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Contribution of a Helix 5 Locus to Selectivity of Hallucinogenic and Nonhallucinogenic Ligands for the Human 5-Hydroxytryptamine$_{2A}$ and 5-Hydroxytryptamine$_{2C}$ Receptors: Direct and Indirect Effects on Ligand Affinity Mediated by the Same Locus

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SUMMARY

An important determinant of the neurobehavioral responses induced by a drug is its relative receptor selectivity. The molecular basis of ligand selectivity of hallucinogenic and nonhallucinogenic compounds of varying structural classes for the human 5-hydroxytryptamine (5-HT)$_{2A}$ and 5-HT$_{2C}$ receptors was investigated with the use of reciprocal site-directed mutagenesis. Because these two closely related receptor subtypes differ in the amino acid present at position 5.46 (residues 242 and 222 in the sequences, respectively), the effects of corresponding substitutions in the 5-HT$_{2A}$ [S5.46(242) → A] and 5-HT$_{2C}$ [A5.46(222) → S] receptors were studied in tandem. By studying both receptors, the direct and indirect effects of mutations on affinity and selectivity can be distinguished. The ergolines studied, mesulergine (selective for the 5-HT$_{2C}$ receptor) and *d*-lysergic acid diethylamide (selective for the 5-HT$_{2A}$ receptor), reversed their relative affinity with mutations in each receptor, supporting a direct role of this locus in the selectivity of these ligands. However, interchange mutations in either receptor led to decreased or unchanged affinity for (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane and ketanserin, which have higher affinity for the 5-HT$_{2A}$ receptor, consistent with little contribution of this locus to the selectivity of these ligands. The indoleamines studied were affected differently by mutations in each receptor, suggesting that they bind differently to the two receptor subtypes. Mutation of this locus in the 5-HT$_{2A}$ receptor decreased the affinity of all indoleamines, whereas the interchange mutation of the 5-HT$_{2C}$ receptor did not affect indoleamine affinity. These results are consistent with a direct interaction between this side chain and indoleamines for the 5-HT$_{2A}$ receptor but not for the 5-HT$_{2C}$ receptor. Furthermore, this analysis shows that the higher affinity of 5-HT and tryptamine for the 5-HT$_{2C}$ receptor than for the 5-HT$_{2A}$ receptor is not due to the difference at this locus. The hallucinogens studied [*d*-lysergic acid diethylamide, psilocin, bufotenin, and (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane] fell into different classes in this analysis. For the classes of ligand studied, the side-chain difference at this position directly determines relative ligand selectivity only for ergolines and may contribute to the specific effects of hallucinogens in this class.

The molecular mechanisms by which hallucinogenic drugs of abuse induce their unique neuropsychological effects have not been elucidated. The initial actions of these chemicals occur at the level of receptor interaction, and their receptor selectivity in humans is likely to play an important role in determining their effects. A series of studies, primarily in rodents (1), implicated the pharmacologically defined 5-HT$_{2A/2C}$ receptors as potentially major loci for the action of hallucinogens. The potency in humans of known hallucinogens correlates with their affinity for the rat brain 5-HT$_{2A}$ receptor (1, 2).

The recent cloning of the various 5-HT receptor subtypes (3) provides the opportunity to evaluate the selectivity of hallucinogenic and nonhallucinogenic serotonergic ligands for human receptor subtypes and to investigate the molecular basis of this selectivity. Significant differences in the affinities of certain ligand classes for particular rat and human 5-HT receptors have been identified, and the sequence differences responsible for these pharmacological distinc-

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; LSD, *d*-lysergic acid diethylamide; DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; TMH, transmembrane helix; 4-HT, 4-hydroxytryptamine.

tions have been determined through the use of site-directed mutagenesis (4–11). For example, a single amino acid change in the TMH 7 domain determines the marked differences in affinity of β-adrenergic antagonists for the rat and human 5-$HT_{1B/D}$ receptors (4–6). If a ligand has significantly different affinities for the same receptor subtype in human and animal species, it follows that the receptor subtype selectivity of particular ligands varies significantly between animal model systems and humans. This situation exists for the rat and human 5-HT_{2A} receptors, which show large differences in the affinities of some ligands (7, 8). It is therefore important to determine the molecular basis for the selectivity of serotonergic ligands for human 5-HT receptor subtypes to achieve an understanding of the actions of hallucinogenic drugs of abuse at the level of receptor/ligand complexing.

A one-amino acid difference between the human and rat 5-HT_{2A} receptors has been reported to account for many of the pharmacological differences between the two homologues (7–10). Alignment of the sequences of the putative TMH 5 domain of the cloned rat and human 5-HT_{2A} and 5-HT_{2C} receptors (Fig. 1) reveals that the two rat receptor subtypes are identical at position 5.46 in TMH 5, but the two human subtypes differ at this locus by having a serine in the 5-HT_{2A} receptor and an alanine at the corresponding sequence in the 5-HT_{2C} receptor. Thus, the sequence difference in this locus between the rat and human 5-HT_{2A} receptor is echoed in the difference between the 5-HT_{2A} and 5-HT_{2C} receptors in humans. Based on the studies of the human and rat 5-HT_{2A} receptors (7–10), we hypothesized that this single substitution may affect the relative affinities of a variety of ligands for the two closely related human receptor subtypes and may contribute to the selectivity of some hallucinogenic drugs of abuse. This hypothesis was investigated by mutating the cognate locus in both human receptor subtypes and by characterizing the pharmacological profile of wild-type and mutant receptors expressed in COS-1 cells. Four of the ligands studied (bufotenin, psilocin, LSD, and DOI) are hallucinogenic drugs.

Through exchange mutations in two related receptors, we attempted to dissect the different effects of mutations on affinity and selectivity. The results are discussed in the context of the difference between direct effects of mutations on ligand/receptor interaction as opposed to the indirect effects that mutations may have on ligand affinity through rearrangements of receptor structure. Our findings suggest that for different ligands and the same receptor mutations, different mechanisms underlie the alterations in affinities that were observed. For some ligands, interchange of the amino acids between the two receptor subtypes reverses the affinities for the receptors, whereas for other ligands, the results of the mutation in each receptor are inconsistent. These results lead to inferences about the nature of the contribution of the amino acid at this locus toward ligand selectivity for these 5-HT receptor subtypes.

