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[^{3}H]Opipramol Labels a Novel Binding Site and σ Receptors in Rat Brain Membranes

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SUMMARY

Opipramol (OP), a clinically effective antidepressant with a tricyclic structure, is inactive as an inhibitor of biogenic amine uptake. [^{3}H]Opipramol binds saturably to rat brain membranes (apparent K_D = 4 nM, B_{max} = 3 pmol/mg of protein). [^{3}H]Opipramol binding can be differentiated into haloperidol-sensitive and -resistant components, with K_i values for haloperidol of 1 nM (B_{max} = 1 pmol/mg of protein) and 350 nM (B_{max} = 1.9 pmol/mg of protein), respectively. The drug specificity of the haloperidol-sensitive component is the same as that of σ receptors labeled with (+)-[^{3}H]3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine. The haloperidol-resistant component does not correspond to any known neurotransmitter receptor or uptake recognition site. It displays high affinity for phenothiazines and related structures such as perphenazine, clopenthixol, and flupenthixol, whose potencies are comparable to that of opipramol. Because certain of these drugs are more potent at the haloperidol-resistant opipramol site than in exerting any other action, it is possible that this opipramol-selective site may mediate their therapeutic effects.

OP, a clinically effective antidepressant whose structure resembles the major tricyclic antidepressants (1, 2), is inactive as an inhibitor of norepinephrine and serotonin uptake (3, 4). Thus, unlike most tricyclic antidepressants, the therapeutic actions of OP do not involve biogenic amine uptake. In a study of [^{3}H]dextromethorphan binding sites, whose drug specificity and localization coincide with those of σ receptor binding sites (5, 6), Craviso and Musacchio (7) observed that OP is notably potent, with a K_i value of about 7 nM. We have characterized the binding of [^{3}H]OP to rat brain membranes. We report that [^{3}H]OP labels two sites with similar high affinity, one corresponding to σ receptors and the other to a novel site that differs from any known receptor.

Experimental Procedures

Materials. (+)-[^{3}H]3-PPP and [^{3}H]OP were obtained from NEN-DuPont (Boston, MA). Coomassie protein assay reagent was obtained from Pierce (Rockford, IL). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). BMY-14802 was a gift of Dr. Duncan Taylor (Bristol-Myers-Squibb, Wallingford, CT).

Binding assays. (+)-[^{3}H]3-PPP binding was assayed as described (8–10). For [^{3}H]OP binding, brain membranes (from 150–200-g Sprague Dawley rats) were prepared as described (9). [^{3}H]OP binding was assayed in a 1-ml volume with a final concentration of 1 nM [^{3}H]OP, 5 mg/ml (wet weight) brain membranes, and 50 mM Tris (pH 7.7, 25°), in the presence or absence of various inhibitors. Nonspecific binding was determined in the presence of 10 μM unlabeled OP or 10 μM haloperidol. Specific binding was linear with respect to tissue concentration over the range of 1–15 mg/ml (wet weight) tissue. Because equilibrium was obtained in 30–60 min at 25°, routine incubations were for 60 min at 25°. Following the appropriate incubation time, the binding reaction was stopped by the addition of 4 ml of ice-cold wash buffer (10 mM Tris, pH 7.7 at 25°) and immediate filtration, under vacuum, onto glass fiber filters that had been soaked in 0.5% polyethyleneimine for 20–40 min before use. Filters were rapidly and immediately washed with 2 × 4 ml of the same ice-cold wash buffer. Radioactivity that was specifically associated with the membranes trapped on the filters was determined by liquid scintillation counting. Protein was determined using the Coomassie protein assay reagent.

Results

Evidence for an interaction of [^{3}H]OP with two distinct binding sites. [^{3}H]OP binds saturably to rat brain membranes. In typical experiments using 1 nM [^{3}H]OP, total binding is about 2000 cpm, whereas nonspecific binding, measured in the presence of either 10 μM OP or 10 μM haloperidol, is about 250 cpm. Saturation analysis using increasing concentrations of either [^{3}H]OP (data not shown) or unlabeled OP (Fig. 1) is consistent with a bimolecular interaction with an apparent single binding site. Scatchard analysis suggests a single component of binding, with an apparent dissociation

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ABBREVIATIONS: OP, opipramol; 3-PPP, 3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine; BMY-14802, α-(4-fluoromethyl)-4-(5-fluoro-2-pyrimidinyl)-1-piperazine; DTG, 1,3-ditolylguanidine.

constant (K_D) of 4 nM, maximal number of binding sites (B_{max}) of 3 pmol/mg of protein, and a pseudo-Hill coefficient of 0.98.

