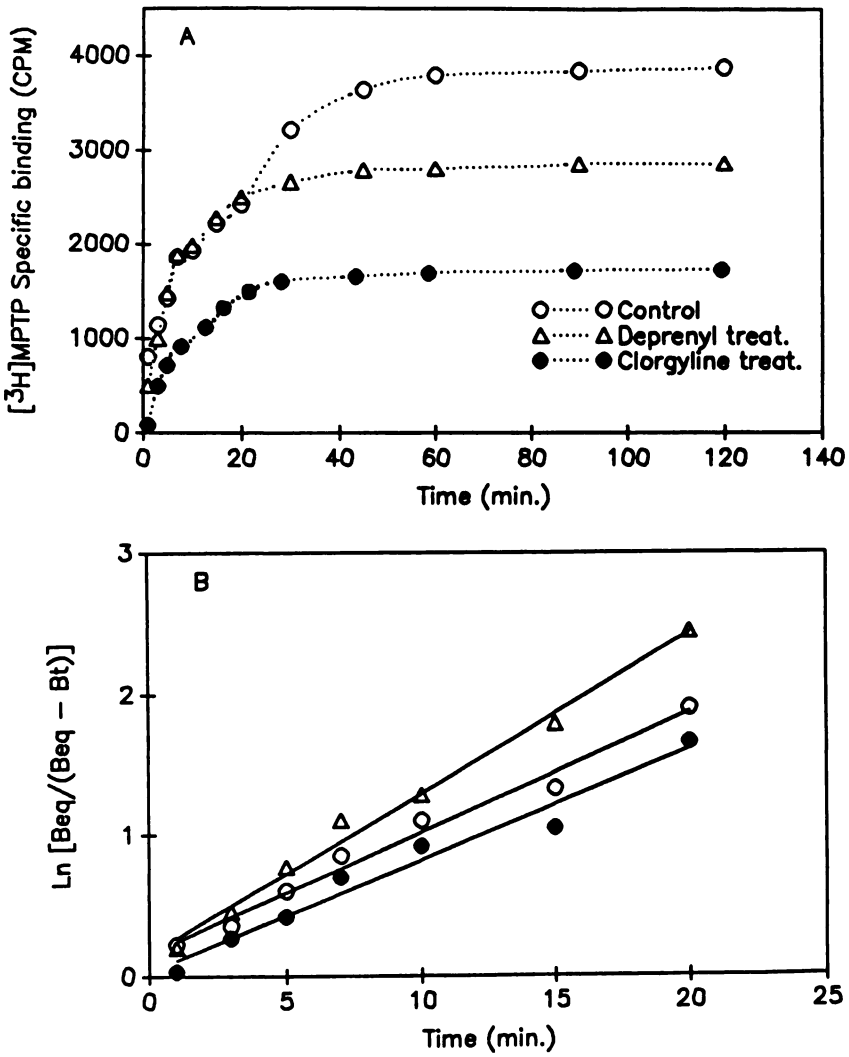

Fig. 6. Association of [^{3}H]MPTP (2.2 nM) binding to control and *in vitro* deprenyl- and clorgyline-treated mouse brain membranes. A, Association time course was carried out at 25°, as described in Experimental Procedures. Results are expressed as specifically bound [^{3}H]MPTP, in cpm. Nonspecific binding, determined in the presence of unlabeled MPTP (50 μM), did not change over the time period examined. B, Pseudo-first-order association rate constants were calculated as described before (28, 31) and are summarized in Table 1. *Beq*, specific binding at equilibrium; *Bt*, binding at time *t*. Results represent a typical experiment, which was repeated three times with similar results (standard error < 10% of mean values).

4-(2′-methylphenyl)- and (2′-ethylphenyl)-1,2,3,6-tetrahydropyridine, which are potent MPTP analogs (37).

The hypothesis that MPTP may exert its effect via multiple binding sites is supported by the findings that MPTP displays higher affinity not only for MAO-A but also for the σ binding sites. The affinity of MPTP for MAO-B is apparently 25- and 60-fold lower than its affinity for σ and MAO-A sites, respectively (Table 2 and 3). These findings are consistent with the observation that all MAO-A inhibitors tested display relatively high affinity for the σ sites and the high affinity [^{3}H]MPTP binding site(s). Most striking is the high correlation between the affinity of these ligands for [^{3}H]MPTP/MAO-A sites and (+)-3-PPP/σ sites (Fig. 5). This correlation, coupled with the finding that certain σ ligands have moderately high affinity for the MPTP/MAO-A binding site(s), suggests a possible association between the σ and MAO-A binding sites. This notion is largely supported by the dissociation experiments indicating that (+)-3-PPP may allosterically modulate the binding of MPTP to MAO-A binding site(s) (Fig. 8). A possible association between MAO activity and σ receptor binding stems also from our preliminary studies indicating that (+)-3-PPP and (+)-SKF 10047 binding in a mitochondrial membrane preparation, which is enriched in MAO activity, is significantly higher than in synaptosomal fractions but only slightly lower (16–18%) than the binding levels observed in the microsomal fraction, where the highest level of σ ligand binding is detected (38).

