HHS Public Access

Author manuscript

Biochem Pharmacol. Author manuscript; available in PMC 2023 June 01.

Published in final edited form as:

Biochem Pharmacol. 2022 June ; 200: 115028. doi:10.1016/j.bcp.2022.115028.

"Selective" serotonin 5-HT_{2A} receptor antagonists

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Abstract

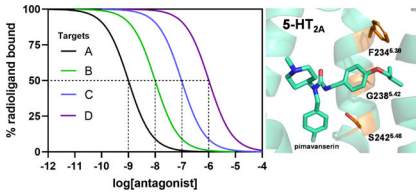
Blockade of the serotonin 5-HT_{2A} G protein-coupled receptor (5-HT_{2A}R) is a fundamental pharmacological characteristic of numerous antipsychotic medications, which are FDA-approved to treat schizophrenia, bipolar disorder, and as adjunctive therapies in major depressive disorder. Meanwhile, activation of the 5-HT_{2A}R by serotonergic psychedelics may be useful in treating neuropsychiatric indications, including major depressive and substance use disorders. Serotonergic psychedelics and other 5-HT_{2A}R agonists, however, often bind other receptors, and standard 5-HT_{2A}R antagonists lack sufficient selectivity to make well-founded mechanistic conclusions about the 5-HT_{2A}R-dependent effects of these compounds and the general neurobiological function of 5-HT_{2A}Rs. This review discusses the limitations and strengths of currently available "selective" 5-HT_{2A}R antagonists, the molecular determinants of antagonist selectivity at 5-HT_{2A}Rs, and the utility of molecular pharmacological and computational methods in guiding the discovery of novel unambiguously selective 5-HT_{2A}R antagonists.

Graphical Abstract

Declarations of interest: The authors declare no conflicts of interest.

CRediT author statement

Austen B. Casey: Writing - Original Draft, Conceptualization, Visualization. **Meng Cui**: Writing - Review & Editing, Resources. **Raymond G. Booth**: Writing - Review & Editing, Funding acquisition. **Clinton E. Canal:** Writing - Review & Editing, Conceptualization, Funding acquisition, Resources, Supervision.



Keywords

5-HT_{2A}; GPCR; antipsychotic; antagonist; selectivity

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is an endogenous signaling molecule that regulates nearly all neurocognitive functions by activating at least 14 genetically-encoded 5-HT receptors, along with dozens of variants generated by post-transcriptional editing or alternative splicing (1, 2). Thirteen genetically encoded 5-HT receptors are G protein-coupled receptors (GPCRs) that, owing to their plasma membrane localization and extensive control over intracellular signaling, are highly accessible and versatile drug targets. Approximately one-third of drugs approved by the United States Food and Drug Administration target GPCRs and ~4% bind to 5-HT_{2A} receptors—arguably the most studied 5-HT receptor (3, 4).

Early antipsychotic medications, known to block dopamine D_2-like GPCRs in the striatum, were coincidentally instrumental in the discovery of 5-HT_{2A}Rs in the cortex. For example, radioligand binding studies with rat brains showed that both spiperone (Figure 1) and haloperidol bound striatal dopamine receptors, whereas only spiperone had high affinity for a population of cortical receptors labeled by the promiscuous (i.e., nonselective) serotonergic compound lysergic acid diethylamide (LSD), suggesting that spiperone-bound 5-HT receptors (5). Electrophysiology experiments then showed that at least two 5-HT receptors in the brain modulate neuronal firing (6). LSD was subsequently reported to bind both receptors in the rat frontal cortex: those demonstrating higher affinity for [^{3}H]5-HT or [^{3}H]spiperone, termed 5-HT_1R and 5-HT_2R, respectively (7). The discovery of distinct 5-HT receptors naturally led to investigations into their behavioral and physiological functions, creating demand for selective antagonists to block 5-HT_2R-mediated effects (8).

