Clozapine: Selective Labeling of Sites Resembling $5HT_6$ Serotonin Receptors May Reflect Psychoactive Profile

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ABSTRACT

Background: Clozapine, the classic atypical neuroleptic, exerts therapeutic actions in schizophrenic patients unresponsive to most neuroleptics. Clozapine interacts with numerous neurotransmitter receptors, and selective actions at novel subtypes of dopamine and serotonin receptors have been proposed to explain clozapine's unique psychotropic effects. To identify sites with which clozapine preferentially interacts in a therapeutic setting, we have characterized clozapine binding to brain membranes.

Materials and Methods: [^{3}H]Clozapine binding was examined in rat brain membranes as well as cloned-expressed 5-HT_6 serotonin receptors.

Results: [^{3}H]Clozapine binds with low nanomolar affinity to two distinct sites. One reflects muscarinic receptors consistent with the drug's anticholinergic actions. The drug competition profile of the second site most closely resembles $5HT_6$ serotonin receptors, though serotonin itself displays low affinity. [^{3}H]Clozapine binding levels are similar in all brain regions examined with no concentration in the corpus striatum.

Conclusions: Besides muscarinic receptors, clozapine primarily labels sites with properties resembling $5HT_6$ serotonin receptors. If this is also the site with which clozapine principally interacts in intact human brain, it may account for the unique beneficial actions of clozapine and other atypical neuroleptics, and provide a molecular target for developing new, safer, and more effective agents.

INTRODUCTION

Clozapine is an important therapeutic agent in treating schizophrenia. Though developed as a neuroleptic, it is unique in its therapeutic profile and may provide major benefits to patients who are resistant to other neuroleptics (1,2). Even in patients who respond to conventional neuroleptics, clozapine may be more efficacious (2). Clozapine appears to relieve negative symptoms, including apathy and emotional withdrawal, that resist conventional neuroleptics and displays a very low incidence of extrapyramidal side effects (EPS). Diminished EPS and greater therapeutic efficacy are also manifested by more recently developed atypical neuroleptics, including risperidone, olanzepine, seroquel, and sertindole (2).

Therapeutic actions of conventional neuroleptics correlate closely with their potencies in blocking dopamine D_2 receptors, which presumably explains their therapeutic and EPS actions (3–5). Imaging D_2 receptors in humans by positron emission tomography reveals less occupancy of D_2 receptors by atypical than conventional neuroleptics, when administered at clinically effective doses (6). The lesser incidence of EPS associated with the atypical drugs may also reflect their greater anticholinergic potencies, as muscarinic anticholinergic drugs are well known to relieve Parkinsonian, EPS symptoms (7,8).

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To explain the greater antischizophrenic actions of atypical neuroleptics, researchers have evaluated effects of these drugs at novel sites. Thus, clozapine displays uniquely high affinity for dopamine D_4 receptors (9). Serotonin $5HT_2$ receptors have also been implicated. Ritanserin, a selective $5HT_2$ antagonist, decreases EPS elicited by haloperidol (10). Mianserin, also a $5HT_2$ antagonist, relieves negative symptoms in schizophrenics receiving conventional neuroleptics (11). Compared with conventional neuroleptics, atypical drugs tend to have higher affinities for $5HT_2$ than D_2 receptors (12). However, there are exceptions such as chlorpromazine and amoxapine, both more potent at $5HT_2$ than D_2 sites (13).

To assess clozapine actions at various receptors, most studies have evaluated clozapine's potency in competing for the binding of radioligands. However, ligands exert "induced fit", altering receptor conformation so that the potency of a drug in competing for binding of a radioligand may not faithfully reflect that drug's propensity to bind to the receptor in vivo. Ideally, one would like to specify the receptors to which clozapine binds when encountering the human brain in vivo.

To evaluate sites to which clozapine binds preferentially, we have examined the binding of [^{3}H]clozapine itself to rat brain membranes. We report labeling of muscarinic cholinergic receptors as well as sites resembling $5HT_6$ serotonin receptors.

