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Molecular Interaction of Serotonin 5-HT$_{2A}$ Receptor Residues Phe339$^{(6.51)}$ and Phe340$^{(6.52)}$ with Superpotent *N*-Benzyl Phenethylamine Agonists[S]

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MOLECULAR PHARMACOLOGY

ABSTRACT

Experiments were conducted to examine the molecular basis for the high affinity and potency of a new class of 5-HT$_{2A}$ receptor agonists, *N*-benzyl phenethylamines. Competition binding assays at several serotonin receptors confirmed that an *N*-arylmethyl substitution was necessary for affinity increases up to 300-fold over simple *N*-alkyl homologs, as well as enhanced selectivity for 5-HT$_{2A}$ versus 5-HT$_{2C}$ and 5-HT$_{1A}$ receptors. PI hydrolysis functional assays confirmed that these *N*-benzyl phenethylamines are potent and highly efficacious agonists at the rat 5-HT$_{2A}$ receptor. Virtual docking of these compounds into a human 5-HT$_{2A}$ receptor homology model indicated that the *N*-benzyl moiety might be interacting with Phe339$^{(6.51)}$, whereas the phenethylamine portion was likely to be interacting with Phe340$^{(6.52)}$. Experiments in h5-HT$_{2A}$ receptors with Phe339$^{(6.51)}$L and Phe340$^{(6.52)}$L mutations seem to support this hypothesis. Dramatic detrimental effects on affinity, potency, and intrinsic activity were observed with the Phe339$^{(6.51)}$L mutation for all *N*-benzyl analogs, whereas most *N*-unsubstituted phenethylamines and traditional agonists were only weakly affected, if at all. Consistent with other published studies, the Phe340$^{(6.52)}$L mutation detrimentally affected affinity, potency, and intrinsic activity of nearly all compounds tested, although a strong change in intrinsic activity was not seen with most *N*-aryl analogs. These data further validate the topology of our h5-HT$_{2A}$ receptor homology model. It is noteworthy that this study is the first to identify a hitherto unrecognized role for residue 6.51 in agonist activation of a serotonin G protein-coupled receptor (GPCR), whereas most previous reports have suggested a varied and sometimes contradictory role in homologous GPCRs.

Agonist activity at the serotonin 2A (5-HT$_{2A}$) receptor is essential for the psychopharmacology of serotonergic psyche-

delics such as LSD, DOI, psilocin, and 5-MeO-DMT, compounds with unique and dramatic effects on certain aspects of consciousness (Nichols 2004). Moreover, we have recently identified a functionally selective 5-HT$_{2A}$ receptor agonist that selectively activates phosphoinositide turnover over production of eicosanoids (McLean et al., 2006a). A key aspect to understanding the effects on consciousness of psychedelics is the study of the receptor-ligand interaction at the molecular level and how it modulates second messenger generation subsequent to receptor activation.

We have been particularly interested in experimental val-

ABBREVIATIONS: LSD, *d*-lysergic acid diethylamide; 5-HT, 5-hydroxytryptamine, serotonin; CHO, Chinese hamster ovary; HEK, human embryonic kidney; psilocin, 4-hydroxy-*N,N*-dimethyltryptamine; 5-MeO-DMT, 5-methoxy-*N,N*-dimethyltryptamine; mescaline, 3,4,5-trimethoxyphenethylamine; DOI, 4-iodo-2,5-dimethoxyphenylisopropylamine; DOI-NBOMe, *N*-(2-methoxybenzyl)-4-iodo-2,5-dimethoxyphenylisopropylamine; 25H, 2,5-dimethoxyphenethylamine; 25H-NMe, *N*-methyl-2,5-dimethoxyphenethylamine; 25H-NPr, *N*-propyl-2,5-dimethoxyphenethylamine; 25H-NB, *N*-benzyl-2,5-dimethoxyphenethylamine; 25H-NBOMe, *N*-(2-methoxybenzyl)-2,5-dimethoxyphenethylamine; 25H-NBOH, *N*-(2-hydroxybenzyl)-2,5-dimethoxyphenethylamine; 24, 2,4-dimethoxyphenethylamine; 24-NB, *N*-benzyl-2,4-dimethoxyphenethylamine; 24-NBOMe, *N*-(2-methoxybenzyl)-2,4-dimethoxyphenethylamine; 24-NBOH, *N*-(2-hydroxybenzyl)-2,4-dimethoxyphenethylamine; 25I, 4-iodo-2,5-dimethoxyphenethylamine; 25I-NB, *N*-benzyl-4-iodo-2,5-dimethoxyphenethylamine; 25I-NNap, *N*-methylnapthyl-4-iodo-2,5-dimethoxyphenethylamine; 25I-NBOMe, *N*-(2-methoxybenzyl)-4-iodo-2,5-dimethoxyphenethylamine; 25I-NBOH, *N*-(2-hydroxybenzyl)-4-iodo-2,5-dimethoxyphenethylamine; 25I-NBF, *N*-(2-fluorobenzyl)-4-iodo-2,5-dimethoxyphenethylamine; 25I-NBMD, *N*-(2,3-methylenedioxybenzyl)-4-iodo-2,5-dimethoxyphenethylamine; PI, phosphatidylinositol(s); TM, transmembrane.

idation of our h5-HT$_{2A}$ receptor homology model (Chambers and Nichols, 2002). Although the model has proven to have predictive power in designing simple conformationally constrained phenethylamine agonists for this receptor (McLean et al., 2006a,b), we were interested in extending it to provide a more comprehensive description of key residues involved in binding and activation. As a member of the type A GPCR family, we reasoned that understanding this receptor would provide general concepts that might extend across the GPCR field.

Thus, we were intrigued when we were alerted to a new class of extremely potent 5-HT$_{2A}$ receptor agonists that are derivatives of the phenethylamines mescaline and 2C-B (Pertz et al., 1999; Elz et al., 2002; K. Ratzeburg, personal communication). Although these novel agonists were characterized in a rat-tail artery model, we decided to examine whether these *N*-benzyl and related analogs had high affinity and potency in our heterologous cell-based assay systems. Furthermore, because *N*-alkyl substitution was known to abolish hallucinogenic activity and binding affinity of phenethylamines (Shulgin and Shulgin, 1992; Glennon et al., 1994), we were particularly curious about the contribution of the *N*-benzyl group, which seemed most likely to be a π-π interaction of some type with an aromatic residue within the receptor.

Based on virtual docking into our homology model of the h5-HT$_{2A}$ receptor, we identified two highly conserved aromatic amino acids, Phe339$^{(6.51)}$ and Phe340$^{(6.52)}$, that were likely to be interacting with these novel ligands. Previous site-directed mutagenesis studies had identified these residues to be within the 5-HT$_{2A}$ agonist binding pocket (Choudhary et al., 1993, 1995; Roth et al., 1997). Phe339$^{(6.51)}$, however, had been indicated to be more important for antagonist than for agonist binding. Substituted cysteine accessibility method studies in the human dopamine D$_2$ (Javitch et al., 1998) and hamster adrenergic β$_2$ (Chen et al., 1999) receptors provided evidence supporting the idea that the Phe339$^{(6.51)}$ cognate residue is solvent-accessible and is probably part of the ligand binding pocket. These two studies disagree, however, as to whether the Phe340$^{(6.52)}$ cognate residue is solvent accessible and thus within the binding pocket or is involved in helical packing. Mutations of the cognate residues in other amine-binding type A GPCRs have provided varied and disparate support for the involvement of either residue 6.51 or 6.52 in the binding and activity of agonists and antagonists at muscarinic acetylcholine (Ward et al., 1999), adrenergic (Chen et al., 1999), bradykinin (Nardone and Hogan, 1994), histamine (Wieland et al., 1999), neurokinin (Huang et al., 1995), and dopamine receptors (Cho et al., 1995; Brown et al., 2004).