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                          5.   5.
                         46   50
HUM5HT2A      NFVLIGSFV S FFIPLTIMVITYFLTIKSLQ
HUM5HT2C      ---------A------------C---YV-R

RAT5HT2A      ---------A----------
RAT5HT2C      ---------A----------YV-R
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Fig. 1. Alignment of the putative TMH 5 domain from the deduced amino acid sequence of the human (*HUM5HT$_{2A}$* and HUM5HT$_{2C}$) and rat (*RAT5HT$_{2A}$* and *RAT5HT$_{2C}$*) 5-HT_{2A} and 5-HT_{2C} receptors (13, 23, 24). *Bold type*, amino acids that were studied through mutagenesis: Ser5.46(242) in the human 5-HT_{2A} and Ala5.46(222) in the human 5-HT_{2C} receptors. *Dashes*, residues that are identical to those in the human 5-HT_{2A} receptor.

Materials and Methods

Chemicals. LSD, bufotenin, and psilocin were obtained from the National Institute on Drug Abuse, National Institutes of Health. All other unlabeled ligands were obtained from Sigma Chemical Co. (St. Louis, MO) or Research Biochemicals International (Natick, MA).

Receptor numbering scheme. To facilitate comparison of cognate residues in different receptors, residues are numbered according to a consensus numbering scheme described in detail previously (12). S5.46(242) → A indicates that Ser242 in helix 5 has been mutated to alanine. The notation 5.46 indicates the position of this residue relative to the most conserved amino acid within helix 5, Pro246, which is designated residue 5.50. The residue number in parentheses indicates the amino acid identity with the use of standard amino terminus-based numbering.

DNA constructs and transfection. The cDNA clones encoding the human 5-HT_{2A} and 5-HT_{2C} receptors were generously provided by Dr. Alan Saltzman (13). Mutations in the human 5-HT_{2A} receptor were introduced as described previously (14). The 5-HT_{2C} receptor clone was digested with *Bam*HI, and the insert was ligated into *Bam*HI-digested pALTER (Promega, Madison, WI). Mutations were introduced following the manufacturer's protocol and confirmed through sequencing. The insert was subcloned into the *Bam*HI site of pCDNAI/Amp for expression.

The presence of the desired mutations were reconfirmed through sequencing of the mutation site in the expression vector. COS-1 cells (American Type Culture Collection, Rockville, MD) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Each 100-mm plate, seeded 24 hr earlier with 3×10^6 cells, was transfected with 8 μg of DNA construct and 48 μl of Lipofectamine (Life Technologies, Gaithersburg, MD).

Phosphastidylinositol hydrolysis assay. Hydrolysis of [^{3}H]phosphatidylinositol was assayed as described previously (14). Briefly, transfected cells were seeded onto two 12-well plates. Thirty-two hours later, the medium was replaced by serum-free medium containing 0.5 μCi/ml *myo*-[^{3}H]inositol (NEN, North Billerica, MA). One day later, the cells were washed and incubated with the desired concentrations of 5-HT in the presence of 20 mM LiCl for 30 min at 37°. Cell extracts, in 10 mM formic acid, were applied to the Dowex ion-exchange column before elution with a buffer containing 1 M ammonium formate and 0.1 M formic acid, and the eluate was counted with a scintillation counter.

Ligand binding assays. Three days after transfection, cells were harvested, and the cell pellets were stored at −70°. All membrane preparation procedures were carried out at 4°. Pellets were thawed and homogenized (Polytron, setting No. 6 for 8 sec) in 20 ml of 50 mM Tris·HCl (pH 7.4 at 25°) buffer. An additional 20 ml of buffer was added, and the homogenates were centrifuged at 35,000 × *g* for 15 min. The membrane pellets were resuspended in buffer with a Teflon-and-glass homogenizer.

[^{3}H]Ketanserin (DuPont-NEN) was used to label 5-HT_{2A} receptors. Saturation and competition assays were carried out as described previously (14). Incubations were carried out for 1 hr at 37° in a total volume of 1 ml of Tris·HCl buffer. Nonspecific binding was defined with the use of 10 μM methysergide. For competition studies, the concentration of [^{3}H]ketanserin was 1.5–2.0 nM. [^{3}H]Mesulergine was used to label 5-HT_{2C} receptors. Binding assays were carried out as described above, except that nonspecific binding was defined with the use of 10 μM mianserin. The concentration of [^{3}H]mesulergine used in competition studies with wild-type receptors was 0.5 nM.

Because mutation of the 5-HT_{2C} receptor resulted in a reduced affinity for [^{3}H]mesulergine, competition studies for the A222S mutant were carried out with 5.0 nM [^{3}H]mesulergine. For both radioligands, assays were terminated by rapid filtration through Whatman GF/C filters that had been presoaked in 3% polyethyleneimine. Protein content was determined according to the method of Lowry *et al.* (15). Each binding assay tube contained 30–60 μg of membrane protein.

Data analysis. All curve fitting was carried out with the graphics software Kaleidagraph (Synergy Software, Reading, PA). For saturation studies, B_{max} and K_d values for [^{3}H]ketanserin and [^{3}H]mesulergine were obtained through a fit of the data to the following equation: Total binding = $\{(B_{max}/[1 + (K_d/D)^n]\} + (m \cdot D)$, where n is the slope factor analogous to the Hill coefficient, m is the slope of a linear regression of the nonspecific binding curve, and D is the concentration of radioligand. Values are given as mean ± standard error. For competition studies, specific binding was expressed as a percentage of the specific binding in the absence of competing ligands and fit to the following equation: Percentage of specific binding = $100 - [100/(1 + (IC_{50}/L)^n]$, where IC_{50} is the concentration of competing drug that produces 50% specific binding, n is the slope factor analogous to the Hill coefficient, and L is the concentration of unlabeled competing ligand. IC_{50} values were converted to apparent K_i values according to the method of Cheng and Prusoff (16) with the K_d values for the radioligands determined from saturation experiments. For some ligands, the fit slope factors were less than unity, which could indicate the existence of more than one site or of different affinity states; however, in these cases, fit of the data to a two-site model did not improve the fit. The addition of guanine nucleotides to the incubation mixtures did not change the slope factors of those competition curves with $n < 1$ (not shown), which suggests that the presence of interconverting affinity states is not the reason for the shallow slope factors. Therefore, "apparent" K_i values are reported to facilitate comparison of ligand affinities when different concentrations of radioligand are used. K_i values were converted to pK_i ($-\log K_i$) to normalize values before analysis of variance followed by Tukey's post-hoc comparison was carried out (SuperANOVA, Abacus Concepts, Berkeley, CA). The significance level was set at $p < .05$.