MATERIALS AND METHODS

All unlabeled drugs were obtained from Research Biochemicals International (Natick, MA, U.S.A.). [^{3}H]Clozapine was generously provided by Dr. S. Hurt NEN-Dupont (Boston, MA, U.S.A.).

Preparation of Rat Brain Membranes

Sprague-Dawley rats (200–300 g) were killed by decapitation. Brains were rapidly removed and specific brain regions dissected. Tissue was homogenized by Polytron in 50 mM Tris HCl, pH 7.4, at 4°C. Homogenates were centrifuged at 48,000 × g for 10 min. Pellets were resuspended and rehomogenized in the same buffer and centrifuged a second time at 48,000 × g for 10 min. Pellets were resuspended in 50 mM Tris HCl, pH 7.4, to a concentration of 15 mg/ml. Except in regional dissection studies, membranes from whole rat brain minus cerebellum were employed for binding assays.

Preparation of Membranes from $5HT_6$ Receptor Expressing Cells

Human embryonic kidney 293 cells stably expressing rat $5HT_6$ receptors were grown in D-modified Eagle's medium with 10% FBS, 1 mM sodium pyruvate, and G418 at 300 μg/ml. Cells were grown to confluence, rinsed with 5 ml 0.5 mM EDTA in phosphate buffered saline (PBS). Cells were then washed for 5 min with an additional 5 ml EDTA/PBS. Cells were released by this treatment and treated as above for brain membranes. Final membrane concentration was ~10.0 mg/ml protein. These cells express approximately 800 fmol/mg protein of $5HT_6$ receptor binding activity (14).

[^{3}H]Clozapine Binding Assays

Binding assays were performed in a final volume of 500 μl Tris HCl, pH 7.4. Membrane preparation (0.25 ml) was added to each tube. Compounds for competition were added in a 50-μl volume. Fifty microliters of a 2% bovine serum albumin (BSA) solution was added to reduce nonspecific filter binding. [^{3}H]Clozapine (specific activity 51.3–89.1 Ci/mmol) in 50 μl was added to give a final concentration of 1 nM. For all experiments performed in the presence of scopolamine, 100 μl was added to give a final concentration of 10 nM. For the initial experiments without scopolamine, 100 μl of distilled water were used. Nonspecific binding was determined by addition of 1 μM (final concentration) unlabeled clozapine. Tubes were incubated for 20 min at 37°C. The incubation was terminated by rapid filtration over 0.5% polyethyleneimine-soaked filters (GF/B) and washed 2× 3 ml with ice-cold 50 mM NaCl.

RESULTS

As an initial screen, we evaluated inhibition of [^{3}H]clozapine binding by agents acting at sites where clozapine is thought to exert effects (Table 1). Scopolamine and atropine are uniquely potent with IC_{50} values of 0.8 nM and 1.0 nM, respectively. Scopolamine maximally inhibits about 60% of [^{3}H]clozapine binding at 5 nM (Fig. 1). Accordingly, in all subsequent experiments we include 10 nM scopolamine so that hereafter [^{3}H]clozapine binding will refer to binding measured in the presence of scopolamine.

[^{3}H]Clozapine binding is saturable with a K_d of 4.5 nM and B_{max} of 380 fmol/mg protein (Fig. 2a). Scatchard analysis of [^{3}H]clozapine binding as well

TABLE 1. Drug effect on [^{3}H]clozapine binding in rat brain membranes

Drug	K_i (nM)
Histamine H1	
Triprolidine	1,000
Adrenergic α 1	
Prazosin	>10,000
Muscarinic Cholinergic	
Scopolamine	0.8
Atropine	1.0
Butyrophenones	
Pipamperone	400
Spiperone	30
Phenothiazines	
Fluphenazine	20
Trifluoperazine	30
Thioridazine	30
Chlorpromazine	20
Tricyclic Antidepressants	
Nortriptyline	30
Imipramine	150
Amitriptyline	30
Ergot Alkaloids	
Ergotamine	10
Dihydroergotamine	20