The present article describes our examination of a series of 5-HT$_{2A}$ receptor agonists, including a new series of *N*-benzyl analogs, and the effects of the mutations F339L and F340L on binding affinity to and functional activity at 5-HT$_{2A}$ receptors. We provide additional new evidence for the role of these two residues in GPCR receptor function.

Materials and Methods

Materials. [^{3}H]Ketanserin, [^{3}H]8-hydroxy-2-(dipropylamino)tetralin, [^{125}I]4-iodo-2,5-dimethoxyphenylisopropylamine (DOI), and [*myo*-^{3}H]inositol were obtained from PerkinElmer Life and Analyti-

cal Sciences (Boston, MA). Serotonin (5-HT) was obtained from Sigma-Aldrich (St. Louis, MO). 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. Full details of chemical syntheses of all novel compounds and characterization of additional analogs will be described elsewhere. Structures of novel compounds used in this study and their abbreviations are shown in Fig. 1. Stock solutions of 5-HT were prepared as the creatine sulfate salt, 5-MeO-DMT and psilocin as the maleate salts, and all other compounds as their HCl salts.

Cell Culture Methods. NIH-3T3 cells stably expressing either the rat 5-HT$_{2A}$ receptor (GF-6; 5500 fmol/mg) or the r5-HT$_{2C}$ receptor (PØ; 7500 fmol/mg) were the kind gift of Dr. David Julius (University of California, San Francisco, CA) and within this article will hereafter be referred to as Nr2A and Nr2C, respectively. CHO cells stably expressing the human 5-HT$_{1A}$ receptor (CHO-1A; 500 fmol/mg) were the kind gift of Upjohn (Puurs, Belgium) and will be referred to as Ch1A. The construction of human embryonic kidney (HEK) 293 cells with high stable expression of wild-type h5-HT$_{2A}$ receptors (Hh2A; 8000 fmol/mg) has been described previously (Parrish et al., 2005) and will be referred to as Hh2A$_{hi}$. Human 5-HT$_{2C}$ receptors were transiently expressed in HEK-293 cells by transfection with a 3:1 (v/w) ratio of Fugene 6 (Roche Biomolecules, Indianapolis, IN) to pcDNA3.1-h5HT$_{2C}$R (Guthrie cDNA Resource Center, Sayre, PA; http://www.cdna.org) mixture in Opti-MEM (Invitrogen, Carlsbad, CA). These membrane preparations will be referred to as Hh2C. All cell types were maintained as described previously (Parrish et al., 2005), except that media for regular growth of cells contained 5% (v/v) bovine calf serum and 5% (v/v) fetal clone serum (VWR, West Chester, PA), whereas media for propagating cells for membrane preparations and PI hydrolysis assays contained 10% (v/v) dialyzed fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). All DMEM media for stably expressing cell lines contained 300 μg/ml G-418 (Sigma-Aldrich) for Nr2A and Nr2C cells, 22.5 units/ml Hygromycin B (Invitrogen) for CHO-1A cells, and 30 μg/ml Zeocin (Invitrogen) for Hh2A, Hh2A/F339L, and Hh2A/F340L cells (described below).

Establishing Stable Wild Type and Mutant Human 5-HT$_{2A}$R Cell Lines. A heterologous cell population with lower expression of the wild-type h5-HT$_{2A}$ receptor (Hh2Ahet3; 1600 fmol/mg) was obtained in a manner similar to the Hh2A cell line described previously (Parrish et al., 2005) without clonal selection and within this article will hereafter be referred to as Hh2A$_{lo}$. The h5HT$_{2A}$R insert was excised from pcDNA3.1-h5HT$_{2A}$R (Guthrie cDNA Resource Center) and subcloned into the pLNCX2 vector (Clontech, Mountain View, CA). Site-directed mutagenesis of pLNCX2-h5HT$_{2A}$R was performed using the QuikChange kit (Stratagene, La Jolla, CA) method of dual complementary mutant primer PCR followed by DpnI digestion of parental vector DNA. The following sense primers were used, along with their corresponding antisense primers (Integrated DNA Technologies, Coralville, IA): GGTGATGTGGTGCCCTTTGTTCATCA-CAAACATCATGGCCG for F339L and GGTGATGTGGTGCCCTT-TCTTGATCACAAACATCATGGCCG for F340L. Mutant inserts verified by primer directed sequencing (Retrogen, San Diego, CA) were then subcloned into the pBudCE4 vector (Invitrogen). HEK-293 cells were transfected, colonies were selected, and receptor expression was verified as described previously (Parrish et al., 2005). The Hh2A/F339L and Hh2A/F340L cell lines were chosen for moderate expression (2200 and 2500 fmol/mg, respectively).

Radioligand Binding Assays. Membrane preparations, saturation isotherm, and competition binding assays were performed as previously described in detail (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 nM (±)-[^{125}I]DOI. Ligands were tested in competition binding assays for their ability to displace 0.25 nM [^{125}I]DOI at Nr2A, Nr2C, Hh2A$_{hi}$, and Hh2A/F339L mem-

branes, 0.5 nM [³H]ketanserin at Hh2A$_{hi}$ and Hh2A/F340L membranes, and 0.5 nM [³H]8-hydroxy-2-(dipropylamino)tetralin at Ch1A membranes.

Inositol Phosphate Accumulation Assays. Compounds were tested for their ability to stimulate hydrolysis of radiolabeled phosphatidylinositides (PI) by measurement of radiolabeled inositol phosphate accumulation in Nr2A, Hh2Ahet3, Hh2A/F339L, and Hh2A/F340L cells, as described previously in detail (Marona-Lewicka et al., 2002; Kurrasch-Orbaugh et al., 2003). Primary comparison of EC$_{50}$ and intrinsic activity was performed between cell lines with approximately equivalent expression of h5-HT$_{2A}$ wild type (1600 fmol/mg), F339L mutant (2000 fmol/mg), and F340L mutant (2500 fmol/mg) receptors. Each assay plate was normalized to wells stimulated with water (0%) and a concentration of serotonin chosen to be maximally stimulating (100%).

Computational Modeling/Virtual Docking. Ligand structures were prepared, virtually docked into an h5-HT$_{2A}$R homology model (Chambers and Nichols, 2002), and local energy minimized-ensemble structures obtained as described previously (Parrish et al., 2005). In brief, energy minimized structures were virtually docked using the GOLD software package (Cambridge Crystallographic Data Center, Cambridge, UK). Docking algorithms were performed without any constraints. Ligand-receptor ensemble structures were each obtained by merging the highest-ranked 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. The molecular dynamics and minimization simulations were performed with constraints only between Asp155$^{(3.32)}$ and the amine nitrogen of the ligand, and Ser239$^{(5.43)}$ and the nearest polar group of the ligand.

