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Drug discovery targeting human 5-HT_{2C} receptors: Residues S3.36 and Y7.43 impact ligand—binding pocket structure via hydrogen bond formation

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Abstract

Specific activation of serotonin (5-HT) 5-HT_{2C} G protein-coupled receptors may be therapeutic for obesity and neuropsychiatric disorders. Mutagenesis coupled with computational and molecular modeling experiments based on the human β_2 adrenergic receptor structure were employed to delineate the interactions of different ligands at human 5-HT_{2C} residues D3.32, S3.36 and Y7.43. No binding of the tertiary amine radioligand (³H]-mesulergine) could be detected when the 5-HT_{2C} D3.32 residue was mutated to alanine (D3.32A). The S3.36A point-mutation greatly reduced affinity of primary amine ligands, modestly reduced affinity of a secondary amine, and except for the 5-HT_{2C}-specific agonist N(CH₃)₂-PAT, affinity of tertiary amines was unaffected. Molecular modeling results indicated that the primary amines form hydrogen bonds with the S3.36 residue, whereas, with the exception of N(CH₃)₂-PAT, tertiary amines do not interact considerably with this residue. The Y7.43A point-mutation greatly reduced affinity of 5-HT, yet reduced to a lesser extent the affinity of tryptamine that lacks the 5-hydroxy moiety present in 5-HT; modeling results indicated that the 5-HT 5-hydroxy moiety hydrogen bonds with Y7.43 at the 5-HT_{2C} receptor. Additional modeling results showed that 5-HT induced a hydrogen bond between Y7.43 and D3.32. Finally, modeling results revealed two low-energy binding modes for 5-HT in the 5-HT_{2C} binding pocket, supporting the concept that multiple agonist binding modes may stabilize different receptor active conformations to influence signaling. Ligand potencies for modulating WT and point-mutated 5-HT_{2C} receptor-mediated phospholipase C activity were in accordance with the affinity data. Ligand efficacies, however, were altered considerably by the S3.36A mutation only.

Keywords

G protein-coupled receptor; molecular modeling; mutagenesis; serotonin 2C receptor

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Conflicts of Interest

The authors declare no conflicts of interest.

1. Introduction

Some of the diverse neurophysiological functions of serotonin (5-hydroxytryptamine, 5-HT) are mediated by the 5-HT₂ G protein-coupled receptors (GPCRs), 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}. The human 5-HT_{2C} receptor (Saltzman et al., 1991), expressed with apparent exclusivity in the central nervous system, regulates ingestive behavior and is proposed to be involved in the pathophysiology of schizophrenia, psychostimulant abuse, and other neuropsychiatric disorders (Bubar and Cunningham, 2008; Jensen et al., 2010; Millan, 2005; Tecott et al., 1995).

Recently, (1*R*, 3*S*)-(-)-*trans*-1-phenyl-3-*N,N*-dimethylamino-1,2,3,4-tetrahydronaphthalene (N(CH₃)₂-PAT) was reported as the first 5-HT_{2C} receptor agonist with 5-HT_{2A} and 5-HT_{2B} receptor inverse agonist activity (Booth et al., 2009), and suggests a class of ligands suitable for pharmacotherapeutic development. In contrast, selective 5-HT_{2C} agonists that also activate 5-HT_{2B} and/or 5-HT_{2A} receptors, may produce cardiopulmonary toxicity (Fitzgerald et al., 2000; Launay et al., 2002; Rothman et al., 2000) and/or hallucinations (Nichols, 2004). For example, lorcaserin is a selective 5-HT_{2C} agonist that also activates 5-HT_{2A} and 5-HT_{2B} receptors (Thomsen et al., 2008) and produces “mood and perceptual adverse events” in humans at a dose four times higher than the recommended anorexiant dose (Arena Pharmaceuticals, 2010).

The molecular determinants that govern the receptor-specific function of N(CH₃)₂-PAT at 5-HT₂ subtypes are not yet clear. It is established that the basic amine moiety of most ligands interacts with the fully-conserved aspartate residue at position 3.32 (D3.32) of aminergic G protein-coupled receptors (Ballesteros et al., 2001a; Kristiansen et al., 2000). In 5-HT₂ receptors, there is also a conserved serine residue, S3.36, approximately one helical turn from D3.32. When the 5-HT_{2A} S3.36 residue is mutated to alanine (S3.36A), affinity of 5-HT, a primary amine, is reduced ~20-fold, whereas affinity of tertiary amines is barely affected. Together with molecular modeling based on homology to the bovine rhodopsin structure, these data suggest the 5-HT_{2A} S3.36 residue is important for hydrogen bond interactions with primary amines, but not for tertiary amine ligands, likely due to steric hindrance of *N*-alkyl groups (Almaula et al., 1996; Ebersole et al., 2003).

Molecular interactions between the ligand amine moiety and the 5-HT_{2C} receptor are less understood. Experiments here used a mutagenesis approach coupled with molecular modeling based on homology to the human β₂ adrenergic receptor structure (Rasmussen et al., 2007) to characterize molecular determinants for ligand binding at 5-HT_{2C} receptors. To probe ligand amine interactions, affinity of the tertiary amine N(CH₃)₂-PAT (Fig. 1), its secondary and primary amine analogs (NH(CH₃)-PAT and NH₂-PAT, respectively, Fig. 1), as well as, reference primary and tertiary amines were assessed at the human, nonedited 5-HT_{2C}-INI (WT) receptor in comparison to the corresponding S3.36A point-mutated receptor. 5-HT_{2C} receptor-mediated phospholipase C signaling data were also collected for class representative ligands. When building the 5-HT_{2C} receptor model, it was observed that amino acid Y7.43 orients in the putative binding pocket such that it could hydrogen bond with a ligand amine moiety. Thus, we extended studies to include the Y7.43A point-mutated 5-HT_{2C} receptor. The critical role of D3.32 in ligand amine binding was confirmed using the D3.32A point-mutated 5-HT_{2C} receptor.

2. Materials and methods

2.1 DNA constructs and transfection of HEK cells

Amino acids were numbered according to the convention proposed for aminergic neurotransmitter G protein-coupled receptors (Ballesteros et al., 2001b). The cDNA

encoding the human, 5-HT_{2C}-INI (WT) receptor was obtained from UMR cDNA Resource Center (Rolla, MO). PCR-based methods were used to create the expression constructs of the S3.36A and Y7.43A point-mutated receptors based on the template WT 5-HT_{2C} receptor. The PCR primers (sense and antisense) used were, 5'-gccccgtctgatttcttagctgtttatttcaacagcg-3' and 5'-gccccgtctggatttcttagatgtttatttcaacagcg-3' and 5'-cgctgttgaaaataaacagctaaagaatccagacggggc-3' for the D3.32A mutation, 5'-ccgtctggatttcttagatgtttatttgaacagcgctccatcat-3' and 5'-atgatggacgctgttgcaataaaaacatctaagaatccagacgg-3' for the S3.36A mutation, and, 5'-tgaatgtttgtttggattggcgtgtttgttcaggaatcaatcctc-3' and 5'-gaggattgattcctgaacaacagcgccaatccaaacaacacattca-3' for the Y7.43A mutation. The PCR primers (sense and antisense) used were, 5'-gccccgtctggatttcttagctgtttatttcaacagcg-3' and 5'-gccccgtctgatttcttagatgtttatttcaacagcg-3' for the D3.32A mutation, 5'-ccgtctggatttcttagatgtttatttgaacagcgctccatcat-3' and 5'-atgatggacgctgttgcaataaaaacatctaagaatccagacgg-3' for the S3.36A mutation, and, 5'-tgaatgtttgtttggattggcgtgtttgttcaggaatcaatcctc-3' and 5'-tgaatgtttgtttggattggctatgtttgttcaggaatcaatcctc-3' for the Y7.43A mutation. The cDNA sequences were verified using the dideoxy chain termination method. PCR was carried out using the QuikChange Kit (Stratagene, Santa Clara, CA) with WT 5-HT_{2C} in the pcDNA3.1+ vector as the template, primers with designated mutations, and a thermocycle of initial denaturing at 95°C for 30 sec, followed by 16 cycles of 95°C for 30 sec, 70°C for 1 min, and 68°C for 3 min. The WT parental DNA was digested by *DpnI*. PCR products were used to transform XL1-Blue Supercompetent Cells. A single colony was sequenced to confirm the designed mutation. HEK-293 cells, 50–80% confluent in a 10 cm plate, were transfected with the isolated DNA constructs by mixing 8–16 µg of construct DNA with 12–16 µl Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Expression of receptors was confirmed by Western-blot (data not shown).

To generate receptors for radioligand binding studies, HEK-293 cells were grown to 90% confluency in DMEM (10-013-CV, Mediatech, Manassas, VA) supplemented with 5% dialyzed fetal bovine serum, 100 I.U./mL penicillin and 100 µg/mL streptomycin (30-0020-CI, Mediatech) in 10 cm plates. Cells were washed 1× with phosphate-buffered saline, and then transfected with 24 µg of human 5-HT_{2C} WT, S3.36A or Y7.43A DNA mixed with 33 µl Lipofectamine 2000 reagent in Opti-MEM and placed in an incubator at 37°C, 5% CO₂, 95% humidity for 24 to 48 hr. Membranes were then collected in 50 mM Tris, 10 mM MgCl₂-6H₂O, 0.1 mM EDTA (assay buffer) using previous methods (Booth et al., 2009) and stored at –80° Celsius until binding assays were performed.

2.2 Compounds

The radioligand [³H]-mesulergine (specific activity 92 Ci/mmol) was purchased from Perkin-Elmer. Serotonin hydrochloride was purchased from Alfa Aesar (Ward Hill, MA), tryptamine hydrochloride and mianserin hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO), mesulergine hydrochloride was purchased from Tocris (Ellisville, MO), and lisuride hydrogen maleate was a gift from Dr. Elaine Sanders-Bush (Vanderbilt University, Nashville, TN). Synthesis of the primary, secondary, and tertiary amine analogues of (1*R*, 3*S*)-(±)-*trans*-1-phenyl-3-*N,N*-dimethylamino-1,2,3,4-tetrahydronaphthalene was performed according to previous laboratory methods (Booth et al., 2009; Bucholtz et al., 1999) to obtain the hydrochloride salt of the *trans*-stereoisomers (ligand docking studies considered only (1*R*, 3*S*)-(–) enantiomer). Ligand structures are given in Fig. 1.

2.3 Radioligand binding assay

Radioligand saturation isotherm and competitive displacement binding assays were performed in 96-well plates, using 3–5 μg of protein per well from membrane samples of HEK cells, similar to laboratory methods used previously (Booth et al., 2009). All binding experiments were performed using duplicates of samples, and each independent binding experiment was repeated a minimum of three times (with an average of six times). Briefly, 0.02 to 8 nM [^3H]-mesulergine was used for saturation binding assays to obtain K_D and B_{max} values, and 1–2 nM [^3H]-mesulergine (ca. K_D) was used for competition binding assays to obtain K_i values for unlabeled ligands. Mianserin hydrochloride (10 μM) was used to define non-specific binding. After a 90 min equilibration period at room temperature, incubation mixtures were rapidly passed through GF/B filters using a Mach 2 cell harvester (Tomtec, Hamden, CT) and subsequently washed five times with 50 mM Tris-HCl at room temperature. Filters containing bound [^3H]-mesulergine were dried, placed in vials containing 2 mL scintillation cocktail (ScintiVerse, Fisher), allowed to equilibrate overnight, and then were counted for ^3H -induced scintillation using a Beckman-Coulter LS6500 counter.