Because of the reported high affinity of OP for σ receptors (7), we examined the influence of haloperidol, a very potent agent at σ binding sites, on [^{3}H]OP binding (Fig. 2). Haloperidol displays a markedly biphasic inhibition of [^{3}H]OP binding, suggesting the presence of two distinct binding sites. Haloperidol inhibits by 50% the high and low affinity components at about 1 nM and 350 nM, respectively. A clear plateau separating the high and low affinity components is apparent between 10 and 100 nM haloperidol. Thus, for experiments selectively examining [^{3}H]OP binding at sites for which haloperidol has low affinity, we routinely include 40–60 nM haloperidol together with [^{3}H]OP.

As expected for multiple binding sites, kinetic experiments reveal a complex pattern of [^{3}H]OP association and dissociation (Fig. 3, A and C). Neither association nor dissociation phases are monophasic (Fig. 3 A and C), and we cannot dissociate them into discrete components with defined kinetic constants. However, in the presence of 60 nM haloperidol, [^{3}H]OP displays apparent monophasic association and dissociation (Fig. 3, B and D). At 25° in the presence of 60 nM haloperidol, [^{3}H]OP reaches equilibrium by 60 min and half-maximal binding levels at about 30 min. The calculated rate constant for association (k_1) is 3.1×10^6 M^1 min^{-1}. Dissociation was initiated with excess (50 μM) haloperidol following incubation to equilibrium. When plotted on semilogarithmic paper, dissociation is monophasic, with a $t_{1/2}$ of 40 min and a k_{-1} of 0.023 min^{-1}. The K_D calculated from the kinetic data using the ratio of k_{-1}/k_1 is 7 nM. This value is very similar to the equilibrium K_D of [^{3}H]OP in the presence of 60 nM haloperidol (Table 1).

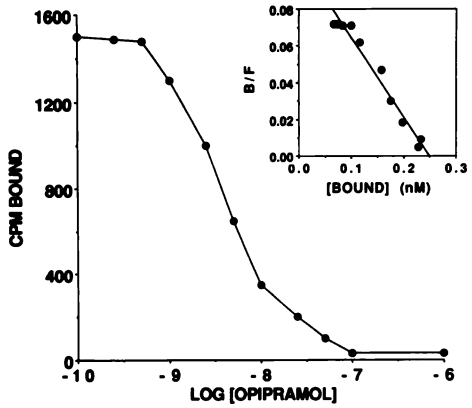

Fig. 1. Equilibrium binding of [^{3}H]OP to rat brain membranes. Binding was assayed as described in Experimental Procedures. Data are the means of triplicate determinations, which varied less than 10%, from a typical experiment. This experiment has been repeated five times with virtually identical results, giving an apparent linear Schatchard plot (*inset*) in all cases. The protein concentration in this experiment was 85 μg/ml.

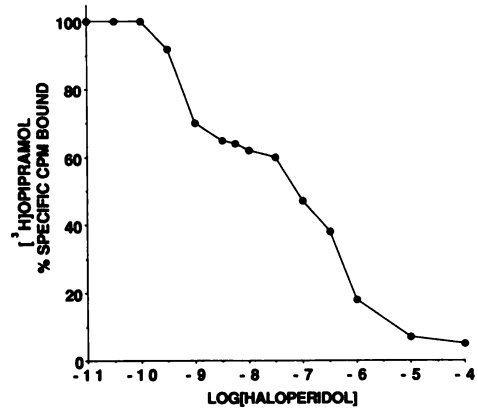

Fig. 2. Inhibition of specific [^{3}H]OP binding to rat brain membranes by haloperidol. As described in the text, haloperidol exhibits a biphasic inhibition of binding, having a high affinity component (K_i = 1 nM) and a low affinity component (K_i = 350 nM). Data shown are the means of triplicate determinations, which varied less than 10%. This experiment has been repeated six times with essentially the same results.