Data Analysis. Prism software (GraphPad Software Inc. San Diego, CA) was used to calculate nonlinear regression curves 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 assays. This software also was used to perform two-way ANOVAs on pEC$_{50}$ and

intrinsic activity values of human wild type and mutant receptors, with a Bonferroni post test to compare replicate mean values of mutant receptors to the wild type. Unpaired two-tailed Student's t-tests were used to compare the pK_i at the mutant receptors to the corresponding wild type for the same competing radioligand. Values obtained from mutant receptors were considered statistically distinguishable from wild type if the models generated $p < 0.01$. Changes in the standard Gibbs free energy ($\Delta\Delta G°$) of binding as a result of the mutations were calculated from the K_i values at 25°C, as follows: $\Delta(\Delta G°) = \Delta G°_{mutant} - \Delta G°_{wild\ type} = RT\ \ln(K_{mutant}/K_{wild\ type})$, where R is the gas constant and T is the absolute temperature. Changes in binding affinity and EC$_{50}$ values were transformed to normalize the scale by taking the difference of the log$_{10}$ value (ΔpK_i and ΔEC$_{50}$, respectively) as follows: ΔpK_i = p$K_{i\text{-}mutant}$ − p$K_{i\text{-}WT}$ = −log$K_{i\text{-}mutant}$ − (−log$K_{i\text{-}WT}$) and ΔpEC$_{50}$ = pEC$_{50mutant}$ − pEC$_{50WT}$ = −logEC$_{50mutant}$ − (−logEC$_{50WT}$). Changes in intrinsic activity (ΔInt.Act.) were calculated as follows: ΔInt.Act. = Int.Act.$_{mutant}$ − Int.Act.$_{WT}$.

An arbitrary threshold of "weak effect" was defined as a change in pK_i or pEC$_{50}$ of ~≤1 log order (10-fold) and a change in intrinsic activity of ≤ 25%. Virtual docking figures were generated using PyMol (DeLano Scientific, San Carlos, CA). Amino acid residues are numbered with their position in the h5-HT$_{2A}$ receptor and their position relative to the most highly conserved residue of that transmembrane region, per Ballesteros and Weinstein (1995).

Results

The Effect of the *N*-Benzyl Moiety Does Not Result Simply from a Hydrophobic Interaction. As shown in Table 1, increasing hydrophobic bulk of the *N*-substituent on (25H) by adding a methyl or *n*-propyl group to yield 25H-NMe and 25H-NPr was detrimental to the binding affinity at all wild type receptors tested. By contrast, the *N*-benzyl group produced a profound increase in affinity (~7- to 300-fold) and potency (~100- − 200-fold) at the r5-HT$_{2A}$ receptor.
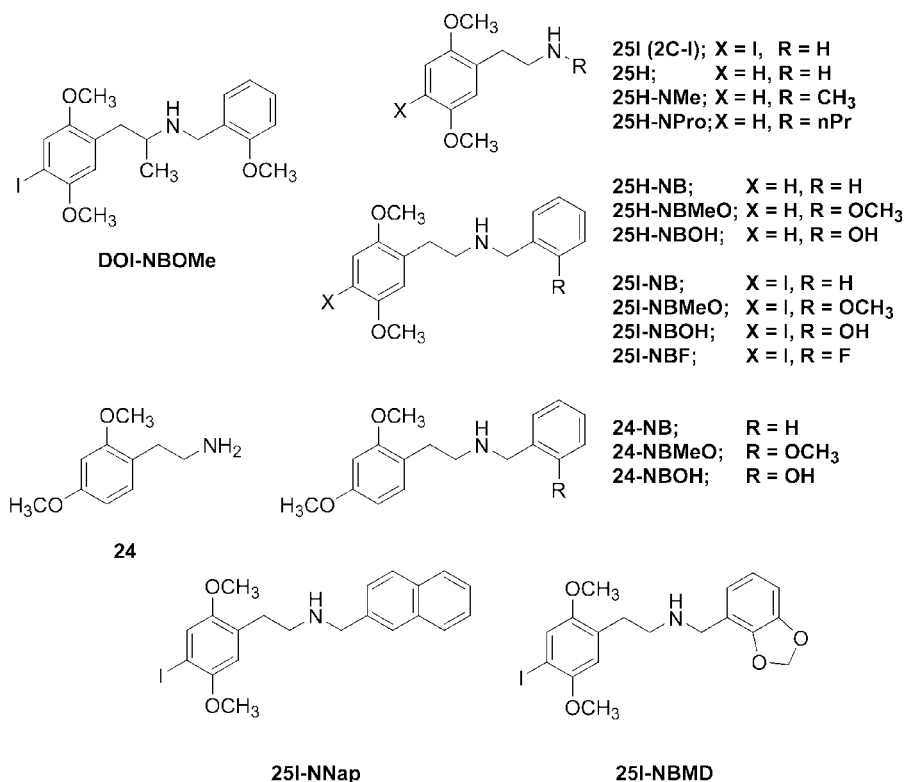
Fig. 1. Structures of molecules used in this study.

Addition of a polar methoxy or hydroxy group at the 2-position of the benzyl group further increased affinities.

Increases in affinity for *N*-benzyl analogs of 4-iodo-2,5-dimethoxyphenethylamine (25I) were not as dramatic (~2- – 7-fold) as the other phenethylamines tested yet were still significant. Addition of an α-methyl to the side chain (DOI-NBOMe) or the large *N*-naphthyl analog (25I-NNap) provided the only *N*-benzyl compounds tested that had decreased activity. Most of the test compounds also were assayed at the r5-HT$_{2C}$, h5-HT$_{2C}$, and 5-HT$_{1A}$ receptors (see Supplemental material). *N*-Substitution with an arylmethyl group seems to maintain if not slightly increase the modest

TABLE 1

Abilities of test compounds to displace (±)-[^{125}I]DOI and activate PI hydrolysis at rat 5-HT$_{2A}$ receptors

Data are represented as the mean and (S.E.M.) from non-linear regression fits of a single binding site model for K_i values and normalize variable slope sigmoidal dosage-response curves for estimates of EC$_{50}$ and intrinsic activity. All data are from at least three independent experiments. A typical experiment would show 10- to 20-fold stimulation by 5-HT over basal for PI hydrolysis assays.

Drug	K_i r5-HT$_{2A}$ (±)-[^{125}I]DOI	r5-HT$_{2A}$R PI Hydrolysis	
		EC$_{50}$	Intrinsic Activity (5-HT)
	nM	*nM*	*%*
25H	227 (39)	12,877 (1930)	82 (8)
25H-NMe	1286 (64)		
25H-NPr	734 (30)		
25H-NB	17.5 (1.9)		
25H-NBOMe	1.19 (0.17)	81.2 (3.8)	81 (0.4)
25H-NBOH	2.76 (0.40)	141 (21)	66 (2)
24	202 (19)	4034 (260)	67 (8)
24-NB	28.5 (2.9)		
24-NBOMe	0.68 (0.12)	51.0 (6.7)	72 (1)
24-NBOH	0.67 (0.01)	74.0 (6.7)	82 (4)
DOI	0.58 (0.06)	19.2 (2.6)	77 (3)
DOI-NBOMe	1.08 (0.21)	36.1 (2.7)	43 (3)
25I	0.62 (0.08)	19.0 (2.6)	59 (4)
25I-NB	0.31 (0.03)	12.0 (0.7)	37 (2)
25I-NNap	3.74 (0.52)	> 1 μM	25 @ 10 μM
25I-NBOMe	0.087 (0.010)	2.50 (0.55)	78 (6)
25I-NBOH	0.12 (0.02)	6.34 (0.18)	71 (2)
25I-NBF	0.28 (0.04)	23.2 (1.2)	32 (3)
25I-NBMD	0.19 (0.02)	8.2 (1.6)	68 (7)

2A versus 2C selectivity of these compounds, but the *N*-benzyl had no effect on 5-HT$_{1A}$ receptor affinity.

