Molecular Pharmacology and Ligand Docking Studies Reveal a Single Amino Acid Difference between Mouse and Human Serotonin 5-HT_{2A} Receptors That Impacts Behavioral Translation of Novel 4-Phenyl-2-dimethylaminotetralin Ligands

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ABSTRACT

During translational studies to develop 4-phenyl-2-dimethylaminotetralin (PAT) compounds for neuropsychiatric disorders, the (2*R*,4*S*)-*trans*-(+)- and (2*S*,4*R*)-*trans*-(-)-enantiomers of the analog 6-hydroxy-7-chloro-PAT (6-OH-7-Cl-PAT) demonstrated unusual pharmacology at serotonin (5-HT) 5-HT₂ G protein-coupled receptors (GPCRs). The enantiomers had similar affinities (*K_i*) at human (h) 5-HT_{2A} receptors (~70 nM). In an in vivo mouse model of 5-HT_{2A} receptor activation [(±)-(2,5)-dimethoxy-4-iodoamphetamine (DOI)-elicited head twitch], however, (-)-6-OH-7-Cl-PAT was about 5-fold more potent than the (+)-enantiomer at attenuating the DOI-elicited response. It was discovered that (+)-6-OH-7-Cl-PAT (only) had ~40-fold-lower affinity at mouse (m) compared with h5-HT_{2A} receptors. Molecular modeling and computational ligand docking studies indicated that the 6-OH moiety of (+)- but not (-)-6-OH-7-Cl-PAT could form a hydrogen bond with serine residue 5.46 of the h5-HT_{2A} receptor. The m5-HT_{2A} as well as m5-HT_{2B}, h5-HT_{2B}, m5-HT_{2C}, and h5-HT_{2C} receptors have alanine at position 5.46, obviating this

interaction; (+)-6-OH-7-Cl-PAT also showed ~50-fold lower affinity than (-)-6-OH-7-Cl-PAT at m5-HT_{2C} and h5-HT_{2C} receptors. Mutagenesis studies confirmed that 5-HT_{2A} S5.46 is critical for (+)- but not (-)-6-OH-7-Cl-PAT binding, as well as function. The (+)-6-OH-7-Cl-PAT enantiomer showed partial agonist effects at h5-HT_{2A} wild-type (WT) and m5-HT_{2A} A5.46S point-mutated receptors but did not activate m5-HT_{2A} WT and h5-HT_{2A} S5.46A point-mutated receptors, or h5-HT_{2B}, h5-HT_{2C}, and m5-HT_{2C} receptors; (-)-6-OH-7-Cl-PAT did not activate any of the 5-HT₂ receptors. Experiments also included the (2*R*,4*S*)-*trans*-(+)- and (2*S*,4*R*)-*trans*-(-)-enantiomers of 6-methoxy-7-chloro-PAT to validate hydrogen bonding interactions proposed for the corresponding 6-OH analogs. Results indicate that PAT ligand three-dimensional structure impacts target receptor binding and translational outcomes, supporting the hypothesis that GPCR ligand structure governs orthosteric binding pocket molecular determinants and resulting pharmacology.

Introduction

Serotonin (5-HT) 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} G protein-coupled receptors (GPCRs) are pharmacotherapeutic targets of interest due in part to their involvement in several psychiatric disorders, as inferred from human genetic studies (Dracheva et al., 2008; Sun et al., 2008; Nichols, 2009; Thanseem et al., 2012). Drug discovery programs targeting

5-HT₂ receptors are focused on treating psychosis (5-HT_{2A} antagonists), sleep disorders (5-HT_{2A} antagonists), cluster headaches (5-HT₂ agonists), anxiety (5-HT_{2B/2C} antagonists), and obesity (5-HT_{2C} agonists) (Sewell et al., 2006; Abbas and Roth, 2008; Smith et al., 2010). In addition, compounds with combined 5-HT_{2C} agonist plus 5-HT_{2A/2B} antagonist activity, similar to certain *trans*-4-phenyl-2-dimethylaminotetralins (PATs), may be useful for treating substance abuse and other compulsive behavioral disorders (Booth et al., 2009; Canal et al., 2013; Cunningham et al., 2013; Higgins et al., 2013; Morgan et al., 2013).

The (±)-(2,5)-dimethoxy-4-iodoamphetamine (DOI)-elicited head-twitch response (HTR) in mice is an animal model that is

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ABBREVIATIONS: ANOVA, analysis of variance; β_2 AR, β_2 -adrenergic receptor; DMEM, Dulbecco's modified Eagle's medium; DOI, (±)-(2,5)-dimethoxy-4-iodoamphetamine; GPCR, G protein-coupled receptor; h5-HT_{2A}, human 5-HT_{2A} receptor; 5-HT, serotonin; HTR, head-twitch response; HTRF, homogeneous time-resolved fluorescence; m5-HT_{2A}, mouse 5-HT_{2A} receptor; MD, molecular dynamics; (+)-6-OH-7-Cl-PAT, (2*R*,4*S*)-*trans*-(+)-6-hydroxy-7-chloro-PAT; (-)-6-OH-7-Cl-PAT, (2*S*,4*R*)-*trans*-(-)-6-hydroxy-7-chloro-PAT; (+)-6-OMe-7-Cl-PAT, (2*R*,4*S*)-*trans*-(+)-6-methoxy-7-chloro-PAT; (-)-6-OMe-7-Cl-PAT, (2*S*,4*R*)-*trans*-(-)-6-methoxy-7-chloro-PAT; PAT, 4-phenyl-2-dimethylaminotetralin (4-phenyl-*N,N*-dimethyl-1,2,3,4-tetrahydronaphthalene-2-amine); PLC, phospholipase C; TMD, transmembrane domain; WT, wild-type.

dependent on 5-HT_{2A} receptor activation, and the HTR is produced reliably by most hallucinogenic 5-HT₂ agonists (Glennon, 1992; Canal and Morgan, 2012; Halberstadt and Geyer, 2013). Thus, use of the DOI-elicited HTR model is expedient for translating drug discovery targeting 5-HT₂ receptors. Although the C57Bl/6 mouse 5-HT_{2A} (m5-HT_{2A}) receptor shares ~99% sequence identity with the human 5-HT_{2A} (h5-HT_{2A}) ortholog in the transmembrane domains (TMDs) where orthosteric ligands bind, there is significant divergence regarding binding affinities (K_i or K_D) of 5-HT_{2A}-targeting ligands at the two receptors (Dougherty and Aloyo, 2011). Similar observations are reported for the rat versus human 5-HT_{2A} receptors (Nelson et al., 1993; Wainscott et al., 1996).