Among the first behavioral activities to be associated with the 5-HT_2R was the ability of laboratory animals to discriminate the subjective effects elicited by serotonergic psychedelics, including LSD, mescaline, and 2,5-dimethoxy-4-methyl-amphetamine, which could be blocked by the 5-HT_2R antagonists pirenperone and ketanserin (described below) (9–12). Soon after this, 5-HT was found to effectuate phosphoinositide turnover in the rat cerebral cortex, and the potencies of 5-HT_2R antagonists to block this effect were correlated with their affinity for these sites (13). The 5-HT_2R was later designated as the 5-HT_{2A}R after the discovery of 5-HT_{2B} and 5-HT_{2C}Rs (14–20), classified together based on extensive similarities in their ligand binding affinities, signal transduction mechanisms, and structural homology (60–70% amino acid identity within structurally conserved regions).

Decades have passed since the seminal discoveries described above, and antagonism of 5-HT_{2A}Rs has become a mainstay of antipsychotic drug action. In fact, so called selective 5-HT_{2A}R antagonists can effectively treat dimensions of psychosis (21–25), without untoward side effects associated with the off-target binding of more promiscuous antipsychotic agents. Additionally, there is new widespread interest in identifying mechanisms of rapid, persistent neuronal and behavioral plasticity elicited by psychedelics that share activation of 5-HT_{2A}Rs, but diverge in their activities at other targets (26–30). The delineation of such mechanisms inherently relies on the use of unambiguously selective 5-HT_{2A}R antagonists to block these effects, yet the utility of currently available antagonists is limited by their selectivity, pharmacological characterization, and structural diversity.

Central to this review, defining the fundamentally vague pharmacological term “selective” remains a formidable challenge. For example, a ligand may bind target A with 10-fold greater potency than targets B and C and be defined as “selective” for target A, albeit, the ligand affinities at targets D and E may not have been considered. In other words, a ligand may be referred to in the literature as “selective” irrespective of the number of off-target proteins against which it has been compared. Thus, it is critical to define under what context a ligand may be “selective”. In this review, we discuss the discovery and relative selectivity of putatively selective 5-HT_{2A}R antagonists. We define here ligands that block 5-HT-elicited $G\alpha_{q/11}$-signaling via 5-HT_{2A}Rs as “antagonists” owing to a paucity of data at alternative G protein or β-arrestin1/2 signaling pathways. Likewise, there is limited evidence of physiological, therapeutic, or structural distinctions between neutral antagonists and inverse agonists, leading us to refer to neutral antagonists and inverse agonists alike as “antagonists”, unless otherwise specified. We also discuss structural mechanisms underlying selective antagonist binding to 5-HT_{2A}Rs, and the potential of molecular modeling and molecular dynamics to advance the design of unambiguously selective 5-HT_{2A}R antagonists.

2. Putatively selective 5-HT_{2A}R antagonists

2.1 Spiperone (spiroperidol)

Spiperone, a butyrophenone discovered by Janssen Pharmaceuticals, was developed as an antipsychotic and binds central dopamine (31) and 5-HT (32) receptors with high affinity. After its affinity for 5-HT receptors was reported, several other antipsychotics were also found to bind 5-HT receptors (5, 7). Spiperone was later found to have >500-fold higher affinity at human 5-HT_{2A}Rs than the highly homologous 5-HT_{2B} and 5-HT_{2C}Rs (33, 34), making it the first commercially-available compound for clearly labeling and distinguishing 5-HT_{2A}Rs from 5-HT_{2B} and 5-HT_{2C}Rs. However, its high affinity at dopamine D_2-like receptors, along with its moderate (K_i = 30–300 nM) to high ($K_i \leq$ 30 nM) affinity at α_{1A}-, α_{1B}-, α_{1D}-adrenergic, 5-HT_{1A}, and 5-HT_7Rs (Table 1) limits its use for interrogating the physiological and behavioral role of 5-HT_{2A}Rs (9).

2.2 Ketanserin (R 48,648)

Since its discovery by Janssen Pharmaceuticals in 1981 (35), the quinazoline derivative ketanserin is among the most widely used tools for probing 5-HT_{2A}R function in preclinical

research (26–28, 36), and the sole antagonist used to delineate the 5-HT_{2A}R-dependent effects of serotonergic psychedelics in humans (37–41). Although ketanserin was the first 5-HT_{2A}R antagonist discovered that lacks high affinity for other serotonin and dopamine receptors, it is less appreciated that it has high affinity at several aminergic receptors, including α_{1A}-, α_{1B}-, α_{1D}-adrenergic, and histamine H_1 receptors (35, 42–44), as well as, moderate affinity at α_{2B}-adrenergic and 5-HT_{2C} receptors (Table 1). These off-target activities limit the utility of ketanserin as a specific tool for assessing 5-HT_{2A}R activity. The off-target activity of ketanserin at adrenergic and histaminic receptors is particularly confounding because α_1-adrenergic receptors colocalize with 5-HT_{2A}R transcripts, and α_1-, α_2-adrenergic and histamine H_1 receptors share signal transduction mechanisms with the 5-HT_{2A}R and modulate the excitability of serotonergic neurons in the dorsal raphe nucleus (45–49).