Increasing concentrations of the indicated compounds were used to inhibit the binding of 1 nM [^{3}H]clozapine to rat brain membranes. K_i values were determined from the IC_{50}'s by the method of Cheng and Prusoff (15). Results are the means of at least two experiments run in duplicate.

as of a displacing curve of unlabeled clozapine competing for [^{3}H]clozapine binding reveals a single binding site with a K_d of 6.7 nM and a B_{max} of 240 fmol/mg protein (Fig. 2b). Since dopamine receptors are most highly concentrated in the corpus striatum, we compared [^{3}H]clozapine binding in the striatum, hippocampus, and cerebral cortex (Table 2). The K_d's are similar in all areas, suggesting similar binding sites, and the B_{max} values are roughly equal, suggesting a regional distribution of [^{3}H]clozapine binding that does not fit with a classical dopamine D_1 or D_2 receptor.

The relative potencies of numerous drugs in inhibiting [^{3}H]clozapine binding provide suggestions about the types of receptors that are most likely involved (Table 1). Triprolidine, a classic histamine H1 antagonist, displays an IC_{50} of 1

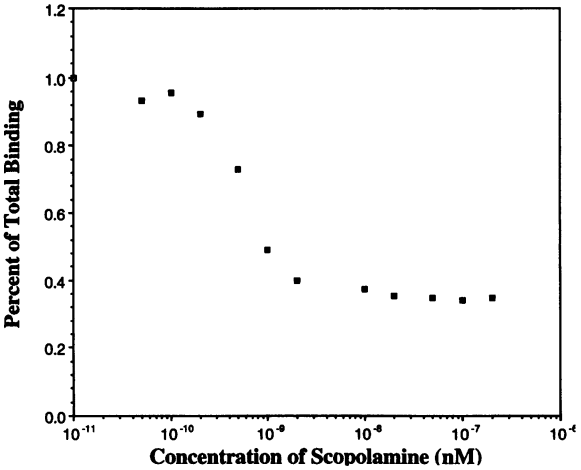

FIG. 1. Inhibition of [^{3}H]clozapine binding to rat brain membranes by scopolamine

Shown is a typical displacement curve for [^{3}H]clozapine bound to rat brain membranes. The data represent the mean percent of maximum specific binding (defined with 1 μM clozapine). The experiment was replicated three times.

μM, almost 1000 times higher than its affinity for histamine H1 receptors. The potent α-1 adrenoceptor antagonist prazosin has an IC_{50} greater than 10 mM. Similarly, agents active at sigma, GABA, and glutamate receptors display low affinity for [^{3}H]clozapine sites.

Clozapine is well known to influence dopamine receptors with an IC_{50} for D_1 and D_2 receptors of 172 nM and 182 nM, respectively (3). Accordingly, we compared the potencies of various dopamine-related agents in competing for [^{3}H]clozapine binding with their affinities for cloned and expressed D_1, D_2, D_3, and D_4 receptors (Table 3). [^{3}H]Clozapine does not seem to bind to D_{1a} or D_{1b} receptors (D_{1b} being the same as D_5). For instance, (+)butaclamol has a K_i value in the low nanomolar range for the D_1 receptor but a K_i of 1 μM for [^{3}H]clozapine binding. D_2, D_3, and D_4 receptors have a fairly similar pharmacologic profile. One major exception is that D_4 receptors display a low affinity for most neuroleptics but relatively high affinity for clozapine (9). Thus, the potency of clozapine at D_4 receptors is consistent with its potency at [^{3}H]clozapine binding sites. However, there are other major differences. For instance, spiperone displays subnanomolar affinity for D_2, D_3, and D_4 receptors but has a K_i of 30 nM at [^{3}H]clozapine sites. Further, raclopride displays low nanomolar potency at D_2, D_3, and D_4 sites, but, at 10 μM concentration, it fails to affect [^{3}H]clozapine sites. Finally, the K_i of dopamine for [^{3}H]clozapine sites is 5 mM, more than 1000 times greater than its K_i for D_2, D_3, and D_4 receptors.