Equilibrium binding demonstrates similar high affinity of OP in the presence or absence of haloperidol, with a K_D of 4 nM in the absence of haloperidol and a K_D of 7 nM with 60 nM haloperidol. The B_{max} value for the haloperidol-resistant OP binding site, calculated from Scatchard data obtained in the presence of 60 nM haloperidol, is 1.9 pmol/mg of protein, whereas the B_{max} for total [^{3}H]OP binding obtained in the absence of haloperidol is 3 pmol/mg of protein. Thus, the haloperidol-sensitive component has a B_{max} of about 1.1 pmol/mg of protein.

[^{3}H]OP labeling of σ binding sites. We examined the influence of a number of σ-selective drugs as competitors for the binding of (+)-[^{3}H]3-PPP, a well established ligand for the σ receptor, and [^{3}H]OP binding in the presence or absence of 60 nM haloperidol (Table 1). In the absence of haloperidol, most of these drugs inhibit [^{3}H]OP binding in a biphasic or shallow fashion (Fig. 4). We have analyzed the inhibition of [^{3}H]OP binding by these various σ receptor ligands utilizing the LIGAND computer program. Computer analysis reveals a statistically better fit of the data to a two-site than a one-site model and resolves the inhibition of [^{3}H]OP binding by these drugs into high and low affinity components (Table 1). The high affinity components of various σ drugs in competing for [^{3}H]OP binding (K_{i_1}) correspond closely (correlation coefficient, 0.90) to their relative potencies at (+)-[^{3}H]3-PPP binding sites, as determined in the present as well as in previous (8, 9) studies. Haloperidol is most potent at this site, with a K_i of 2 nM, whereas OP has an affinity of 6 nM. Butaclamol isomers display stereoselectivity similar to what has been previously reported at σ binding sites, with the (−)-isomer being about 5-fold more potent that the (+)-isomer. (+)-3-PPP is about twice as potent as (−)-3-PPP. DTG is similar in potency to (−)-3-PPP, whereas (±)-pentazocine is about 4 times more potent than DTG.

We examined levels of [^{3}H]OP binding in the presence or absence of 60 nM haloperidol and of (+)-[^{3}H]3-PPP binding in seven dissected regions of rat brain (Fig. 5). As observed previously (10), the highest levels of (+)-[^{3}H]3-PPP binding occur in the brain stem, with values almost double those of the cerebral cortex, whose levels resemble thalamic values. Relative levels of (+)-[^{3}H]3-PPP binding and haloperidol-sensitive [^{3}H]OP binding are quite similar, with a correlation coefficient of 0.98. In contrast, no statistically significant correlation is apparent between (+)-[^{3}H]3-PPP binding in various brain regions and binding levels of [^{3}H]OP associated with the haloperidol-resistant site. Moreover, the B_{max} of haloperidol-sensitive [^{3}H]OP binding (1.1 pmol/mg of protein) is very similar to the B_{max} for (+)-[^{3}H]3-PPP binding (8), although the B_{max} for haloperidol-resistant [^{3}H]OP binding (1.9 pmol/mg of protein) is significantly greater. The very close correspondence between the regional distribution of haloperidol-sensitive [^{3}H]OP binding