Molecular modeling. The models of the TMH bundle of the 5-HT_{2A} and 5-HT_{2C} receptors were constructed according to the criteria and procedures described previously (12) based on sequence conservation (17), degree of residue polarity (18), and incorporation of a large number of experimental constraints reviewed elsewhere (12). The criteria for predicting the TMH helix boundaries included the arginine/lysine motif at the cytoplasmic side (12). Sequences were aligned with the use of Oxford Molecular Software, and energy minimizations were performed with the Quanta/Charmm molecular modeling package. The LSD conformation was derived from the crystal structure (19). LSD was docked into the 5-HT_{2A} model in proximity to the proposed TMH 3 and TMH 5 contact sites and according to previous structure-activity data (20), and its position was refined through energy minimization. The mesulergine/5-HT_{2C} receptor complex model was obtained by mapping the corresponding molecular fragments from the LSD/5-HT_{2A} receptor complex.

Results

Binding of [^{3}H]Ketanserin and [^{3}]Mesulergine

The wild-type and mutant human 5-HT_{2A} and 5-HT_{2C} receptors were expressed and characterized in COS-1 cells. All constructs were functional and mediated phosphoinositol turnover when exposed to 5-HT (not shown). Saturation binding is shown in Fig. 2. For the 5-HT_{2A} receptor, the B_{max}

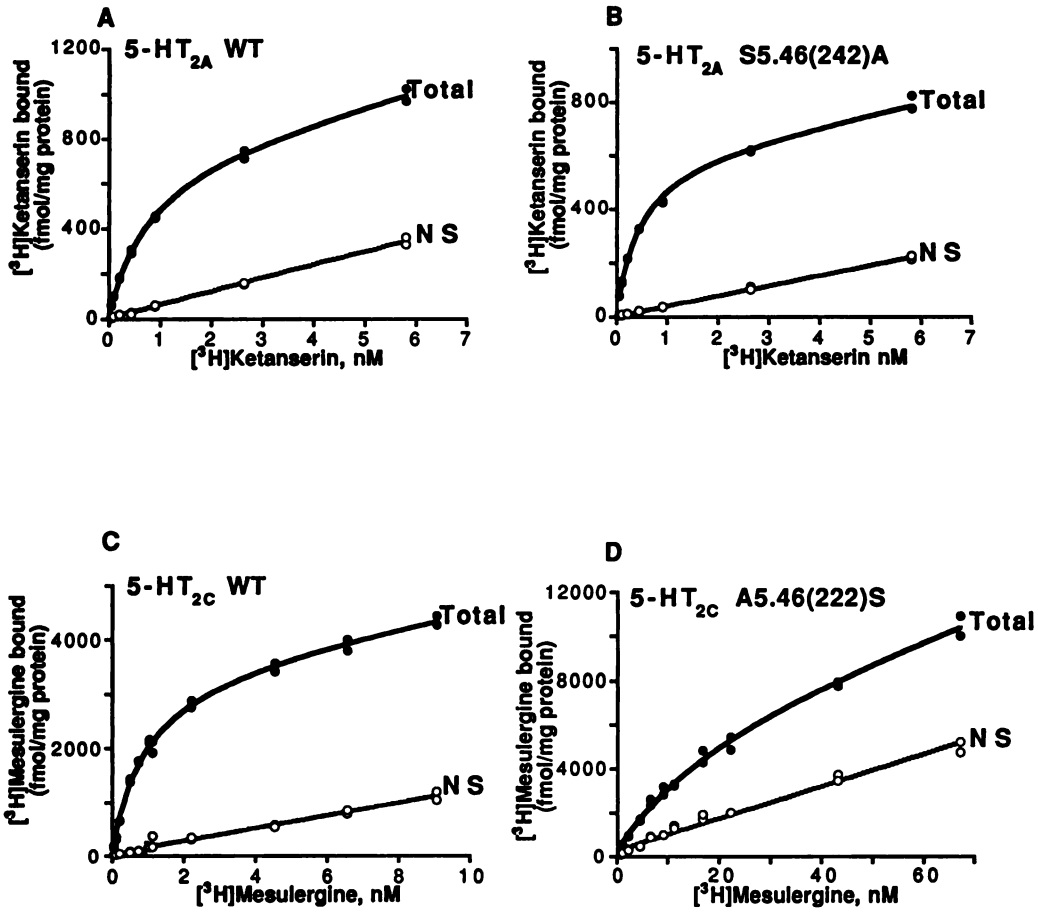

Fig. 2. [^{3}H]Ketanserin saturation binding to the wild-type and mutant 5-HT_{2A} receptors (A and B) and [^{3}H]mesulergine saturation binding to the wild-type and mutant 5-HT_{2C} receptors (C and D). *Total*, total radioligand binding; *NS*, nonspecific binding.

for binding of [^{3}H]ketanserin was 853 ± 98 fmol/mg protein for the wild-type receptor and 746 ± 95 fmol/mg protein for the S5.46(242) → A mutant (three experiments). For the 5-HT$_{2C}$ receptor, the B_{max} for binding of [^{3}H]mesulergine was 2939 ± 489 fmol/mg protein for the wild-type receptor and 6561 ± 174 fmol/mg protein for the A5.46(222) → S mutant (two experiments). The affinity of [^{3}H]ketanserin for the S5.46(242) → A mutant 5-HT$_{2A}$ receptor was unchanged relative to the wild-type receptor (Table 1). In contrast, the affinity of [^{3}H]mesulergine for the A5.46(222) → S mutant 5-HT$_{2C}$ receptor was decreased 28-fold.