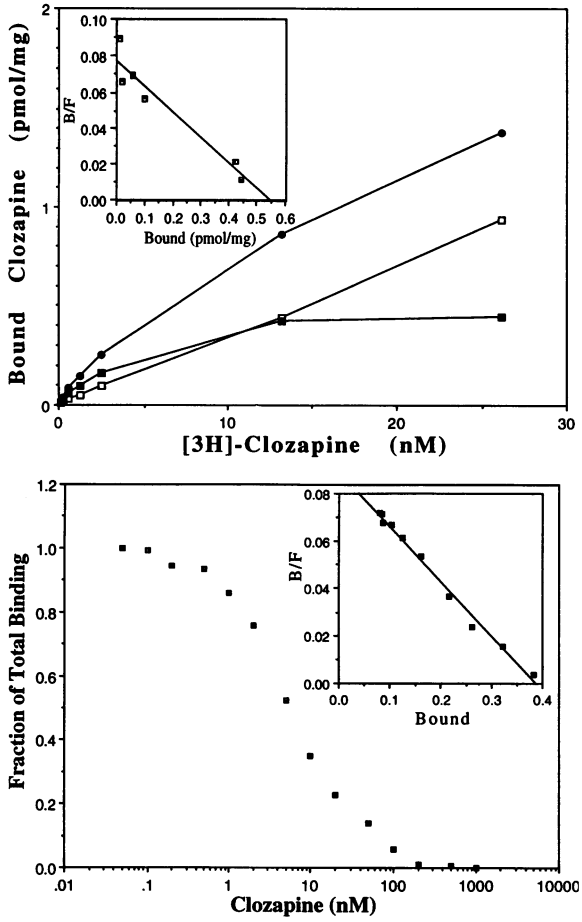

FIG. 2. (a) Saturation binding plot of [^{3}H]clozapine to rat brain membranes and (b) displacement curve and Scatchard analysis for unlabeled clozapine competing with [^{3}H]clozapine.

Data in Panel a were plotted using Cricketgraph. Shown are total (----●----), nonspecific (----□----), and specific (----■----) binding in pmol/mg protein as well as Scatchard analysis of this data. In Panel b, data are presented as fraction of total specific binding defined by 1 μM unlabeled clozapine. The experiment was replicated three times.

A substantial number of serotonin receptor subtypes have been differentiated by ligand binding studies. Molecular cloning has led to the demonstration of an even greater heterogeneity so that many of the sites labeled in brain membranes by ligand binding actually represent a mixture of 5HT receptor subtypes. To compare drug potencies at 5HT receptor subtypes with [^{3}H]clozapine binding sites, we have summarized reports from the literature on molecularly cloned and expressed receptors (Table 4). [^{3}H]Clozapine sites in brain are not likely to involve $5HT_{1C}$ receptors, as spiperone is almost

TABLE 2. Regional distribution of [^{3}H]clozapine binding

Region	K_d (nM)	B_{max} (pmol/mg)
Cerebral cortex	6.7	0.24
Striatum	7.3	0.17
Hippocampus	4.3	0.11

Rats were killed by decapitation, their brains rapidly removed and dissected on ice. Regions were homogenized separately and pellets were resuspended to approximately the same protein concentration (~15 mg/ml). Binding was assessed at varying [^{3}H]clozapine concentrations. K_i and B_{max} values were determined by Scatchard analysis. Data are the means of three determinations with results varying less than 15%.

fifty times more potent at clozapine binding sites than at $5HT_{1C}$ receptors. In addition, mesulergine is more than 300 times as potent at $5HT_{1C}$ sites than at [^{3}H]clozapine sites. The $5HT_{1E}$ site is also excluded, as ergotamine has a 60-fold higher affinity for [^{3}H]clozapine sites than for the cloned $5HT_{1E}$ receptor. The $5HT_2$ receptor can likewise be excluded, since it has a dramatically higher affinity for ritanserin than [^{3}H]clozapine binding sites. The $5HT_7$ receptor does not fit, because mesulergine's affinity for $5HT_7$ receptors is 50 times greater than for [^{3}H]clozapine sites.