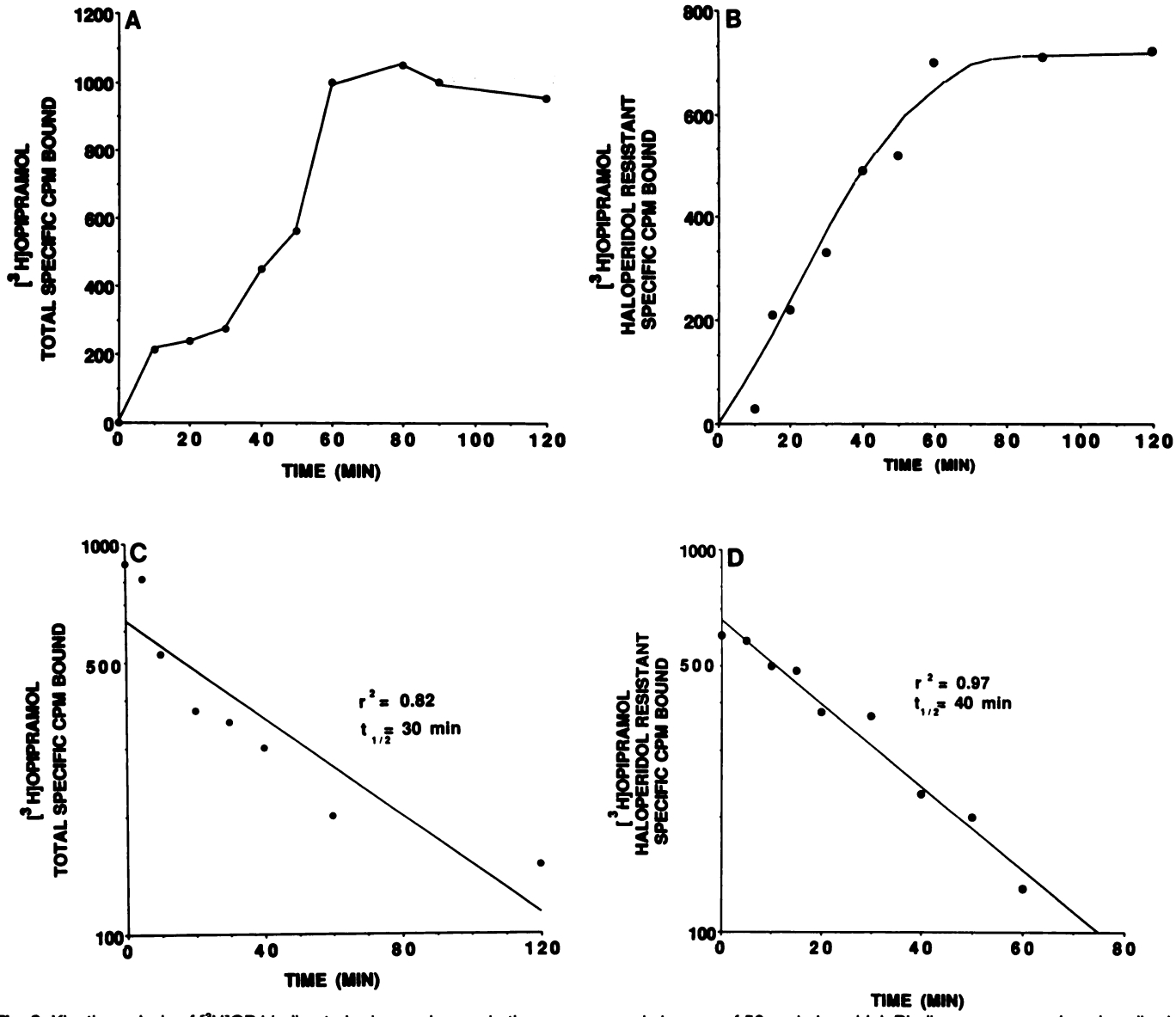

Fig. 3. Kinetic analysis of [^{3}H]OP binding to brain membranes in the presence and absence of 50 nM haloperidol. Binding was assayed as described in Experimental Procedures. Membranes were incubated for various lengths of time in the presence or absence of 10 μM OP or haloperidol. Nonspecific binding was determined for all time points shown. Dissociation was initiated, following incubation to equilibrium, by the addition of excess inhibitor (50 μM haloperidol or 10 μM OP). Data shown are the means of triplicate determinations, which varied less than 10%, from typical experiments. These experiments have been performed three times with the same results. A, Association kinetics of [^{3}H]OP binding in the absence of haloperidol, demonstrating complex kinetics suggestive of multiple receptor sites (see text). B, Association kinetics of [^{3}H]OP binding in the presence of 60 nM haloperidol, which blocks the σ receptor component of binding (see text). C, Dissociation kinetics of binding in the absence of haloperidol. D, Dissociation kinetics of binding in the presence of 60 nM haloperidol.

and (+)-[^{3}H]3-PPP binding, the similar B_{max} values, and their very similar affinities for numerous drugs indicate that the haloperidol-sensitive component of [^{3}H]OP binding involves σ binding sites, as labeled by (+)-[^{3}H]3-PPP. Accordingly, hereafter we designate the haloperidol-sensitive component of [^{3}H]OP binding as the OP-σ binding site and the haloperidol-insensitive component as the OP-OP site.