Competition Binding

Representative competition binding curves are shown in Fig. 3, and ligand binding data for all constructs are summarized in Tables 1 and 2. The compounds studied had varying selectivities for either the 5-HT$_{2A}$ or 5-HT$_{2C}$ receptor subtypes. The changes in affinity observed for the mutant receptors varied; for some compounds, the interchange of amino acids at position 5.46 resulted in reciprocal changes in the affinity of the ligand for the receptor subtype, indicating that this locus is the major determinant of selectivity. For other compounds, the changes in affinity were not defined by the amino acid at position 5.46, suggesting an indirect effect of this locus on the selectivity of the ligand (see Discussion). Based on the selectivity of the ligands for the wild-type receptors and on the effects of mutations on affinity and selectivity, the ligands studied can be classified into four categories.

Group I. This group of ligands includes mesulergine, LSD, lisuride, and ergonovine. All ergolines studied were selective for one of the two receptor subtypes, and significant changes in their affinities were observed with mutations at position 5.46. The introduction of the 5-HT$_{2C}$ residue (alanine) into position 5.46 of the 5-HT$_{2A}$ receptor altered their affinities, bringing them closer to those of the 5-HT$_{2C}$ receptor. The reverse was found with mutation of the 5-HT$_{2C}$ receptor (Tables 1 and 2). For example, the affinity of mesulergine for the wild-type 5-HT$_{2C}$ receptor (K_d = 0.74 nM) is ~40-fold higher than that for the wild-type 5-HT$_{2A}$ receptor (K_i = 28 nM). Mutation of the 5-HT$_{2C}$ receptor (A5.46(222) → S) decreased the affinity of mesulergine (K_d = 21 nM) to a value similar to that for the wild-type 5-HT$_{2A}$ receptor. Conversely, the mutant 5-HT$_{2A}$ receptor (S5.46(242) → A) had an increased affinity for mesulergine (K_i = 3.9 nM), a value closer to that of this ligand for the 5-HT$_{2C}$ receptor. These changes in affinity suggest a relationship between the selectivity of mesulergine for the 5-HT$_{2C}$ receptor and the nature of the side chain at position 5.46. The symmetrical nature of the observed affinity changes suggests a direct interaction between mesulergine and the residue at this locus in both receptor subtypes (see Discussion).

In contrast to mesulergine, the ergoline derivatives LSD, lisuride, and ergonovine had higher affinity for the wild-type 5-HT$_{2A}$ receptor than for the wild-type 5-HT$_{2C}$ receptor. The reciprocal serine/alanine mutations in the two receptors caused parallel and reciprocal changes in the affinities of these ligands. All exhibited an increased affinity for the mutant 5-HT$_{2C}$ receptor and a decreased affinity for the mutant 5-HT$_{2A}$ receptor. The affinity of ergonovine was most affected by the substitution of alanine for serine in the 5-HT$_{2A}$ receptor (~20-fold decrease in affinity). This is consistent with the low affinity of ergonovine for the wild-type 5-HT$_{2C}$ receptor and suggests a relatively large contribution of the serine at this locus to the receptor affinity of this ligand.

Group II. Ligands in this group, including 4-HT, psilocin (4-hydroxy-*N*,*N*-dimethyltryptamine), and bufotenin (5-hydroxy-*N*,*N*-dimethyltryptamine), exhibited no significant differences in their affinities for the wild-type 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors. Mutation of the 5.46 locus in the 5-HT$_{2C}$ receptor had no significant effect on the affinities of these drugs. However, all three drugs had a significant decrease in affinity with mutation of the serine at 5.46 to alanine in the 5-HT$_{2A}$ receptor. The asymmetry in the effects of mutation in the two receptors, showing that these ligands were sensitive to the side chain in this position only in the 5-HT$_{2A}$ receptor, suggests that these compounds bind differently to the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors.

Group III. The ligands in this group, 5-HT and tryptamine, have higher affinity for the 5-HT$_{2C}$ than for the

TABLE 1

Affinities of the wild-type and mutant 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors

Binding affinities (K_i values) and slopes obtained from competition binding experiments with [^{3}H]ketanserin for the 5-HT$_{2A}$ receptors and [^{3}H]mesulergine for the 5-HT$_{2C}$ receptors. The values represent the means ± standard error from three to six experiments, except for [^{3}H]mesulergine for which there were two experiments. For the wild-type 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors, K_d values (**bold**) were obtained from saturation binding experiments.

	5-HT$_{2A}$				5-HT$_{2C}$			
	Wild-type		S5.46A		Wild-type		A5.46S	
	K_i/(K_d)	Slope	K_i/(K_d)	Slope	K_i/(K_d)	Slope	K_i/(K_d)	Slope
	nM		nM		nM		nM	
Mesulergine	28 ± 2	0.96 ± 0.04	3.9 ± 0.2	1.00 ± 0.15	**0.74 ± 0.26**	1.0 ± 0.08	**21 ± 3**	1.15 ± 0.11
LSD	0.24 ± 0.05	1.12 ± 0.09	2.1 ± 0.4	1.06 ± 0.13	6.4 ± 0.7	1.14 ± 0.22	1.3 ± 0.3	0.9 ± .3
Ergonovine	0.46 ± 0.12	0.87 ± 0.08	9.9 ± 1.5	0.80 ± 0.01	19 ± 4	0.91 ± 0.09	3.1 ± 0.9	0.72 ± 0.07
Lisuride	0.33 ± 0.05	0.95 ± 0.04	2.0 ± 0.1	0.80 ± 0.04	6.1 ± 1.2	1.04 ± 0.06	1.4 ± 0.4	0.83 ± 0.09
Bufotenin	182 ± 35	0.91 ± 0.07	473 ± 49	0.87 ± 0.07	234 ± 27	1.04 ± 0.08	176 ± 20	0.81 ± 0.11
Psilocin	81 ± 12	1.04 ± 0.09	259 ± 26	1.05 ± 0.14	140 ± 12	1.23 ± 0.07	73 ± 13	1.02 ± 0.14
4-HT	410 ± 74	1.16 ± 0.08	1023 ± 155	1.31 ± 0.17	695 ± 33	1.02 ± 0.07	330 ± 48	0.71 ± 0.08
Tryptamine	929 ± 285	0.89 ± 0.03	3493 ± 420	0.83 ± 0.02	284 ± 43	0.85 ± 0.08	174 ± 28	0.78 ± 0.07
5-HT	316 ± 33	0.81 ± 0.03	1150 ± 149	0.75 ± 0.01	126 ± 20	0.92 ± 0.05	76 ± 16	0.74 ± 0.05
Ketanserin	**0.75 ± 0.07**	0.93 ± 0.06	**0.49 ± 0.02**	0.83 ± 0.04	28 ± 3	0.89 ± 0.08	94 ± 19	0.82 ± 0.06
DOI	15 ± 1	0.81 ± 0.01	51 ± 5	0.80 ± 0.03	41 ± 2	0.85 ± 0.04	120 ± 39	0.68 ± 0.03