The $5HT_6$ receptor displays high affinity for a variety of psychotropic drugs (14, 35). We utilized cell lines expressing molecularly cloned rat $5HT_6$ receptors and in our laboratory compared drug affinities for [^{3}H]clozapine, [^{3}H]5HT, and [^{3}H]LSD binding to the expressed $5HT_6$ receptors with [^{3}H]clozapine binding in rat cerebral cortex membranes (Table 5). The similarities in the pharmacology of the $5HT_6$ receptor and [^{3}H]clozapine sites are striking. Tricyclic structures, including tricyclic antidepressants and phenothiazines, display nanomolar potencies at $5HT_6$ receptors and [^{3}H]clozapine sites. Some structures related to ergots, such as methiothepin, are extremely potent at $5HT_6$ receptors and clozapine sites, whereas mesulergine, which is in the same structural class, is about 100 times less potent at both sites. For some drugs, affinities vary depending on whether the ligand is [^{3}H]clozapine, [^{3}H]5HT, or [^{3}H]LSD. However, none of these differences are marked. One notable exception is 5HT itself whose K_i at [^{3}H]clozapine sites is 0.1–0.2 mM (data not shown) whereas it displays a 0.15 μM K_i at $5HT_6$ receptors.

The relatively low potency of 5HT itself at [^{3}H]clozapine binding sites remains a puzzle. Po-

TABLE 3. Comparison of drug affinities for [^{3}H]clozapine binding sites in rat brain and cloned dopamine receptors

Ligand	D_1 nM[a,b]	D_2 nM[c]	D_3 nM[c]	D_4 nM[d]	[^{3}H]Clozapine
SCH 23390	0.11	1,000	—	—	—
(+) Butaclamol	0.90	0.83	—	40	1,000
Ketanserin	190	>1,000	—	148	—
Spiperone	220	0.07	0.61	0.05	30
Apomorphine	210	24	20	4.1	50,000
Dopamine	2,500	474	25	28	500,000
Haloperidol	203	0.45	9.8	5.1	400
Bromocriptine	—	5.3	7.4	340	200
Domperidone	—	0.3	9.5	—	6,000
Thioridazine	—	3.3	7.8	12	30
Chlorpromazine	—	2.8	6.1	37	20
Raclopride	—	1.8	3.5	237	>10,000
Eticlopride	—	—	—	2.1	>10,000
Fluphenazine	—	—	—	46	20
Trifluoperazine	—	—	—	3	30
Clozapine	—	56	180	9	10

All numbers represent K_i values from the literature as noted or [^{3}H]clozapine to rat brain membranes. Values for drug potencies at [^{3}H]clozapine binding sites are means of two to three determinations that varied less than 20%.

[a]Deary et al. (16)

[b]Zhou et al. (17)

[c]Sokoloff et al. (18)

[d]Van Tol et al. (9)

tencies of neurotransmitters and other agonists in competing for [^{3}H]antagonists are often relatively weak and vary considerably depending on the ligand employed. Antagonist ligands such as [^{3}H]clozapine may fix the conformation of the receptor in an antagonist preferring conformation that diminishes affinity of the agonist. Alternatively, sites labeled by [^{3}H]clozapine may not be $5HT_6$ receptors but instead a distinct receptor with similar drug specificity.