[^{3}H]OP labeling of a novel binding site (the OP-OP site). Drug potencies clearly differentiate OP-OP and OP-σ sites (Table 1). Haloperidol is about 160-fold more potent at the OP-σ site than at the OP-OP site. Butaclamol is 20–30-fold more potent at the OP-σ than the OP-OP site, with less stereoselectivity at the OP-OP than the OP-σ site. (±)-Pentazocine is 250-fold more potent at the OP-σ than the OP-OP site. The absolute and relative potencies of the drugs at the OP-OP site and in competing for [^{3}H]OP binding in the presence of 60 nM haloperidol are very similar, with a correlation coefficient of 0.92. In contrast to the OP-σ site, the regional distribution of the OP-OP binding site is essentially homogeneous, with no more than a 2-fold difference between any of the seven brain regions.

To ascertain the identity of the OP-OP site, we evaluated a wide range of ligands for known receptor sites. We explored drugs that act at uptake sites or receptors for known neurotransmitters such as histamine, serotonin, dopamine, norepinephrine, γ-aminobutyric acid, glutamate, and acetylcholine, as well as a number of neuropeptides (Table 1, legend). None of these agents influence the OP-OP site with a potency cor-

TABLE 1

Drug affinities for (+)-[^{3}H]3-PPP and [^{3}H]OP binding sites

Binding was assayed as described in Experimental Procedures. σ Binding sites were labeled with (+)-[^{3}H]3-PPP. K_i^1 and K_i^2 were obtained with computer assistance using the EBDA/LIGAND program (25, 26), which revealed a statistically significant better fit of the binding data to a two-site model. In all cases the pseudo-Hill coefficients were not statistically different from unity. [^{3}H]OP binding was measured in the absence (control column) or presence of haloperidol. The following drugs/neurotransmitters at 10 μM concentrations did not affect haloperidol-resistant [^{3}H]OP binding: mepyramine, prazosin, apomorphine, clonidine, alprenolol, spiperone, mazindol, sulpiride, propranol, isoproterenol, citalopram, serotonin, γ-aminobutyric acid, phencyclidine, glutamate, aspartate, scopolamine, carbachol, atropine, ω-conotoxin, endothelin (1,2,3), sarafotoxin, neurotensin, physalaemin, proctolin, ocytocin, *m*-enkephalin, substance P, angiotensin II, His-Pro-diketoperazine, naloxone, peptide YY, and neuropeptide Y. Data shown are the average of three separate experiments, in which individual data points were determined in triplicate. The K_i from the three experiments varied less than 20% in all cases.

	[^{3}H]OP			
	Control		+60 nM Haloperidol, K_i	(+)-[^{3}H]3-PPP, K_i
	K_i^1	K_i^2		
	nM			
OP	4	6	6	6
Haloperidol	2	358	321	3
(±)-Pentazocine	22	5,253	10,000	24
BMY-14802	63	461	438	71
DTG	103	5,198	5,971	58
(+)/3-PPP	58	2,788	2,468	40
(−)/3-PPP	112	5,500	7,597	155
(−)/Butaclamol	80	4,175	3,521	147
(+)/Butaclamol	1,157	8,928	>10,000	1,800