N-Benzyl Analogs of Phenethylamines Are Potent Agonists at the r5-HT$_{2a}$ Receptor. Several of the test ligands were examined at cloned r5-HT$_{2A}$ receptors for their ability to stimulate the hydrolysis of radiolabeled phosphatidyl inositides (PI), as also shown in Table 1. All compounds were relatively potent with high intrinsic activity in the PI hydrolysis assay, except for DOI-NBOMe, 25I-NNap, 25I-NB, and 25I-NBF, which were partial agonists. Again, the *N*-benzyl analogs of 25H and 24 displayed dramatic increases in PI hydrolysis potency (~100- to 200-fold), relative to their simple phenethylamine counterparts. Only modest improvement of potency and efficacy were generally observed in the series of *N*-arylmethyl substituted of 25I (~2- to 8-fold), except for 25I-NNap and 25I-NBF, which both lost potency and efficacy. The *N*-(2-methoxy)benzyl analog of DOI, DOI-NBOMe, did not have improved potency or efficacy.

Virtual docking of *N*-benzyl Analogs to an in Silico-Activated h5-HT$_{2A}$ Receptor Homology Model Reveals Potential Aromatic Residues Interacting with These Ligands. As the example in Fig. 2 illustrates, unconstrained virtual docking and subsequent energy minimization simulations of several *N*-benzyl analogs of phenethylamines produced orientations that placed the aromatic ring of the classic phenethylamine pharmacophore in a position to interact with Phe340[(6.52)], similar to previously observed orientations (Parrish et al., 2005). A further potential π-π interaction was identified between the novel aromatic *N*-benzyl moiety and Phe339[(6.51)], a residue previously identified only to affect antagonist binding in the 5-HT$_{2A}$ receptor (Roth et al., 1997). It was predicted that this residue might play a key role in interacting with this new class of 5-HT$_{2A}$ agonists.

The Phe339[(6.51)]L and Phe340[(6.52)]L mutations within the h5-HT$_{2A}$ receptor both strongly affect the binding affinities of the *N*-benzyl analogs, whereas Phe340[(6.52)]L strongly affects only the smaller classic ligands. K_D values for [^{125}I]DOI at Hh2A and Hh2A/F339L membrane preparations were 0.78 ± 0.01 and 0.86 ± 0.14 nM, respectively. K_D values for [^{3}H]ketanserin at Hh2A,
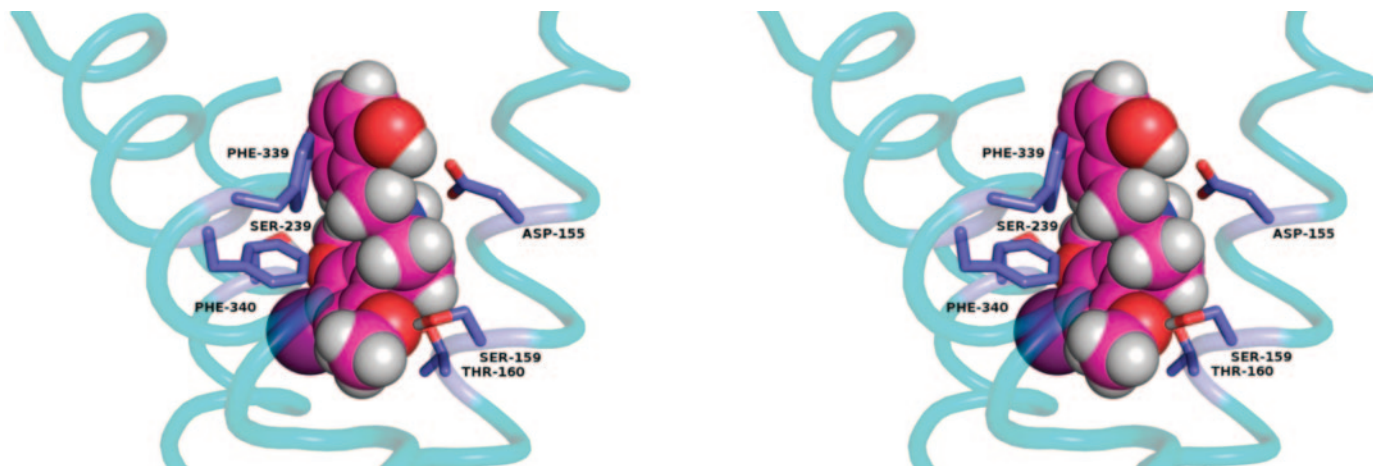

Fig. 2. Illustrative cross-eyed stereopair representation of ligand pose from virtual docking experiments with 25I-NBOH in the h5-HT$_{2A}$ receptor, showing proposed π-π interactions between the *N*-benzyl moiety and Phe339, and the aryl portion of the phenethylamine and Phe340. The ligand is shown as space-filling spheres, and receptor residues believed to be interacting with the ligand are displayed as sticks. The view is within the membrane, with TM6 in the left foreground, TM5 in the left background, TM3 on the right, 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 Phe340[(6.52)] may interact with the aryl ring of the phenethylamine pharmacophore and Phe339[(6.51)] may interact with the *N*-benzyl moiety.

Hh2A/F339L, and Hh2A/F340L membrane preparations were 1.10 ± 0.12, 11.1 ± 3.6, and 0.40 ± 0.02 nM, respectively. As anticipated from the work of Roth et al. (1997), it was not possible to determine a K_D value for [125I]DOI at h5-HT$_{2A}$/F340L receptors. Furthermore, because of the high K_D (11 nM) for [3H]ketanserin at h5-HT$_{2A}$/F339L, it was not practical to use this radioligand for competition binding at this receptor. Therefore, competition binding assays were performed with [125I]DOI at h5-HT$_{2A}$/F339L receptors, [3H]ketanserin at h5-HT$_{2A}$/F340L receptors, and both radioligands at wild-type receptors. To control for differences in wild-type K_i values from different radioligand displacement, K_i values were compared only with wild type receptors using the same radioligand.

As Table 2 and Fig. 3 show, the Phe339(6.51)L mutation had relatively weak (< 10-fold) detrimental effects on the affinities of all the classic and smaller ligands except for the phenethylamine 25H and the 5-substituted tryptamines 5-HT and 5-MeO-DMT. Except for LSD, all compounds tested had binding affinities that were statistically distinguishable from wild type. The mutation had weak detrimental effects on binding when a hydrophobic N-alkyl substitution on the amine of 25H was present (~5- –6-fold), but when an N-arylmethyl group was attached, as seen with 25H-NB, 25H-NBOMe, and 25H-NBOH, there was a dramatic ~40- to 700-fold decrease in affinity compared with the wild type. A similar marked detrimental effect was observed between 24 and 24-NB, 24-NBOMe, and 24-NBOH, with a ~100-fold decrease in affinity for the latter three N-benzyl analogs. The N-benzyl analogs of 25I were not as strongly affected, with 25I-NNap, 25I-NBOMe, 25I-NBOH, and 25I-NBF showing 30- to 50-fold decreases in binding affinities, and 25I-NB and 25I-NMD showing only ~10-fold decreases in affinity.