To demonstrate that ketanserin interacts with high affinity at sites independent of the 5-HT_{2A}R, we present an autoradiography experiment wherein mouse brain slices were incubated with 1 nM [^{3}H]ketanserin in the presence or absence of 1 μM M100,907 (Figure 2), another putatively selective 5-HT_{2A}R antagonist (discussed below). Our results demonstrate that [^{3}H]ketanserin prominently labels sites in the striatum that are not specific to 5-HT_2-type receptors, consistent with earlier work in rat brain using other 5-HT_2-type receptor antagonists (50, 51). Dopaminergic, adrenergic, or histaminergic antagonists were also ineffective at blocking striatal [^{3}H]ketanserin binding (50, 51). Further experiments using rat brains (52), bovine chromaffin granule membranes (53), or heterologous cell systems (54) demonstrate that [^{3}H]ketanserin binds the vesicular monoamine transporter 2 with moderate affinity (K_i, K_d = 22–540 nM). Therefore, studies investigating the effects of 5-HT_{2A}R agonists should use caution when interpreting results obtained with ketanserin as a pharmacological tool since its pharmacodynamic effects are incompletely understood.

2.3 M100,907 (MDL100,907, Volinanserin)

Driven by work suggesting that 5-HT_{2A}R antagonists elicit antipsychotic-like behavioral effects with a low risk of extrapyramidal symptoms compared to antipsychotics that primarily block D_2Rs, researchers at Hoechst Marion Roussel in the early 1990s developed the piperidine derivative M100,907 (Figure 1) for the treatment of schizophrenia (55, 56). Until this time, 5-HT_{2A}R antagonists demonstrated only modest selectivity (< 30-fold) over 5-HT_{2C}, α_1-adrenergic, H_1, and dopamine D_2 receptors. Thus, Hoechst Marion Roussel broke ground by reproducibly showing that M100,907 had sub-nanomolar affinity at 5-HT_{2A}Rs, with >100-fold selectivity over these off-target GPCRs, along with numerous other receptors and ion channels (57, 58).

An important caveat when considering the selectivity of M100,907 is that much of the work characterizing its binding profile is derived using a variety of species, tissue sources, and radioligands (57, 58). Thus, the apparent ligand affinity may be impacted by differences in the ligand binding pocket between species (59–61), plasma membrane composition (62), and/or radiolabeling discrepancies in native tissue (e.g., [^{3}H]spiperone binds non-selectively to several dopamine and serotonin receptors, Table 1). Moreover, GPCRs exist in a dynamic equilibrium of conformational states often determined by the prevalence of guanine

nucleotides and G proteins (63, 64). Agonists tend to recognize these conformational states with variable affinity, making radioligand-based affinity measurements sensitive to differences in the conformation stabilized by the radioligand and competitor ligand. For example, 5-HT has much higher affinity for [^{3}H]5-HT-labeled than [^{3}H]ketanserin-labeled 5-HT$_{2A}$Rs (K_i = 3.8 and 144 nM, respectively) (65). Accordingly, the off-target binding of M100,907 is less certain than one might assume by glancing at available data in Table 1, and since competitive radioligand displacement data provides little information on ligand functional activity, some skepticism is warranted regarding the selectivity of M100,907 at 5-HT$_{2A}$Rs (see ref. (66) for review on defining ligand activity).