The only two residues within the TMDs that differ between the m5-HT_{2A} and h5-HT_{2A} receptors are at positions (Ballesteros and Weinstein, 1995) 1.42 (alanine in human and tyrosine in mouse) and 5.46 (serine in human and alanine in mouse). Position 1.42 is far from the orthosteric ligand binding pocket (Córdova-Sintjago et al., 2012a), and the functional and/or structural role of amino acids at position 1.42 is unknown. Amino acid residues in TMD5, however, have received attention due to their importance in ligand binding and function of GPCRs (Almaula et al., 1996a; van Rhee and Jacobson, 1996; Wang et al., 2013). Pointedly, residue 5.46 of aminergic neurotransmitter GPCRs (serotonin, dopamine, norepinephrine, and histamine) is an important component for binding of certain ligands in the orthosteric binding pocket (van Rhee and Jacobson, 1996; Shapiro et al., 2000; Armbruster et al., 2007; Cummings et al., 2010; Istyastono et al., 2011).

Mutagenesis studies have revealed that in vitro pharmacological properties of some 5-HT_{2A} ligands are impacted by the difference in residue 5.46 at rodent versus human 5-HT_{2A} receptors (Kao et al., 1992; Johnson et al., 1994). Few studies, however, have examined species differences in receptor protein structure-function with regard to in vivo behavioral pharmacology (Miller et al., 2009; Suratman et al., 2011), with most reports just including a comparison of ligand-receptor in vitro pharmacology. Even among in vitro studies, there are no reports that delineate molecular determinants for binding of different enantiomers of the same compound with 5-HT_{2A} residue 5.46.

Using a structure-based drug design approach, novel PATs targeting 5-HT₂ receptors are being developed to treat compulsive behavioral and other neuropsychiatric disorders (Rowland et al., 2008; Booth et al., 2009; Canal et al., 2013; Morgan et al., 2013). Reported here are results from in silico m5-HT_{2A} and h5-HT_{2A} molecular modeling/ligand docking studies, in vitro molecular pharmacology studies, and in vivo neurobehavioral studies using the DOI-elicited HTR model for the *trans*-($-$)- and *trans*-($+$)-enantiomers of two PAT-type drug candidates, 6-OH-7-Cl-PAT and 6-OMe-7-Cl-PAT (Fig. 1). In preliminary studies, it was observed that for 6-OMe-7-Cl-PAT, both enantiomers have similar affinity across h5-HT₂ subtypes, and there was no difference between the enantiomers regarding potency and efficacy for attenuating the DOI-elicited HTR in mice.

For 6-OH-7-Cl-PAT, the ($-$)-enantiomer had significantly higher affinity than the ($+$)-enantiomer at h5-HT_{2B} and h5-HT_{2C} receptors, but there was no difference in affinity for the two enantiomers at the h5-HT_{2A} receptor. Nevertheless, the ($-$)-enantiomer showed significantly greater potency and

Fig. 1. Structures of PAT ligands tested.

efficacy for attenuating the 5-HT_{2A}-dependent DOI-elicited HTR in mice. Docking studies of the 6-OH-7-Cl-PAT enantiomers at molecular models of 5-HT_{2A} receptors (Córdova-Sintjago et al., 2012a) led to an examination of the putative role of 5-HT_{2A} residue 5.46 in mediating ($+$)-6-OH-7-Cl-PAT ligand affinity and function. The results pinpoint a single amino acid difference at position 5.46 in the m5-HT_{2A} and h5-HT_{2A} receptor that impacts translation of novel PAT drug candidates for neuropsychiatric disorders.

Materials and Methods

Compounds

Structures of ($+$)- and ($-$)-6-OH-7-Cl-PAT and 6-OMe-7-Cl-PATs used in the studies are shown in Fig. 1. The PATs were synthesized in our laboratory as racemic mixtures. Single enantiomers were resolved by chiral stationary-phase high-performance liquid chromatography and converted to hydrochloride salts as previously described (Bucholtz et al., 1999; Booth et al., 2009; Vincek and Booth, 2009). DOI and mianserin hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). 5-HT hydrochloride was purchased from Alfa Aesar (Ward Hill, MA). [³H]Ketanserin, [³H]mesulergine, and [³H]myoinositol were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). All solid compounds were weighed on an XP26 microanalytical balance (Mettler-Toledo, Columbus, OH).

Human and Mouse Receptor Constructs and Point Mutations

A description of the wild-type (WT) m5-HT₂ and h5-HT₂ receptor cDNA constructs used was reported recently (Canal et al., 2013). Point mutations of residue 5.46 of both m5-HT_{2A} and h5-HT_{2A} receptors were made by polymerase chain reactions using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. WT m5-HT₂ receptor cDNAs, cloned in the pCMV6 vector, were purchased from OriGene (Rockville, MD). WT h5-HT₂ receptor cDNAs, cloned in the pcDNA3.1+ vector, were purchased from Missouri S&T cDNA Resource Center (Rolla, MO; <http://www.cdna.org/>). Mutagenesis and sequencing primers were obtained from Life Technologies (Carlsbad, CA). Primers used to make the m5-HT_{2A} A5.46S point mutation were 5'-cctcattagctctttgtgtcattttcatccccaac-3' (sense) and 5'-gttagggggatgaaaatgacacaaaagagcctatgagg-3' (antisense). Primers used to make the h5-HT_{2A} S5.46A point mutation were 5'-cctgatcgctctttgtggcattttcattcccttaac-3' (sense) and 5'-gttaagggatgaaaatgccacaaaagccgatcagg-3' (antisense). Polymerase chain reaction was performed as previously described, with optimization (Canal et al., 2011). Parental DNA in the reaction mixture was digested using DpnI at 37°C for 1 hour. Digestion mixture (2 μ l) was transformed into 50 μ l of XL1-Blue supercompetent cells by heat pulse at 42°C for 45 seconds. The

transformed reaction was incubated in Super Optimal broth with Catabolite repression medium (Sigma-Aldrich), then plated onto LB agar plates containing 100 μ g/ml kanamycin (mouse 5-HT_{2A} WT and A5.46S mutant receptor) or 100 μ g/ml ampicillin (human 5-HT_{2A} WT and S5.46A mutant receptor). Single colonies were selected for sequencing, and point mutations were confirmed by Genewiz (South Plainfield, NJ).

