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Assessment of the Roles of Serines 5.43(239) and 5.46(242) for Binding and Potency of Agonist Ligands at the Human Serotonin 5-HT_{2A} Receptor

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ABSTRACT

We assessed the relative importance of two serine residues located near the top of transmembrane helix 5 of the human 5-HT_{2A} receptor, comparing the wild type with S5.43(239)A or S5.46(242)A mutations. Using the ergoline lysergic acid diethylamide (LSD), and a series of substituted tryptamine and phenethylamine 5-HT_{2A} receptor agonists, we found that Ser5.43(239) is more critical for agonist binding and function than Ser5.46(242). Ser5.43(239) seems to engage oxygen substituents at either the 4- or 5-position of tryptamine ligands and the 5-position of phenylalkylamine ligands. Even when a direct binding interaction cannot occur, our data suggest that Ser5.43(239) is still important for receptor activation. Polar ring-substituted tryptamine ligands also seem to engage Ser5.46(242), but tryptamines lacking such a substituent may adopt an alternate binding orientation that does not engage this residue. Our results are consistent with the role of Ser5.43(239) as a hydrogen bond donor, whereas Ser5.46(242) seems to serve as a hydrogen bond acceptor. These results are consistent with the functional topography and utility of our in silico-activated homology model of the h5-HT_{2A} receptor. In addition, being more distal from the absolutely conserved Pro5.50, a strong interaction with Ser5.43(239) may be more effective in straightening the kink in helix 5, a feature that is possibly common to all type A GPCRs that have polar residues at position 5.43.

The serotonin 2A (5-HT_{2A}) receptor is a member of the monoamine family A type GPCRs. It seems to play an essential role in cognition, memory, and consciousness (Nichols, 2004). Although no crystal structure exists for this or any of the monoamine GPCRs, advances in X-ray crystallography have provided structures for the dark-adapted inverse agonist form of bovine rhodopsin (Palczewski et al., 2000; Li et al., 2004), creating opportunities to develop homology models of general GPCR structure. Given sufficient sequence similarity/identity, the accuracy and reliability of comparative homology models is generally believed to be superior to de novo models (Baker and Sali, 2001)

As an example of this approach, the in silico-activated homology model of the h5-HT_{2A} receptor developed in our laboratory (Chambers and Nichols, 2002) has been used to predict several ligand-receptor interactions. Our results thus far have provided qualitative support for the model and have identified directions for further investigation of the structure-activity relationships of agonist ligands (Parrish et al., 2005; Braden et al., 2006; McLean et al., 2006a,b). Although such homology receptor models must be viewed with caution, they can often be validated by receptor mutagenesis experiments, along with complementary changes in ligand structure.

To refine our homology model and provide empirical support for the receptor functional topography it defines, we extended results derived from previous mutagenesis studies

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ABBREVIATIONS: 1-iPr-5-MeO-T, 1-isopropyl-5-methoxytryptamine; 1-Me-5-HT, 1-methyl-5-hydroxytryptamine (1-methylserotonin); 2CH, 2,5-dimethoxyphenethylamine; 2CI, 2,5-dimethoxy-4-iodophenethylamine; 2-Et-DOM, 2-ethyl-5-methoxy-4-methylphenyl-isopropylamine; 5-Et-DOM, 5-ethyl-2-methoxy-4-methylphenylisopropylamine; 5-H-DOM, 2-methoxy-4-methylphenylisopropylamine; 5-HT, 5-hydroxtryptamine (serotonin); 5-Me-T, 5-methyltryptamine; 5-MeO-DMT, 5-methoxy-*N*,*N*-dimethyltryptamine; 5-MeO-T, 5-methoxytryptamine; DMT, *N*,*N*-dimethyltryptamine; DOH, 2,5-dimethoxyphenyl-isopropylamine; DOI, 4-iodo-2,5-dimethoxyphenylisopropylamine; DOM, 2,5-dimethoxy-4-methylphenylisopropylamine; PI, phosphoinositide; TM, transmembrane; GPCR, G protein-coupled receptor; LSD, lysergic acid diethylamide; ANOVA, analysis of variance; WT, wild type.

of 5-HT$_{2A}$ receptors. Agonist ligands for this receptor are of two chemical types: tryptamines and phenylalkylamines, the former being analogs of the endogenous ligand serotonin (5-hydroxytryptamine; 5-HT) and the latter comprising phenethylamines and phenylisopropylamines, all typically with polar substituents attached to the aromatic ring. A polar oxygen atom in the 4- or 5-position of tryptamines generally confers high affinity and activity at this receptor (McKenna and Towers, 1984; Nichols and Glennon, 1984). Tricyclic ergolines such as LSD can be considered special cases of rigidified tryptamines and are particularly interesting because they lack polar aromatic ring substituents. Extensive studies of the phenylalkylamine pharmacophore have revealed optimal polar ring substitution when methoxy groups are located at the 2- and 5-positions (Nichols, 1981; Glennon et al., 1986, 1989; Shulgin and Shulgin, 1991; Nichols, 1994, 1997; Glennon, 1999). Although most previous pharmacological investigations of 5-HT$_{2A}$ receptor agonists have been carried out in rats or in rat-derived preparations, we believe that the mechanistic effects and possible clinical utility of these compounds must be based on an understanding of the human receptor.

Before the advent of computationally derived homology models, putative binding sites had been identified using site-directed mutagenesis data. A strong consensus exists for the small molecule agonist binding site of these receptors to be nestled within the transmembrane domains (TM) of the seven heptahelical bundle, with contacts primarily between the ligand and residues in helices 3, 5, and 6 (van Rhee and Jacobsen, 1996; Roth et al., 1998).

The present study focused on serine residues at positions 5.43 [Ser5.43(239)] and 5.46]Ser5.46(242)] in TM5 of the 5-HT$_{2A}$ receptor [see Ballesteros and Weinstein (1995) for numbering scheme]. These two residues are located one and two turns, respectively, above the highly conserved proline residue Pro5.50(246) in TM5, toward the extracellular face of the receptor. In other homologous GPCRs with a polar residue at position 5.43, mutagenesis to a nonpolar residue dramatically reduced the affinity and activity of nearly all agonists tested (Strader et al., 1989; Ho et al., 1992; Pollock et al., 1992; Wess et al., 1992) or increased the affinity of antagonists or was involved in species selectivity (Link et al., 1992).

Mutation of a polar residue at position 5.46 in nonserotonin aminergic GPCRs also decreased affinity of agonists (Strader et al., 1989; Wang et al., 1991; Pollock et al., 1992), affected the selectivity of agonists or antagonists (Mansour et al., 1992), and/or decreased the affinity of antagonists (Gantz et al., 1992; Leurs et al., 1994). Functional activity was generally unaffected (van Rhee and Jacobsen, 1996).

Previous mutagenesis studies have examined the S5.43(239)A mutant rat 5-HT$_{2A}$ receptor, and Ser5.46(242) in the human and Ala5.46(242) in the rat 5-HT$_{2A}$ receptors have been reciprocally mutated (Kao et al., 1992; Johnson et al., 1993, 1994, 1997; Shapiro et al., 2000). There is some disagreement, however, among these studies as to the binding orientation of tryptamines and/or their interactions with these residues. Furthermore, these residues have not been investigated in parallel in any 5-HT$_{2A}$ receptor, and only one phenylalkylamine was tested in these mutant rat 5-HT$_{2A}$ receptors. In this study, we wished to clarify discrepancies in the earlier work and extend the data to include more phenylalkylamines by mutating residues Ser5.43(239) and Ser5.46(242) to alanine in the human 5-HT$_{2A}$ receptor and assessing the effects on selected structurally modified tryptamine and phenylalkylamine derivatives.