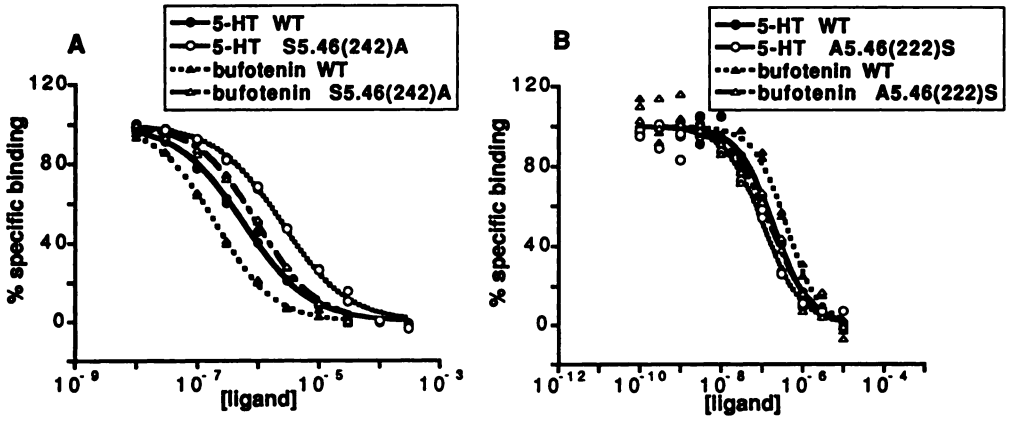

Fig. 3. Competition by 5-HT and bufotenin for [^{3}H]ketanserin binding to the wild-type and mutant 5-HT$_{2A}$ receptors (A) and competition by 5-HT and bufotenin for [^{3}H]mesulergine binding to the wild-type and mutant 5-HT$_{2C}$ receptors (B).

TABLE 2

Differences and changes in the affinities of ligands between the wild-type and mutant receptors. In column 1 a significant difference in affinity between the wild-type receptors is indicated. In columns 2 and 3, a significant effect of the mutation on affinity (increasing or decreasing affinity) is indicated by arrows.

Ligand	2AWT:2CWT	2AMUT:2AWT	2CMUT:2CWT
Mesulergine	<	↑	↓
LSD	>	↓	↑
Ergonovine	>	↓	↑
Lisuride	>	↓	↑
4-HT	NS	↓	NS
Psilocin	NS	↓	NS
Bufotenin	NS	↓	NS
Tryptamine	<	↓	NS
5-HT	<	↓	NS
Ketanserin	>	NS	↓
DOI	>	↓	↓

NS, no significant difference observed; 2AWT, wild-type 5-HT$_{2A}$ receptor; 2AMUT, mutant 5-HT$_{2A}$ receptor; 2CWT, wild-type 5-HT$_{2C}$ receptor; 2CMUT, mutant 5-HT$_{2C}$ receptor; ↑, increasing affinity; ↓, decreasing affinity.

5-HT$_{2A}$ receptor. However, the changes observed with mutation of locus 5.46 in the two receptors indicate that this locus does not account for the selectivity of these ligands. Introduction of alanine into position 5.46 of the 5-HT$_{2A}$ receptor decreased the affinity, thus increasing the difference in affinity from the 5-HT$_{2C}$ receptor. Furthermore, the replacement of alanine by serine in the corresponding position of the 5-HT$_{2C}$ receptor induced no significant change in the affinity of these ligands. These results suggest that ligands in this group may bind differently to the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors and that other loci and/or interactions must be important in determining the differences in the affinities of these ligands for the two receptors. As found for ligands in group II, these results are consistent with a direct interaction of this locus of the 5-HT$_{2A}$ receptor alone.

Group IV. The ligands in this group, DOI and ketanserin, show differences in affinity between the wild-type receptor subtypes. However, the affinities of these drugs were decreased or unaffected by corresponding mutations of the receptors. Thus, DOI had a higher affinity for the wild-type 5-HT$_{2A}$ receptor (K_i = 15 nM) than for the wild-type 5-HT$_{2C}$ receptor (K_i = 41 nM). The affinity of DOI for the mutant 5-HT$_{2A}$ receptor and the mutant 5-HT$_{2C}$ receptor was decreased ~3-fold. Ketanserin had a much higher affinity for the wild-type 5-HT$_{2A}$ receptor (K_d = 0.75 nM) than for the 5-HT$_{2C}$ receptor (K_i = 28 nM), yet its affinity for the mutant 5-HT$_{2A}$ receptor did not change significantly (K_d = 0.49 nM), whereas the affinity for the mutant 5-HT$_{2C}$ receptor decreased slightly (K_i = 94 nM). Thus, the identity of the residue at this position is not contributing to the relative affinity of ketanserin for the two receptors. This pattern suggests that the selectivity of DOI or ketanserin is not mediated by the nature of the residue at this position in either receptor subtype and that the affinities are altered indirectly by the mutation of the residue at this locus.