The Phe340(6.52)L mutation had comparatively weak (<10-

fold) detrimental effects only on the phenethylamines mescaline, 25H, 24, and 25I, in addition to the aliphatic analogs 25H-NMe and 25H-NPro, and the aromatic analog 25I-NBMD. Most of the other N-arylmethyl derivatives had ~100-fold decreases in binding affinities. The strongest detrimental effects were seen with 5-HT (>1000-fold), psilocin (160-fold), and 5-MeO-DMT (500-fold). The aromatic analogs followed a trend similar to that seen with the phenethylamines in the Phe339(6.51)L mutation. All compounds tested had binding affinities that were statistically distinguishable from the wild type.

Both the Phe339(6.51)L and Phe340(6.52)L Mutations within the h5-HT$_{2A}$ Receptor Adversely Affect to Different Degrees the Ability of Compounds Tested to Stimulate PI Hydrolysis. It has been shown that large differences in expression level can have marked effects on potency and intrinsic activity (Esbenshade et al., 1995; Zhong et al., 1996; Kenakin, 1997; Roth et al., 1997), so a new HEK-293 cell line was developed with wild-type expression comparable with the mutant h5-HT$_{2A}$ receptor expressing cell lines. The Hh2A$_{hi}$ cell line established previously (Parrish et al., 2005) has a [3H]ketanserin B_{max} of ~9000 fmol/mg. The newly constructed Hh2A$_{lo}$ cell population had a B_{max} of ~1600 fmol/mg, compared with expression levels of ~2200 and 2500 fmol/mg, respectively, for the mutant Hh2A/F339L and Hh2A/F340L cell lines.

As Table 3 and Fig. 4 indicate, the Phe339(6.51)L mutation had relatively weak detrimental effects on the potency of LSD and the unsubstituted phenethylamine agonists mescaline, 25H, 24, and 25I. The tryptamines 5-HT, psilocin, and 5-MeO-DMT were moderately affected. This mutation, however, produced strong detrimental effects (~100-fold or greater) with all of the N-benzyl phenethylamine analogs. Some detrimental effects on intrinsic ac-

TABLE 2
Abilities of test compounds to displace (±)-[125I]DOI or [3H]ketanserin at wild-type and mutant h5-HT$_{2A}$ receptors

Data are represented as the mean and (S.E.M.) of K_i values from non-linear regression fits of a single binding site model from at least three independent experiments. $\Delta\Delta G°$ values are calculated from K_i values at 25°C, except where noted. $P < 0.01$ for all values of Δ pK_i from unpaired two-tailed student t tests between mutant and wild-type receptors tested with the same radioligand.

Drug	(±)-[125I]DOI			[3H]Ketanserin		
	h5-HT$_{2A}$ K_i	h5-HT$_{2A}$/F339L K_i	$\Delta\Delta G°$	h5-HT$_{2A}$ K_i	h5-HT$_{2A}$/F340L K_i	$\Delta\Delta G°$
	nM	nM	kcal/mol	nM	nM	kcal/mol
5-HT	4.84 (0.2)	59.6 (10.0)	1.5	77.6 (13.8)	192,725 (36,305)	4.6
LSD	0.40 (0.02)	0.60 (0.12)‡	0.2	0.81 (0.16)	13.01 (1.09)	1.6
Psilocin	11.8 (1.2)	28.6 (4.3)	0.5	22.8 (4.0)	3659 (243)	3.0
5-MeO-DMT	7.54 (1.06)	129 (15)	1.7	49.2 (3.2)	23,726 (4726)	3.7
Mescaline	1499 (245)	4488 (608)	0.6	14,640 (2447)	62,425 (10,485)	0.9
25H	377 (67)	5786 (734)	1.6	1999 (311)	16,001 (3163)	1.2
25H-NMe	1907 (254)	8719 (671)	0.9	5934 (92)	43,918 (2271)	1.2
25H-NPr	1295 (151)	7863 (769)	1.1	3597 (642)	9815 (943)	0.6
25H-NB	68.1 (10.6)	2722 (470)	2.2	184 (33)	6698 (1031)	2.1
25H-NBOMe	2.83 (0.31)	1435 (192)	3.5	11.0 (0.5)	689 (107)	2.5
25H-NBOH	3.73 (0.45)	2642 (455)	3.9	11.6 (1.7)	277 (40)	1.9
24	298 (29)	1013 (190)	0.7	999 (182)	8391 (1200)	1.3
24-NB	26.6 (2.7)	1768 (339)	2.5	71.9 (3.0)	3316 (356)	2.3
24-NBOMe	1.71 (0.34)	252 (49)	3.0	5.24 (1.01)	703 (14)	2.9
24-NBOH	1.51 (0.20)	306 (57)	3.1	2.83 (0.36)	292 (14)	2.7
25I	0.73 (0.06)	2.63 (0.32)	0.8	4.52 (0.30)	28.9 (4.8)	1.1
25I-NB	0.25 (0.05)	3.1 (0.1)	1.3	0.28 (0.02)	27.0 (1.8)	2.7
25I-NNap	4.83 (0.55)	157 (31)	2.1	6.68 (1.02)	268 (268)	2.1
25I-NBOMe	0.044 (0.006)	2.08 (0.35)	2.3	0.15 (0.03)	4.3 (0.76)	2.1
25I-NBOH	0.061 (0.012)	1.84 (0.16)	2.0	0.068 (0.012)	1.58 (0.17)	1.9
25I-NBF	0.26 (0.05)	15.2 (1.7)	2.4	0.19 (0.03)	37.9 (1.3)	3.1
25I-NBMD	0.049 (0.008)	0.29 (0.03)	1.1	0.21 (0.03)	0.94 (0.17)	0.9

‡ $P > 0.01$.

tivity of the smaller agonists were observed with this mutation but generally were weak (<10-fold). As evident in the EC$_{50}$ values, stronger detrimental effects on intrinsic activity