The data from the present study support the hypothesis that Ser5.43(239) serves as the most critical residue engaged by substituted tryptamine and phenylalkylamine agonists in the process of receptor binding and activation. We find that most oxygen-substituted tryptamines interact with both Ser5.43(239) and Ser5.46(242), whereas unsubstituted tryptamines probably adopt alternate binding orientations. The potent hallucinogen LSD seems to engage only Ser5.46(242) directly, whereas the phenylalkylamines engage only Ser5.43(239).

Materials and Methods

Materials. [^{3}H]Ketanserin, [^{125}I]4-iodo-2,5-dimethoxyphenylisopropylamine ([^{125}I]DOI), and [*myo*-^{3}H]inositol were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). 5-HT, 5-methoxytryptamine (5-MeO-T), 5-methyltryptamine (5-Me-T), and tryptamine were obtained from Sigma-Aldrich (St. Louis, MO). 5-Methoxy-*N*(1)-isopropyltryptamine and 1-methylserotonin) were obtained from the National Institute of Mental Health Chemical Synthesis and Drug Supply Program (http://nimh-repository.rti.org). All other test ligands used in this study were synthesized in our laboratory using standard methods. The purity and identity of synthesized compounds were verified with thin layer chromatography, melting point, NMR, mass spectrometry, and elemental analysis. Structures of compounds used in this study and their abbreviations are shown in Fig. 1. Stock solutions of compounds were prepared as the following salts: 5-HT as the creatine sulfate salt; 5-MeO-DMT, 1-methylserotonin, and psilocin as the maleate salts; 5-methoxy-1-

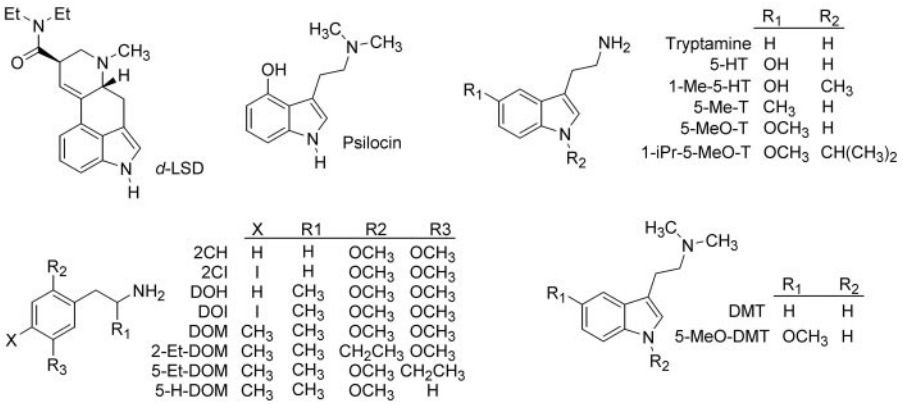

Fig. 1. Structures of compounds used in this study.

isopropyltryptamine as the oxalate salt; and all other compounds as their HCl salts. All phenylisopropylamines were prepared as their racemates.

Cell Culture Methods. The construction of human embryonic kidney 293 human embryonic kidney cells with stable expression of wild-type h5-HT_{2A} receptors at high ($Hh2A_{hi}$; 8000 fmol/mg) and moderate ($Hh2A_{lo}$; 1600 fmol/mg) expression levels has been described previously (Parrish et al., 2005; Braden et al., 2006). All cell types were maintained in complete Dulbecco's modified Eagle's medium with 30 μg/ml Zeocin (Invitrogen) as described previously (Parrish et al., 2005; Braden et al., 2006). The $Hh2A_{hi}$ cell line was used for competition binding assays and the $Hh2A_{lo}$ cell line was used for functional assays.

Establishing Mutant Human 5-HT_{2A} Receptor Cell Lines. Vector construction and site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA) method were performed as described previously (Braden et al., 2006) with the following sense primers and corresponding antisense primers (Integrated DNA Technologies, Coralville, IA): S5.43(239)A, CTTTGTCCTGATCGGCTCTTTTGTGTCATTTTTCATTCCC; S5.46(242)A, CCTGATCGGCTCTTTTGTGTCATTTTTCATTCCCTTAACC. Mutant inserts verified by primer-directed sequencing (Retrogen, San Diego, CA) were then subcloned into the pBudCE4 vector (Invitrogen, Carlsbad, CA). Human embryonic kidney 293 cells were transfected, colonies selected, and receptor expression verified as described previously (Parrish et al., 2005). The Hh2A/S5.43(239)A and Hh2A/S5.46(242)A cell lines were chosen for moderate expression (3800 and 3200 fmol/mg, respectively).

Radioligand Binding Assays. Membrane preparations, saturation isotherm, and competition binding assays were performed as described in detail previously (Chambers et al., 2002; Marona-Lewicka et al., 2002). Saturation isotherm binding assays used 0.25 to 10 nM [^{3}H]ketanserin or 0.125 to 5.0 nM (±)-[^{125}I]DOI. Ligands were tested in competition binding assays for their ability to displace 0.25 nM [^{125}I]DOI. Binding assays at wild-type receptors used the high-expressing cell line ($Hh2A_{hi}$; 8000 fmol/mg); those at mutant receptors used the cell lines described above.

Inositol phosphate accumulation assays. Compounds were tested for their ability to stimulate hydrolysis of radiolabeled phosphatidyl inositides (PI) by measurement of radiolabeled inositol phosphate accumulation as described previously in detail (Marona-Lewicka et al., 2002; Kurrasch-Orbaugh et al., 2003). Each assay plate was normalized to wells stimulated with water (0%) and a concentration of serotonin chosen to be maximally stimulating (100%). PI accumulation assays at wild-type receptors used the lower expressing cell line ($Hh2A_{lo}$; 1600 fmol/mg); assays at mutant receptors used the cell lines described above.

Computational Modeling/Virtual Docking. Ligand structures were virtually docked into an in silico-activated h5-HT_{2A}R homology model constructed from the published X-ray crystal structure of bovine rhodopsin after undergoing rigid-body molecular dynamics subsequent to isomerization of the bound 11-*cis*-retinal to all-*trans*-retinal (Chambers and Nichols, 2002). Local energy-minimized ensemble structures were obtained as described previously (Parrish et al., 2005; Braden et al., 2006). In brief, energy-minimized structures were virtually docked using the GOLD software package (Cambridge Crystallographic Data Center, Cambridge, UK). Docking algorithms for phenylalkylamines and ergolines were performed without constraints. Docking algorithms for tryptamines were performed with the following constraints: 1) a distance constraint of 1.5 to 3.5Å between the ligand amine (nonindole) nitrogen and the carbonyl carbon of the side chain of Asp3.32(155); 2) a protein hydrogen bond constraint for the side-chain oxygen of Ser5.46(242); and 3) a protein hydrogen bond constraint for the side-chain OH hydrogen of Ser5.43(239). The latter two constraints favorably weight the scoring of docking orientations that place any polar ligand atom in proximity to the protein atoms that could form a possible hydrogen bond without constraining a particular ligand atom. Ligand-receptor ensemble structures were each obtained by merging the highest ranked docking output ligand orientation structures with the input h5-HT_{2A} homology model structure using the SYBYL software package (Tripos, St. Louis, MO), followed by energy minimization, molecular dynamics, and a final energy minimization simulation. Aggregates for molecular dynamics and minimization simulations were defined as residues more than 6 Å from the ligand as well as the backbone atoms. The molecular dynamics and minimization simulations were performed with constraints between Asp3.32(155) and the ligand aliphatic amine, and between any polar residue within 2.5 Å of a polar ligand group, including Ser3.36(159), Thr3.37(160), Ser5.43(239), or Ser5.46(242). Final energy minimization simulations also used these constraints.