2.4 Phosphoinositide hydrolysis assay

HEK cells grown to approximately 80% confluency in DMEM containing 10% fetal bovine serum and 1% antibiotic in 10 cm plates at 37°C, 5% CO_2 (incubator) were washed one time with PBS, then transfected with 20 μg 5-HT $_2\text{C}$ pcDNA (5-HT $_2\text{C}$ -INI, 5-HT $_2\text{C}$ -INI S3.36A, or 5-HT $_2\text{C}$ -INI Y7.43A), and 30 μl lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) in 5 mL DMEM containing 5% dialyzed fetal bovine serum and 5 mL Opti-MEM (transfection media). Cells were placed in an incubator, and approximately 16 hr. later, transfection media was removed and replaced with 16 mL inositol-free DMEM containing 2.5% dialyzed fetal bovine serum. Cells were then detached by vigorous pipetting, 0.1 $\mu\text{Ci/mL}$ [^3H]-myo-inositol was added to the mixture (labeling media), and cells were seeded 300 μl per well into 48 well CellBind® plates (Corning, Lowell, MA), and placed in an incubator. 24 hr. later, plates were centrifuged at 2500 r.p.m. for 10 min. at room temperature, labeling media was discarded, and 450 μL inositol-free, serum-free DMEM was added to each well. Cells were placed in an incubator for one hour. Cells were then incubated for 30 min. with increasing concentrations of compounds (1 nM to 10 μM), 5-HT, $\text{N}(\text{CH}_3)_2\text{-PAT}$, or mesulergine, diluted in inositol-free, serum-free DMEM containing a final concentration of 50 mM LiCl and 10 μM pargyline per well. Plates were again centrifuged at 2500 r.p.m. for 10 min. at room temperature, drug incubation media was discarded, and 400 μl of 50 mM formic acid was added to each well to lyse cells. one hour later, 200 μl of 150 mM NH_4OH was added to each well to neutralize cells, and plates were stored at -20°C overnight. After thawing, 500 μl of solution from each well was added to individual anion-exchange columns to separate [^3H]-inositol phosphates formed from [^3H]-myo-inositol. Following a 10 mL wash with dH_2O , bound [^3H]-inositol phosphates were eluted with 4 mL 800 mM ammonium formate into vials. 1 mL of eluate was added to 10 mL scintillation fluid (ScintiVerse Cocktail, Fisher). After mixing, ^3H -induced scintillations were counted with a Beckman-Coulter LS6500 counter. Experiments were performed with duplicates for 5-HT and triplicates for $\text{N}(\text{CH}_3)_2\text{-PAT}$ and mesulergine, and each independent experiment was performed a minimum of three times.

2.5 Statistical analysis

Saturation and competition, radioligand binding data were analyzed using nonlinear regression curve-fitting algorithms in GraphPad Prism, 5.03 for Windows (San Diego, CA). Data points were limited (six to eight points per experiment), thus Hill slopes were not calculated (Motulsky and Christopoulos, 2003); data were fit using the “one site fit- K_i ” model that constrains the Hill slope to 1.0. Two-site curve-fitting did not result in an

improved fit (data not shown). Ligand affinity is expressed as an approximation of K_i values by conversion of the IC_{50} data using the equation $K_i = IC_{50}/(1 + L/K_D)$ where L is the concentration of radioligand (Cheng and Prusoff, 1973). K_D values for [3H]-mesulergine at 5-HT_{2C} WT, S3.36A and Y7.43A receptors determined experimentally were used in calculating K_i values of cold ligands tested. Analysis of variance tests were performed for each ligand, comparing K_i or K_D (affinity), EC_{50} or IC_{50} (potency), and E_{MAX} or I_{MAX} (efficacy) values obtained at WT 5-HT_{2C}, S3.36A and Y7.43A receptors. Tukey's multiple comparison post-hoc tests were performed when one-way analysis of variance tests resulted in P values less than 0.05, and tested for statistical differences in affinities, potencies, or efficacies at WT compared to S3.36A, WT compared to Y7.43A, and S3.36A compared to Y7.43A 5-HT_{2C} receptors.

2.6 Homology modeling

The 3-D molecular models of the human WT and D3.32A, S3.36A, and Y7.43A point-mutated 5-HT_{2C} receptor were constructed by homology modeling based on the crystal structure of the human β_2 adrenergic receptor/T4-lysozyme chimera (Protein Data bank entry 2rh1) (Rasmussen et al., 2007). The 5-HT_{2C} WT native sequence was aligned to the β_2 adrenergic receptor sequence using Clustal W multiple sequence alignment (Larkin et al., 2007). The transmembrane domains (without T4L) were built using the Biopolymer module of Sybyl 8.1 (Tripos, St. Louis, MO), and point-mutations, additions and deletions were performed as needed according to the native amino acid sequence of the receptor after alignment. The inverse agonist carazolol, present in the crystal structure of the human β_2 adrenergic receptor (2rh1) was deleted, and the resulting seven transmembrane domain bundle was optimized using Tripos force field and atomic charges. Then, the 5-HT_{2C}N-terminus, C-terminus, extracellular and intracellular loop residues were added. The conserved bond formed between the cysteine amino acids C3.25 (at the end of transmembrane domain3) and C6.31 (in extracellular loop 3) present in the β_2 adrenergic receptor structure was included in the 5-HT_{2C} model. Water molecules present in the β_2 adrenergic receptor structure were not incorporated in the 5-HT_{2C} receptor model.

The crude models were minimized using the Powell method implemented in Sybyl with Tripos force field and atomic charges. The resulting model was inserted into a rectangular box containing a pre-equilibrated 1-palmitoyl-2-oleyl-*sn*-glycero phosphatidyl choline (POPC) bilayer (Heller, 1993), consisting of 200 lipid molecules, forming a rectangular patch, and 5,483 water molecules covering the head groups on each side of the bilayer. The system containing the 5-HT_{2C} WT receptor model within the simulated membrane contained 37,775 atoms. The system was minimized initially using the Tripos force field to a convergence 0.05 Kcal/Å mol prior to molecular dynamics simulation in the PoPC membrane for 5000 ps, time step 1 fs, with snapshots collected every 5 fs. Run parameters used the NVT canonical ensemble, 300K temperature, Boltzmann initial velocities, with non-bonded cutoff set at 8 Å. Constraints for alpha carbons in the transmembrane domain were used. Finally the constraints were removed in 1000 ps runs, and the final structure was obtained from the average of the last 10 ps of the simulation, optimized using the Tripos force field to a convergence of 0.05 Kcal/Åmol.

2.7 Ligand docking and molecular dynamics simulations

Flexible ligand docking was performed with the FlexiDock utility in Sybyl 8.1 that uses an algorithm to probe the conformational space defining possible interactions between the ligand and its putative binding site. Ligand structures were built as monocations using HyperChem 8.0.8 and optimized using a PM3 model. The binding site was defined using residue D3.32 as definitive binding site interaction, and residues within a 7 Å radius (Kristiansen and Dahl, 1996; Kristiansen et al., 2000). Structures were prepared assigning

amber atom types to both the receptor and the ligands. Amber charges were used for the protein and Gesteiger-Marsili charges for the ligand. The ligand was pre-positioned into the putative binding site, and the position of the ligand was refined prior to docking to ensure the interaction between the carboxylate oxygen at D3.32 and the protonated amine of the ligand, an interaction necessary for protonated amine ligands to bind to G protein-coupled receptors (Kristiansen and Dahl, 1996; Kristiansen et al., 2000). All rotatable bonds in the ligand and the side chains of the residues defining the active site were screened for optimal positioning of the ligand and the side chains in the conformational space. Default FlexiDock parameters were set at 80,000-generation, and the best docking solution, according to the highest FlexiDock score, was minimized using the Amber force field.

Using the selected high-score pose of the docked ligand, molecular dynamics was performed. Simulation times were 500 ps, with snapshots collected every 5 fs. Run parameters used the NVT canonical ensemble, 300K temperature, Boltzmann initial and scaling velocities, with non-bonded cutoff set at 8 Å. The final structure of the ligand docked into the receptor was obtained from the last 10 ps of the dynamics simulation and optimized as described above.

2.8 Quantum mechanics model

Partial ligand–receptor models for the two poses of 5-HT in the 5-HT_{2C} binding pocket were built for quantum mechanics calculations. These models utilized the 5-HT_{2C} binding pocket–ligand structure extracted from the full receptor models docked with one or the other of the two 5-HT poses. Missing valences of amino acids resulting from breaking of peptide bonds during the extraction process were restored by adding the acetyl (COCH₃) moiety to the N-terminus and the methyl amino (NHCH₃) moiety to the C terminus. The structures were optimized using the semi-empirical PM3 Hamiltonian model to a gradient of 0.01 Kcal/mol to obtain the final energies, constraining the alpha-carbon atoms of the residues to preserve the configuration of the binding pocket.

3. Results

3.1 Affinity of ligands at S3.36A and Y7.43A point-mutated 5-HT_{2C} receptors

Table 1 shows the mean and S.E.M. K_D values derived from saturation binding isotherms of [³H]-mesulergine labeled WT, S3.36A and Y7.43A 5-HT_{2C} receptors. No specific binding of [³H]-mesulergine to the D3.32A point-mutated 5-HT_{2C} receptor was detected (not shown), consistent with results previously reported (Muntasir et al., 2006). Affinity of [³H]-mesulergine at the WT, S3.36A and Y7.43A point-mutated receptors was not significantly different (mean (S.E.M.) K_D values = 1.8 (0.3) WT, 2.0 (0.4) S3.36A, and 3.0 (0.6) Y7.43A; $F(2, 10)=2.14$, $P = 0.18$). The B_{max} values varied across experiments, and generally were 2–4 times higher for WT versus the point-mutated 5-HT_{2C} receptors. The highest B_{max} values for WT, S3.36A and Y7.43A receptors were 16.7, 7.6 and 2.6 pmol/mg of protein, respectively.

Table 2 shows the mean and S.E.M. of K_i values, as well as fold-differences in K_i values (mean K_i at the point-mutated receptor divided by the mean K_i at the WT receptor) derived from test ligand competitive inhibition of [³H]-mesulergine binding at WT, S3.36A and Y7.43A point-mutated 5-HT_{2C} receptors expressed in HEK cells. The affinity of the primary amine 5-HT for the receptors was significantly different ($F(2, 10) = 23.8$, $P < 0.001$). The affinity of 5-HT was significantly less, approximately 13- and 17-times lower, at S3.36A and Y7.43A point-mutated 5-HT_{2C} receptors, respectively, versus the WT receptor (post-hoc P values < 0.05). The affinity of tryptamine was also significantly different at WT and point-mutated receptors ($F(2, 12) 133.6$, $P < 0.001$). The affinity of tryptamine was

significantly less, approximately 30- and 5-times lower, at S3.36A and Y7.43A receptors, respectively, versus the WT receptor (post-hoc P values < 0.05). In contrast, affinity of the tertiary amine lisuride at the S3.36A receptor was not significantly different from its affinity at the WT receptor (post-hoc P value > 0.05), but the affinity of lisuride was slightly, but significantly increased at the Y7.43A point-mutated receptor (post-hoc P values < 0.05) relative to the WT receptor ($F(2, 12) = 11.2, P < 0.05$). Representative competition binding curves for 5-HT, tryptamine and lisuride are shown in Figs. 2, 3 and 4.