Radioligand Binding Assays

Radioligand saturation isotherm and competitive displacement binding assays were performed in 96-well plates using 5-HT_{2A}, 5-HT_{2B}, or 5-HT_{2C} receptors transiently expressed in HEK293 cells as previously described (Canal et al., 2011, 2013); 10 μ g of *htr2A*, *htr2B*, or *htr2C* cDNA and 20 μ l of Lipofectamine 2000 (Life Technologies) or TurboFect Transfection Reagent (Thermo Scientific, Waltham, MA) was used for transfections. For saturation binding assays, 0.02–16 nM [³H]ketanserin was used to obtain K_D values at the WT and mutant 5-HT_{2A} receptors. The BCA protein assay kit (Thermo Scientific) was used to quantify protein concentration in receptor-expressing cell membranes, for later determination of m5-HT_{2A} and h5-HT_{2A} WT receptor binding site densities, B_{max} values, calculated using GraphPad software (GraphPad Software, La Jolla, CA; <http://graphpad.com/quickcalcs/radcalcform/>). For competition binding assays, 1–3 nM [³H]ketanserin or [³H]mesulergine ($\sim K_D$ concentration) was used to obtain the K_i values for unlabeled ligands at each of the 5-HT₂ receptor subtypes. Nonspecific binding was determined in the presence of 10 μ M mianserin. Each assay was performed a minimum of three times, using at least sample triplicates for each ligand concentration.

Phosphoinositide Hydrolysis Assay

Receptor-mediated phosphoinositide hydrolysis by phospholipase C (PLC) was measured as previously described (Canal et al., 2013). In brief, HEK293 cells transiently expressing a 5-HT₂ receptor were incubated with 16 ml inositol-free Dulbecco's modified Eagle's medium (DMEM) containing 1 μ Ci/ml [³H]myoinositol. Cells were apportioned into 48-well CellBind plates (Corning, Lowell, MA), and [³H]myoinositol incorporation continued for 24 hours in an incubator. Cells were then acclimated to serum-free media, treated for 30 minutes with experimental drugs that had been prediluted in media to contain a final concentration of 50 mM LiCl per well. Media were discarded, and 50 mM formic acid was added to lyse cells. Plates were stored at -80°C for at least 24 hours. After thawing, solution from each well was added to individual anion-exchange columns. Bound [³H]inositol phosphates were eluted with 800 mM ammonium formate, and eluate was added to scintillation fluid (ScintiVerse Cocktail; Thermo Fisher Scientific, Pittsburgh, PA). ³H-Induced scintillations were counted with a scintillation analyzer. Experiments were performed with duplicates for 5-HT and triplicates for all other drugs, and each independent experiment was performed a minimum of three times.

IP-One HTRF Assay

5-HT_{2A} receptor-mediated inositol phosphate 1 production was measured using the IP-One HTRF assay kit (Cisbio, Bedford, MA). These assays were performed during a transition from using the time-consuming method of the phosphoinositide hydrolysis assay, as described above. We have found consistent results between the two assays in the rank order of potency and efficacy of all ligands tested relative to 5-HT at 5-HT_{2A} receptors, and others also report congruency between the IP-One HTRF and Ca^{2+} flux methods at 5-HT_{2A} receptors (Jensen et al., 2013). The assay was performed according to the Cisbio protocol yet with the following optimization. HEK293 cells transiently transfected with 5-HT_{2A} WT or 5.46 mutant receptors were incubated in serum-free DMEM for 1 hour. Cells were then harvested and pelleted by centrifugation at 200g at

37°C for 5 minutes, followed by resuspension in serum-free DMEM. Cell number was counted using a hemacytometer (Hausser Scientific, Horsham, PA), and 500 cells/ μ l DMEM were used. Five *microliters* of cell suspension and 5 μ l of test compounds in stimulation buffer (included in kit and containing 50 mM LiCl) were added to each well of a 384-well plate (Greiner Bio-one, Monroe, NC). The plate was incubated at 37°C for 2 hours. After incubation, the reaction was terminated by adding 5 μ l of the donor and acceptor fluorescent conjugates in lysis buffer. The plate was incubated at room temperature for 1 hour to reach equilibrium. Inositol phosphate 1 levels were assessed by the ratio of fluorescence emission at 665 nM and 620 nM (fluorescence resonance energy transfer, HTRF) using a Synergy H1 plate reader with an HTRF filter cube (BioTek, Winooski, VT). Experiments were performed with duplicates for 5-HT and triplicates for all other drugs, and each independent experiment was performed a minimum of three times.

Animal Subjects

Male C57Bl/6J mice, purchased at 60 days old from The Jackson Laboratory (Bar Harbor, ME), were used in all experiments. Mice were housed in standard cages with ad libitum access to food and water and were acclimated in the vivarium for at least 4 days before testing. Animal procedures were approved by the Institutional Animal Care and Use Committee and are in accordance with the principles in the National Institutes of Health *Guide for Care and Use of Laboratory Animals* (Publication No. 85-23, revised 1985).

DOI-Elicited HTR

All compounds were dissolved in sterile Milli-Q water (EMD Millipore, Billerica, MA), which served as the vehicle control. All compounds were administered subcutaneously at a volume of 10 ml/kg. A single dose of DOI (1 mg/kg) was used for all experiments, as it is known to reliably elicit a robust, consistent, and submaximal number of HTRs in C57Bl/6J mice (Canal et al., 2010; Canal and Morgan, 2012). Two or three doses were used to test the efficacy of novel PAT ligands for attenuating the DOI-elicited HTR. Doses for the compounds were as follows: (–)- and (+)-6-OMe-7-Cl-PAT, 3 and 5.6 mg/kg; (–)- and (+)-6-OH-7-Cl-PAT, 1.5, 3, and 5.6 mg/kg. Solutions of all compounds were made fresh on the day of behavioral testing. Drug or vehicle was injected 10 minutes prior to injection of DOI. Mice were placed in an activity chamber (43 $\times$ 43 cm; Med Associates, Inc., St. Albans, VT) 10 minutes later, and HTRs were counted for the next 10 minutes by an observer blind to the experimental treatment.

Statistical Analyses

Competition and saturation binding data were analyzed using nonlinear regression commands in GraphPad Prism 6.02 for Windows (GraphPad Software). Binding data were fit using the “one-site” models. Two-site curve-fitting did not result in an improved fit (data not shown). Approximate K_i values were determined by conversion of the IC_{50} data using the equation $K_i = \text{IC}_{50}/(1 + L/K_D)$, where L is the concentration of radioligand (Cheng and Prusoff, 1973). Calculations of unpaired t tests and one-way analyses of variance (ANOVAs) with Tukey's multiple-comparison post hoc tests were performed to compare calculated K_i and EC_{50} values of individual compounds for receptors. Ligand efficacies (Tables 1 and 2) are presented as the mean ($\pm$ S.E.M.) of the percentages of maximal 5-HT response from all assays; 5-HT served as a positive control, full agonist in all functional assays.

Separate ANOVAs were performed to analyze effects of experimental compounds on the DOI-elicited HTR. Dunnett's post-test compared the number of HTRs elicited by DOI after pretreatment with each experimental compound to the number of HTRs elicited by DOI and vehicle administration. Two-way ANOVA and Student-Newman-Keuls comparisons were used to identify dose-dependent effects and differences in efficacy across test compounds.