Data Analysis. Prism software (GraphPad Software Inc., San Diego, CA) was used to calculate nonlinear regression curves based on the Cheng-Prusoff equation for a one-site model to obtain K_i (affinity) values for radioligand displacement and variable slope sigmoidal dose-response curves for EC_{50} (potency) and intrinsic activity from PI hydrolysis. This software package was also used to perform two-way ANOVA calculations with Bonferroni post-tests comparing wild-type and mutant pK_i, pEC_{50}, and intrinsic activity values. Values obtained from the mutant receptors were considered statistically distinguishable from wild-type if the models generated $p < 0.05$. Change in the standard Gibbs free energy ($\Delta\Delta G°$) of binding due to the mutation was calculated from the K_i values at 25°C as follows: $\Delta(\Delta G°) = \Delta G°_{mutant} - \Delta G°_{wild\text{-}type} = RT \ln(K_{mutant}/k_{wild\text{-}type})$, where R is the gas constant, and T is absolute temperature. Changes in binding affinity and EC_{50} were transformed to normalize the scale by taking the difference of the $\log_{10}$ value (ΔpK_i and ΔEC_{50}, respectively) as follows: $\Delta pK_i = pK_{i\text{-}mutant} - pK_{i\text{-}WT} = -\log K_{i\text{-}mutant} - (-\log K_{i\text{-}WT})$ and $\Delta pEC_{50} = pEC_{50\text{-}mutant} - pEC_{50\text{-}WT} = -\log EC_{50\text{-}mutant} - (-\log EC_{50\text{-}WT})$. Changes in intrinsic activity (ΔInt.Act.) were calculated as follows: ΔInt.Act. = $Int.Act._{mutant} - Int.Act._{WT}$.

Virtual docking figures were generated using pyMol (DeLano Scientific, San Carlos, CA; http://www.pymol.org). Amino acid residues are numbered with their position relative to the most highly conserved residue of that transmembrane region and their sequence position in the h5-HT_{2A} receptor (Ballesteros and Weinstein, 1995).

Results

Human S5.43(239)A and S5.46(242)A Mutant Receptors Possessed Appropriate Affinity and Expression Levels. Table 1 presents the results from saturation binding experiments with membrane preparations of stable cell lines expressing wild-type and mutant h5-HT_{2A} receptors using a radiolabeled antagonist, [^{3}H]ketanserin, or agonist, [^{125}I]DOI. As Table 1 indicates, we were able to detect and characterize the mutant receptors with both radioligands, and all mutant receptors exhibited acceptable receptor expression. Note that cells with moderate WT h5-HT_{2A} receptors (1600 fmol/mg) were used in functional assays, whereas those with high expression (8800 fmol/mg) were used only for binding assays.

Virtual Docking of Tryptamines, Ergolines, and Phenylalkylamines to an in Silico-Activated h5-HT_{2A} Receptor Homology Model Indicated That Ser5.43(239) Interacted with Polar Substituents on the Aromatic Moiety of the Ligand, Whereas Ser5.46(242) Interacted with the Indole Nitrogen of Tryptamines and Ergolines, but Did Not Interact with Phenylalkylamines. Fig. 2 illustrates representative binding poses for virtual docking and subsequent energy minimization simulations of tryptamines, ergolines, and phenylalkylamines in an in silico-activated homology model of the h5-HT_{2A} receptor. Vir-

tual docking of LSD or the phenylalkylamines consistently oriented the ligand amine near Asp3.32(155) and the polar ligand substituents near polar residues in TM3 and TM5. Initial unconstrained docking orientations for tryptamines, however, were highly variable. Few of these orientations positioned the ligand so that the strong ionic interaction could occur between the protonated amine of the ligand and the highly conserved acidic residue at position 3.32, Asp3.32(155), believed to be the counter-ion in all biogenic amine-binding GPCRs (Ho et al., 1992; Mansour et al., 1992; Kristiansen et al., 2000; Abekawa et al., 2003). Applying a distance constraint between the side-chain nitrogen of the ligand and the carbonyl carbon of the side chain of Asp3.32(155) gave more reasonable and less variable highest-ranked output structures from docking simulations, although many structures were still oriented out of the putative small agonist binding pocket. Thus, additional docking constraints for tryptamines were introduced to encourage any hydrogen bonding between the ligand and either Ser5.43(239) and Ser5.46(242) but without forcing a particular receptor-ligand interaction.

We observed final low-energy binding poses for ligands that position their polar aromatic substituents in regions and orientations able to interact with h5-HT$_{2A}$ receptor residues identified to be potentially important for agonist binding and activity (Chambers and Nichols, 2002). Of particular relevance for this study, the 4- or 5-oxygen atom of ring-substituted tryptamines is positioned to engage in hydrogen bonding with Ser5.43(239). Moreover, virtual docking simulations are consistent with an interaction between Ser5.46(242) and the indole nitrogen of the tryptamines, as suggested by others (Kao et al., 1992; Johnson et al., 1994). As Fig. 2 further illustrates, LSD, an ergoline that has structural features similar to those of the tryptamines, is also oriented to allow an interaction between the indole nitrogen and Ser5.46(242); however, there are no polar groups in LSD positioned to interact with Ser5.43(239). The 2-methoxy and 5-methoxy groups of phenylalkylamines are positioned to indicate hydrogen bonding with Ser3.36(159) and Ser5.43(239), respectively. Virtual docking simulations, however, indicate no interaction between Ser5.46(242) and phenylalkylamines.

The S5.43(239)A Mutation Detrimentally Affected the Binding and Activity of Tryptamines and Phenylalkylamines Predicted to Interact with This Residue. Table 2 and Fig. 3 present the results of competition binding assays with wild-type, S5.43(239)A mutant, and S5.46(242)A mutant h5-HT$_{2A}$ receptors. To aid visual interpretation, a loss in binding affinity is defined as a negative $\Delta(\Delta G°)$. Binding of tryptamines with a polar aromatic substituent, such as 5-MeO-T, 5-HT, 5-MeO-DMT, psilocin, and 1-Me-5-HT, was attenuated by the S5.43(239)A mutation to a degree consistent with the loss of a hydrogen bond (0.5 to 1.5 kcal/mol; Fersht, 1988), although 1-iPr-5-MeO-T was surprisingly unaffected. Both 5-HT and 5-MeO-DMT suffered the greatest

TABLE 1

[^{3}H]-Ketanserin and [^{125}I]DOI saturation binding at wild type and mutant receptors

Data are represented as the mean ± S.E.M. of K_d and B_{max} from nonlinear regression fits of a single binding site model from at least three independent experiments. Cell lines used are from isolated colonies of human embryonic kidney 293 cells with stable receptor expression.