were observed with all of the *N*-benzyl analogs of phenethylamines. For some of the *N*-benzyl analogs, this change is so dramatic that they are transformed from nearly full agonists at
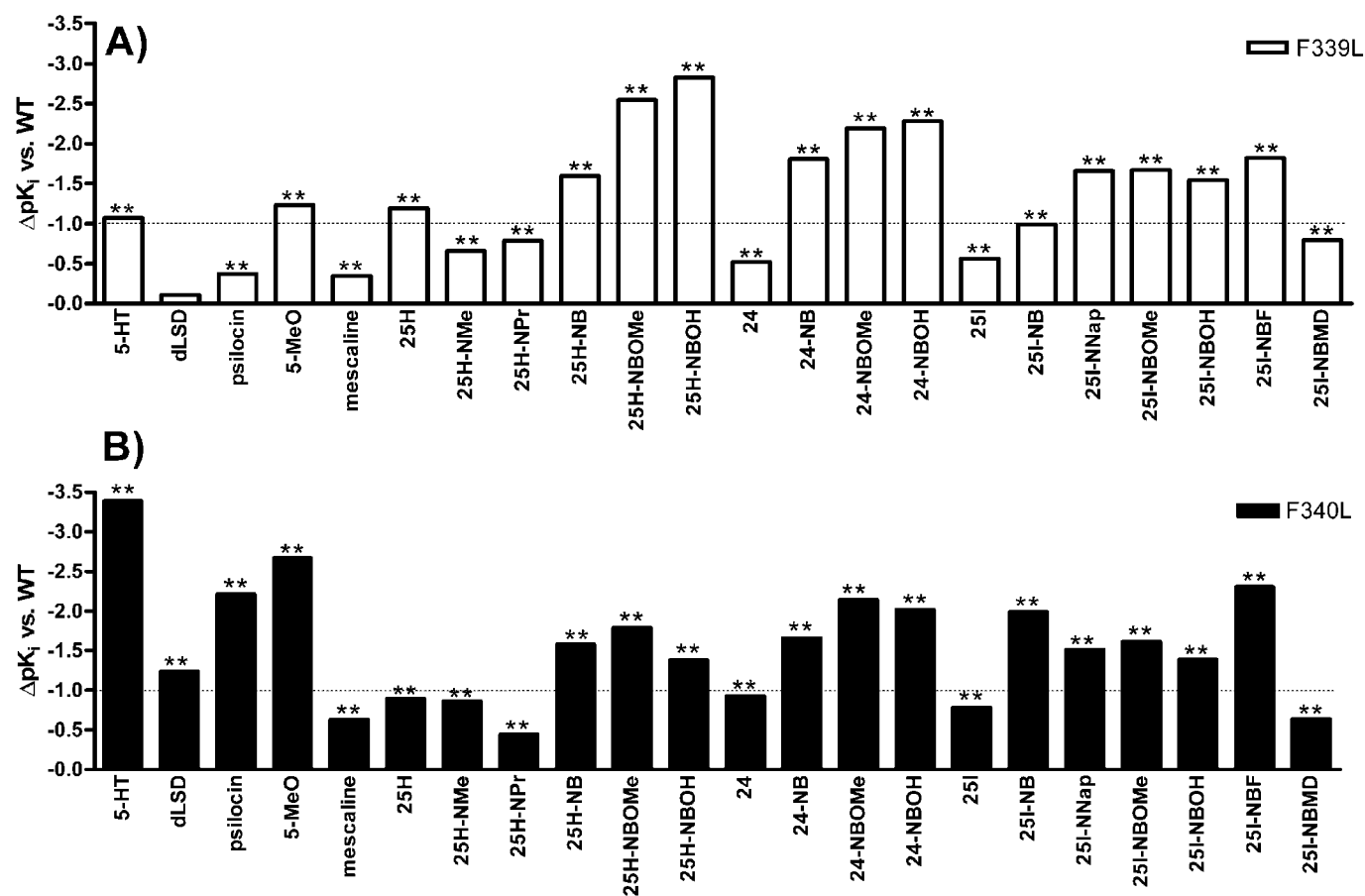

Fig. 3. Effects on binding affinities of the Phe339[(6.51)]L (A) and Phe340[(6.52)]L (B) mutations in the h5-HT$_{2A}$ receptor. These bar graphs display the ΔpK_i values derived from the data of Table 3 (see *Materials and Methods*). Larger negative (upward) values in these graphs indicate a greater negative effect of the mutation on binding affinity. The dashed line at -1.0 indicates an arbitrary threshold for "weak" effects. ∗∗, $p < 0.01$ for values of ΔpK_i from unpaired two-tailed Student T-tests.

TABLE 3

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

Data are represented as the mean and (S.E.M.) of computer-derived estimates of EC$_{50}$ and intrinsic activity values from at least three independent experiments. A typical experiment would show 4- to 10-fold stimulation by 5-HT over basal. Unless otherwise noted, $P < 0.01$ for ΔpEC_{50} and ΔInt.Act. from two-way ANOVA with Bonferroni post-tests.

Drug	h5-HT$_{2A}$		h5-HT$_{2A}$/F339L		h5-HT$_{2A}$/F340L	
	EC$_{50}$ PI Hydrolysis	Intrinsic Activity (5-HT)	EC$_{50}$ PI Hydrolysis	Intrinsic Activity (5-HT)	EC$_{50}$ PI Hydrolysis	Intrinsic Activity (5-HT)
	nM	%	*nM*	%	*nM*	%
5-HT	5.17 (0.97)	100	92.4 (10.5)	100	9840 (458)	100[‡]
LSD	0.22 (0.04)	84 (3)	1.36 (0.23)	55 (5)	15.7 (2.9)	20 (5)
Psilocin	7.29 (0.72)	105 (9)	129 (18)	44 (8)	4529 (813)	9 (1)
5-MeO-DMT	4.33 (0.78)	98 (4)	416 (71)	74 (5)	5255 (969)	15 (4)
Mescaline	1117 (223)	83 (5)	11,333 (991)	82 (7)[‡]	78,795 (3869)	30 (1)
25H	1021 (14)	96 (10)	10,353 (1652)	78 (1)[‡]	141,033 (39,537)	12 (4)
25H-NBOMe	15.3 (3.7)	88 (6)	3407 (390)	27 (4)	1341 (340)	43 (5)
25H-NBOH	23.5 (1.8)	100 (6)	11,267 (758)	32 (6)	2156 (503)	28 (3)
24	832 (200)	83 (5)	4077 (579)	66 (4)[‡]	109,311 (37,671)	17 (1)
24-NBOMe	4.00 (0.80)	89 (6)	1436 (281)	55 (5)	2029 (199)	66 (8)[‡]
24-NBOH	5.42 (0.66)	84 (4)	5623 (29)	49 (8)	696 (139)	31 (3)
25I	2.54 (0.18)	82 (3)	22.8 (2.7)	72 (5)[‡]	99.5 (5.3)	38 (2)
25I-NB	1.96 (0.12)	66 (2)	1093 (353)	14 (2)	263 (40)	82 (1)[‡]
25I-NBOMe	0.44 (0.07)	81 (4)	28.0 (5.2)	51 (4)	26.8 (4.2)	84 (7)[‡]
25I-NBOH	0.19 (0.03)	86 (5)	42.3 (6.5)	45 (6)	14.6 (2.9)	82 (7)[‡]
25I-NBF	1.55 (0.21)	87 (11)	150 (25)	8 (1)	410 (33)	81 (6)[‡]
25I-NBMD	1.07 (0.20)	72 (3)	91.0 (30.9)	11 (1)	145 (25)	70 (5)[‡]

[‡] $P > 0.01$.

the wild-type receptor into very weak partial agonists by the mutation.

The Phe340$^{(6.52)}$L mutation had marked detrimental effects on the potency of all compounds tested, particularly the tryptamines 5-HT, psilocin, and 5-MeO-DMT. Because of the extreme loss of potency for 5-HT at this mutant receptor and solubility limitations of 5-HT, the normalization concentration of serotonin may not be maximally stimulating and thus all intrinsic activity values indicated at this mutant receptor may be slightly elevated and all negative ΔInt.Act. values may therefore be underestimated as well. With this caveat, the Phe340$^{(6.52)}$L mutation had strong detrimental effects on intrinsic activity (efficacy) of all the smaller classic agonists. It is noteworthy that the *N*-benzyl analogs of 25H and one analog of 24 also showed relatively strong (~100-fold or greater) decreases in intrinsic activity, whereas none of the 25I analogs showed any strong decreases. As with the Phe339$^{(6.51)}$L mutation, the compounds most dramatically affected by the Phe340$^{(6.52)}$L mutation were transformed from nearly full agonists at the wild-type receptor to very weak partial agonists in the mutant receptor.