Regarding the PAT analogs, relative to WT 5-HT_{2C} receptors, the affinity of the tertiary amine N(CH₃)₂-PAT was significantly lower at both the S3.36A (post-hoc P value < 0.05 , about 3-times lower) and the Y7.43A (post-hoc P values < 0.05 , about 5-times lower) point-mutated receptors ($F(2, 21) = 35.3, P < 0.001$). Affinity of the secondary amine NH(CH₃)-PAT was also significantly different at WT and point-mutated receptors ($F(2, 29) = 41.4, P < 0.001$), having about 4- and 3-times lower affinity for the S3.36A and Y7.43A receptors, respectively (post-hoc P values < 0.05). Finally, the primary amine NH₂-PAT showed significant differences in affinity at WT and point-mutated receptors ($F(2, 24) = 108.9, P < 0.001$), but the effects on affinity of the mutations were different relative to the tertiary and primary amine PAT compounds. NH₂-PAT demonstrated a significant reduction in affinity for the S3.36A point-mutated receptor relative to the WT receptor (post-hoc P value < 0.05 , about 6-times lower). Affinity of NH₂-PAT, however, was not affected by the Y7.43A mutation (post-hoc P value > 0.05). Representative competition binding curves for the PATs are shown in Figs. 5–7.

3.2 Functional activity of ligands at S3.36A and Y7.43A point-mutated 5-HT_{2C} receptors

WT, S3.36A and Y7.43A 5-HT_{2C} receptors transiently expressed in HEK cells were examined for their ability to stimulate the phospholipase C signaling pathway by measuring the accumulation of [³H]-inositol phosphates from [³H]-myo-inositol following incubation of cells with either the primary amine agonist, 5-HT, the tertiary amine agonist, N(CH₃)₂-PAT, or the tertiary amine inverse agonist, mesulergine. Results from these studies are shown in Table 3, and include the calculated potency values, represented by half-maximal concentrations (EC₅₀ values for agonists and IC₅₀ values for inverse agonists), efficacy values, represented by maximal response levels (E_{MAX} values for agonists and I_{MAX} values for inverse agonists, representing percent change from basal counts per minute), as well as fold-differences in EC₅₀ or IC₅₀ values (the mean EC₅₀/IC₅₀ of a ligand at the point-mutated receptor divided by the mean EC₅₀/IC₅₀ of the ligand at the WT receptor) of ligands to alter 5-HT_{2C}-receptor mediated inositol phosphate production.

Results revealed that both the S3.36A and Y7.43A mutations led to significant decreases in the potency of 5-HT to stimulate production of [³H]-inositol phosphates (post-hoc P values < 0.05 ; $F(2,7) = 29.1, P < 0.001$). Paralleling the decrease in affinity of 5-HT at the point-mutated receptors, the potency of 5-HT at both point-mutated receptors was more than 25-times lower than its potency at the WT receptor. The potency of N(CH₃)₂-PAT to stimulate [³H]-inositol phosphate production also was significantly reduced at the point-mutated compared WT receptors (post-hoc P values < 0.05 ; $F(2,6) = 23.0, P < 0.002$). Like 5-HT, the potency of N(CH₃)₂-PAT at WT versus point-mutated receptors also paralleled its affinities for these receptors; relative to WT, both mutations led a greater than 8-fold reduction in potency of N(CH₃)₂-PAT. The potency of mesulergine was not significantly different between WT, S3.36A and Y7.43A receptors ($F(2,8) = 0.9, P = 0.45$), consistent with the lack of change in affinity of mesulergine at point-mutated compared to WT receptors.

The efficacy values of 5-HT were significantly different between receptors ($F(2,8) = 44.6, P < 0.001$). The E_{MAX} of 5-HT at the WT receptor compared to the Y7.43A point-mutated

receptor was not significantly different (post-hoc P value > 0.05). The E_{MAX} value of 5-HT at the S3.36A point-mutated receptor, however, was significantly increased (nearly than 5-times higher) compared to the WT receptor (post-hoc P value < 0.05). Notably, the absolute values for basal and maximally stimulated [3H]-inositol phosphate accumulation (in counts per minute) from WT receptors was ~ 2500 and 5000 , respectively, whereas, from S3.36A receptors, these values were ~ 800 and ~ 8000 (data not shown), suggesting the effect was not caused solely by reduced basal signaling of S3.36A receptors. The same trend was observed for $N(CH_3)_2$ -PAT. Its efficacy was not different between WT and Y7.43A receptors (post-hoc P value > 0.05). At the S3.36A receptor, however, $N(CH_3)_2$ -PAT had a significantly higher E_{MAX} value than at the WT receptor (post-hoc P value < 0.05 ; $F(2,6) = 81.0$, $P < 0.001$). This was also observed in absolute values (data not shown). Relative to 5-HT, $N(CH_3)_2$ -PAT behaved as a partial agonist at all three receptors. Mesulergine behaved as an inverse agonist at both the WT and Y7.43A receptors, decreasing basal signaling about 20 percent. At the S3.36A receptor, however, mesulergine behaved as a very low efficacy partial agonist, and this change from inverse agonism at WT and Y7.43A receptors to partial agonism at S3.36A receptors was statistically significant (E_{max} vs I_{max} values; post-hoc P values < 0.05 ; $F(2,9) = 75.1$).

3.3 Molecular models of ligand binding to WT and point-mutated 5-HT_{2C} receptors¹

3.3.1 5-HT and tryptamine—As shown in Fig. 8, ligand docking experiments revealed two interchangeable, low-energy poses for 5-HT in the putative binding pocket of the WT 5-HT_{2C} receptor. Additional analyses using quantum mechanics calculations (described in Methods) indicated that the energy difference between structures representing modeling poses 1 and 2 was only about 34 Kcal/mol, suggesting both poses were plausible. Residues in the binding pocket in close proximity to serotonin in pose 1 (Fig. 8A) were: D3.32, V3.33, S3.36, L5.33, D5.35, W6.48, F6.51, F6.52, N6.55, V7.39, and Y7.43. Residues in close proximity to 5-HT in pose 2 (Fig. 8B) were: W3.28, I3.29, L3.31, D3.32, S3.36, L5.33, M6.47, W6.48, C6.49, V7.39, W7.40, and Y7.43. In both poses, the protonated amine group of 5-HT apparently acted as a hydrogen bond donor to the carboxylate side chain of D3.32 (1.85 Å, pose 1 and 2), to the hydroxyl group of S3.36 (2.20 Å, pose 1, and 2.08 Å, pose 2), and to the hydroxyl moiety of Y7.43 (3.05 Å pose 1, and 3.02 Å, pose 2). Consistent with these computational results, experimental results showed that the S3.36A point-mutated 5-HT_{2C} receptor had a large reduction in affinity for 5-HT (Table 2). Also in both poses, binding of 5-HT facilitated a closer hydrogen bond interaction between the D3.32 carboxylate moiety and the Y7.43 *para*-hydroxy moiety (2.50 Å), whereas in the unbound (native) 5-HT_{2C} and β_2 adrenergic receptor models (not shown), the interaction between D3.32 and Y7.43 occurred over a longer distance (3.38 Å) and presumably was weaker.

There were important differences between the two 5-HT poses. In pose 1 (Fig. 8A), the 5-HT indole moiety positioned close to transmembrane domain 6, near the indole of W6.48 (4.30 Å) and between the aromatic rings in F6.51 (3.81 Å) and F6.52 (4.61 Å), possibly forming π - π interactions. The 5-hydroxy group was near to N6.55 (4.28 Å) and D5.35 (5.31 Å), likely forming electrostatic interactions, but too far for hydrogen bonding. Finally, in pose 1, there was no apparent interaction of the 5-hydroxy group with residues in transmembrane domains 3 and 7 (e.g., D3.32, S3.36, and Y7.43). In pose 2 (Fig. 8B) the configuration of 5-HT was flipped relative to pose 1. The 5-HT indole moiety was oriented closer to transmembrane domain 7, and significantly, the 5-hydroxy group was capable of forming a hydrogen bond with Y7.43 (2.46 Å). Experimental evidence indicated that loss of this interaction and loss of the 5-HT binding-induced D3.32-Y7.43 interaction (described

¹Molecular modeling figures are presented in color in the online version of this paper.

earlier) at the Y7.43A point-mutated 5-HT_{2C} receptor resulted in a large reduction in 5-HT affinity (Table 2).

Docking results for tryptamine interactions at the 5-HT_{2C} receptor also indicated two interchangeable, low-energy poses (Fig. 9). Like 5-HT, binding of tryptamine in both poses facilitated hydrogen bonding between the 5HT_{2C} receptor residues D3.32 and Y7.43 (2.85 Å in pose 1 and 2.09 Å in pose 2). Also in pose 1 (Fig. 9A), the tryptamine protonated amine group was capable of forming a hydrogen bond with D3.32 (2.03 Å) and S3.36 (2.18 Å) but was much further away from Y7.43 (5.53 Å) than was the case for the 5-HT amine moiety; consistent with this binding mode is the much larger reduction in affinity for tryptamine at the S3.36A (~30-times) compared to the Y7.43A (~5-times) point-mutated receptors (Table 2). In pose 2 (Fig. 9B), the primary amine moiety of tryptamine docked similarly as in pose 1, and although the position of the aromatic indole moiety is shifted, there are no hydrogen bonding possibilities for the 5-position as is the case for 5-HT in pose 2 (Fig. 8B), which may explain the difference in degree of affinity loss between 5-HT (about 17-times less) and tryptamine (about 5-times less) at the Y7.43A point-mutated receptor relative to the WT receptor.

3.3.2 Lisuride and mesulergine—Fig. 10 shows the calculated lisuride pose docked closely to transmembrane domains 3, 5 and 6 at the WT 5-HT_{2C} receptor. Similar to the binding of 5-HT, lisuride binding facilitated hydrogen bond formation between the 5-HT_{2C} Y7.43 and D3.32 residues (1.52 Å), but loss of this hydrogen bond possibility at the Y7.43A point-mutated receptor did not strongly affect the affinity of lisuride, which was slightly enhanced at this receptor (Table 2). The protonated tertiary amine group of lisuride acted as a hydrogen bond donor to 5-HT_{2C} D3.32 (1.56 Å), as was the case for the primary amine group of 5-HT. Unlike 5-HT, the lisuride amine moiety was too far from S3.36 (7.21 Å) and Y7.43 (3.95 Å) for significant hydrogen bonding interactions, consistent with experimental results indicating no change in affinity of lisuride at the S3.36A mutated receptor and only a minor change at the Y7.43A mutated receptor (Table 2). Although the phenyl ring of F6.52 is in a T-configuration for π - π stacking with respect to the lisuride indole system, the distance is 6.2 Å, preventing significant interactions. Overall, aromatic binding interactions between lisuride and 5-HT_{2C} residues W6.48, F6.51, and F6.52 were not apparent. Studies docking lisuride at the S3.36A 5-HT_{2C} and Y7.43A 5-HT_{2C} receptor models (not shown) indicated the location of the ligand was similar to the orientation at the WT receptor. A notable difference was that the sterically small alanine residue allowed for increased binding pocket space in the point-mutated receptors. This was more apparent for the Y7.43A receptor due to the greater size differential between tyrosine and alanine compared to serine and alanine, i.e. the S3.36A receptor. In the Y7.43A mutant, lisuride was able to form a hydrogen bond with the carbonyl oxygen to the amide side chain in N5.66; this interaction was not present in the WT receptor, potentially explaining the slightly enhanced affinity of lisuride observed at the Y7.43A.