Drug	h5-HT$_{2A}$			
	WT (moderate)	WT (high)	S5.43(239)A	S5.46(242)A
Ketanserin				
K_d (nM)	0.7 ± 0.1	1.1 ± 0.1	2.2 ± 0.2	0.7 ± 0.1
B_{max} (fmol/mg)	1620 ± 80	8800 ± 860	3790 ± 560	3215 ± 530
DOI				
K_d (nM)	N.D.	0.78 ± 0.01	2.15 ± 0.31	1.74 ± 0.17
B_{max} (fmol/mg)	N.D.	625 ± 110	519 ± 65	300 ± 25

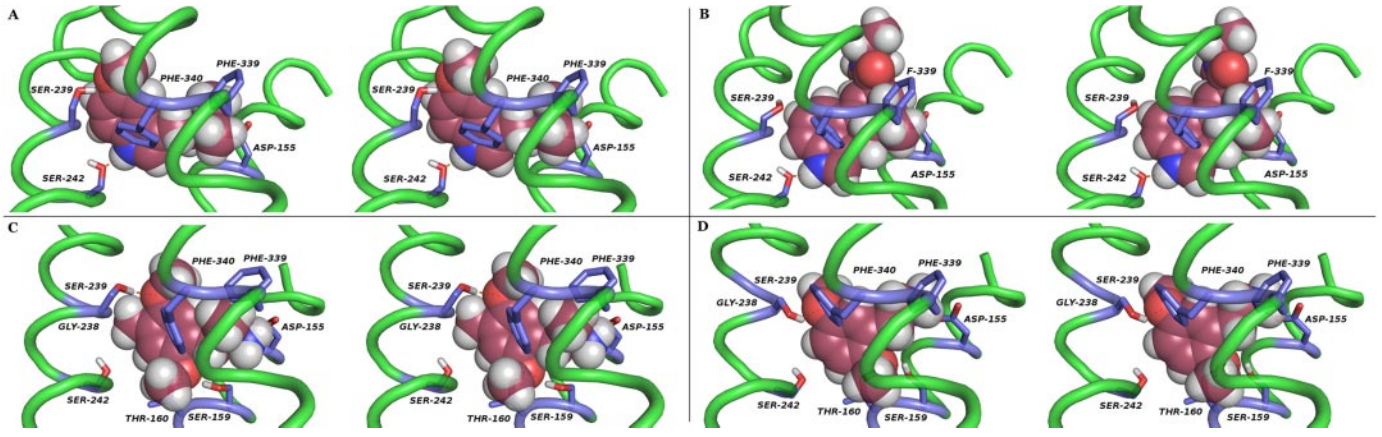

Fig. 2. Illustrative cross-eyed stereopair representation of ligand poses from virtual docking experiments with 5-MeO-DMT (A), *d*-LSD (B), (*R*)-DOM (C), and 2CH (D) in the h5-HT$_{2A}$ receptor showing predicted polar interactions between the ligand and receptor residues. Ligands are shown as space-filling spheres, whereas receptor residues believed to be interacting with the ligand are displayed as sticks. The view is within the membrane, with TM5 on the left, TM6 in the foreground, TM3 in the right background, and the extracellular face of the receptor toward the top of the figure. TMs 1, 2, 4, and 7 are not displayed. Energy-minimized ensemble structures indicate that Ser5.46(242) is interacting with the indole nitrogen of LSD and the tryptamines and Ser5.43(239) is interacting with the 4- and 5-oxygen of tryptamines. Only Ser5.43(239) is able to interact with the 5-oxygen atom of DOM or 2CH.

losses of affinity, approximately 14- and 11-fold (~1.5 kcal/mol), respectively. The affinities of the phenylisopropylamines DOI, DOM, and DOH were detrimentally affected by the S5.43(239)A mutation, with $\Delta\Delta G°$ well within the range given above for hydrogen bonds. The phenethylamines 2CI and 2CH also were detrimentally affected, but the $\Delta\Delta G°$ values were less than 0.5 kcal/mol, and not statistically discernible from wild-type.

Table 3 and Fig. 4 present measures of functional activity of the test compounds using PI hydrolysis assays at wild-type and mutant receptors. In general, the trends observed in affinity changes for polar-substituted tryptamines were maintained in shifts of functional potency, as defined by ΔpEC_{50}. All compounds were affected by this mutation to a certain degree, although in some cases the effect was very slight (e.g., 5-MeO-T). Again, 5-MeO-DMT and 5-HT were the polar-substituted tryptamines most affected, their potencies being reduced by approximately 20- and 35-fold, respectively. Psilocin was the only tryptamine to show a statistically discernible change in intrinsic activity (Table 3; ~33% compared with the wild type) as well as a ~30-fold decrease in potency.

By contrast, LSD lacks a polar substituent in the area indicated by virtual docking experiments to be near Ser5.43(239) and, not surprisingly, this mutation had no effect on its affinity and only a weak effect on its potency. Likewise, tryptamine and DMT also lack a polar substituent in this region; their binding was relatively unaffected and potency was only weakly affected. Although affinity of 5-Me-T also was unaffected, we were surprised to observe a dramatic 65-fold decrease in potency.

All phenylalkylamines tested showed significant decreases in potency (ΔpEC_{50}) at the S5.43(239)A mutant receptor compared with wild type. DOM and DOH were the most markedly affected ligands, with decreases of ~60- and ~220-fold, respectively. Of all the phenylalkylamines tested at all the mutant receptors in this study, we observed a decrease in efficacy (ΔInt.Act. $\approx -35\%$) only with 2CI at the S5.43(239)A receptor.

The S5.46(242)A Mutation in the h5-Ht$_{2A}$ Receptor Attenuated the Affinity and Activity of Some Tryptamines Predicted to Interact with This Residue, but Enhanced the Binding and Activity of Tryptamines with Alkyl Substitution on the Indole (N1) Nitrogen and Did Not Affect Phenylalkylamines. As Fig. 3 illustrates, the S5.46(242)A mutation attenuated the affinity only of LSD, 5-HT, and 5-MeO-DMT to a degree consistent with the loss of a hydrogen bond (Fersht, 1988). Changes to the binding energetics of psilocin and 5-MeO-T approach the lower limit of this energy range (0.5–1.5 kcal/mol). The affinity of other tryptamines, particularly those lacking a polar ring substituent, was not significantly affected by the S5.46(242)A mutation. By contrast, the tryptamines with alkyl substitution on the indole (N1) nitrogen, 1-Me-5-HT and 1-iPr-5-MeO-T, showed 12- and 21-fold increased affinity, respectively.

Similar trends were generally observed in functional potency, as illustrated in Fig. 4. With the exception of 5-MeO-T, the potency of all the tryptamines with polar aromatic substituents, as well as LSD, was weakly to moderately decreased. No shifts in potency were observed in any of the ring-unsubstituted tryptamines 5-Me-T, tryptamine, or DMT. Again, we observed dramatic 45- and 51-fold increases in potency for the N(1)-alkyl analogs 1-Me-5-HT and 1-iPr-5-MeO-T, respectively.