TABLE 1

Summary of in vitro pharmacology results for (+)-6-OH-7-Cl-PAT, (–)-6-OH-7-Cl-PAT, (+)-6-OMe-7-Cl-PAT, and (–)-6-OMe-7-Cl-PAT

K_i and receptor-mediated PLC activity values (mean $\pm$ S.E.M.) obtained from at least three independent experiments. [3 H]Ketanserin and [3 H]mianserin were used to label 5-HT_{2A} and 5-HT_{2C} receptors, respectively. E_{max} was defined as the percent of maximal response to 5-HT. K_i values and functional outcomes of each compound at h5-HT_{2B} receptors were also obtained (see Results). Note the distinct difference in pharmacology between the (+)- and (–)-enantiomers of 6-OH-7-Cl-PAT at h5-HT_{2A} compared with m5-HT_{2A} receptors. (+)-6-OH-7-Cl-PAT showed partial agonist activity at h5-HT_{2A} receptors but was inactive at all other WT 5-HT₂ receptors tested.

Ligand	h5-HT2A					m5-HT2A					h5-HT2C					m5-HT2C				
	K_i	Function	EC ₅₀	E_{max}	%	K_i	Function	EC ₅₀	E_{max}	%	K_i	Function	EC ₅₀	E_{max}	%	K_i	Function	EC ₅₀	E_{max}	%
5-HT	128^a	Full agonist	127 (24)	100	100	138^a	Full agonist	56 (7.5)	100	100	15^a	Full agonist,	11 (2.7)	100	100	50^a	Full agonist	18 (2.8)	100	100
(+)-6-OH-7-Cl-PAT	80 (4.4)	Partial agonist	125 (32)	36 (10)	3005 (321) ^{***}	3005 (321) ^{***}	No activation				1079 (42.9)	No activation				3651 (481) ^{**}	No activation			
(-)-6-OH-7-Cl-PAT	69 (4.9)	No activation				58 (6.8)	No activation				21 (2.4)	No activation				51 (9.5) [*]	No activation			
(+)-6-OMe-7-Cl-PAT	176 (18)	No activation				173 (29)	No activation				47 (5.3)	No activation				243 (22) ^{**}	Partial agonist	1359 (178)	59 (4.0)	
(-)-6-OMe-7-Cl-PAT	134 (18)	No activation				119 (12)	No activation				30 (3.3)	Partial agonist	127 (8.6)	71 (8.2)	71 (8.2)	98 (15) [*]	Partial agonist	29 (5.4)	80 (5.4)	

^a From Canal et al., 2013.

^{*} $P < 0.05$; ^{**} $P < 0.01$; ^{***} $P < 0.001$ vs. human ortholog.

Molecular and Computational Modeling

Homology Modeling. A β_2 -adrenergic receptor (β_2 AR)-based (Protein Data Bank entry: 2RH1) homology model of the h5-HT_{2A} receptor was used in docking and molecular dynamics (MD) studies. Detailed homology modeling procedures are described elsewhere (Córdova-Sintjago et al., 2012b). In brief, the 5-HT_{2A} native sequence (WT) was aligned to the β_2 AR sequence using ClustalW multiple sequence alignment (Thompson et al., 1994; Larkin et al., 2007). Other structures (e.g., the inverse agonist carazolol, T4-lysozyme, and cholesterol molecules) present in the β_2 AR/T4-lysozyme chimera crystal structure were deleted. To obtain the 5-HT_{2A} receptor model, point mutations were performed as needed, followed by the appropriate sequence additions and deletions to match the WT amino acid sequence. TMDs and loops were built using the Biopolymer module of Sybyl-X 2.0 (Tripos International, St. Louis, MO), and the crude models of the unbound receptor were minimized using the Powell method with Tripos force field (Clark et al., 1989) and AMBER charges (Cornell et al., 1995) followed by equilibration in a 1-palmitoyl-2-oleyl-*sn*-glycero phosphatidyl choline bilayer (Heller et al., 1993). The system was relaxed using the Tripos force field to a gradient 0.05 kcal/Å.mol prior to MD simulations in the 1-palmitoyl-2-oleyl-*sn*-glycero phosphatidyl choline membrane. MD simulation conditions were time run for 5 microseconds, time step 1 femtosecond, with snapshots collected every 5 femtoseconds. Other parameters were the canonical ensemble (number of particles, volume, and temperature were constants), 300K temperature, Boltzmann initial velocities, and nonbonded cutoff set at 8 Å. Constraints for alpha carbons in the TMDs were employed. Subsequently, the constraints were removed for a 1000-picosecond MD simulation run. The final unbound WT 5-HT_{2A} homology model was obtained from the median structure after clustering analysis of the frames from the last 10 picoseconds of the MD simulation, and optimized using the Tripos force field to a convergence of 0.05 kcal/Å.mol. The stability and validation of the model were tested as described previously (Córdova-Sintjago et al., 2012b).

While this article was in preparation, the crystal structure of h5-HT_{2B} GPCR was reported (Wang et al., 2013). To assess the validity of our h5-HT_{2A} model using β_2 AR as a template, we similarly built an h5-HT_{2B} homology model and compared it to the crystal structure of the chimeric h5-HT_{2B}-BRIL in complex with ergotamine, Protein Data Bank code 4IB4, by aligning the alpha carbon atoms. The root mean square deviation of 3.34 Å indicates that the h5-HT_{2B} homology model is similar to the crystal structure and validates the strategy used to generate the h5-HT_{2A} model (Fig. 6). A similar conclusion was reached when comparing the β_2 AR-based human histamine H₁ homology model to its crystal structure (Córdova-Sintjago et al., 2012c).

Ligand Docking. The ligands (+)- and (–)-6-OH-7-Cl-PAT were built as monocations (protonated amines) using HyperChem 8.0 (Hypercube, Gainesville, FL) and optimized using PM3 model Hamiltonian to a gradient of 0.01 kcal/Å mol. The binding site was defined by assigning residue D3.32 as a definitive binding site interaction point with the PAT ligand's protonated amine moiety and including residues within a 7-Å radius (Kristiansen et al., 2000; Canal et al., 2011). The initial ligand-receptor complex configuration was used for flexible ligand docking with FlexiDock in Sybyl-X 2.0. Structure preparation was carried out prior to docking studies by assigning AMBER charges (Cornell et al., 1995) for the protein and Gasteiger-Marsili charges (Gasteiger and Marsili, 1980) for the ligand. Rotatable bonds in the ligand and the side chains of residues defining the receptor putative active site were screened for optimal positioning of the ligand and side chains in the conformational space; remaining residues were frozen during docking. Default FlexiDock parameters were set at 80,000-generation. The best docking solution, according to the highest FlexiDock score, was minimized using the Tripos force field to a gradient of 0.05 kcal/Å.mol, prior to MD simulation.