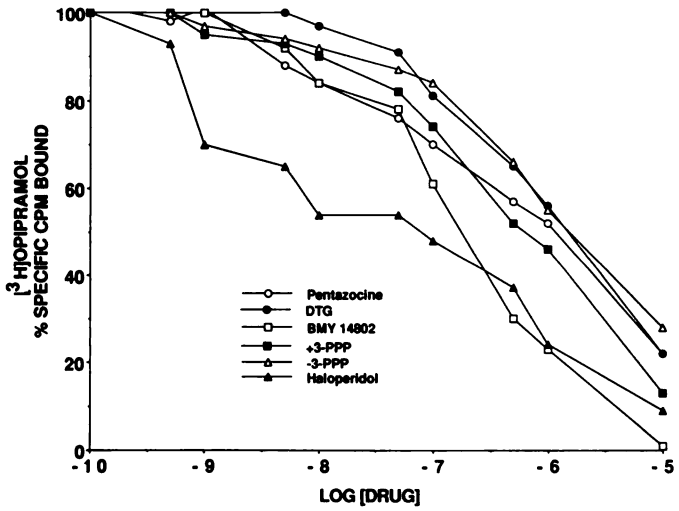

Fig. 4. Inhibition of [^{3}H]OP binding by various σ receptor ligands. Binding was assayed as described in Experimental Procedures. Data shown are means of triplicate determinations from a typical experiment, whose results varied less than 10%. This experiment has been repeated three times with the same results.

responding to their known effects at uptake or receptor sites for known neurotransmitters.

To characterize the OP-OP site in detail, we examined structure-activity relationships for numerous drugs. A number of drugs in the phenothiazine and tricyclic classes display substantial potency for the OP-OP site (Fig. 6). For instance, perphenazine is similar in potency to OP itself. Among the phenothiazines, greater potency occurs with piperazine than alkylamino side chains. Piperazines possessing an ethanol substituent on the piperazine ring are more potent than those with a corresponding methyl group, e.g., perphenazine is more potent than prochlorperazine. A substituent at the 2-position of the

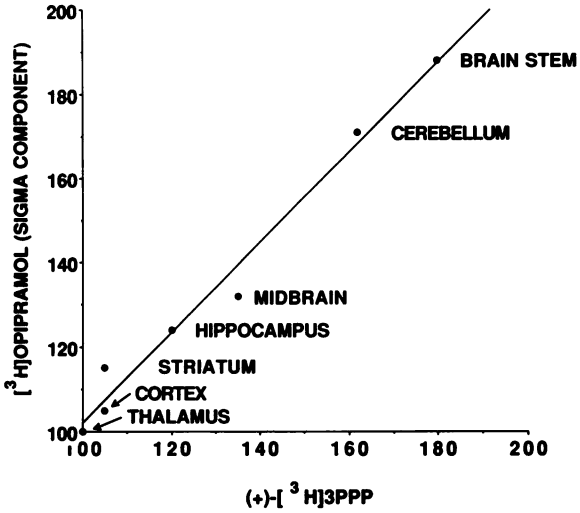

Fig. 5. Regional distribution of the OP-σ (haloperidol sensitive) component of [^{3}H]OP binding and (+)-[^{3}H]3-PPP binding. Binding was assayed as described in Experimental Procedures. Data shown are mean cpm/μg of protein for triplicate determinations, which varied less than 10%. This experiment has been performed three times with the same results.

phenothiazine ring enhances potency, inasmuch as chlorpromazine is more potent than promazine. A chlorine substituent provides greater potency than a trifluomethyl, as reflected by the greater potency of chlorpromazine than triflupromazine and of perphenazine compared with fluphenazine.

Among tricyclic compounds, greatest potency occurs with drugs possessing a piperazine-ethanol substituent, such as OP itself, clopenthixol, and α-flupenthixol. For the tricyclic drugs with an alkylamino side chain, greater potency occurs with *N*-dimethyl than *N*-monomethyl substituents, inasmuch as imipramine and amitriptyline are, respectively, more potent than desipramine and nortriptyline.

Benztropine, a bicyclic structure with an tropane side chain, is notably potent, as is hydroxyzine, which is also bicyclic with a piperazine side chain. Iprindole, a clinically effective antidepressant that does not inhibit biogenic amine uptake (3, 4), displays a K_i value of 122 nM.

The importance of side chain structure is evident in the inactivity of phenothiazine-related agents such as clozapine, mesoridazine, and thioridazine, which have phenothiazine-like ring structures but side chains that differ from the piperazine or alkylamino pattern. The importance of a three-ring system is apparent in the very weak activity of bicyclic drugs with alkylamino side chains, such as the antihistamines chlorpheniramine, diphenhydramine, and mepyramine.