Discussion

Our primary goal with this study was to explore further the topology of the human 5-HT$_{2A}$ receptor by characterizing the pharmacology of a new class of 5-HT$_2$ receptor agonists. At the start, we wished to determine whether the beneficial effects of *N*-(2-methoxy)-benzyl substitution on phenethylamines reported by Pertz et al. (1999) and Elz et al. (2002) would be observed in our heterologous expression systems. We also compared two *N*-alkyl substituted phenethylamines to ascertain that the effect of the *N*-benzyl was not simply due to a hydrophobic interaction.

As Table 1 indicates, these analogs are high-affinity, selective, potent, and highly efficacious agonists that depend on an *N*-arylmethyl substitution for their exceptionally high activity. The *N*-methyl and *n*-propyl analogs of 25H had decreased affinity at all receptors tested. Yet, *N*-benzyl compound 25H-NBOMe had more than 100-fold increased affinity compared with the unsubstituted 25H. *N*-(2-Methoxy)-benzyl compound 24-NBOMe, displayed an increase in affinity of up to 300-fold compared with the unsubstituted 24. We recognized, therefore, that the aromatic ring of the *N*-
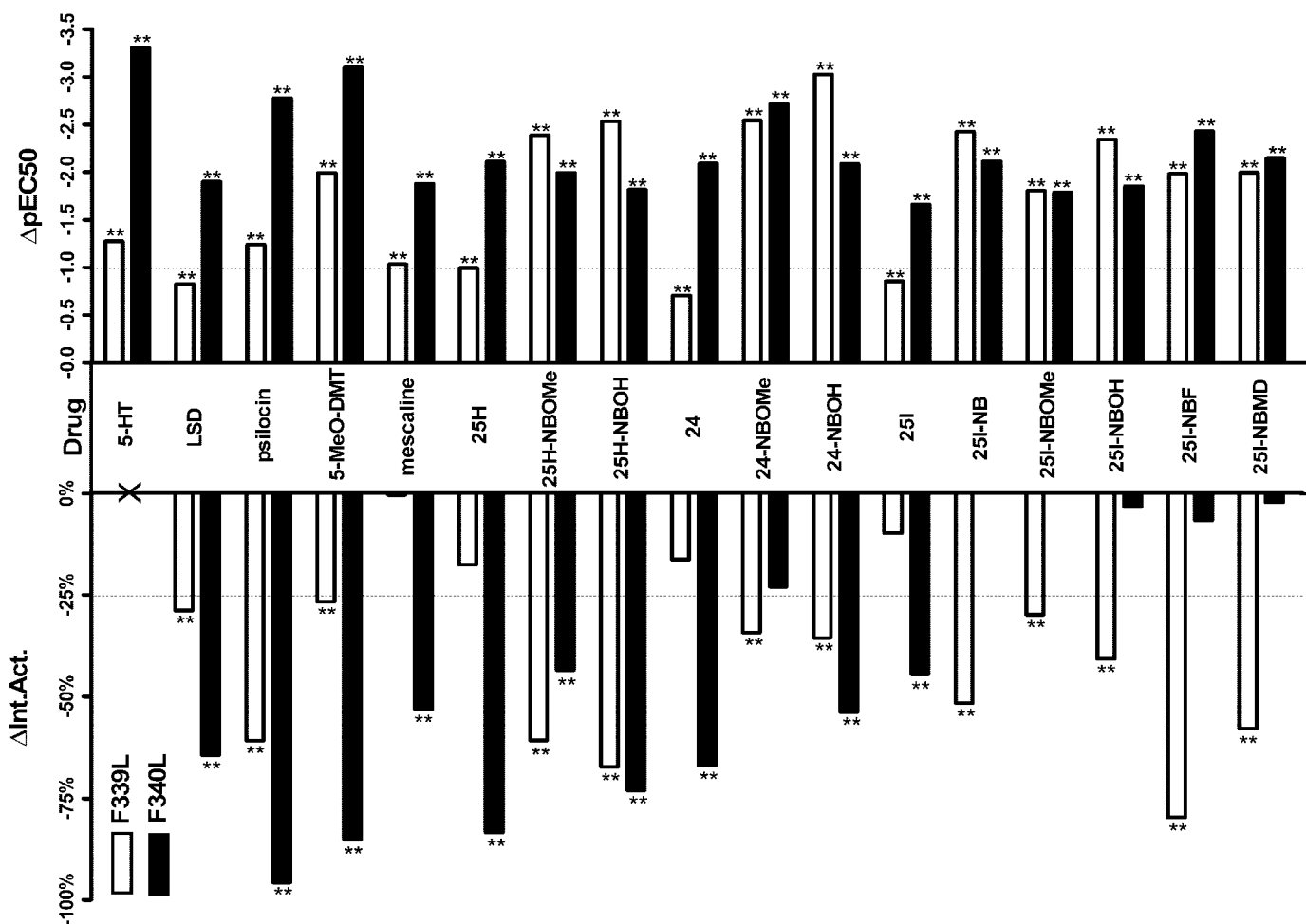

Fig. 4. Effects on EC$_{50}$ and intrinsic activity of PI hydrolysis functional activity by Phe339$^{(6.51)}$L (open bars) and Phe340$^{(6.52)}$L (solid bars) mutations in the h5-HT$_{2A}$ receptor. These bar graphs display the ΔpEC$_{50}$ and ΔInt. Act. values derived from the data of Table 3 (see *Materials and Methods*). All intrinsic activity values are normalized to serotonin stimulation and thus it is not possible to obtain ΔInt.Act. values for 5-HT. Positive ΔInt.Act. values for 25I-NB and 25I-NBOMe at h5-HT$_{2A}$/F340L receptors were not significantly different from WT and are not shown for formatting reasons. Dashed lines at −1.0 and −25% indicate an arbitrary threshold for "weak" effects. ∗∗, $p < 0.01$ for values of ΔpEC$_{50}$ and ΔInt.Act. from two-way ANOVA tests with Bonferroni post tests.

benzyl moiety was enhancing activity through some specific biophysical property, which we believe to be a π-π interaction with an aromatic residue in the receptor. These data are consistent with a previous study evaluating amine substitution on phenylalkylamines and indolealkylamines (Glennon et al., 1994). In that study, however, N-arylmethyl analogs of the phenethylamine 2C-B showed only ~2- to 3-fold increased affinity. The underlying basis for this effect was not evaluated and none of their ligands were tested for functional activity.

Dramatic increases also were observed in the potency of these compounds to stimulate PI hydrolysis. Although N-arylmethyl analogs of 25I did not show as marked an increase in affinity or potency, they still possessed affinities at the h5-HT$_{2A}$ receptor as high as 40 pM. We speculate that the decreased sensitivity of the 4-iodo series to N-benzyl substitution may be due to a reorientation of the ligand within the receptor binding domain to accommodate the large iodine atom. This change may force the ligand to adopt a binding pose that displaces the N-benzyl from an optimal position for interaction with Phe339$^{(6.51)}$.