The finding that the binding of (+)-[^{3}H]-3-PPP in C57BL/6 mouse brain membranes is consistent with the properties of "σ receptor" binding was documented previously (25, 26). In the present study we did not attempt to investigate the binding sites labeled with DTG, which is also considered a σ receptor ligand (39). We have recently demonstrated that DTG binding in C57BL/6 mouse brain membranes is associated with sites that are not susceptible to dextrorotatory isomers of drugs considered to be prototypic σ ligands and that also display low sensitivity to MAO inhibitors, suggesting that [^{3}H]DTG labels distinct sites from those labeled with (+)-3-PPP (25). Several lines of evidence suggest that the (+)-3-PPP/σ sites are distinct from the MAO-A binding sites. As indicated in Table 2, the affinities of the σ ligands tested for the MPTP/MAO-A site(s) are markedly lower than their affinities for (+)-3-PPP binding sites (26, 28, 40). Unlike the MAO inhibitors, the σ ligands do not fit in the correlation between affinities for the σ binding sites and MAO-A sites, because the σ ligands apparently do not interact "directly" with the enzyme site. This notion is sup-

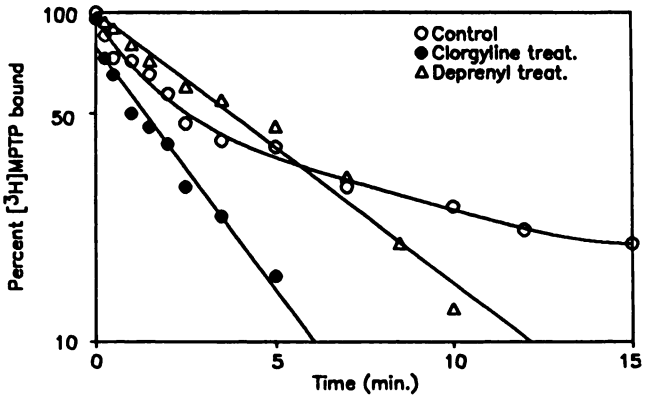

Fig. 7. Dissociation of [^{3}H]MPTP binding from control and *in vitro* deprenyl- and clorgyline-treated mouse brain membranes. Membranes were incubated with [^{3}H]MPTP (2.2 nM) for 60 min at 25°. At time 0, unlabeled MPTP (2.2 μM) was added to initiate dissociation, and samples of 1 ml were filtered at the indicated time intervals. Results are expressed as percentage of specific binding at time 0. Nonspecific binding did not change over the time period examined. In control membranes, the best fit was for a biexponential decay, determined by nonlinear regression as described previously (28). In the clorgyline- and deprenyl-treated membranes, the lines represent a fit for a monoexponential decay, because the equation for a biexponential decay (28) did not fit significantly better. Results represent a typical experiment, which was repeated four times with similar results (standard error < 10%).

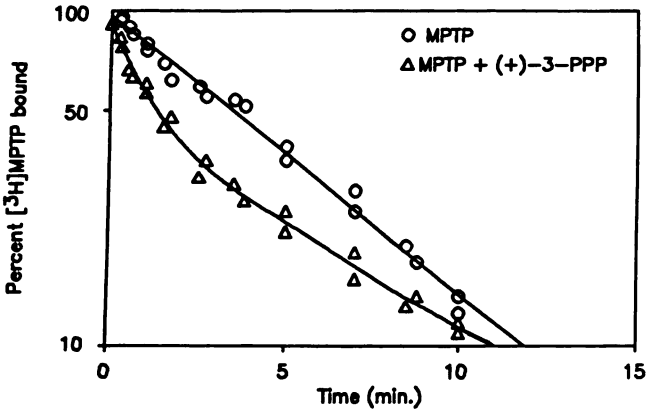

Fig. 8. Effect of (+)-3-PPP on the dissociation of [^{3}H]MPTP binding from the high affinity site. Assays were carried out in mouse brain membranes pretreated *in vitro* with (−)-deprenyl (1 μM) to block binding to MAO-B binding sites, as described in Experimental Procedures. Membranes were incubated with 2.2 nM [^{3}H]MPTP for 60 min at 25°. Dissociation was initiated by addition of either unlabeled MPTP (2.2 μM) alone or unlabeled MPTP (2.2 μM) plus (+)-3-PPP (10 μM) simultaneously. Results are expressed as percentage of specific binding at time 0. Nonspecific binding did not change over the time period examined. In the presence of MPTP alone, the line represents the best fit for a monoexponential decay, as described in the legend to Fig. 7. The first-order dissociation rate constant (K_{-1}) is 0.135 ± 0.01 min^{-1}. In the presence of both unlabeled MPTP and (+)-3-PPP, the best fit was for a biexponential decay ($p < 0.005$), determined by nonlinear regression. The corresponding K_{-1} values are 0.57 and 0.18 min^{-1} for the fast and slow dissociation components, respectively. Results represent two separate experiments.

ported by the finding that σ ligands do not inhibit either MAO-A or MAO-B activity in enzyme experiments (38).

Although further studies are required to establish a possible association between the function of the σ receptors and MAO-A, the lack of radiolabeled selective and reversible MAO-A inhibitors may currently hamper such studies. Nevertheless, under appropriate conditions, we have now demonstrated that [^{3}H]MPTP may be a useful ligand to further explore the relationship between MAO-A and σ binding sites. Because the σ receptors are speculated to be involved in psychiatric disorders (24, 25) and MAO-A is responsible for the therapeutic effects of some antidepressants, further studies may elucidate the physiological role of the σ receptors and their association with other neurotransmitter systems.

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