Fig. 11 shows mesulergine docked closely to WT receptor transmembrane domains 3, 5 and 6, similar to 5-HT, tryptamine, and lisuride. In contrast to the 5-HT_{2C} agonists, 5-HT, tryptamine, and lisuride, the inverse agonist mesulergine did not facilitate hydrogen bond formation between the 5-HT_{2C} D3.32 and Y7.43 residues. In fact, binding of mesulergine increased the distance between these two residues (6.61 Å, compared to 3.38 Å in the unbound receptor). The protonated tertiary amine group of mesulergine apparently acted as a strong hydrogen bond donor to D3.32 (1.90 Å), consistent with experimental evidence that indicated no detectable binding of [³H]-mesulergine at the D3.32A point-mutated 5-HT_{2C} receptor. The distance between the mesulergine tertiary amine moiety and S3.36 (8.68 Å) was too large and also the orientation did not allow for hydrogen bonding, consistent with experimental results that indicated no change in K_D for [³H]-mesulergine at the S3.36A and

WT receptors (Table 1). The aromatic indole group of mesulergine oriented parallel to the indole side chain of W6.48 (3.4 Å), likely participating in forming π - π stacking interactions, however, it was relatively far away (5.40 Å) from Y7.43 and not in a suitable orientation for π - π or T-stacking interactions; experimental results indicated that the Y7.43A mutation did not significantly affect [³H]-mesulergine binding (Table 1).

3.3.3 Primary, secondary and tertiary amine PATs—Fig. 12 shows that the tertiary amine analog, 5-HT_{2C} agonist, N(CH₃)₂-PAT, docked at the WT receptor close to transmembrane domains 3, 6, and 7. Interestingly, binding of N(CH₃)₂-PAT did not alter the hydrogen bonding distance between Y7.43 and D3.32 (3.38 Å) that existed in the unbound 5-HT_{2C} receptor and the β_2 adrenergic receptor (not shown). This result contrasted with the binding of the other agonists (5-HT, tryptamine, and lisuride) that decreased the distance between these two residues, as well as, with the inverse agonist mesulergine that increased the distance.

The N(CH₃)₂-PAT protonated tertiary amine group appeared to form a tight hydrogen bond to D3.32 (1.84 Å), whereas weak hydrogen bonding was possible between the tertiary amine and S3.36 (3.27 Å) as well as Y7.43 (2.96 Å). The N(CH₃)₂-PAT tetrahydronaphthalene system aligned parallel to the aromatic ring of Y7.43, apparently forming π - π stacking binding interactions. These docking results were consistent with experimental results that indicated affinity of N(CH₃)₂-PAT was significantly diminished at the S3.36A and Y7.43A point-mutated 5-HT_{2C} receptors (Table 2). The N(CH₃)₂-PAT pendant phenyl moiety oriented close to the W6.48, F6.51, and F6.52 aromatic amino acids in transmembrane domain 6, presumably forming aromatic π - π stacking binding interactions. The configuration of N(CH₃)₂-PAT docked in the β_2 adrenergic receptor-based 5-HT_{2C} model has some differences compared to the previously reported N(CH₃)₂-PAT docked in Rho-based 5-HT_{2C} model (Booth, et al., 2009). The main difference is the location of the pendant phenyl of N(CH₃)₂-PAT in the pocket; in contrast to the results described above for the N(CH₃)₂-PAT docked in β_2 adrenergic receptor-based 5-HT_{2C} model, in the Rho-based 5-HT_{2C} model, the location of the PAT pendant phenyl moiety does not allow for interactions with W6.48, F6.51, and F6.52.

Fig. 13 shows that the secondary amine analog NH(CH₃)-PAT bound similar to N(CH₃)₂-PAT, near transmembrane domains 3, 6, and 7, and formed a strong hydrogen bond to D3.32 (1.97 Å). Binding of NH(CH₃)-PAT appeared to facilitate weak hydrogen bond interactions with 5-HT_{2C} residues S3.36 (2.84 Å) and also with Y7.43 (2.78 Å). Furthermore, NH(CH₃)-PAT binding interactions between the tetrahydronaphthalene system and pendant phenyl moieties and nearby 5-HT_{2C} residues were similar to N(CH₃)₂-PAT. Experimental results indicated a significant loss of affinity of NH(CH₃)-PAT at both the Y7.43A and S3.36A point-mutated receptors (Table 2), consistent with hydrogen bonding interactions between these residues and the PAT amine moieties.

Fig. 14 shows the primary amine analog NH₂-PAT bound close to transmembrane domains 3, 6, and 7. In particular, NH₂-PAT formed a hydrogen bond with D3.32 (1.93 Å). Moreover, similar to the secondary and tertiary PATs, the pendant phenyl of NH₂-PAT was in close proximity to W6.48. However, there was a difference in the overall position of NH₂-PAT in the binding pocket compared to NH(CH₃)-PAT and N(CH₃)₂-PAT that can be attributed to its lack of amine methyl substituents. Relative to NH₂-PAT, the methyl groups on NH(CH₃)-PAT and N(CH₃)₂-PAT increase amine basicity, likely enhancing electronic interactions with binding pocket residues to produce higher affinity (Table 2). Furthermore, there were weak interactions between the amine moiety of NH₂-PAT and Y7.43 (3.70 Å), and the tetrahydronaphthalene moiety was not in a position suitable for π - π stacking interactions with the Y7.43 aromatic ring. Thus, mutation of the 5-HT_{2C} Y7.43 residue to

alanine did not affect NH₂-PAT affinity (Table 2, Fig. 6). Meanwhile, the amine of NH₂-PAT could form a hydrogen bond with S3.36 (2.62 Å), and the S3.36A point-mutated receptor showed about 6-times lower affinity for the ligand compared to the WT receptor (Table 2, Fig. 6). These data suggest that the hydrogen bond between the PAT primary amine moiety and S3.36 assumes added importance given the absence of compensatory π - π stacking between the NH₂-PAT tetrahydronaphthalene moiety and Y7.43. Compared to 5-HT and tryptamine, however, NH₂-PAT binds farther from the 5-HT_{2C} S3.36 residue, and accordingly, its affinity was reduced to a lesser extent when S3.36 was mutated to alanine (Table 2).

4. Discussion

Measurements of ligand affinity, potency, and efficacy at WT compared to point-mutated 5-HT_{2C} receptors, coupled with computational experiments and molecular modeling based on homology to the human β_2 adrenergic receptor structure, indicated an important role for hydrogen bonding in ligand amine moiety interactions at the 5-HT_{2C-INI} receptor. The molecular rigidity of the PAT chemical scaffold provided a known 3-dimensional arrangement of the aromatic ethylamine pharmacophore moiety to delineate the impact of amine substitution on interactions with the 5-HT_{2C} receptor. This approach avoided the confounding entropy factors inherent in highly flexible tryptamine analogs that have been used in corresponding studies of the 5-HT_{2A} receptor (Almaula et al., 1996; Ebersole et al., 2003). When the 5-HT_{2C} S3.36 residue was mutated to alanine (S3.36A), affinity of the primary amine NH₂-PAT (as well as the primary amines, 5-HT and tryptamine) was reduced to a greater extent than the secondary amine NH(CH₃)-PAT, that in turn was reduced more than the tertiary amine N(CH₃)₂-PAT. Similarly, affinity of the tertiary amines lisuride and mesulergine were not reduced by the S3.36A mutation. The potencies for ligand modulation of 5-HT_{2C} receptor-mediated phospholipase C signaling also were consistent with the corresponding ligand affinities at WT and S3.36A receptors, i.e., the potency of 5-HT was reduced more than N(CH₃)₂-PAT, yet the potency of mesulergine was unaffected by the mutation. Modeling and computational results indicated that in comparison to the secondary and tertiary amine ligands, primary amine ligands interact closely with the S3.36 residue.

Modeling results showed the methyl groups of the tertiary amine, N(CH₃)₂-PAT, form pi-pi interactions with the Y7.43 residue, which were lost when methyl groups were not present. Experimentally, N(CH₃)₂-PAT showed a greater loss in affinity relative to NH(CH₃)-PAT at the Y7.43A point-mutated receptor, and NH₂-PAT was unaffected by the mutation. Furthermore, the effect of the Y7.43A mutation on ligand affinity was consistent with its effect on ligand potency. Notably, tertiary amine substitution on the PAT scaffold provides enhanced basicity and increased electronic interactions with binding pocket residues that account for the higher affinity of N(CH₃)₂-PAT at the WT 5-HT_{2C} receptor relative to the secondary and primary amines. Apparently, the increase in overall receptor binding interactions that accompanies increased basicity of tertiary amines can compensate for loss of hydrogen bonding between a primary amine and certain residues.

To our knowledge, this is the first report that utilized both mutagenesis experiments and molecular modeling based on the human β_2 adrenergic receptor structure to estimate where the 5-hydroxy group of 5-HT binds at the human WT 5-HT_{2C} receptor. Results indicated the 5-hydroxy group of 5-HT forms a hydrogen bond with the 5-HT_{2C} receptor residue Y7.43. When this residue is mutated to alanine, 5-HT exhibits a much greater loss of affinity compared to tryptamine that lacks the 5-hydroxy group. In contrast, a computational study based on the structure of bovine rhodopsin suggested that the 5-hydroxy group of 5-HT interacts with 5-HT_{2C} residues S3.39 and W6.48 (Bray and Goddard, 2008). There are some differences in the overall structure of the β_2 adrenergic receptor-based 5-HT_{2C} model used

in the present studies relative to our previously reported rhodopsin-based 5-HT_{2C} homology model (Booth et al., 2009). For example, there was a more pronounced kink in transmembrane domain 5 near P5.50 in the rhodopsin-based model, and a stronger helix kink in transmembrane domain 6 near P6.50 in the present model, among other differences. These variations may account for the differences in the interactions of 5-HT at the 5-HT_{2C} receptor reported here relative to previous reports (Bray and Goddard, 2008). Comparative information for the 5-HT_{2A} receptor, from mutagenesis studies and modeling based on bovine rhodopsin, suggests that the 5-hydroxy group of 5-HT interacts with 5-HT_{2A} residues S5.43 and S5.46 (Braden and Nichols, 2007).

Although the 5-HT₂ receptor family shares approximately 80% transmembrane sequence identity and a primary signal transduction pathway involving G $\alpha_{q/11}$ proteins and activation of phospholipase C (Hoyer et al., 1994; Raymond et al., 2001), most 5-HT₂ ligands demonstrate differential 5-HT₂-subtype affinity and potency regarding 5-HT₂-mediated intracellular signaling (Egan et al., 2000; Knight et al., 2004; Porter et al., 1999). Even the small, flexible 5-HT molecule distinguishes between 5-HT_{2A} and 5-HT_{2C} receptors such that its affinity and potency to activate phospholipase C signaling is at least two-times higher at 5-HT_{2C-INI} compared to 5-HT_{2A} receptors (Egan et al., 2000; Knight et al., 2004; Porter et al., 1999). These differences likely result from unique binding pocket conformations stabilized by a given ligand. Thus, the putatively subtle, structural differences between 5-HT₂ receptor subtypes can be exploited to develop 5-HT₂ subtype-specific agonists.