TABLE 2

Summary of the pharmacology for (+)-6-OH-7-Cl-PAT and (–)-6-OH-7-Cl-PAT at h5-HT_{2A} S5.46A and m5-HT_{2A} A5.46S point-mutated receptors. Comparing data in Table 1, note that the pharmacology of (+)-6-OH-7-Cl-PAT at the h5-HT_{2A} S5.46A receptor is similar to its pharmacology at the m5-HT_{2A} WT (A5.46) receptor. Also, its pharmacology at m5-HT_{2A} A5.46S is similar to its pharmacology at h5-HT_{2A} WT (S5.46). Structures highlight stereochemistry and substituents in the 6,7 positions. Data are K_i and receptor-mediated PLC activity values (mean $\pm$ S.E.M.) obtained from at least three independent experiments.

Ligand	Structure	h5-HT _{2A} S5.46A		m5-HT _{2A} A5.46S			
		K_i	Function	K_i	Function	EC ₅₀	E_{max}
		nM		nM		nM	%
(+)-6-OH-7-Cl-PAT		2813 (266)	No activation	66 (10)***	Partial agonist	213 (32)	20 (1.7)
(–)-6-OH-7-Cl-PAT		61 (0.8)	No activation	35 (1.6)***	No activation		

*** $P < 0.001$ vs. human S5.46A.

Molecular Dynamics. The selected high-score pose of the docked ligand was subjected to an MD simulation run for 500 picoseconds, with other parameters the same as above, to allow adjustment of the positions of side chains and helices. The final structure of the ligand docked at the receptor model was obtained from the average of the last 10 picoseconds of the MD simulation.

Results

Affinity and Function of 6,7-Substituted PATs at Mouse and Human WT 5-HT_{2A} Receptors. Four PAT compounds were tested for affinity and function at each of the m5-HT₂ and h5-HT₂ receptor subtypes, excluding m5-HT_{2B} (which was not procured). 5-HT was used as a positive control, full agonist for assessing the functional efficacies and potencies of PAT compounds. Representative binding curves for (–)- and (+)-6-OH-7-Cl-PAT at m5-HT_{2A} and h5-HT_{2A} are shown in Fig. 2. A summary of all K_i results (mean $\pm$ S.E.M.), excluding h5-HT_{2B} data, is shown in Table 1. The mean $\pm$ S.E.M. K_i of (–)-6-OH-7-Cl-PAT at h5-HT_{2B} receptors was 102.4 ± 2.6 nM, whereas (+)-6-OH-7-Cl-PAT had a K_i of 1018 ± 144.8 nM. The mean $\pm$ S.E.M. K_i of (–)-6-OMe-7-Cl-PAT at h5-HT_{2B} receptors was 29.3 ± 4.8 nM, whereas (+)-6-OMe-7-Cl-PAT had a K_i of 72.7 ± 10.3 nM.

ANOVA of K_i values of (–)-6-OH-7-Cl-PAT at each receptor revealed a significant main effect of receptor subtype ($F_{4,16} = 18.71$; $P < 0.0001$). The only post-test comparisons that did not reach a significant difference were the affinities at m5-HT_{2A} versus h5-HT_{2A} receptors, m5-HT_{2C} versus h5-HT_{2A} receptors, and m5-HT_{2A} versus m5-HT_{2C} receptors; similar results were obtained for (+)-6-OH-7-Cl-PAT ($F_{4,12} = 51.46$; $P < 0.0001$). Only h5-HT_{2B} versus h5-HT_{2C} and m5-HT_{2A} versus m5-HT_{2C} K_i values did not reach a significant difference. Unpaired t tests comparing K_i values of the (–)- versus (+)-enantiomers at each of the 5-HT₂ subtypes revealed that the h5-HT_{2A} receptor was the only receptor where the K_i values were not significantly different. Regarding functional effects, the (+)-6-OH-7-Cl-PAT compound was a partial agonist at h5-HT_{2A} receptors only (Table 1). Neither 6-OH-7-Cl-PAT enantiomer showed activity up to $10 \mu\text{M}$ at all other 5-HT₂ receptors screened, including m5-HT_{2A}.

Separate ANOVAs of K_i values of (–)-6-OMe-7-Cl-PAT and (+)-6-OMe-7-Cl-PAT at each receptor revealed a significant main effect of receptor subtype ($F_{4,15} = 20.40$; $P < 0.0001$ and $F_{4,11} = 17.43$; $P < 0.0001$, respectively). Post-test comparisons that did not reach a significant difference were the same across the two enantiomers: m5-HT_{2A} versus h5-HT_{2A} receptors, h5-HT_{2B} versus h5-HT_{2C} receptors, h5-HT_{2A} versus m5-HT_{2C} receptors, and m5-HT_{2A} versus m5-HT_{2C}. Unpaired t tests comparing K_i values of the (–)- versus (+)-enantiomers at each of the 5-HT₂ subtypes revealed that the K_i values of the enantiomers were statistically different only at h5-HT_{2B} receptors and m5-HT_{2C} receptors ($P < 0.05$). Regarding functional effects, (–)-6-OMe-7-Cl-PAT was a partial agonist at both m5-HT_{2C} and h5-HT_{2C} receptors yet was inactive up to $10 \mu\text{M}$ at all other 5-HT₂ receptors (Table 1). (+)-6-OMe-7-Cl-PAT was a nonpotent, partial agonist at m5-HT_{2C} receptors and was inactive up to $10 \mu\text{M}$ at all other 5-HT₂ receptors (Table 1).

B_{max} values for [³H]ketanserin-labeled h5-HT_{2A} and m5-HT_{2A} receptors were obtained from HEK293 cells transfected separately from cells used for competition binding and functional assays, yet transfection procedures for all assays were the same. m5-HT_{2A} and h5-HT_{2A} receptor B_{max} values varied across transfections; the mean $\pm$ S.E.M. values were 1311 ± 137.9 and 1046 ± 595.8 fmol/mg, respectively, which were not statistically different ($P > 0.05$).