Discussion

In an attempt to elucidate the molecular basis of the selectivity of various hallucinogenic and nonhallucinogenic ligands for the human 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors, we used exchange mutations at homologous positions in both receptors. The analysis of the effects of exchange mutations on

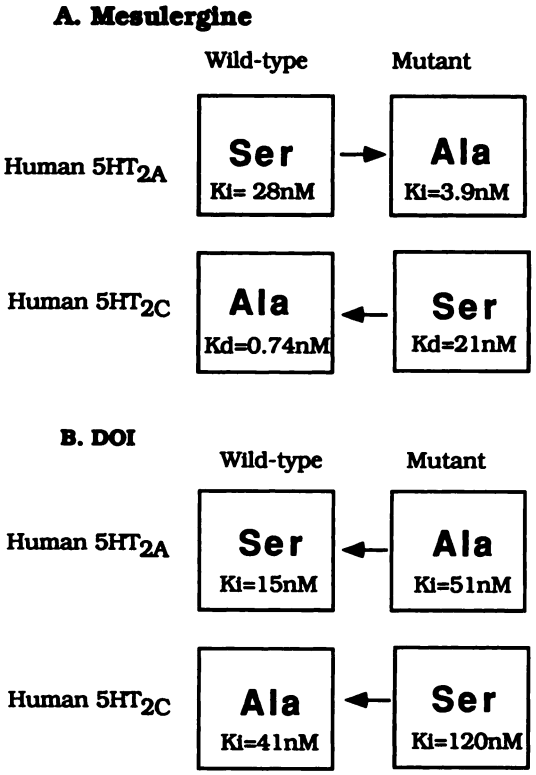

Fig. 4. Schematic of the changes in affinity of mesulergine and DOI for the wild-type and mutant receptors. *Boxes*, amino acid at position 5.46 for each receptor subtype; *Arrows*, significant increases in affinity.

ligand affinity in both receptors studied in tandem allows more insight into the structural basis for the effect on affinity than would a study of the effects of mutations in only one receptor. The selectivity of a ligand for a particular receptor subtype may result from a direct favorable or unfavorable interaction between a functional moiety of the ligand and an amino acid residue in the receptor that is absent in other receptor subtypes. Alternatively, selectivity may arise from more subtle differences in the overall conformation of the binding pocket that are not obvious from sequence comparison of the two receptors.

If the selectivity for a receptor subtype is mediated by a single amino acid that provides a favorable interaction, the introduction of this functional group through mutagenesis into a receptor subtype lacking this moiety would be predicted to increase the affinity of the ligand for the receptor. Correspondingly, the removal of this group from the receptor for which the ligand is selective would result in a decreased affinity. More significant for selectivity is the realization that if a single amino acid site can produce an unfavorable interaction (e.g., from steric clash), the introduction of such a functional group through mutagenesis into a receptor subtype lacking this moiety will decrease the affinity of the ligand for this construct.

When exchange mutations in two receptors are studied in tandem, the changes in affinity are likely to be quantitatively similar and opposite in direction if the affinity of the ligand is largely mediated by the amino acid residue at this locus in both receptor subtypes. If the only effect of an exchange of the residue in this locus is to decrease affinity for the same ligand in one or both receptors, a direct interaction between this locus in the receptor and a ligand in both receptors is less likely. Rather, this pattern suggests that the mutation may affect affinity for this ligand through indirect perturbations of the binding pocket. Thus, through examination of the effect of interchange mutations in both receptors, the nature of the contribution of the amino acid substitution at this locus in each receptor may be inferred more readily than through similar mutation in any one receptor.

Based on the differences in affinities of the ligands for the wild-type receptors and the changes in affinity observed for the mutant receptors, the ligands studied have been divided into four categories. Two examples of this classification are presented schematically in Fig. 4. Mesulergine (Fig. 4A; group I ligand) shows a much higher affinity for the 5-HT_{2C} receptor. Introduction of a serine at position 5.46 into the 5-HT_{2C} receptor, which introduces the side chain found at this position in the 5-HT_{2A} receptor, also reduces the affinity to a value closer to that of the 5-HT_{2A} receptor. Importantly, the results of the parallel experiment are consistent with a direct effect of the residue at this position on the ligand/receptor interaction. Thus, introduction of the residue found at this position in the 5-HT_{2C} receptor into the same position of the 5-HT_{2A} receptor increases the affinity, bringing the affinity closer to that of the 5-HT_{2C} receptor. These results suggest that mesulergine binds similarly to the two receptors and that the side chain at this position is the major determinant of ligand selectivity between the two receptors. This conclusion is consistent with the inference reached in a previous study (8) that mesulergine has a direct interaction at this locus of the human and rat 5-HT_{2A} receptors, where the indole methyl group has an unfavorable steric clash with Ser5.46 and a favorable Van der Waals interaction with the smaller and hydrophobic Ala5.46 side chain (Fig. 5).

In contrast, the changes in affinity observed for the binding of DOI to the mutant receptors (Fig 4B.; group IV) are not consistent with this locus playing an important or a direct role in DOI selectivity. The affinity of DOI for both mutant receptors was decreased to the same extent. The findings that serine is preferred in the 5-HT_{2A} receptor and alanine is preferred in the 5-HT_{2C} receptor suggest that either this ligand may bind quite differently to the two receptors or that the side chain at this position is affecting DOI affinity indirectly via a perturbation of the binding cleft to which DOI is sensitive. Analysis of the results with ketanserin, which has a more marked selectivity, leads to similar conclusions. It is thus unlikely that either DOI or ketanserin interacts directly with the amino acid residue at this position. The changes in their affinities for the mutant receptors most probably arise from indirect effects on the binding pocket for these two ligands. DOI and ketanserin are structurally different from the indole ring-containing ligands, and it is not surprising that they do not share binding determinants with the tryptamines and the ergolines.