Interestingly, the current data show that the S3.36A point-mutation alters the phospholipase C signaling properties of the 5-HT_{2C-INI} receptor. Notably, the efficacy of 5-HT, N(CH₃)₂-PAT, and mesulergine were not different at WT compared to Y7.43A receptors. At the S3.36A receptor, however, the efficacy of each ligand was potentiated in the direction of activation. Thus, the E_{MAX} values for the agonists 5-HT and N(CH₃)₂-PAT were greatly enhanced at the S3.36A receptor (relative to the WT), and the inverse agonist (at WT 5-HT_{2C}) mesulergine displayed low efficacy partial agonism at the S3.36A receptor. This phenomenon, at least for agonists at WT receptors, is not unusual for aminergic G protein-coupled receptors, including the 5-HT_{2A} receptor (Almaula et al., 1996). Speculatively, the S3.36A receptor may reveal an active conformation that is not possible in the WT receptor and that can be stabilized by mesulergine.

Mutagenesis and modeling approaches are useful for predicting G protein-coupled receptor ligand affinity, however, molecular determinants governing ligand binding that lead to stabilization of agonist vs. inverse agonist or neutral antagonist conformation states are poorly understood. Even recent studies that report the active state β_2 adrenergic receptor structure (Rasmussen et al., 2011; Rosenbaum et al., 2011) cannot predict unique functional outcomes that are dependent on the cellular milieu, as well as different receptor conformation(s) stabilized by different ligands. Indeed, a single G protein-coupled receptor can couple to multiple signaling pathways, and a ligand can modulate activity of each pathway with dissimilar potencies and efficacies (Kenakin, 1995; Urban et al., 2007). For example, it is known that 5-HT can activate 5-HT_{2C} receptors linked to phospholipase C activation, as well as, 5-HT_{2C} receptors linked to phospholipase A activation (Berg et al., 1999; Moya et al., 2007). The modeling results obtained here indicated that 5-HT can bind to the 5-HT_{2C} receptor in at least two configurations, leading to speculation that individual ligand poses may stabilize a receptor conformation favoring a particular signaling pathway, e.g. biased agonism, and that agonist ligands with fewer feasible (or restricted) docking poses may be more selective for activating particular pathways. Notably, in both poses, 5-HT docked differently than the other 5-HT_{2C} agonists tested, lisuride and N(CH₃)₂-PAT.

The current data support the hypothesis that different agonist ligands can stabilize different receptor binding pocket structures, suggesting a molecular basis for conformationally-selective receptor binding, activation, and type of signaling (Kenakin, 1995; Urban et al., 2007). $N(\text{CH}_3)_2$ -PAT may demonstrate a unique 5-HT_{2C} receptor functional profile compared to 5-HT and lisuride. $N(\text{CH}_3)_2$ -PAT is known to be a functionally-selective ligand at the histamine H₁ G protein-coupled receptor (Moniri et al., 2004) that is phylogenetically closely related to 5-HT_{2C} receptors (Smit et al., 1999). Thus, $N(\text{CH}_3)_2$ -PAT is an agonist with potency and efficacy similar to histamine regarding H₁-mediated activation of adenylyl cyclase, but it is an antagonist (and inverse agonist) regarding H₁-mediated activation of phospholipase C.

Finally, mesulergine, a WT 5-HT_{2C} receptor inverse agonist (Berg et al., 1999; Moya et al., 2007), stabilized a 5-HT_{2C} receptor binding pocket structure that was different from the conformation(s) stabilized by binding of the agonists 5-HT and lisuride. When mesulergine was docked to the WT 5-HT_{2C} receptor, the distance between the D3.32 and Y7.43 side chains was about 6 Å, too far for a hydrogen bond. When 5-HT or lisuride was docked to the 5-HT_{2C} receptor model, the distance between D3.32 and Y7.43 was about 2 Å, suggesting a strong hydrogen bond was formed. Thus, results here support the hypothesis that hydrogen bond formation between D3.32 and Y7.43 may be involved in 5-HT or lisuride-induced activation of the 5-HT_{2C} receptor. This hypothesis is consistent with recent results from mutations of the M(3) muscarinic acetylcholine receptor (Han et al., 2005) and structural results using an engineered activated state of the β₂ adrenergic receptor that showed activation is associated with inward movement of transmembrane domains 3 and 7 (Rasmussen et al., 2011; Rosenbaum et al., 2011).

In conclusion, drug discovery targeting 5-HT_{2C} receptors has centered on agonists that produce anorexigenic effects to treat obesity (Rowland et al., 2008; Smith et al., 2010). 5-HT_{2C} agonists with 5-HT_{2A} receptor and 5-HT_{2B} receptor inverse agonist properties, similar to $N(\text{CH}_3)_2$ -PAT, may prove to be suitable medicines as they may not cause hallucinations (Nichols, 2004) or cardiopulmonary toxicity (Fitzgerald et al., 2000; Launay et al., 2002; Rothman et al., 2000) associated with activation of 5-HT_{2A} and 5-HT_{2B} receptors, respectively. Further clarifications of ligand molecular interactions that determine the uncommon pharmacology of PAT at 5-HT₂ receptors will likely guide development of novel, clinically useful 5-HT_{2C}-specific ligands.

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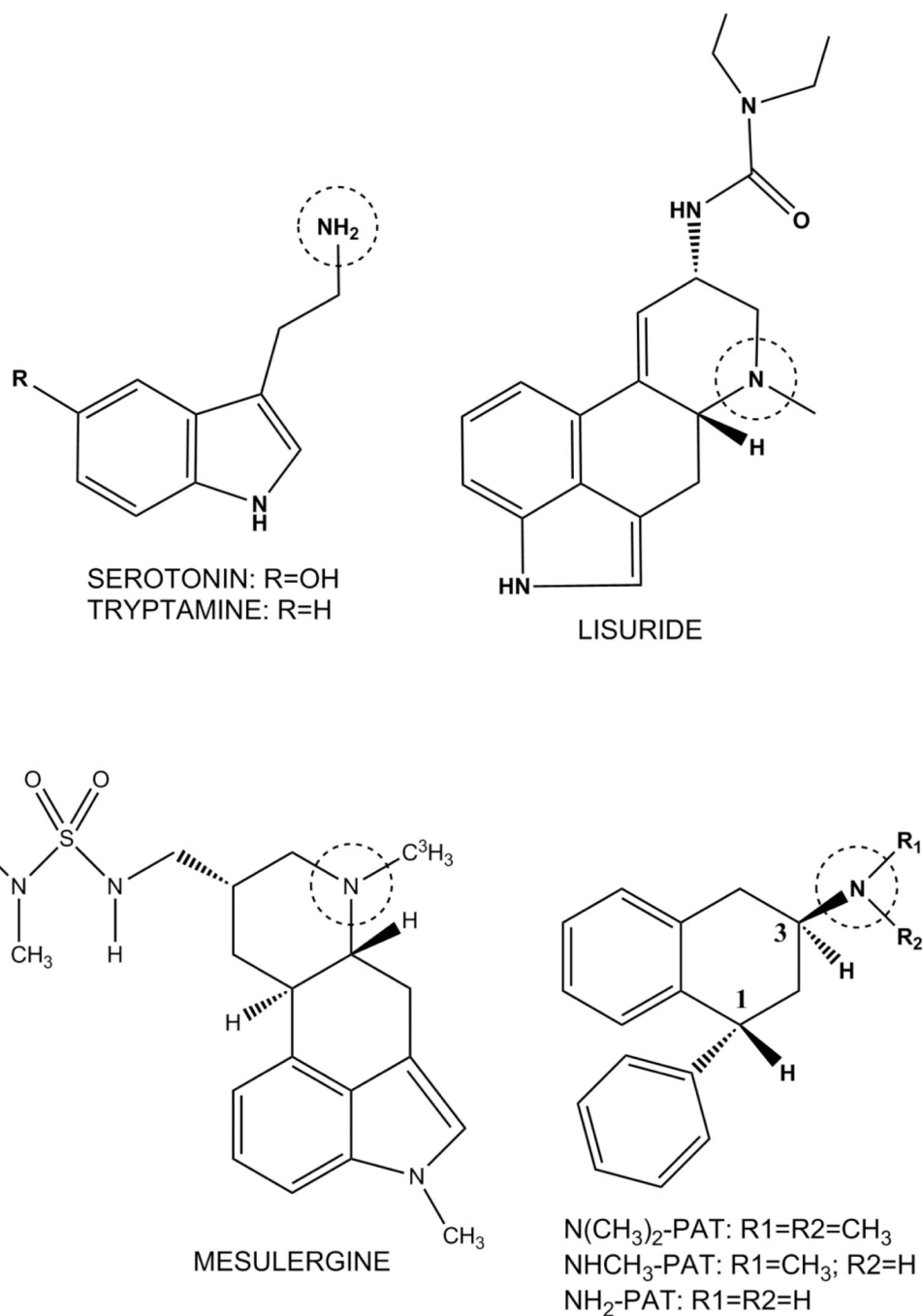


Fig. 1. Structures of test compounds. Circled on each compound is the basic nitrogen atom that bonds with the D3.32 residue of the 5-HT_{2C} receptor.

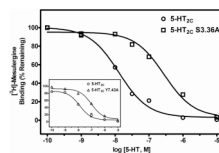


Fig. 2. Representative radioligand competition displacement curves for the primary amine 5-HT at WT vs. S3.36A and Y7.43A (inset) point-mutated 5-HT_{2C} receptors. Data show means and S.E.M. from an individual experiment of percent specific binding of [³H]-mesulergine. The curves for the point-mutated receptors are significantly ($P < 0.05$) shifted to the right compared to the WT receptor.

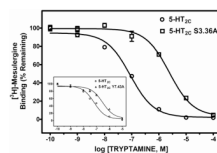


Fig. 3. Representative radioligand competition displacement curves for the primary amine tryptamine at WT vs. S3.36A and Y7.43A (inset) point-mutated 5-HT_{2C} receptors. Data show means and S.E.M. from an individual experiment of percent specific binding of [³H]-mesulergine. The curves for the point-mutated receptors are significantly ($P < 0.05$) shifted to the right compared to the WT receptor.