Affinity and Function of (+)- and (–)-6-OH-7-Cl-PATs at Mouse and Human 5.46 Point-Mutated 5-HT_{2A} Receptors. The K_D value of [³H]ketanserin at mutant m5-HT_{2A} A5.46S and h5-HT_{2A} S5.46A receptors was not statistically different. The mean $\pm$ S.E.M. K_D values of [³H]ketanserin at m5-HT_{2A} A5.46S and h5-HT_{2A} S5.46A receptors were 1.2 ± 0.3 and 1.6 ± 0.4 nM, respectively, closely matching many reported literature values (Almaula et al., 1996a; Braden and Nichols, 2007) and not statistically different from 5-HT_{2A} WT receptors ($P > 0.05$). The K_D value of [³H]ketanserin at m5-HT_{2A} WT and h5-HT_{2A} WT receptors was 1.3 ± 0.2 and 1.6 ± 0.5 , respectively. Figure 2 shows representative competition binding curves for (–)- and (+)-6-OH-7-Cl-PAT at each of the [³H]ketanserin-labeled mutant receptors—only these two enantiomers were screened at the mutant

Fig. 2. Representative radioligand competition displacement curves. Data show mean $\pm$ S.E.M. from an individual experiment of percent specific binding of [3 H]ketanserin. Note that only (+)-6-OH-7-Cl-PAT is affected by the point mutations. Its affinity (K_i) is decreased at h5-HT2A S5.46A receptors and enhanced at m5-HT2A A5.46S receptors relative to WT receptors.

receptors, because only they showed unusual differences in pharmacology between m5-HT2A and h5-HT2A WT receptors. K_i values are shown in Table 2. The K_i value of (+)-6-OH-7-Cl-PAT at h5-HT2A S5.46A receptors increased 30-fold relative to 5-HT2A WT; this value, 2813 ± 266 nM, was not statistically different from m5-HT2A WT, 3005 ± 321 nM ($P > 0.05$). Similarly, the K_i value of (+)-6-OH-7-Cl-PAT at m5-HT2A A5.46S receptors decreased 45-fold relative to the WT receptor, and this value, 66 ± 10 nM, was not statistically different from h5-HT2A WT, 80 ± 4.4 nM ($P > 0.05$). Consistent with the results from WT receptors, the point mutations did not greatly affect the K_i values of (–)-6-OH-7-Cl-PAT; only the affinity at m5-HT2A A5.46S receptors, 35 ± 1.6 nM, was statistically different relative to WT, 61 ± 0.8 nM ($P < 0.05$).

Finally, in congruence with the similarities in 6-OH-7-Cl-PAT pharmacology between the h5-HT2A WT and m5-HT2A A5.46S receptors, (+)-6-OH-7-Cl-PAT was a partial agonist at m5-HT2A A5.46S receptors (Fig. 3; Table 2). Furthermore, the EC_{50} (nM) and E_{max} (% of maximum 5-HT response) values of this compound at h5-HT2A WT and m5-HT2A A5.46S receptors were not statistically different ($P > 0.05$). Finally, (–)-6-OH-7-Cl-PAT showed no activity up to 10μ M at both of the 5-HT2A point-mutated receptors.

Molecular Modeling and Ligand Docking. Selected low-energy poses of (–)- and (+)-6-OH-7-Cl-PAT docked at the h5-HT2A receptor model, as viewed from the extracellular domain, are shown in Figs. 4 and 5. Only these two enantiomers were docked at the h5-HT2A receptor, because (–)- and

(+)-6-OMe-7-Cl-PAT did not show unusual differences in pharmacology between mouse and h5-HT2A WT receptors. Alignment of h5-HT2A and m5-HT2A receptors using Clus-talW 2.1 showed that only two residues in the TMD helices 1 and 5 were different, 1.42 and 5.46. Residue 1.42 is far from the orthosteric pocket; therefore, the analysis of the drug-receptor interactions after docking and MD does not include residue 1.42.

For both 6-OH-7-Cl-PAT enantiomers, the protonated (at physiologic pH) dimethylamine moiety is capable of ionic interaction with the D3.32 carboxylate (Kristiansen et al., 2000; Ballesteros et al., 2001; Canal et al., 2011). Overall, however, the two enantiomers docked differently. For example, the 6-OH and 7-Cl substituents of (–)-6-OH-7-Cl-PAT are close to TMD7 (Fig. 4, left panel), whereas for the (+)-enantiomer the 6-OH and 7-Cl substituents are close to TMD5 (Fig. 4, right panel). Further examination of the docking results (Fig. 5) indicated that the 6-OH group of the (+)-enantiomer is able to form a hydrogen bond with the side chain of S5.46, whereas this interaction is not possible for the (–)-enantiomer. Both the 6-OH and 7-Cl substituents in the (+)-enantiomer (right panel) can interact with the hydroxyl group of S5.46. Conversely, the 6-OH group of the (–)-enantiomer (left panel) is close to Y7.43 in TMD7, far from S5.46. In addition, such a hydrogen bonding interaction is precluded at the m5-HT2A receptor model (not shown) where residue 5.46 is alanine, and similarly is precluded at the mouse and human 5-HT2B and 5-HT2C receptors that also have an alanine at

Fig. 3. Representative 5-HT_{2A}-mediated PLC functional data showing the effects of 5.46 point mutations on activity of the enantiomers of 6-OH-7-Cl-PAT. Note that only the (+)-6-OH-7-Cl-PAT shows partial agonist activity at h5-HT_{2A} WT (S5.46) receptors and m5-HT_{2A} A5.46S point-mutated receptors.

position 5.46. After MD simulations for each 6-OH-7-Cl-PAT enantiomer docked at the h5-HT_{2B} receptor, important binding site residues change orientation; for example, W6.48 and F6.51 are close to the pendant phenyl of the (–)-enantiomer, which is not observed for the (+)-enantiomer (Fig. 6).

DOI-Elicited HTR. When vehicle (“Veh” in Fig. 7) preceded DOI (1.0 mg/kg) administration, there were 35–40 HTRs observed in a 10-minute period (Fig. 7). Pretreatment with (–)-6-OH-7-Cl-PAT resulted in a dose-dependent and complete attenuation of the DOI-elicited HTR, with the lowest dose tested (1.5 mg/kg), producing an ~50% effect. In contrast, 1.5 mg/kg (+)-6-OH-7-Cl-PAT failed to alter the effects of DOI, while a 50% attenuation was achieved with the highest dose tested, 5.6 mg/kg. Comparison of the two dose-effect curves revealed a significant main effect of enantiomer ($F_{1,28} = 91.3$; $P < 0.001$) and a significant main effect of dose ($F_{2,28} = 31.66$; $P < 0.001$), with all doses being different from each other. Relative to vehicle administration, all doses of the (–)- and the two higher doses of the (+)-enantiomer resulted in statistically significant decreases in HTRs ($F_{6,41} = 37.51$; $P < 0.001$). Both enantiomers of 6-OMe-7-Cl-PAT produced similar effects, with significant decreases at both doses relative to DOI preceded by vehicle administration. Comparison of the two dose-response curves revealed a significant effect of dose ($F_{1,16} = 8.9$; $P = 0.009$) but no interaction or difference between the enantiomers.