DISCUSSION

The main finding of this study is abundant, high-affinity binding of [^{3}H]clozapine to rat brain membranes. Our results are in agreement with other studies of [^{3}H]clozapine binding showing two high-affinity binding sites (34, 35). The first is a muscarinic cholinergic receptor which has low nanomolar affinity and represents the majority ~60% of [^{3}H]clozapine binding in the brain. The second site demonstrates a pharmacology similar to that we have seen for the cloned $5HT_6$ receptor. It is possible that other sites, such as the D_4 receptor, may be labeled by [^{3}H]clozapine but would escape detection because of low abundance in brain or because of overlapping pharmacology as occurs with multiple serotonin receptors.

One notable feature of [^{3}H]clozapine binding is its equal distribution in the cortex, hippocampus, and striatum. In one published study of cloned $5HT_6$ receptors, mRNA levels were roughly equal in these three regions (33). While Sibley and collaborators (14) initially described high levels of $5HT_6$ mRNA in the corpus stiatum with negligible levels in cortex and hippocampus, reexamination using in situ hybridization indicates substantially higher levels of $5HT_6$ receptor mRNA in cortex and hippocampus than first reported (D. R. Sibley and J. E. Lachowitz, unpublished observations). Thus, both on the basis of drug specificity and regional distribution, the [^{3}H]clozapine binding sites not involving muscarinic receptors resemble the $5HT_6$ receptor.

TABLE 4. Comparison of drug affinities at [^{3}H]clozapine binding sites in rat brain and cloned serotonin receptors

Ligand	$5HT_{1a}$[a,b] (nM)	$5HT_{1B}$[c,d,e] (nM)	$5HT_{1C}$[f] (nM)	$5HT_{1E}$[g,h] (nM)	$5HT_{1F}$[i] (nM)	$5HT_2$[j] (nM)	$5HT_{5A}$[k] (nM)	$5HT_{5B}$[k] (nM)	$5HT_7$[l,m,n] (nM)	[^{3}H]Clozapine (nM)
8-OH DPAT	0.06	>10,000	—	—	>1,000	>1,000	>10,0	398	35–52	5,000
5HT	0.27–1.66	16–48	—	8.14	70–125	250	251	251	0.6–1.8	200,000
Spiperone	63–134	>10,000	957	—	9.9–20	0.5	—	—	9.9–20	24.6
Mesulergine	400	—	3.0	>1,000	>1,000	—	—	—	21.1	1,000
Methysergide	—	436–1,823	—	300	6.3–14	1.0–5.0	63	126	13.4	8.2
Ketanserin	2,100–23,000	>10,000	28.5	—	3,162	1.0–5.0	>10,000	1,584	265–37,500	81.9
Ritanserin	—	—	2.7	—	—	0.2	—	—	15	123
Methiothepin	—	13	—	6.7	>1,000	0.4	700	15.8	0.3–8.8	9.8
Ergotamine	—	—	—	600	50–55	—	3.9	3.2	—	9.1
Sumatriptan	—	465	—	2,300	67–79	—	3.9	3.2	1,000	>1,000
Clozapine	—	4.2	7.2	—	—	5.0	—	—	4.0	5.3

All numbers represent K_i values from the literature as noted or [^{3}H]clozapine binding to rat brain membranes. Drug affinities for [^{3}H]clozapine binding sites are mean values of two to three determinations varying less than 20%.

[a]Fargin et al. (19)
[b]Albert et al. (20)
[c]Maroteaux et al. (21)
[d]Adham et al. (22)
[e]Voigt et al. (23)
[f]Roth et al. (24)
[g]Zgombick et al. (25)
[h]McAllister et al. (26)
[i]Amlaiky et al. (27)
[j]Meltzer et al. (28)
[k]Matthes et al. (29)
[l]Roth et al. (30)
[m]Ruat et al. (31)
[n]Shen et al. (32)

TABLE 5. Comparison of drug affinities for [^{3}H]clozapine binding sites in rat brain membranes and cloned 5HT$_6$ receptors