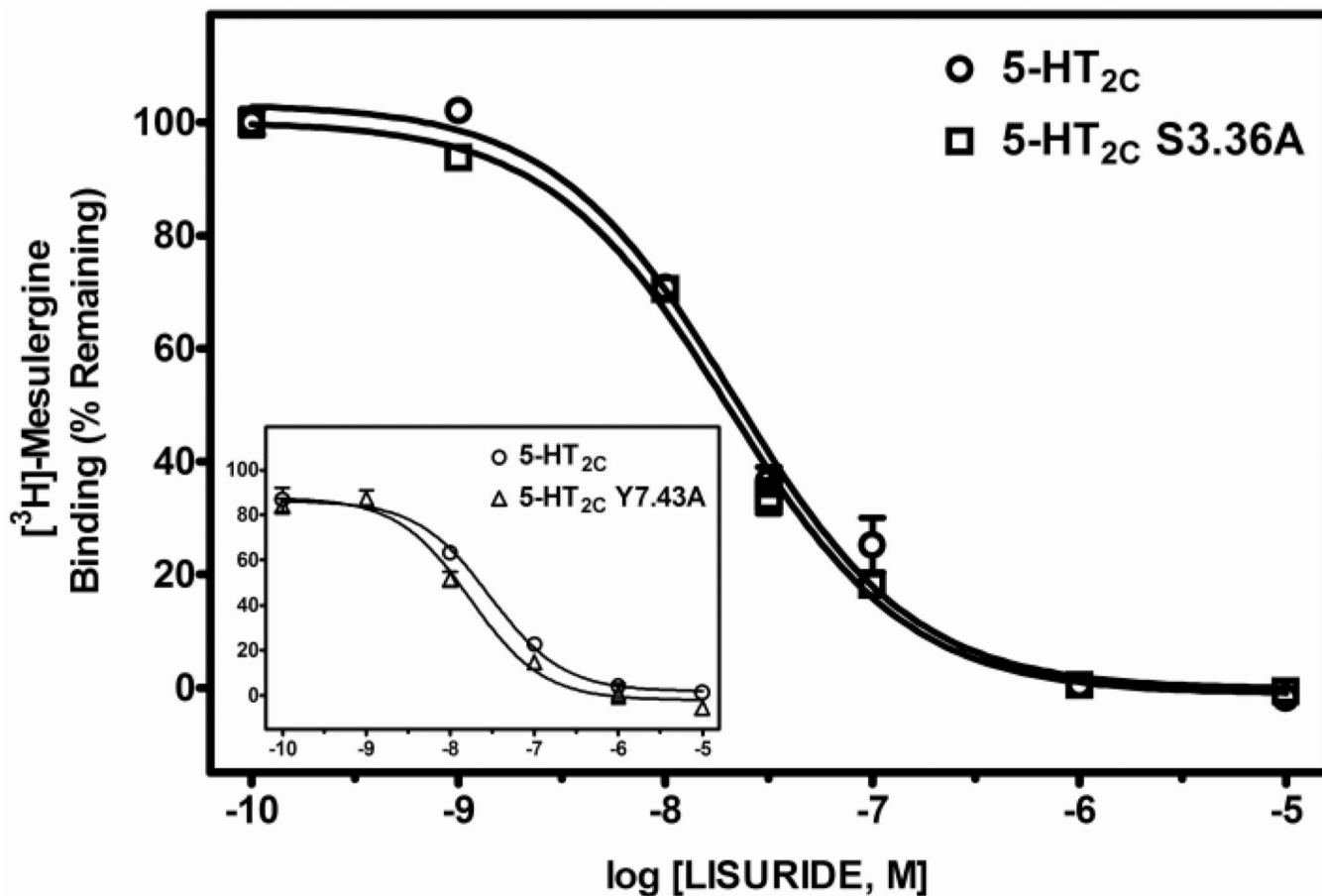


Fig. 4. Representative radioligand competition displacement curves for the tertiary amine lisuride at WT vs. S3.36A and Y7.43A (inset) point-mutated 5-HT_{2C} receptors. Data show means and S.E.M. from an individual experiment of percent specific binding of [³H]-mesulergine. There is no difference ($P = 0.12$) between the curves for the WT and S3.36A receptor. Lisuride, however, showed a slight, yet significant ($P < 0.05$) enhancement in affinity for the Y7.43A relative to the WT receptor.

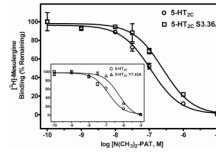


Fig. 5. Representative radioligand competition displacement curves for the tertiary amine $N(\text{CH}_3)_2$ -PAT at WT vs. S3.36A and Y7.43A (inset) point-mutated 5-HT_{2C} receptors. Data show means and S.E.M. from an individual experiment of percent specific binding of [³H]-mesulergine. The curves for the point-mutated receptors are significantly ($P < 0.05$) shifted to the right compared to the WT receptor.

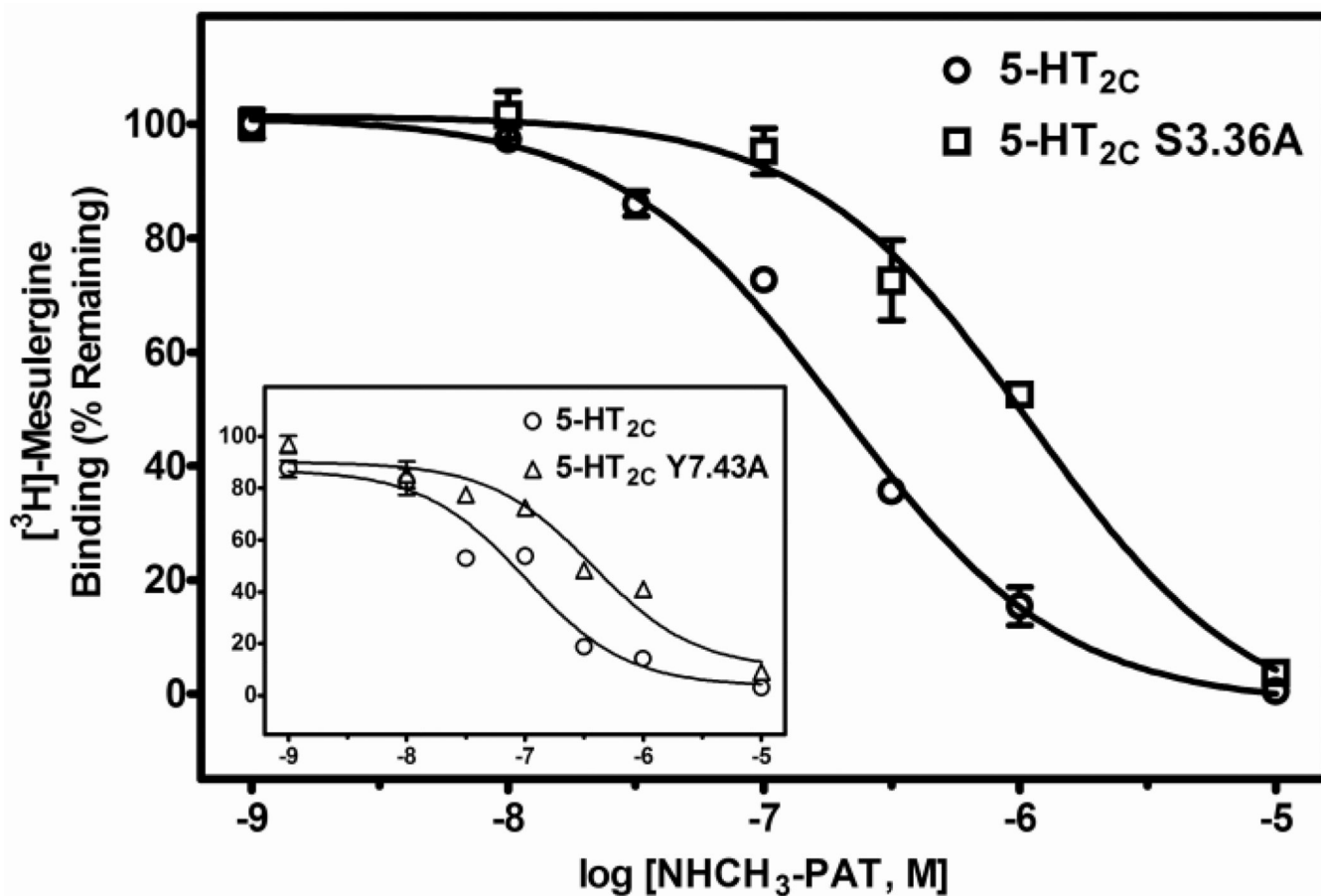


Fig. 6. Representative radioligand competition displacement curves for the secondary amine NH(CH₃)-PAT at WT vs. S3.36A and Y7.43A (inset) point-mutated 5-HT_{2C} receptors. Data show means and S.E.M. from an individual experiment of percent specific binding of [³H]-mesulergine. The curves for the point-mutated receptors are significantly ($P < 0.05$) shifted to the right compared to the WT receptor.

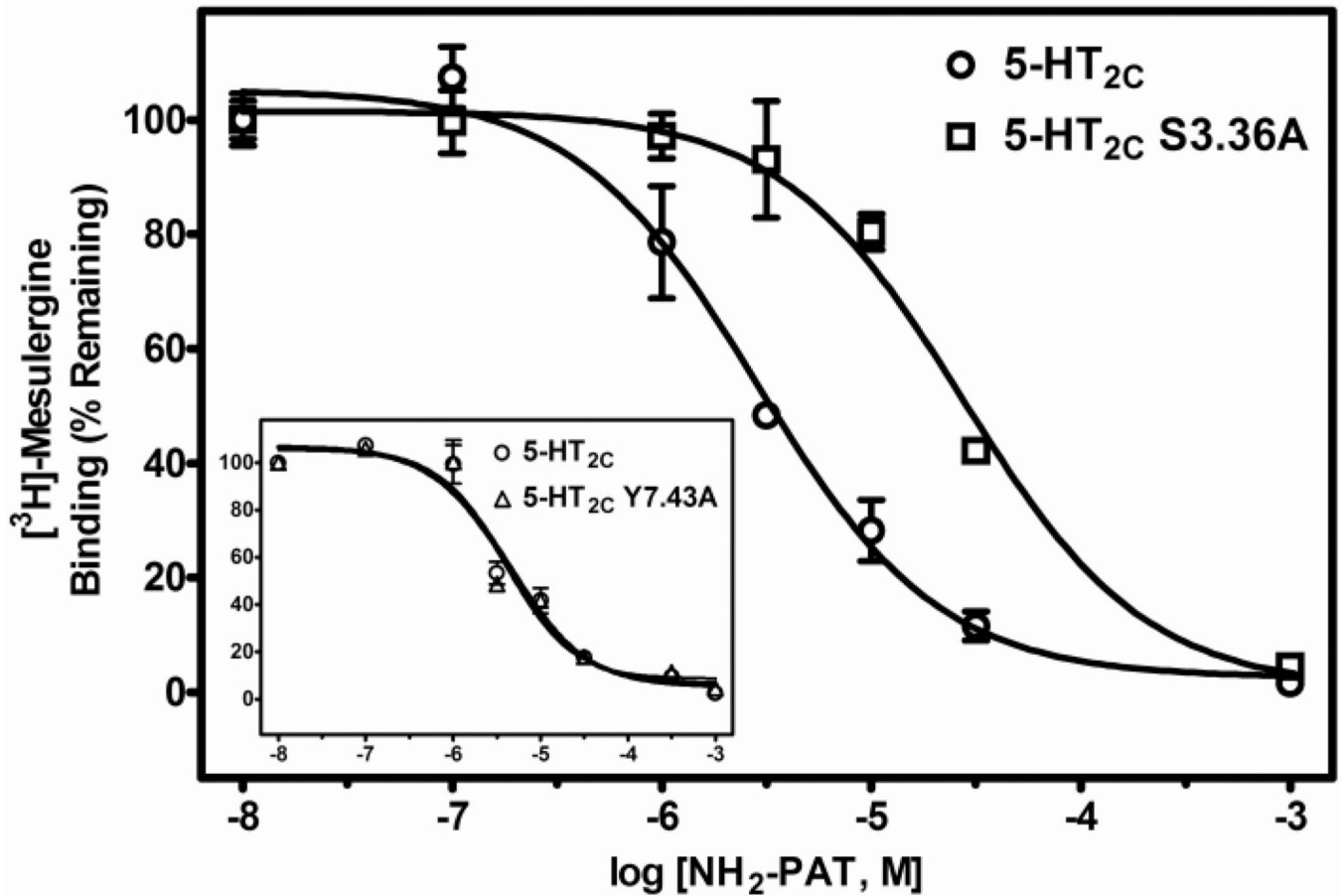


Fig. 7. Representative radioligand competition displacement curves for the primary amine NH₂-PAT at WT vs. S3.36A and Y7.43A (inset) point-mutated 5-HT_{2C} receptors. Data show means and S.E.M. from an individual experiment of percent specific binding of [³H]-mesulergine. The curve for the S3.36A receptor is significantly ($P < 0.05$) shifted to the right, whereas the curve for the Y7.43A receptor is not different compared to the WT receptor.