Discussion

While developing 5-HT₂-targeting PAT compounds for neuropsychiatric disorders, it was discovered that 6-OH-7-Cl-PAT demonstrates unexpected 5-HT₂ pharmacology. For other PATs, the enantiomer that demonstrates highest affinity at h5-HT_{2A} reliably translates as the more potent and efficacious enantiomer in mouse neurobehavioral models of 5-HT₂ receptor activation, including the DOI-elicited HTR (Canal et al., 2013; Morgan et al., 2013). In addition, PAT enantiomers generally retain their affinity (K_i) differences across 5-HT₂ subtypes, e.g., if one enantiomer is more potent at 5-HT_{2A}, it is predictably more potent at 5-HT_{2B} and 5-HT_{2C}, probably because of relatively high sequence homology (~75%) in 5-HT₂ family TMDs.

Unusual was the observation that both (+)- and (–)-6-OH-7-Cl-PAT had indistinguishable affinity (K_i) at h5-HT_{2A}, i.e., about 70 nM. Their affinities, however, at h5-HT_{2B} and h5-HT_{2C} diverged substantially. Thus, at h5-HT_{2B} and h5-HT_{2C}, affinity of (+)-6-OH-7-Cl-PAT was about 1 μ M, whereas affinity of (–)-6-OH-7-Cl-PAT was ~100 and 20 nM, respectively. Furthermore, (+)-6-OH-7-Cl-PAT was a partial agonist at h5-HT_{2A}, whereas (–)-6-OH-7-Cl-PAT did not activate h5-HT_{2A}; neither enantiomer activated h5-HT_{2B} nor h5-HT_{2C}.

Given that the DOI-elicited HTR in mice is principally a 5-HT_{2A} receptor-mediated effect, the greater potency of

Fig. 4. (–)-6-OH-7-Cl-PAT (left) and (+)-6-OH-7-Cl-PAT (right) docked at the h5-HT2A receptor, built by homology to the β_2 AR structure (see *Materials and Methods*). The view is extracellular looking intracellular. TMDs are spectrum color-coded, from TMD1 (blue) counterclockwise to TMD7 (red faded). Important residues in the orthosteric pocket are labeled using Ballesteros nomenclature. Note that the 6-OH moiety of (–)-6-OH-7-Cl-PAT (left panel) interacts closely with residues in TMD7, far from S5.46, whereas the 6-OH group of (+)-6-OH-7-Cl-PAT (right panel) interacts closely with residues in TMD5, including S5.46.

(–)-6-OH-7-Cl-PAT over (+)-6-OH-7-Cl-PAT in this assay was unexpected in view of their similar affinities at h5-HT2A. These results initially suggested that other receptors, including 5-HT2C, could have played a modulating role in the behavior (Canal et al., 2010, 2013) and/or that the 5-HT2A partial agonist activity of (+)-6-OH-7-Cl-PAT negatively impacted its efficacy to attenuate the DOI-elicited HTR. Results from binding and functional assays using the m5-HT2A and m5-HT2C, however, were parsimonious with another hypothesis that accounted for the behavioral data of 6-OH-7-Cl-PAT, i.e., the (+), but not the (–), enantiomer has different

pharmacology at m5-HT2A compared with h5-HT2A (see Table 1). Affinity (K_i) of (+)-6-OH-7-Cl-PAT was nearly 40 times lower at m5-HT2A compared with h5-HT2A, likely accounting for its lower potency versus (–)-6-OH-7-Cl-PAT for attenuating the DOI-elicited HTR. In contrast, potencies of (–)- and (+)-6-OMe-7-Cl-PAT for attenuating the DOI-elicited HTR were not different, probably caused by their indistinguishable affinities and lack of activity (e.g., neutral antagonism) at m5-HT2A receptors.

With the exception of unrestricted rotation of the C(4) phenyl group and limited boat/chair conformational flexibility

Fig. 5. An alternative viewpoint of (–)-6-OH-7-Cl-PAT (left) and (+)-6-OH-7-Cl-PAT (right) docked at the h5-HT2A receptor. TMDs are spectrum color-coded, from TMD1 (blue) to TMD7 (red faded). Important residues in the orthosteric pocket are labeled using Ballesteros nomenclature.

Fig. 6. Superimposition of the β_2 AR-based h5-HT_{2B} homology model (green) on the 4IB4 crystal structure of the chimeric h5-HT_{2B}-BRIL in complex with ergotamine (aqua blue) (Wang et al., 2013). (Left) View from the extracellular domain; (right) transmembrane side view. The h5-HT_{2B} homology model was generated as described for the h5-HT_{2A} homology model. Root mean square difference = 3.34 Å.

of the cyclohexyl moiety, the PAT molecular scaffold is relatively rigid. Moreover, PATs are chiral and can serve as templates to define the three-dimensional arrangement of 5-HT₂ receptor amino acids involved in ligand binding, leading to inferences about receptor structure and conformational changes required for activation. Thus, the PATs are especially well suited for ligand docking studies at 5-HT₂ receptor molecular models (Córdova-Sintjago et al., 2012a,b).

The m5-HT_{2A} and h5-HT_{2A} vary at only one amino acid, residue 5.46, in their putative orthosteric binding pockets, where there is serine in the human receptor and alanine in

the mouse receptor. Results from molecular docking studies indicated that the 6-OH substituent of (+)-6-OH-7-Cl-PAT could form a hydrogen bond with the side chain of S5.46 when docked at the h5-HT_{2A} receptor, similar to the hydrogen bond interaction observed between the catechol *para*-hydroxyl group of norepinephrine and residue S5.46 of β AR (Strader et al., 1989). An equivalent hydrogen bonding interaction is not possible for (+)-6-OH-7-Cl-PAT at m5-HT_{2A} (A5.46), likely accounting for its nearly 40-fold lower affinity and lack of partial agonist functional activity at the mouse compared with the human receptor, as well as its relatively low potency in the mouse DOI-elicited HTR assay. In contrast, (–)-6-OH-7-Cl-PAT docked differently than the (+)-enantiomer at m5-HT_{2A} and h5-HT_{2A}, having little interaction with TMD5, instead docking close to TMD7, with the 6-OH and 7-Cl substituents close to residue Y7.43. Thus, a single amino acid at position 5.46 likely accounts for the differences in (+)-6-OH-7-Cl-PAT pharmacology at m5-HT_{2A} versus h5-HT_{2A} and for the relatively low potency of (+)-6-OH-7-Cl-PAT in the mouse DOI-elicited HTR assay. Additionally, the low affinity of (+)-6-OH-7-Cl-PAT at 5-HT_{2B} and 5-HT_{2C} may be related to the presence of alanine at residue 5.46 in these receptors.