The S5.46(242)A mutation did not affect the binding of any of the phenylalkylamines tested to a degree statistically discernible from wild type. At the S5.46(242)A mutant receptor, DOM, DOH, and 2CH suffered generally weak losses in potency (2-, 6-, and 5-fold, respectively), whereas the potency of DOI was slightly increased (~3-fold). This result is consistent with our observation that there is no significant difference between the affinity of phenethylamine agonists at the human and rat 5-HT$_{2A}$ receptors, which differ only in that the human receptor has a serine at this position, whereas the rat receptor has an alanine. The S5.46(242)A mutation did not lead to a significant change in efficacy for any of the phenylalkylamines tested.

TABLE 2

Abilities of test compounds to displace (±)-[^{125}I]DOI at wild-type and mutant h5-HT$_{2A}$ receptors

Data are represented as the mean (S.E.M.) of K_i values from non-linear regression fits of a single binding site model from at least three independent experiments.

Drug	K_i		
	WT	S5.43(239)A	S5.46(242)A
		nM	
5-MeO-DMT	7.54 (1.06)	105 (19)*	36.0 (1.9)*
5-HT	4.84 (0.20)	53.2 (8.0)*	20.2 (0.9)*
5-MeO-T	1.34 (0.22)	4.17 (0.68)*	2.70 (0.29)
Psilocin	11.8 (1.2)	58.0 (3.7)*	23.1 (3.3)
5-Me-T	11.7 (0.6)	10.9 (1.3)	25.8 (3.9)
Tryptamine	29.7 (4.4)	50.2 (6.6)	36.0 (6.0)
DMT	75.1 (6.0)	116 (12)	135 (20)
1-Me-5-HT	70.0 (1.2)	320 (37)*	5.73 (0.83)*
1-iPr-5-MeO-T	494 (91)	786 (85)	24.1 (0.8)*
LSD	0.40 (0.02)	0.41 (0.08)	1.66 (0.32)*
DOI	0.64 (0.06)	2.19 (0.26)*	0.83 (0.11)
DOM	5.91 (0.97)	46.4 (5.9)*	5.04 (0.55)
2CI	0.73 (0.06)	1.20 (0.22)	1.28 (1.28)
2-Et-DOM	91.1 (9.7)	460 (92)*	38.5 (4.1)*
5-Et-DOM	22.4 (1.2)	32.2 (1.9)	31.5 (5.0)
DOH	245 (28)	2246 (144)*	294 (54)
5-H-DOM	169 (12)	343 (41)	164 (7)
2CH	377 (67)	782 (19)	440 (23)

* $P < 0.05$ for values of Δ pK_i from ANOVA with Bonferroni post tests comparing mutant and wild-type values

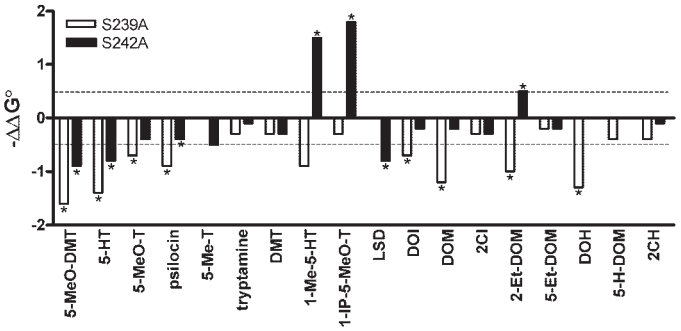

Fig. 3. Effects on binding affinities of the S5.43(239)A (open bars) and S5.46(242)A (closed bars) mutations in the h5-HT$_{2A}$ receptor. This bar graph displays the $-\Delta\Delta G°$ values derived from the data of Table 2 (see *Materials and Methods*). Negative values in these graphs indicate an adverse effect of the mutation on affinity. Positive values indicate that binding is enhanced by the mutation. The dashed line at 0.5 indicates a lower threshold for the energetics of a hydrogen bond (Fersht, 1988). * indicates $p < 0.05$ for values of $\Delta G°$ from two-way ANOVA with Bonferroni post tests comparing mutant to wild-type values.

A Complementary Alteration of Phenylalkylamine Structure Could Produce Binding and Activity Changes at the WT h5-HT$_{2A}$ Receptor Similar to Those of the Unmodified Ligand at the S5.43(239)A Mutation and Could Rescue Some Binding and Activity at the S5.43(239)A Mutant. As Table 4 indicates, removal of a polar group from the aromatic ring of the phenylisopropylamine DOM to yield 2-Et-DOM, 5-Et-DOM, or 5-H-DOM gave changes in affinities at the wild-type receptor consistent with the energetics of a lost hydrogen bond (Fersht, 1988). These shifts are similar in magnitude to those observed when comparing the polar-substituted ligand homologues in wild-type versus S5.43(239)A mutant receptors [e.g., the effect of the S5.43(239)A mutation on DOI or DOM in Fig. 3]. Removal of the polar group at the 5-position of DOM to give 5-Et-DOM did not detrimentally affect binding at the S5.43(239)A mutant receptor. This mutant was still sensitive to the complete removal of the 5-methoxy to give 5-H-DOM ($-\Delta\Delta G° = 1.2$). It was not, however, as sensitive as the wild-type receptor ($-\Delta\Delta G° = 2.0$).

Table 4 also illustrates the difference in functional potency between wild-type and S5.43(239)A mutant receptors when a polar group is removed from phenylisopropylamines. Similar to the binding energies, the functional potencies at h5-HT$_{2A}$ wild-type receptors are sensitive to the removal of a polar group either at the 2- or 5-position of phenylalkylamines. By contrast, the S5.43(239)A mutant receptor is generally less sensitive or even insensitive to the loss of a polar group, but only at the ligand 5-position. This insensitivity is clearly evident in the comparison of DOM and 5-Et-DOM. Although we did not expect 2-Et-DOM and DOM to have significantly different potencies in the S5.43(239)A mutant receptor, the loss of sensitivity to substituting the 2-oxygen (2-Et-DOM; $\Delta pEC_{50} = 1.2$ versus 0.7) is not as great as seen with either substitution (5-Et-DOM; $\Delta pEC_{50} = 1.4$ versus 0.0), or removal (5-H-DOM $\Delta pEC_{50} = 2.3$ versus 0.6) of the 5-oxygen substituent.

Discussion

The goal of the current study was to test the hypothesis that Ser5.43(239) in the h5-HT$_{2A}$ receptor is critical for engaging a polar 5-oxygen of phenylalkylamines and a 4- or 5-oxygen substituent of tryptamines, including serotonin, the endogenous agonist ligand for this receptor. Moreover, a parallel hypothesis was that Ser5.46(242) interacts with the N(1)H of the indole ring in tryptamines, yet does not engage phenylalkylamines. With these experiments, we sought to replicate, expand, and more clearly elucidate results from previous studies mutating these residues in the rat and human 5-HT$_{2A}$ receptors (Kao et al., 1992; Johnson et al., 1993, 1994, 1997; Shapiro et al., 2000).