Discussion

The major finding of the present study is that [^{3}H]OP labels two binding sites in rat brain membranes, with similar high affinity. One of these involves σ receptors, as labeled with (+)-[^{3}H]3-PPP. Thus, the drug specificity and regional distribution of (+)-[^{3}H]3-PPP binding and haloperidol-sensitive [^{3}H]OP binding are very similar. There is evidence for subtypes of σ receptors (11). Drug specificity for haloperidol-sensitive [^{3}H]OP binding resembles that for sites labeled with (+)-[^{3}H]3-PPP more than that for sites labeled with [^{3}H]DTG.

The pharmacologic consequences of OP interactions with σ sites are unclear. σ Binding sites were first investigated as a

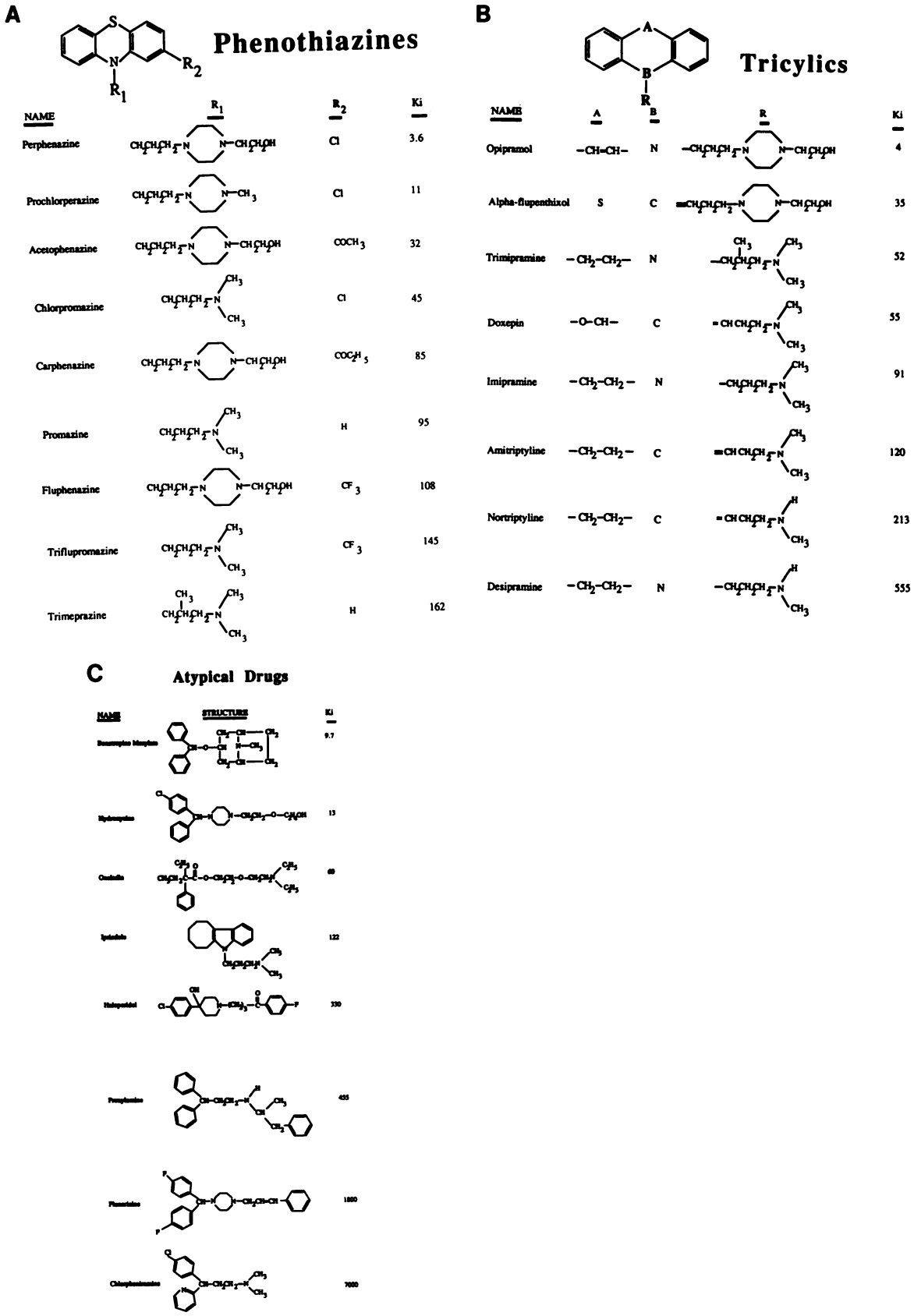

Fig. 6. Structure-activity relationships for the OP-OP binding site. A, Phenothiazine derivatives. B, Tricyclic derivatives. C, Atypical drugs. Binding was assayed as described in Experimental Procedures. Data shown are the means of triplicate determinations, which varied less than 10%. K_i values were determined from displacement curves of 10–14 different concentrations of inhibitor, using the EBDA/LIGAND computer program (25, 26). The pseudo-Hill coefficients of all inhibitors did not differ significantly from unity. These experiments have been performed three times with the same results. The following drugs had IC_{50} values in the concentration range indicated, at the OP-OP site: 100 μM, yohimbine, hydrastine, levorphanol, phenytoin, acetazolamide, diphenoxylate; 50–100 μM, buspirone, mesoridazine, diltiazem, noscapine, pirenzipine; 10–50 μM, progesterone, papaverine, quinine, flufenamic acid, clopimozide, (+)-SKF3893, (−)-SKF3893, fetoxylate; 5–10 μM, mianserin, nitrendipine, (+)-SKF10,047, antazoline, carbinoxamine, chlorpheniramine; 1–5 μM, brompheniramine, lidoflazine, verapamil, diphenhydramine, clozapine, quinidine, flunarizine; 300 nM to 1 μM, fenfluramine, cinanserin, trifluperidol, dextromethorphan, prenylamine, fluoxetine, thioridazine.

biochemical reflection of the psychotomimetic actions of "σ-opiates" (12–14). However, relative psychotomimetic actions of drugs in numerous instances do not correspond closely to affinities for σ binding sites (13). Although OP has been employed extensively as an antidepressant, we are not familiar with any investigations of its actions in schizophrenic patients.