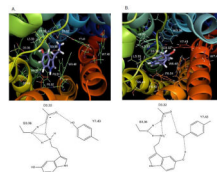


Fig. 8. 5-HT

A. Molecular model graphic pose 1 of 5-HT docked to the WT 5-HT_{2C} receptor and directly underneath, schematic of pose 1. B. Molecular model graphic pose 2 of 5-HT docked to the WT 5-HT_{2C} receptor and directly underneath, schematic of pose 2.

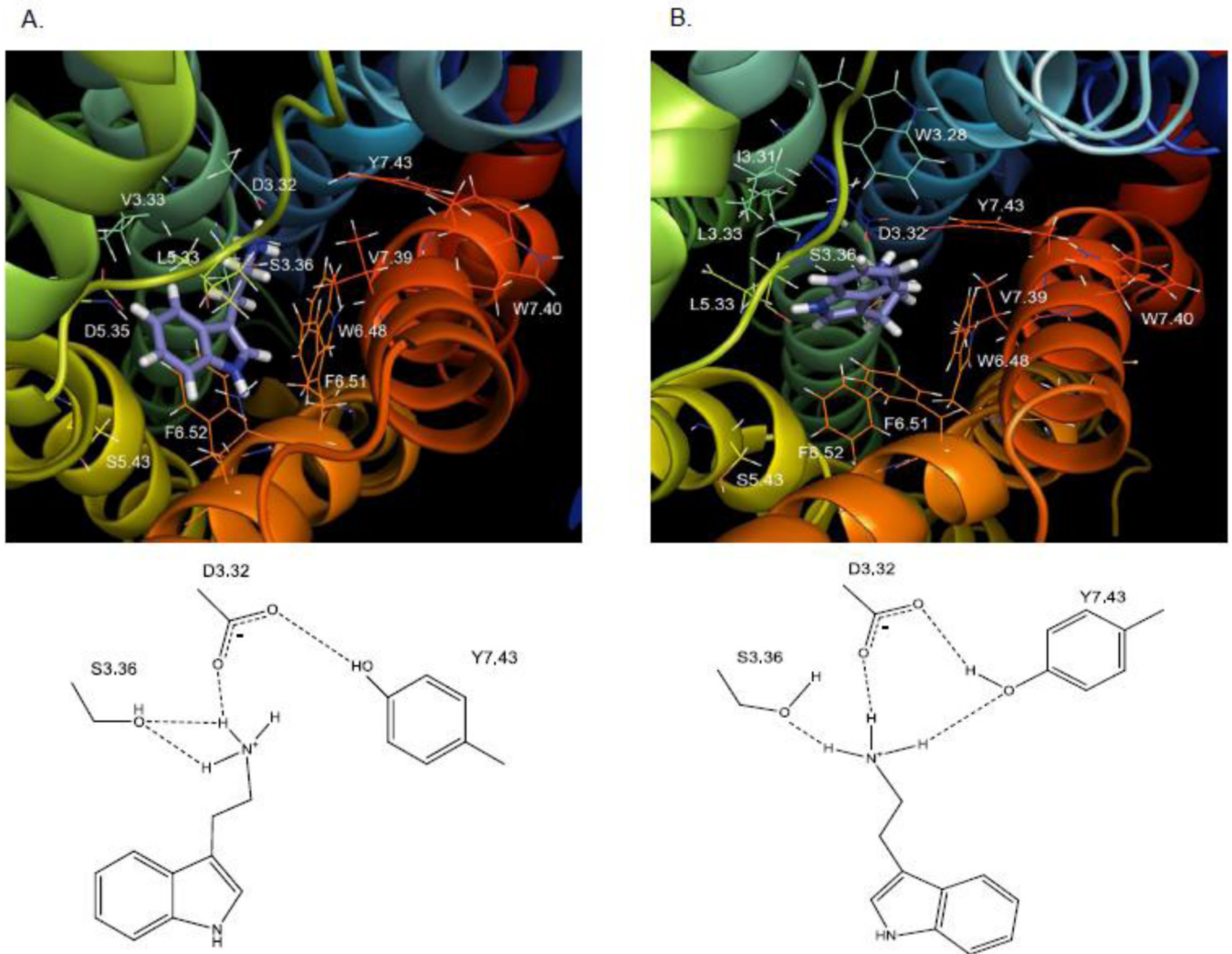


Fig. 9. Tryptamine

A. Molecular model graphic pose 1 of tryptamine docked to the WT 5-HT_{2C} receptor and directly underneath, schematic of pose 1. B. Molecular model graphic pose 2 of tryptamine docked to the WT 5-HT_{2C} receptor and directly underneath, schematic of pose 2.

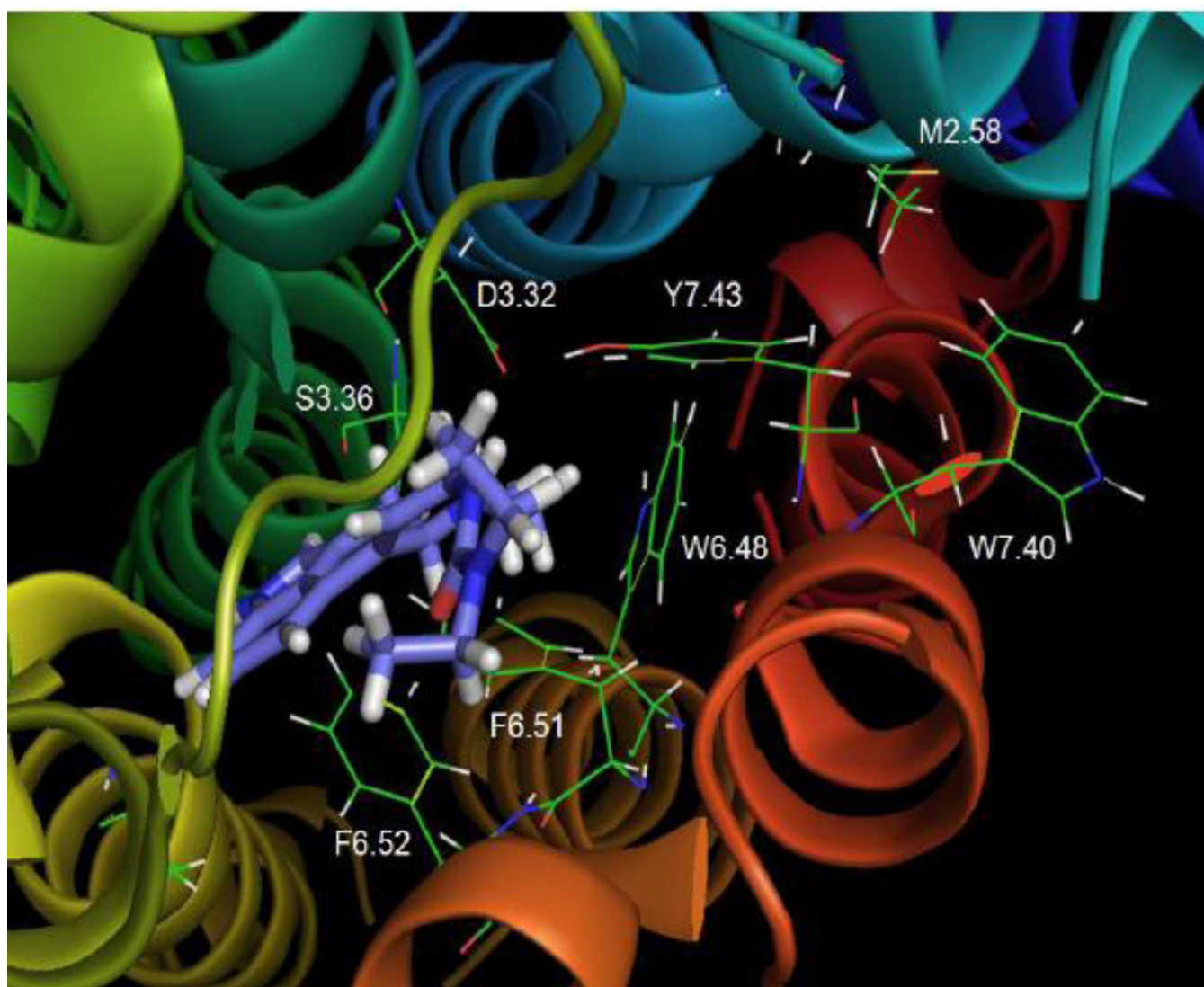


Fig. 10. Lisuride
Lisuride docked to the WT 5-HT_{2C} receptor.