A nondisruptive mutation to an alanine was chosen for each residue because it was anticipated that this change would abolish specific ligand-receptor interactions without affecting global receptor structure (Fersht et al., 1987). Indeed, as Table 1 indicates, only a 2-fold shift in K_D was observed for [^{3}H]ketanserin at S5.43(239)A receptors (1.1 versus 2.2 nM) and a slight enhancement at S5.46(242)A receptors (1.1 versus 0.71 nM). A shift of ~3-fold in the K_D for [^{125}I]DOI was observed at S5.43(239)A receptors (0.78 versus 2.19 nM) and approximately 2-fold at S5.46(242)A receptors (0.78 versus 1.7 nM). The mutant S5.43(239)A and S5.46(242)A receptors still were able to induce the release of radiolabeled inositol phosphates in a dose-dependent manner that was approximately comparable in magnitude with that of the wild-type receptor (data not shown), although functional potencies were slightly decreased for all chemical classes tested at the S5.43(239)A receptor. Although these findings indicate that the S5.43(239)A mutation may have slightly altered G-protein

TABLE 3

Ability of compounds to activate PI hydrolysis at wild type and mutant h5-HT$_{2A}$ receptors

Data are represented as the mean (SEM) of computer-derived estimates of EC_{50} and Intrinsic Activity values from at least three independent experiments. Except where indicated (†), all EC50 values were statistically distinguishable from wild-type as defined by $P < 0.05$ values from ANOVA calculations with Bonferroni post-tests between mutant and wild-type receptors, whereas * indicates $P < 0.05$ values for Δ Int.Act. values using the same statistical comparison.

Drug	WT h5-HT$_{2A}$		S5.43(239)A		S5.46(242)A	
	EC_{50} PI Hydrolysis	Intrinsic Activity	EC_{50} PI Hydrolysis	Intrinsic Activity	EC_{50} PI Hydrolysis	Intrinsic Activity
	nM	*% 5-HT*	*nM*	*% 5-HT*	*nM*	*% 5-HT*
5-MeO-DMT	4.3 (0.78)	98 (4)	150 (25)	88 (7)	31 (4.5)	91 (5)
5-HT	5.2 (0.97)	100	100 (8)	100	19 (3.4)	100
5-MeO-T	5.2 (0.48)	99 (4)	26 (1.2)	100 (7)	5.0 (0.45)†	100 (6)
Psilocin	7.3 (0.72)	110 (9)	210 (25)	72 (8)*	45 (8.4)	92 (3)
5-Me-T	18 (3.4)	110 (8)	1200 (184)	99 (2)	21 (1.0)†	90 (3)
Tryptamine	94 (18)	100 (0)	230 (41)†	97 (3)	95 (17)†	96 (8)
DMT	190 (6)	70 (1)	790 (73)†	91 (3)	200 (20)†	95 (4)
1-Me-5-HT	310 (30)	100 (3)	1800 (200)	101 (3)	6.9 (1.1)	95 (4)
1-iPr-5-MeO-T	2400 (180)	100 (1)	7900 (1400)†	83 (8)	48 (6.7)	97 (3)
LSD	0.22 (0.04)	84 (3)	0.66 (0.10)	80 (5)	1.10 (0.12)	86 (2)
DOI	3.79 (0.36)	95 (7)	38.9 (8.9)	92 (12)	1.37 (0.06)	92 (2)
DOM	2.81 (0.22)	87 (3)	620 (103)	87 (3)	6.46 (1.23)	91 (2)
2CI	4.81 (0.89)	87 (7)	71.8 (13.6)	52 (5)*	5.93 (0.58)†	86 (3)
2-Et-DOM	49.8 (7.0)	81 (2)	2975 (546)	75 (6)	22.6 (2.8)	93 (2)
5-Et-DOM	71.9 (10.8)	84 (10)	571 (78)	81 (6)	62.0 (10.8)†	89 (7)
DOH	284 (22)	104 (5)	17669 (2290)	95 (6)	1793 (298)	101 (7)
5-H-DOM	533 (64)	106 (5)	2532 (108)	98 (1)	245 (40)	98 (1)
2CH	1021 (14)	96 (10)	13053 (416)	100 (4)	4917 (346)	92 (5)

* Int. Act. $P < 0.05$.
† EC50 $P > 0.05$.

coupling, we do not believe it produces a significant global disruption of receptor structure. Thus, our initial hypothesis was that any differences in ligand affinity or activity between the wild-type and S5.43(239)A or S5.46(242)A mutant receptors would mainly result from a loss of direct interaction between the ligand and those serine residues.

The clearest results were obtained with the potent hallucinogen LSD, which was predicted not to interact with Ser5.43(239). The S5.43(239)A mutation had no effect at all on affinity or intrinsic activity and only a minor 3-fold effect on potency (Figs. 3 and 4). By contrast, the S5.46(242)A mutation caused a 4-fold shift in affinity (Table 2), resulting in a change of the standard free energy of binding ($-\Delta\Delta G°$) of -0.8 kcal/mol (Fig. 4), within the -0.5 to -1.5 kcal/mol range determined for a hydrogen bond (Fersht, 1988). This mutation similarly caused a shift of approximately 6-fold in potency for LSD, although it had no effect on intrinsic activity (Fig. 4). Our previous virtual docking studies (Chambers and Nichols 2002) placed LSD into the agonist binding domain such that there was no interaction with Ser5.43(239), whereas the indole N(1)H engaged Ser5.46(242), located approximately one turn below Ser5.43(239). The present data are consistent with this predicted binding pose. The fact that LSD is such a rigid molecule indicates that the bound ligand-ensemble has little conformational freedom to involve Ser5.43(239) in binding. The slight loss of potency in the S5.43(239)A mutant does suggest, however, that even when a ligand does not directly engage this residue, it may still be important for secondary intramolecular interactions that create an "efficient" activated receptor state.

A second compound where there was no difference in affinity between the wild-type and mutant receptors is 5-Me-T, an example of a "mutated" ligand chosen to mimic the loss of interaction between Ser5.43(239) and the tryptamine 5-position oxygen. We initially envisioned that addition of the 5-methyl group to tryptamine might allow a favorable van der Waals interaction between the methyl group and A5.43(239). Indeed, although there is no difference in affinity of 5-Me-T in the wild-type and S5.43(239)A receptors (Fig. 3), this ligand does have higher affinity at the S5.43(239)A receptor compared with both 5-HT and tryptamine (Table 2).

We were surprised to find that affinity and potency at the S5.46(242)A mutant receptor indicate that 5-Me-T does not engage this residue (Figs. 3 and 4). This finding may indicate that this ligand does not bind in an orientation similar to other tryptamines. Further confounding interpretation is the fact that although this ligand has the same intrinsic activity at the wild-type and either of the two mutant receptors, there is a loss of potency in the S5.43(239)A mutant of more than 100-fold (Fig. 4). As was the case for LSD, we believe this result suggests that there may be additional indirect effects of the S5.43(239)A mutation that affect the ability of the ligand-receptor ensemble to adopt or maintain an "activated" state.

The use of "mutated" ligands and shifts in sensitivity indicated by Table 4 yields the most compelling evidence for the interaction of Ser5.43(239) with the phenylalkylamines, particularly the phenylisopropylamine subclass. At wild-type receptors, the data provide strong support for the necessity of the polar oxygen at the ligand 5-position, reflected in the sensitivity to its removal from DOM in the 5-ethyl or 5-hydrogen homologues 5-Et-DOM and 5-H-DOM, respectively. Moreover, the binding energetics and functional potencies at the S5.43(239)A mutant receptor indicate insensitivity to the absence of a 5-oxygen atom in 5-Et-DOM, the most direct 5-desoxy homolog of DOM. This mutant receptor is not completely insensitive to this change, as seen with 5-H-DOM, and also has reduced sensitivity to 2-Et-DOM. The magnitude of sensitivity loss is greatest, however, with the 5-position homologues. It should not be surprising that 5-H-DOM has lower affinity and potency than DOM in the S5.43(239)A mutant receptor, in that there are likely entropic and/or solvation factors with the removal of so many ligand atoms. A diagram of this interaction is provided in Fig. 5.