OP also binds to a site that is relatively resistant to haloperidol and that we have designated the OP-OP site. The affinity of OP for the OP-OP site is the same as for the OP-σ site, as reflected in the monophasic inhibition of [^{3}H]OP binding by OP. At therapeutic doses of OP, one would anticipate a substantial level of occupancy of both OP-OP and OP-σ sites.

The OP-OP site does not appear to correspond to a receptor or uptake site for any known neurotransmitter. Numerous phenothiazines and tricyclic antidepressants display substantial affinity for the OP-OP site. Several antidepressants, including imipramine, amitriptyline, doxepin, and trimipramine, are comparably potent at the OP-OP site and at binding sites associated with serotonin and norepinephrine uptake (15–17). OP, clopenthixol, and α-flupenthixol are significantly weaker at norepinephrine, serotonin, and dopamine uptake sites than at the OP-OP site (18). The atypical antidepressant iprindole, like OP itself, is also inactive in inhibiting biogenic amine uptake (3, 4, 15–17), with greater potency at the OP-OP site than at any of the uptake sites. Whereas drugs like desipramine, imipramine, and nortriptyline have K_i values of 1–10 nM for inhibition of catecholamine uptake, atypical antidepressants like iprinole and OP have K_i values that are at least 1000-fold higher (3, 4, 15–17). This suggests that the OP-OP site may mediate some of the antidepressant actions of these drugs.

A role for the OP-OP site in antidepressant drug effects is further supported by the actions of other drugs. Numerous phenothiazines have demonstrable efficacy in treating depression. For instance, flupenthixol and clopenthixol are phenothiazine related but are more widely used as antidepressants than antipsychotics. In some instances, their therapeutic effects are greatest in anxious depressives (19). In certain studies, the antidepressant effects of phenothiazines are indistinguishable from those of tricyclic antidepressants (20–22). Of the phenothiazines evaluated in depressive patients, some of the clearest antidepressant efficacy has occurred with perphenazine and chlorpromazine, agents with high affinity for OP-OP receptors (19, 23). Indeed, perphenazine is more potent at OP-OP sites than at dopamine D_2 receptors, where it presumably exerts its neuroleptic antipsychotic actions (24). Thus, the greater relative efficacy of these phenothiazines in depression may relate to their high affinity at the OP-OP site.

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