	[^{3}H]Clozapine K_i (nM)		[^{3}H]5HT (nM)	[^{3}H]LSD (nM)
	5HT$_6$ Clone	Cortex	5HT$_6$ Clone	
Clozapine	10	10	20	13
Methiothepin	9	7	0.4	2
Lisuride	20	15	5	8
Dihydroergotamine	25	40	5	13
Clomipramine	150	80	—	54
Lergotrile	150	250	—	36
Loxapine	100	15	56	65
Nortriptyline	400	30	—	148
Amitriptyline	100	10	82	70
Fluoxetine	4,000	5,000	—	1,700
Ritanserin	1,000	150	16	44
Mesulergine	2,000	1,000	—	1,700
Thioridazine	300	25	—	—
Ketanserin	1,000	80	—	—
Ergotamine	10	12	—	—
Risperidone	>1,000	100	—	—
Metergoline	950	1,000	—	—
Methysergide	800	1,000	—	—

K_i values are means of two to three determinations that varied less than 20%.

Compared with conventional neuroleptics, clozapine and other atypical neuroleptics display two unique properties, a low incidence of EPS and greater therapeutic efficacy for negative and possibly even for positive symptoms of schizophrenics. This enhanced efficacy was first shown in patients resistant to conventional neuroleptics (1), but may hold for nonresistant patients as well (1,36). Properties unique to the atypical drugs may thus clarify neural mechanisms mediating negative symptoms as well as modulating EPS. Influences on 5HT receptors appear relevant to clozapine actions, as clozapine displays very great potency at most 5HT receptors, and 5HT receptors modulate dopamine systems involved in EPS. Thus, chronic administration of conventional neuroleptics decreases firing rates of the A_{10} dopamine cells that project to the limbic system and the A_9 cells of the substantia nigra that project to the corpus striatum. Presumably, the slowed firing rate of the dopamine cells accentuates the effects of direct dopamine receptor blockade. The observations that clozapine (37) and other atypical neuroleptics (13,38,39) slow A_{10} but not A_9 cells could explain the lowered EPS incidence associated with clozapine. Metabolic measures showing greater dopamine release in the corpus striatum following atypical than conventional neuroleptics support the neurophysiologic data (36,40). A link to 5HT$_2$ receptors comes from observations that combinations of the 5HT$_2$ selective drug ritanserin and the D_2 antagonist haloperidol elicit striatal dopamine metabolic patterns resembling clozapine (41). Similarly, administration of ritanserin alleviates EPS provoked by haloperidol (10). The similarity of 5HT$_6$ and 5HT$_2$ receptors suggests that pharmacologic data implicating 5HT$_2$ receptors in actions of atypical neuroleptics would apply also to 5HT$_6$ receptors. Interestingly, ritanserin's affinity for 5HT$_6$ receptors varies depending on the ligand employed with K_i values of 16 nM with [^{3}H]5HT and 1000 with [^{3}H]clozapine. At [^{3}H]clozapine binding sites in cortical membranes, ritanserin has a K_i of 150 nM.

Notably, while clozapine has low nanomolar affinity for numerous serotonin as well as other receptors, the predominant receptors labeled by

[^{3}H]clozapine in the present study were muscarinic cholinergic and 5HT$_6$-like. In part, this may reflect receptor density in that the very low density of D_4 receptors in rat brain (9) may have precluded their labeling. Alternatively, conformational alterations in receptors elicited by a radioligand may influence a drug's apparent affinity. Accordingly, the best indication of sites with which the drug will interact in vivo will come from properties of a radiolabeled drug's binding in the intact human brain. Whether the same sites are labeled by [^{3}H]clozapine in rat brain membranes is unclear. Nonetheless, sites labeled by [^{3}H]clozapine, whether reflecting 5HT$_6$ or related receptors, may provide a model system for identifying candidate atypical neuroleptics and clarifying their unique psychoactive properties.

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