The S5.43(239)A and S5.46(242)A mutations had the most dramatic effects on the oxygen-substituted tryptamines 5-HT, 5-MeO-DMT, and psilocin. Although the binding energetics of psilocin were not as strongly affected, as illustrated in Fig. 3, its functional potency was attenuated to a degree similar to the other ring-substituted tryptamines; psilocin was one of the few compounds where we observed a dramatic loss of intrinsic ac-

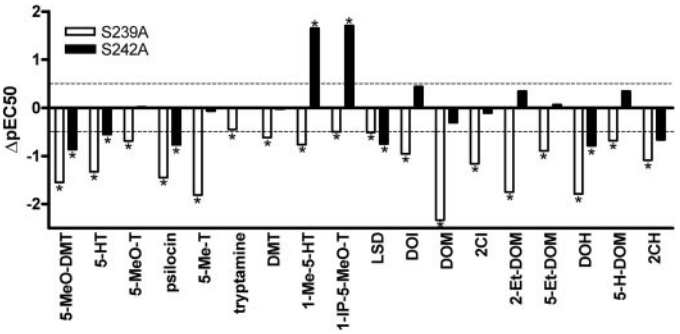

Fig. 4. Effects on EC_{50} PI hydrolysis functional activity by the S5.43(239)A (open bars) and S5.46(242)A (closed bars) mutations in the h5-HT_{2A} receptor. These bar graphs display the ΔpEC_{50} values derived from the data of Table 3 (see *Materials and Methods*). Positive ΔpEC_{50} values indicate a beneficial effect of the mutation on functional potency, whereas a negative value indicates a detrimental effect due to the mutation. The dashed line at 0.5 indicates an arbitrary delimiter for "weak" effects. * indicates $p < 0.05$ for values of ΔpEC_{50} from two-way ANOVA with Bonferroni post tests comparing mutant and wild-type values.

TABLE 4

Effects of change in ligand structure on binding affinity and functional potency at wild-type and S5.43(239)A mutant h5-HT_{2A} receptors

Data are represented as $-\Delta\Delta G°$ or ΔpEC_{50} values based on data from Tables 1 and 2. Negative values indicate a loss in binding affinity or functional potency as a result of the change in ligand structure. Values near 0 indicate the receptor is insensitive to the change of ligand structure being compared. Except where indicated (†), all values for $-\Delta\Delta G°$ or ΔpEC_{50} generated $P < 0.05$ from unpaired two-tailed Student's t tests between ligands at the particular receptor compared (column heading).

Drug	$-\Delta\Delta G°$		ΔpEC_{50}	
	WT h5-HT_{2A}	S5.43(239)A	WT h5-HT_{2A}	S5.43(239)A
DOM vs. 2-Et-DOM	−1.6	−1.4	−1.2	−0.7
DOM vs. 5-Et-DOM	−0.8	0.2†	−1.4	0.0†
DOM vs. 5-H-DOM	−2.0	−1.2	−2.3	−0.6

† $P > 0.05$

tivity, as indicated in Table 3. Furthermore, with the exception of 5-MeO-T, the results support the hypothesized interaction of polar-substituted tryptamines with residue Ser5.43(239). These conclusions are consistent with the previous observation that 5-MeO-T may not be interacting with Ser5.43(239) in the rat 5-HT$_{2A}$ receptor (Johnson et al., 1997) but are in contrast to the conclusion that Ser5.43(239) is either not accessible in the binding site or is interacting with the indole nitrogen of tryptamines (Shapiro et al., 2000).

As further illustrated in Fig. 3, the classic phenylalkylamines affected by the S5.43(239)A mutation were limited to the phenylisopropylamine subclass (i.e., DOI, DOM, and DOH), whereas the S5.46(242)A mutation had no effect on the binding of all but one of the phenylalkylamines tested. This trend is maintained in the functional data, as illustrated in Fig. 4.

Compared with phenethylamines, the phenylisopropylamines DOI and DOM have additional steric bulk on the α carbon that can alter their conformation. We hypothesized that the S5.43(239)A mutation would eliminate the favorable interaction with the 5-methoxy of these ligands. Not surprisingly, we observed a 3-fold and nearly 8-fold loss of affinity at the mutant receptor for DOI and DOM, respectively, compared with the wild type (Table 2). This difference can be related to the nature of the nonpolar 4-substituent, which is known to affect both affinity and potency (Nichols and Glennon, 1984). That is, DOI has nearly 10-fold higher affinity than DOM in the wild-type receptor. Thus, the 4-iodo substituent adds binding energy, probably through a Van der Waals interaction, so that the affinity of DOI is less affected by the S5.43(239)A mutation than DOM. A parallel to this reasoning in seen in the comparison of the potencies of these two ligands, as illustrated in Fig. 4. The potency of DOM in the S5.43(239)A mutant receptor is reduced more than 200-fold, whereas the potency of DOI is reduced only approximately 10-fold.

The phenethylamines 2CI and 2CH also were predicted to interact with Ser5.43(239), yet the changes in energetics of binding are not consistent with the range of energy for a hydrogen bond (Fig. 3) (Fersht, 1988). One observes, however, that even though an interaction with the phenethylamines 2CI and 2CH is not apparent in the affinity assays, a moderate effect on the functional potency of these compounds is observed at the S5.43(239)A receptor, surpassing that of DOI. In addition, 2CI was the only phenylalkylamine tested that had significant and dramatically decreased intrinsic activity in the S5.43(239)A mutant (Table 3). In general, and as hypothesized, it seems that Ser5.43(239) is a key interaction site for most phenylalkylamines, whereas Ser5.46(242) does not interact with phenylalkylamine ligands in a manner that affects binding or potency at the h5-HT$_{2A}$ receptor.

The interaction of all the tryptamines with residue Ser5.46(242) is not so clearly evident. Indeed, it would seem that only oxygen-substituted tryptamines are detrimentally affected to a degree that would suggest loss of a hydrogen bond (Figs. 3 and 4). It seems possible that the strong polar interaction with Ser5.43(239) serves to orient the ligand, placing the indole N(1)H in proximity to Ser5.46(242), where a much weaker hydrogen bond is formed. In the absence of a polar ring substituent, the indole N(1)H interaction with Ser5.46(242) may be too weak, forcing those tryptamines to adopt a different binding orientation. Consistent with that argument, the ring-unsubstituted tryptamine and DMT, as well as the 5-methyl substituted 5-Me-T, were unaffected by the S5.46(242)A mutation. Moreover, the two polar substituted tryptamines with N(1)-alkylation, 1-Me-5-HT and 1-iPr-5-MeO-T, had dramatic increases in affinity and potency at the S5.46(242)A receptor compared with wild-type (Figs. 3 and 4). Additional support for an interaction between the tryptamine N(1)-substituent and either Ser5.46(242) or Ala5.46(242) in human or rat 5-HT$_{2A}$ receptors, respectively, was provided by the studies of Johnson et al. (1994).