The role of residue 5.46 in the observed differences in PAT 5-HT₂ pharmacology was confirmed by assessing affinity and function at the m5-HT_{2A} A5.46S and h5-HT_{2A} S5.46A point-mutated receptors. The mutations impacted the pharmacology of (+)-6-OH-7-Cl-PAT but did not alter the pharmacology of (–)-6-OH-7-Cl-PAT. For (+)-6-OH-7-Cl-PAT, the h5-HT_{2A} S5.46A mutation essentially rendered a receptor that was indistinguishable from the m5-HT_{2A} WT receptor; affinity (K_i) of (+)-6-OH-7-Cl-PAT was about 3 μ M at the h5-HT_{2A} S5.46A receptor, similar to its affinity at the m5-HT_{2A} WT receptor. The converse was also true. Affinity (K_i) of (+)-6-OH-7-Cl-PAT was about 70 nM at the m5-HT_{2A} A5.46S receptor,

Fig. 7. HTRs in C57BL/6J mice. Shown are the effects of DOI when preceded by vehicle (Veh) administration (left panel), various doses of 6-OH-7-Cl-PAT (center panel), and two doses of 6-OMe-7-Cl-PAT (right panel). Statistically significant differences are described in text.

similar to its affinity at the h5-HT2A WT receptor. Thus, the interaction of the (+)-6-OH-7-Cl-PAT 6-OH moiety with S5.46 was crucial for its binding, whereas this amino acid hardly impacted binding of (–)-6-OH-7-Cl-PAT. This observation is unusual compared with most other 5-HT2A-targeting compounds reported (Almaula et al., 1996a; Braden and Nichols, 2007; Shan et al., 2012), including DOI. For example, of more than 20 compounds tested, only the ergot derivatives, e.g., ergonovine, showed major losses in affinity at the human S5.46A 5-HT2A receptor (Almaula et al., 1996a; Braden and Nichols, 2007).

In addition to binding, the interaction of the (+)-6-OH-7-Cl-PAT 6-OH moiety with S5.46 also impacted its function at the 5-HT2A receptor. The (+)-6-OH-7-Cl-PAT enantiomer had nil agonist activity at the m5-HT2A WT receptor (A5.46); however, it demonstrated partial agonist activity at the m5-HT2A A5.46S point-mutated receptor, similar to its partial agonist activity at the h5-HT2A receptor (S5.46). Similarly, activation of β ARs by catecholamines involves hydrogen bonding between the catechol OH– group in the *para* position and the OH– in S5.46 (Strader et al., 1989; Warne and Tate, 2013). The similar $B_{\max}$ values for m5-HT2A and h5-HT2A WT receptors suggested that the partial agonist activity of (+)-6-OH-7-Cl-PAT at h5-HT2A but not m5-HT2A WT receptors was not due to receptor reserve.

These results add to the growing literature that crucial points of interaction between a ligand and the 5-HT2A receptor are ligand-dependent, even within a restricted orthosteric binding pocket, and these unique interactions could contribute to different physiologic outcomes, e.g., hallucinogenic or not (Almaula et al., 1996b; Weinstein, 2005). For example, results here with (+)-6-OH-7-Cl-PAT are the opposite of results reported recently by another group testing the h5-HT2C-preferring ligand (*R*)-9-ethyl-1,3,4,10b-tetrahydro-7-trifluoromethylpyrazino[2,1-*a*]-isoindol-6(2*H*)-one (Miller et al., 2009). This ligand exhibits low affinity and efficacy at h5-HT2A (S5.46) yet is a potent and efficacious agonist at rat 5-HT2A (A5.46) and shows 5-HT2A agonist activity in vivo in rats. The in vitro WT receptor pharmacology of the ligand was reversed at the h5-HT2A S5.46A and rat 5-HT2A A5.46S point-mutated receptors. Thus, the interaction of this compound with S5.46 is detrimental to binding affinity and function, putatively due to a reduction in binding pocket size and negative steric interaction between ligand and receptor when serine exists instead of alanine at position 5.46 (Miller et al., 2009), similar to results from certain indole 5-HT2A agonists (Ebersole et al., 2003).

The complete set of data here converge on evidence that the difference in residue 5.46 between m5-HT2A and h5-HT2A accounts for the difference in behavioral potencies of the 6-OH-7-Cl-PAT enantiomers to attenuate the DOI-elicited HTR. The behavioral data, however, are not completely accounted for by ligand interaction with 5-HT2A, as (+)-6-OH-7-Cl-PAT, with essentially nil affinity at the m5-HT2A and m5-HT2C receptor subtypes, also attenuated the DOI-elicited HTR at higher but behaviorally relevant doses, comparable to the 6-OMe-7-Cl-PAT enantiomers. Thus, other mechanisms that modulate the effects of DOI may have come into play. For example, α -adrenergic receptors may modulate activity of the 5-HT2 circuitry or be direct targets of DOI (Darmani, 1993; Ray, 2010). Relevant here is that an early (1993) NovaScreen of racemic 6-OH-7-Cl-PAT reported appreciable

($K_i \sim 70$ nM) at rat brain α_1 -adrenergic receptors yet negligible affinity for all other aminergic neurotransmitter receptors and two dozen other central nervous system receptor sites, with the known exceptions of histamine H_1 (Bucholtz et al., 1999) and 5-HT2 receptors. Pharmacological studies are under way to assess the role of specific α_1 -adrenergic receptor subtypes in regulating the DOI-elicited HTR, and molecular modeling studies are in progress to assess interactions between 6-OH-7-Cl-PAT enantiomers and α_1 -adrenergic receptor TMD amino acids. Regarding known affinity of PAT ligands for H_1 receptors, it is also acknowledged that H_1 receptor actions do not seem to affect the DOI-elicited HTR (Canal and Morgan, 2012).

Finally, we are unaware of any compound, other than (+)-6-OH-7-Cl-PAT reported here, that possesses appreciable potency to bind and activate h5-HT2A together with negligible affinity and no ability to activate h5-HT2B or h5-HT2C. Given that (+)-6-OH-7-Cl-PAT penetrates mammalian brain (i.e., modulates the DOI-elicited HTR), it could be a useful tool to study the neurobehavioral effects of specific 5-HT2A receptor partial agonism in humans without the confound of 5-HT2B and/or 5-HT2C receptor binding and activation. Notably, partial agonist activity at 5-HT2A is possessed by classic hallucinogens, including LSD (Marek and Aghajanian, 1996; Egan et al., 1998; Ebersole et al., 2003), yet these drugs also bind and activate 5-HT2B and 5-HT2C (Porter et al., 1999), posing a challenge to distinguish specific receptor activation effects without using selective antagonists that may possess behavioral effects of their own.

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Authorship Contributions

Participated in research design: Canal, Cordova-Sintjago, Kim, Booth.

Conducted experiments: Canal, Cordova-Sintjago, Kim, Liu, Morgan.

Contributed new reagents or analytic tools: Kim.

Performed data analysis: Canal, Cordova-Sintjago, Liu, Morgan.

Wrote or contributed to the writing of the manuscript: Canal, Cordova-Sintjago, Liu, Morgan, Booth.

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