The molecular models of the interactions of LSD and mesulergine with the two wild-type receptors illustrate the proposed simultaneous interactions with the TMH 3 aspartic acid and with Ser5.46 (Fig. 5B). They are consistent with the structural constraints suggested by double revertant mutant experiments (14) as well as by experimental data on similar receptors (12). In the energy-minimized model of the receptor/ligand complex, the N1-unsubstituted nitrogen of LSD is optimally located for hydrogen bond formation with the Ser5.46 side chain (Fig. 5C). Fig. 5D shows that in the 5-HT_{2C} receptor/LSD complex model, the hydrogen bond at position 5.46 is no longer feasible due to the presence of alanine. Mesulergine is methyl substituted at the N1 position (Fig. 5A). When mesulergine is superimposed onto the LSD/5-HT_{2A} receptor complex, a significant steric clash appears between the N1 methyl of mesulergine and the hydroxyl of Ser5.46 (Fig. 5E). However, Fig. 5F illustrates the favorable Van der Waals interactions of the N1 methyl group of mesulergine with the Ala5.46 side chain of the 5-HT_{2C} receptor.

It should be noted that the side chain at position 5.46 cannot be the sole determinant of the higher affinity of mesulergine for the human 5-HT_{2C} receptor, as demonstrated by the lack of absolute identity of the affinities of mesulergine for the 5-HT_{2C} and for the mutant 5-HT_{2A} receptors. Study of the ligand/receptor models should facilitate the identification of the other sites in the receptors responsible for differences in ligand affinities for each subtype. For the other ergoline ligands in group I, the changes in affinity of LSD, ergonovine, and lisuride for the mutant receptors are consistent with the favorable hydrogen-bond interaction between the serine side chain at this locus and the indole N—H group of the ligand (see Fig. 5).

Previous studies have demonstrated the importance of the side chain at locus 5.46 in determining the pharmacological differences between the rat and human 5-HT_{2A} receptors. Kao *et al.* (7) reported that mutation of Ser5.46(242) → Ala in the human receptor caused the receptor to assume binding properties of the rat receptor for mesulergine. In a series of structure-activity studies, Johnson *et al.* (8) provided convincing evidence that although the Ser5.46 side chain in the

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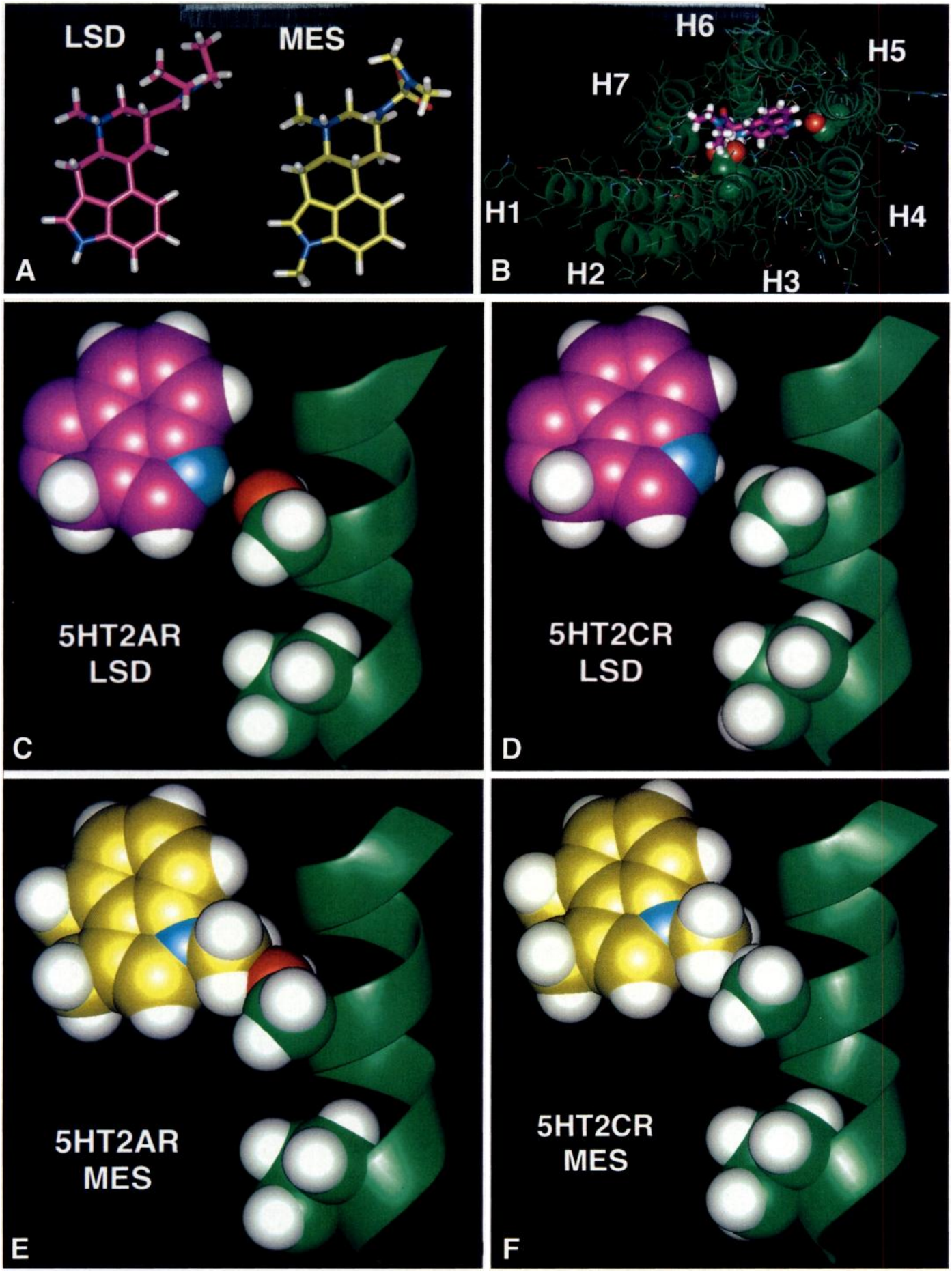

5-HT_{2A} receptor and the N1 hydrogen of N1-unsubstituted ergolines and indoleamines form a hydrogen-bonding interaction, the N1-substituted ergolines would clash with Ser5.46 but have a favorable interaction with Ala in this position.