Another earlier investigation developed de novo models of the rat 5-HT$_{2A}$ receptor, where both Ser5.43(239) and Ala5.46(242) were inaccessible to binding and were instead projected toward TM4, or had Ser5.43(239) engaged with the tryptamine indole N(1) hydrogen (Shapiro et al., 2000). Our

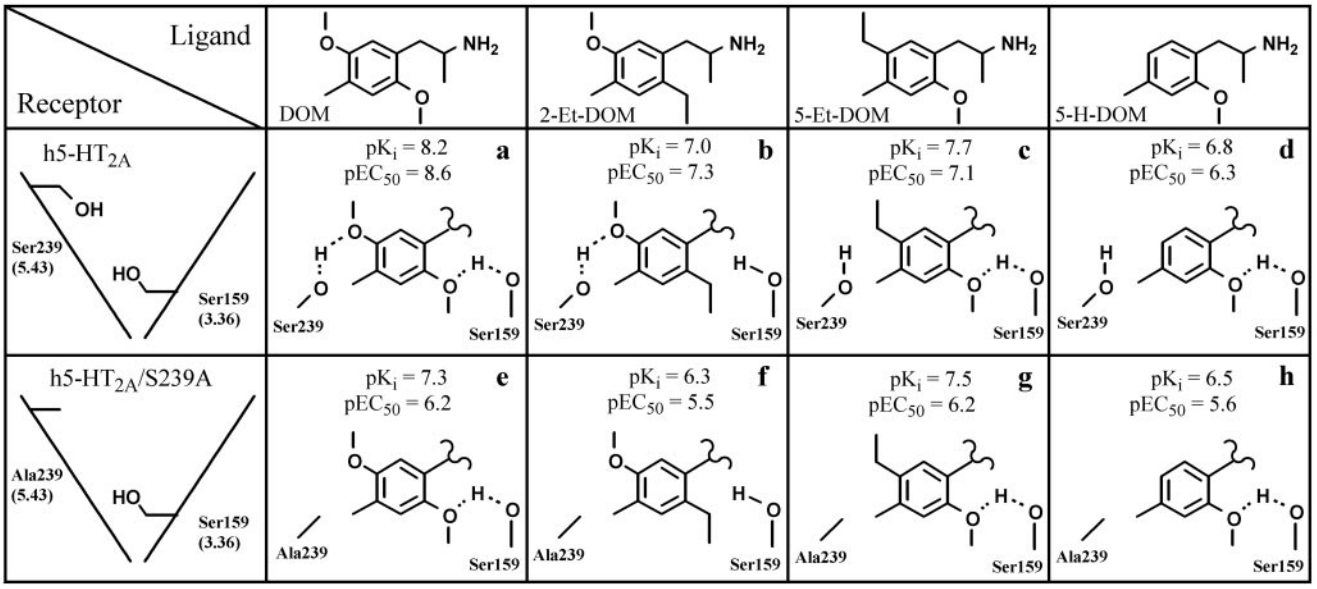

Fig. 5. Proposed interactions of phenethylamine ligands with receptor residues are illustrated by a cartoon structure for each panel. Wild-type receptors are sensitive to the removal of both the 2- and 5-oxygens of phenylisopropylamines (a → b/c/d), whereas the S5.43(239)A mutant receptor is insensitive to the removal of the 5-oxygen of phenylisopropylamines (e → g/h).

data are not consistent with such an orientation of TM5 in the human 5-HT_{2A} receptor, although we agree with the conclusion of that study that not all tryptamines may be binding in the same orientation. Instead, we believe our data support the hypothesis that Ser5.43(239) is directly engaged when binding tryptamines with polar substituents at either the 4- or 5-position. Although the ergoline LSD lacks a polar substituent to interact with Ser5.43(239), it has other structural features that may orient it within the binding site, so that it can engage Ser5.46(242). Even when a direct ligand-receptor interaction cannot occur, our data suggest that residue Ser5.43(239) may be involved in additional indirect interactions essential for potency at the 5-HT_{2A} receptor. These indirect interactions are difficult to model and indicate that our model may not be fully accounting for all possible "active" receptor conformations. Moreover, it may be possible, as Strader et al. (1989) suggested with the β-adrenergic receptor, that some ligands are able to bind to the mutant receptors in orientations that are competitive but not able to affect receptor activation.

Polar residues have been shown to be critical for agonist activity in a variety of monoamine GPCRs, as well as muscarinic acetylcholine receptors; representative TM5 sequences are illustrated in Fig. 6. In particular, in catecholamine receptors, the serine(s) at position 5.43 (and/or Ser5.42) probably engages the "meta" OH of the catechol moiety, a key structural feature of catecholamine agonists recognized for many years (e.g., Strader et al., 1989).

Our results also are consistent with the role of Ser5.43(239) as a hydrogen bond donor for both phenylalkylamines and tryptamines, whereas it seems more likely that Ser5.46(242) serves as a hydrogen bond acceptor for tryptamines and ergolines. This idea is reinforced by the presence of an alanine at position 5.46 in the human 5-HT_{2B}, 5-HT_{2C}, and 5-HT_{1A}, as well as the rat 5-HT_{2A} receptors. Furthermore, our modeling of the h5-HT_{2A} receptor suggests that Ser5.46(242) may form an intrahelical hydrogen bond to the backbone carbonyl of G5.42(238). From a mechanical point of view, if Pro5.50(246) serves to allow the top of TM5 to undergo movement upon ligand binding, engaging a polar residue more distal from Pro5.50(246) would provide greater mechanical force to displace the helix, relative to a residue closer to Pro5.50(246). The global change helical tilt angle indicative of receptor-activation may be related to the loss of a kink near Pro5.50(246) in helix 5 (Crozier et al., 2007).

Our data also indicate that not all tryptamines interact with the receptor in the same way, a conclusion reached by previous studies based on interactions with residue Ser5.43(239) in the rat 5-HT_{2A} receptor (Johnson et al., 1997; Shapiro et al., 2000). The present results extend previous investigations into the role of these residues in the h5-HT_{2A} receptor. As with investigations by our and other laboratories into the aromatic contacts of TM6 (Choudhary et al., 1993, 1995; Roth et al., 1997; Braden et al., 2006), it seems that the functional topography of the receptor binding site is such that a contact with TM5 is necessary for high affinity and functional potency of agonists. These results further support the topography and utility of our in silico-activated homology model of the h5-HT_{2A} receptor, and we anticipate that the present findings can be extended to the understanding of other family A monoamine-binding GPCRs.

	5.43	5.46	5.50
5-HT2A	FVLIG	**S**	FV**S**FFI**P**L
5-HT2B	FMLFG	**S**	LAAFFT**P**L
5-HT2C	FVLIG	**S**	FVAFFI**P**L
5-HT1A	YTIY	**ST**	FGAFYI**P**L
D1	YAIS	**S**	SVISFYI**P**V
D5	YAIS	**S**	SLISFYI**P**V
D2	FVVY	**S**	SIVSFYV**P**F
D3	FVIY	**S**	SVVSFYL**P**F
D4	YVVY	**S**	SVCSFFL**P**C
β1	YAIA	**S**	SVVSFYV**P**L
β2	YAIA	**S**	SIVSFYV**P**L
α2A	YVIS	**S**	CIGSFFA**P**C
AChM1	ITFG	**T**	AMAAFYL**P**V

Fig. 6. Selected examples of sequence homology in the ligand-specific portion of transmembrane helix 5 for several type A family GPCRs.

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