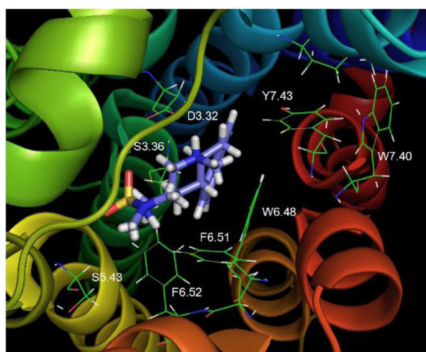


Fig. 11. Mesulergine
Mesulergine docked to the WT 5-HT_{2C} receptor

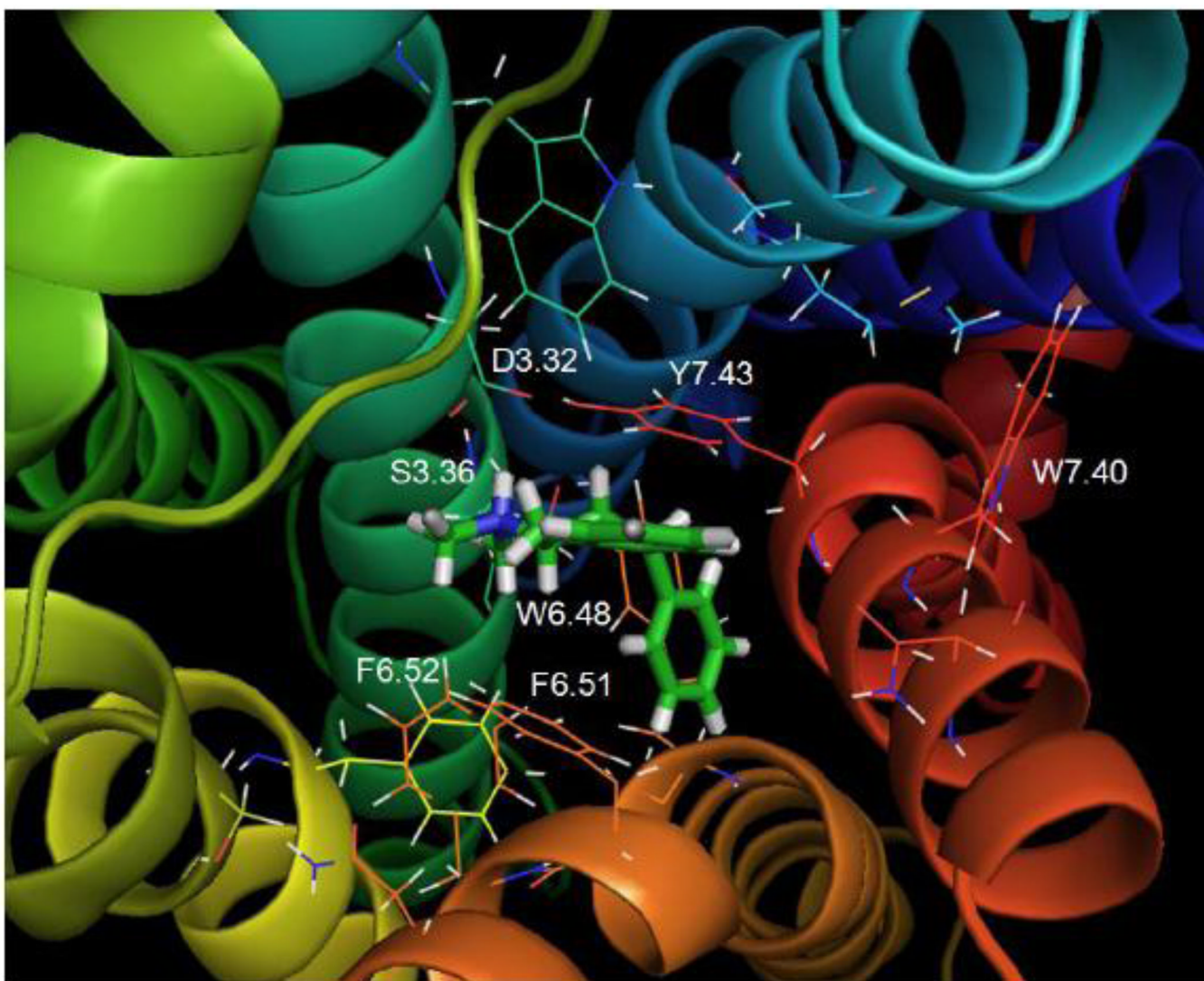


Fig. 12. N(CH₃)₂-PAT
N(CH₃)₂-PAT docked to the WT 5-HT_{2C} receptor

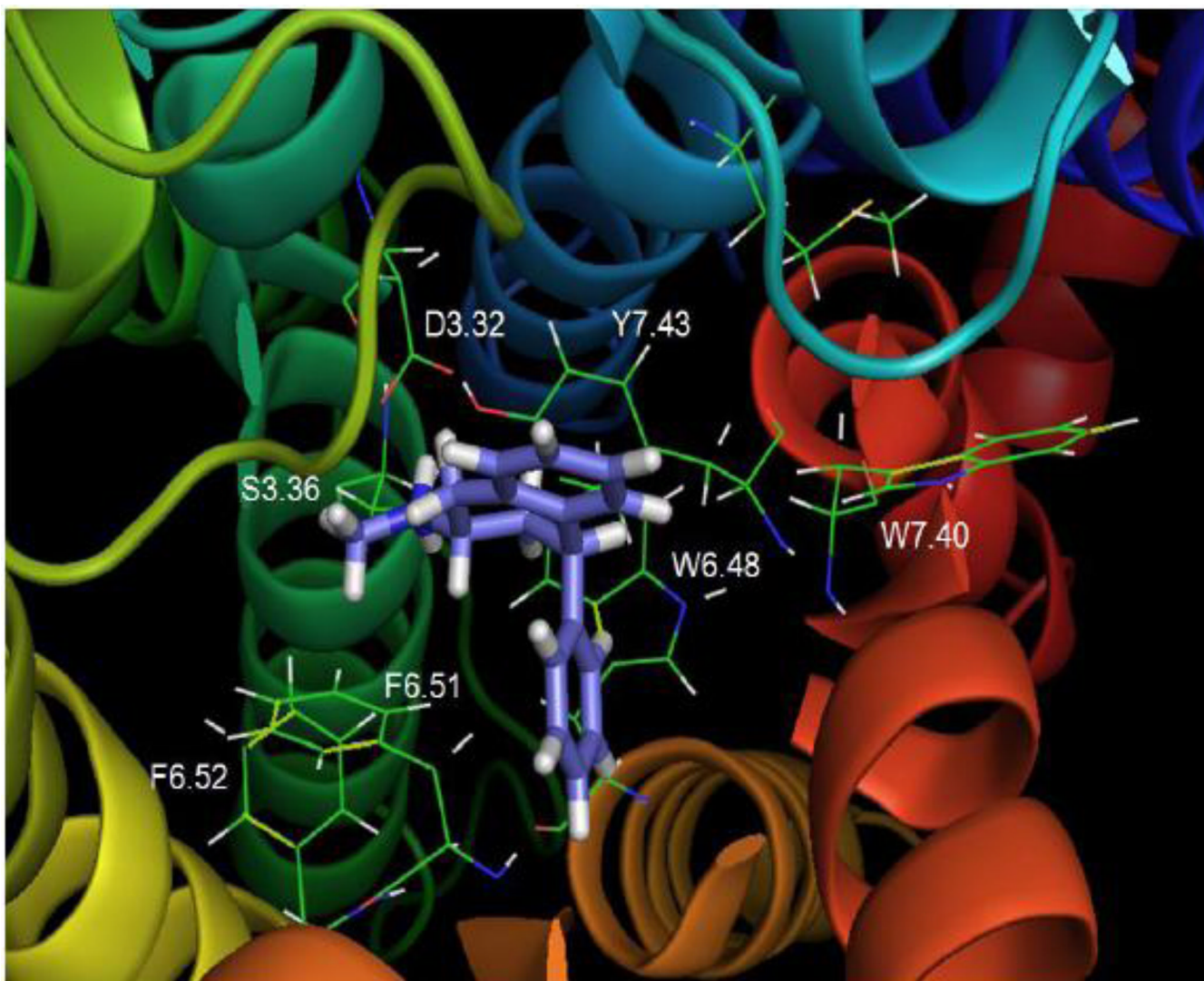


Fig. 13. NH(CH₃)-PAT
NH(CH₃)-PAT docked to the WT 5-HT_{2C} receptor

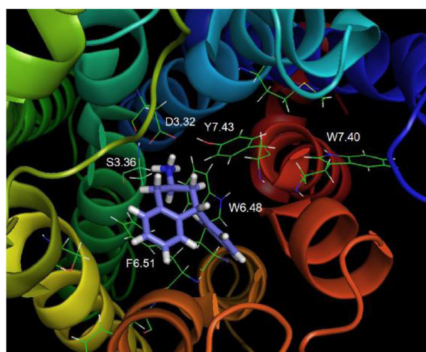


Fig. 14. NH₂-PAT
NH₂-PAT docked to the WT 5-HT_{2C} receptor.

Table 1

Affinity of [³H]-mesulergine for WT, S3.36A and Y7.43A 5-HT_{2C} receptors. *K_D* values were derived from saturation binding isotherms of [³H]-mesulergine at WT, S3.36A, and Y7.43A 5-HT_{2C} point-mutated receptors expressed in HEK cells. Data shown are means and S.E.M. (in parentheses) of at least 3 independent experiments performed with triplicates.

Receptor	[³ H]-Mesulergine
	<i>K_d</i> (nM)
WT 5-HT _{2C}	1.8 (0.3)
S3.36A	2.0 (0.4)
Y7.43A	3.0 (0.6)

Table 2

Affinities of ligands for human WT, S3.36A, and Y7.43A 5-HT_{2C} receptors. K_i values were derived from test ligand competitive inhibition of [³H]-mesulergine binding at the WT, S3.36A and Y7.43A point-mutated 5-HT_{2C} receptors expressed in HEK cells. Data shown are means and S.E.M. (shown in parentheses) of at least 3 independent experiments performed with duplicates. Fold change represents the quantitative fold difference between K_i values of WT and S3.36A or WT and Y7.43A 5-HT_{2C} receptors, respectively.

LIGAND	WT 5-HT _{2C}	S3.36A	Y7.43A	FOLD CHANGE
K_i (nM) K_i (nM)	K_i (nM)			
Serotonin	9.0 (1.6)	118.2 (21.7) ^a	152.0 (37.0) ^a	13.1, 16.9
Tryptamine	57.1 (7.1)	1513 (107.4) ^a	298.5 (72.8) ^a	29.5, 4.5
Lisuride	11.4 (0.6)	11.3 (0.4)	7.9 (0.5) ^a	1.0, 0.7
N(CH₃)₂-PAT	78.4 (9.6)	210 (21.9) ^a	354 (46.8) ^a	2.7, 4.5
NHCH₃-PAT	145.1 (16.7)	636.1 (69.6) ^a	357.3 (45.6) ^a	4.4, 2.5
NH₂-PAT	1555 (146.6)	9037 (820) ^a	1353 (170.3)	5.8, 0.9

^a significantly different from WT 5-HT_{2C}.

Table 3

EC/IC₅₀ and E/I_{MAX} values of ligands to activate/inactivate 5-HT_{2C} receptor-mediated phospholipase C signaling. Functional assays were performed in HEK cells transiently expressing WT, S3.36A or Y7.43A 5-HT_{2C} receptors. E_{MAX}/I_{MAX} values represent percent change from baseline. Data shown are means and S.E.M. (shown in parentheses) of at least 3 independent experiments performed with triplicates. EC/IC₅₀ fold change represents the quantitative fold difference between EC/IC₅₀ values of WT and S3.36A or WT and Y7.43A 5-HT_{2C} receptors, respectively.

LIGAND	WT 5-HT _{2C}		S3.36A		Y7.43A		FOLD CHANGE	
	EC/IC ₅₀ (nM)	E/I _{max}	EC/IC ₅₀ (nM)	E/I _{max}	EC/IC ₅₀ (nM)	E/I _{max}	EC/IC ₅₀	E/I _{max}
Serotonin	5.5 (1.3)	204.7 (11.6)	289.1 (40.4) ^a	998.4 (137.1) _b	158.7 (19.9) ^a	223.4 (22.7)	52.6, 28.9	
N(CH₃)₂-PAT	61.1 (18.2)	163.0 (9.7)	682.5 (102.5) ^a	588.0 (62.0) _b	519.7 (98.8) ^a	180.8 (14.2)	11.2, 8.5	
Mesulergine	23.9 (8.3)	82.9 (1.9)	19.9 (8.2)	152.0 (9.2) _b	38.9 (13.3)	79.3 (2.5)	0.8, 1.6	

^a significantly different from WT 5-HT_{2C}.