The in silico-activated homology model of the h5-HT$_{2A}$ receptor developed in our laboratory (Chambers and Nichols, 2002) was used in an attempt to provide some qualitative explanation of the high affinity, potency, and intrinsic activity of this novel class of compounds. Previous virtual dockings of phenethylamines and related classic agonists have produced low-energy ensemble structures with orientations placing Phe340$^{(6.52)}$ in a position to interact with the aromatic ring of the ligand through a π-π interaction (Parrish et al., 2005). Those findings were consistent with previous mutagenesis results indicating the cognate residue in other receptors is solvent-accessible (Javitch et al., 1998) and is involved in an essential π-π interaction with agonists (Choudhary et al., 1993, 1995; Cho et al., 1995; Huang et al., 1995; Roth et al., 1997). Other mutagenesis studies, however, indicate that this residue is not solvent-accessible (Chen et al., 1999), does not interact with agonists, and/or may be interacting with some antagonists (Choudhary et al., 1993, 1995; Cho et al., 1995; Wieland et al., 1999) or is not involved in receptor activation (Ward et al., 1999). Docking orientations produced in this study again positioned the aromatic ring of the phenethylamine pharmacophore in a position to interact with Phe340$^{(6.52)}$, as well as positioning the polar methoxy groups in ways that indicated interactions with other residues identified by site-directed mutagenesis to be involved in 5-HT$_{2A}$ agonist binding (Wang et al., 1993; Kristiansen et al., 2000; Shapiro et al., 2000) and observed previously in our work (Parrish et al., 2005).

In the present work, a potential π-π interaction was identified between Phe339$^{(6.51)}$ and the novel N-benzyl moiety of the ligand. Mutation of the cognate residue in a variety of other GPCRs has indicated that residue 6.51 is 1) located near retinal in bovine rhodopsin (Nakayama and Khorana, 1991), 2) solvent accessible (Javitch et al., 1998; Chen et al., 1999), and 3) may be essential for a π-π interaction with both agonists and antagonists (Nardone and Hogan, 1994; Cho et al., 1995; Huang et al., 1995; Chen et al., 1999; Ward et al., 1999; Brown et al., 2004). With respect specifically to the 5-HT$_{2A}$ receptor, previous data seemed to indicate that this residue was not within the agonist-binding pocket and was not involved in the binding of agonists or partial agonists

(Choudhary et al., 1993) but rather stabilized binding of the antagonist ketanserin (Choudhary et al., 1995; Roth et al., 1997).

To test the hypothesis that both Phe339$^{(6.51)}$ and Phe340$^{(6.52)}$ might be contributing to a π-π interaction with the N-benzyl analogs of phenethylamines, mutations of these two residues were performed in the h5-HT$_{2A}$ receptor. A semiconservative phenylalanine to leucine mutation was chosen to eliminate aromaticity while conserving steric bulk and hydrophobicity of the residues to reduce possible global effects of the mutations (Fersht et al., 1987). Concerns about potential global structural change were addressed by including several different chemical classes of traditional 5-HT$_2$ agonists. Differential shifts in binding affinities between ligand classes should indicate more regional changes in the receptor structure rather than a global one affecting all ligand classes.

Differences in binding affinities between mutant and wild type receptors were assessed, as well as the functional repercussions of the Phe339$^{(6.51)}$L and Phe340$^{(6.52)}$L mutations in the PI hydrolysis second messenger system. The results of these experiments are illustrated in Figs. 3 and 4, based on the data from Tables 2 and 3. The trends observed in these figures seem to indicate that both Phe339$^{(6.51)}$ and Phe340$^{(6.52)}$ are interacting with the N-benzyl phenethylamines, whereas Phe340$^{(6.52)}$ seems to be interacting mainly with N-unsubstituted phenethylamines and the other classic agonists. The $\Delta\Delta G°$ values of ~2 to 4 kcal/mol are in agreement with the energy of a "stacked" or "T-shape" π-π interaction (Jorgensen and Severance, 1990). This trend is particularly evident in the weak effects of the Phe339$^{(6.51)}$L mutation on EC$_{50}$ and near absence of effects on intrinsic activity of the N-unsubstituted phenethylamines 25H, 24, and 25I. In stark contrast, the Phe339$^{(6.51)}$L mutation had profound effects on both measures of function for the N-benzyl analogs of these phenethylamines, which were nearly converted into antagonists in this mutant receptor.

The Phe340$^{(6.52)}$L mutation seems to affect the affinity and potency of nearly all the ligands tested, although to a different degree across different ligand classes. It seems still able to couple to activation of PI hydrolysis, thus reducing concerns of massive global change of the receptor structure. The simple classic agonists also are turned nearly into antagonists by this mutation. It is surprising that the Phe340$^{(6.52)}$L mutation does not seem to have an effect on intrinsic activity of the N-benzyl analogs of 25I, yet the binding and potency are shifted to a degree similar to the other N-benzyl analogs. Thus, an interaction with Phe339$^{(6.51)}$ in this series may be sufficient to produce a fully "active" receptor state.

Phe339$^{(6.51)}$ and Phe340$^{(6.52)}$ are highly conserved and make up an evolutionarily constrained cascade of aromatic residues that mediate allosteric communication and receptor activation in GPCRs (Süel et al., 2003). These residues reside within transmembrane domain 6 (TM6), which is directly coupled to the third intracellular loop (IL3), between TM5 and TM6. This loop is implicated as being important for the ability of the 5-HT$_{2A}$ receptor (and all related GPCRs) to interact with and activate the appropriate G proteins and thus affect second messenger production, particularly Gα_q activation of PI hydrolysis (Kubo et al., 1988; Wess et al., 1989, 1990; Oksenberg et al., 1995; Hill-Eubanks et al., 1996). It would seem that agonist or antagonist character,

affinity, and potency of a particular ligand is dependent on the ability of the ligand to interact with specific TM6 residues and induce or suppress movement of this helix. Although there is disagreement about the individual contributions of Phe$^{(6.51)}$ and Phe$^{(6.52)}$ toward the binding and activity of agonists and antagonists across several amine-binding type A GPCRs, it seems from our data that within the h5-HT$_{2A}$ receptor, involvement of either residue is sufficient for agonist binding and receptor activation.

Moreover, although we have provided further evidence that Phe340$^{(6.52)}$ is probably not involved in the binding of the antagonist ketanserin (Roth et al., 1997), our results do not preclude the possibility that this residue interacts with other classes of structurally diverse antagonists or partial agonists. We also have expanded and extended the findings of Pertz et al. (1999), and Elz et al. (2002), that *N*-benzyl phenethylamines, particularly those with a 2-methoxy or 2-hydroxy function on the benzyl moiety, represent a novel class of high affinity, potent, and modestly selective 5-HT$_{2A}$ receptor agonists. We believe our data are consistent with the general topology for the h5-HT$_{2A}$ receptor reflected in our in silico-activated homology model of this receptor (Chambers and Nichols, 2002). Finally, and perhaps most important, we believe it may be possible to exploit cognate residue in 6.51 in other GPCRs to design agonists with increased potency and intrinsic activity.

Acknowledgments

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