Our results indicate that the role of this locus in determining subtype selectivity between the human 5-HT_{2A} and human 5-HT_{2C} receptors is more complex than its contribution to the 5-HT_{2A} rat and human pharmacological differences. In the case of ergolines, the differences in pharmacology between the two human subtypes and the rat receptor are predominantly attributable to the side chain at this locus. In the case of the indoleamines, however, in no situation does the side chain at position 5.46 represent a significant determinant of ligand selectivity between the two human receptor subtypes. Furthermore, the lack of reciprocal changes in affinity with mutation for any of these ligands suggests that different positioning of the N1 nitrogen with respect to this locus is achieved through receptor/ligand complexes in the two receptor subtypes. The indoleamine ligands in group II, bufotenin, psilocin, and 4-HT, showed no significant receptor selectivity. The pattern of change with mutation of this locus was not consistent when the results obtained from the two receptors are compared. The affinity decreased with the mutation in the 5-HT_{2A} receptor, but there was no significant change with the mutation in the 5-HT_{2C} receptor, suggesting that these compounds bind differently to the 5-HT_{2A} and 5-HT_{2C} receptors and therefore are affected differently by alteration of this locus in the two receptors. A similar pattern was seen for the group III indoleamines, 5-HT and tryptamine. These compounds were selective for the 5-HT_{2C} receptor, but this selectivity could not be due to the side chain at this position because the only significant changes in affinity after the interconversion of residues at this locus increase the differences in affinity between the two subtypes. Although our results are consistent with the hypothesis developed by Nelson *et al.* (10) that the indole N1 nitrogen hydrogen-bonds with the side chain of Ser5.46 in the human 5-HT_{2A} receptor, the role of this locus in determining the affinity of indoleamines for the 5-HT_{2C} receptor seems to be different. The current results may be useful for future studies in identifying and designing ergolines that are selective for either human 5-$HT_{2A/2C}$ subtype. The presence of an N1 methyl group should confer 5-HT_{2C} selectivity, and its absence should confer 5-HT_{2A} selectivity.

The affinities of agonists determined through competition binding differ according to whether antagonist or agonist radiolabel is used. The relative affinities of specific compounds for the high and low affinity states may vary considerably for different compounds and for different receptors. Leonhardt *et al.* (21) reported that 5-HT has a 250-fold higher affinity for the rat 5-HT_{2A} receptor labeled with the agonist DOI than labeled with the antagonist ketanserin. However, the relative affinities of LSD for the two 5-HT_{2A} receptor states were found to be nearly equivalent. The differences in agonist affinity for agonist- and antagonist-labeled rat 5-HT_{2C} receptors were, in general, also smaller. In the current study, radiolabeled antagonist competition assays were used for both receptors. We showed based on analysis of the structural response of molecular models of 5-HT receptors to agonist binding that the nature and extent of rearrangement corresponding to receptor activation are ligand dependent (18). The measured affinity includes the effects of this rearrangement. Consequently, any mutations, including those studied here, could have different effects on the different conformational states that the receptor adopts on ligand binding (14, 18), as expressed in the measured affinity. Thus, the possibility exists that the mutations studied have different effects on the ability of some of the compounds studied to stabilize the high affinity forms of these receptors, thus producing ligand-dependent effects of the mutations.

To develop a complete structural understanding of the interaction between these ligands and the binding pockets of the two receptors, the direct and indirect roles of sequence differences must be defined. The hypotheses developed regarding specific differences between positions of different ligands with respect to this locus in the two receptors will guide the testing and refinement of the molecular models of the receptor binding pockets and activation mechanisms and the manner in which they account for binding selectivity and differential effects on coupling to effectors (14, 22) .

One goal of this study was to explore the role of this locus in determining the selectivity of serotonergic hallucinogenic drugs of abuse. The hallucinogenic drugs studied, bufotenin, psilocin, LSD, and DOI, fall into three different categories with respect to the role of the locus studied in ligand selectivity. Only for LSD, as for the nonhallucinogenic ergolines, did the side chain at position 5.46 determine the ligand selectivity between the two receptors. This locus may play an important role in determining the neurobehavioral effects of hallucinogens of the ergoline class. Further studies will investigate whether other components of the binding pocket of these receptors are particularly important for the interaction with hallucinogens of various structures.

Interestingly, rat 5-$HT_{2A/2C}$ receptors lack the serine/alanine difference (see Fig. 1), and accordingly, the relative receptor selectivity of some of the ligands studied may differ in rodents and humans. The current results illustrate the potential difficulties of extrapolating those results to humans for some ligands because of potential differences in the relative effect of agonists on these two receptors. In particular, results obtained in the rat for relative actions of ergolines with varying N1 substitutions may require reevaluation. This is especially intriguing because the pharmacological actions of hallucinogens in this chemical class has been characterized with rat models.

Fig. 5. Molecular modeling of ligand/receptor complexes. A, Chemical structures of LSD and mesulergine (*MES*). B, LSD/5-HT_{2A} receptor complex. *Helical ribbons*, protein backbone. Two receptor sites of interactions, Asp3.32 and Ser5.46, are shown as space-filling residues. C, Detail of the LSD/Ser5.46 interaction in the 5-HT_{2A} receptor (*5HT2AR*); only the indole moiety of LSD is shown. LSD, Ser5.46, and Pro5.50 are shown as space-filling models. Note the favorable hydrogen-bonding contact between receptor and ligand. D, Detail of LSD/Ala5.46 interaction in the 5-HT_{2C} receptor (*5HT2CR*). E, Detail of the mesulergine (*MES*)/Ser5.46 interaction in the 5-HT_{2A} receptor (*5HT2AR*); only the indole moiety of mesulergine is shown. Note the steric clash between the N1 methyl carbon (yellow) of mesulergine and the oxygen (red) of the Ser5.46 side chain, as indicated by obliteration of the rounded surface of each atom. F, Detail of mesulergine (*MES*)/Ala5.46 interaction in the 5-HT_{2C} receptor (*5HT2CR*). Note the favorable Van der Waals surface contact between the N1 methyl group of mesulergine and the Ala5.46 side chain.

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