Among the currently known off-targets of M100,907 are sigma, 5-HT$_{2C}$, α_{1A}-, and α_{2B}-adrenergic receptors, with lower affinity at the latter receptors than is observed for ketanserin (Table 1). While selectivity over H$_1$Rs has been challenged (67), most work supports that M100,907 is selective at binding 5-HT$_{2A}$ over H$_1$Rs. (57, 58, 68). Autoradiography studies further support 5-HT$_{2A}$R engagement by [^{3}H]M100,907 at 0.4 nM in rat brain, exhibiting dense labeling in the frontal cortex, motor cortex, and claustrum (51). Rodent studies indicate that M100,907 does not antagonize apomorphine induced-climbing or phenylephrine lethality, indices of *in vivo* D$_2$ and α_1-adrenergic receptor engagement, respectively, and may only engage 5-HT$_{2C}$Rs at elevated doses (58). Notably, some studies report M100,907 is not a potent modulator of mouse locomotor activity (55, 58, 69), while others report robust locomotor suppressing effects (70, 71), suggesting that different animal strains and/or test conditions are involved in the confounding results reported for M100,907 behavioral pharmacology.

In preclinical studies, M100,907 exhibited promising antipsychotic-like behavioral effects in models of psychomotor stimulation and sensorimotor gating deficits, with a favorable side-effect profile (55, 58, 72, 73). Positron emission tomography (PET) studies in humans confirmed that M100,907 exhibited high levels of apparent 5-HT$_{2A}$R occupancy in the cortex (74) for over 24 hours, far outlasting its presence in plasma (75). However, clinical trials for schizophrenia were discontinued due to a failure to meet endpoints (76). In summary, M100,907 appears to offer advantages over ketanserin for evaluating 5-HT$_{2A}$R function *in vivo*. However, the lack of broad receptor panel screening data with a consistent tissue-source suggests exercising caution when interpreting data reported for M100,907.

2.4 Pimavanserin (ACP-103, Nuplazid®)

Since their development, so-called “atypical” antipsychotic medications (e.g., clozapine) have been noted to demonstrate distinct therapeutic and side-effect profiles compared to their “typical” counterparts (e.g., haloperidol), which were defined primarily by potent dopamine D$_2$R antagonist activity. While differences in D$_2$R occupancy at clinically effective doses or polypharmacology (e.g., partial agonism of 5-HT$_{1A}$Rs) likely contribute to these differences, scientists at Acadia Pharmaceuticals identified functionally distinct effects at the 5-HT$_{2A}$R as being, at least in part, responsible for atypical antipsychotic therapeutic outcomes (68). Key to this was the development of a sensitive, high-throughput chemical genomics platform (Receptor Selection and Amplification Technology™; R-SAT™) enabling reliable detection of 5-HT$_{2A}$R constitutive activity (i.e., ligand-independent G protein

activation), historically difficult to detect through 5-HT_{2A}Rs. By screening numerous antipsychotics via the R-SAT™ platform, it was found that nearly all atypical antipsychotics, as well as spiperone, ketanserin, and M100,907, demonstrated inverse agonist activity (i.e., a concentration-dependent reduction in constitutive activity) at 5-HT_{2A}Rs (68). This finding invigorated a high-throughput screening (HTS) campaign that identified several hits for potent 5-HT_{2A}R inverse agonism (77). After thorough selectivity screening and iterative rounds of lead optimization, pimavanserin (ACP-103, Figure 1) was identified as a novel putatively selective 5-HT_{2A}R inverse agonist with preclinical efficacy in multiple models of psychosis, and was devoid of untoward motor side effects (78). Notably, pimavanserin exhibits $G\alpha_q$-mediated inverse agonist activity in clonal cells expressing wild type (WT) 5-HT_{2A}Rs or constitutively activated 5-HT_{2A}R variants (79, 80). A recent study using human and mouse brain tissue, however, reported that pimavanserin may act as a neutral antagonist and inverse agonist of $G\alpha_{q/11}$- and $G\alpha_{i1}$-mediated signal transduction, respectively, *in vivo*, apparently via 5-HT_{2A}Rs (81). Nevertheless, although the therapeutic relevance of inverse agonism at 5-HT_{2A}Rs is uncertain, pimavanserin is approved to treat hallucinations and delusions in Parkinson's disease patients (22, 82). Pimavanserin also has shown efficacy in clinical trials to treat dementia-related psychosis (23, 24), negative symptoms of schizophrenia (25), and major depression (83).

A broad affinity profiling screen of over 75 GPCRs, ion channels, transporter proteins, and enzymes, indicated that pimavanserin had >1,000-fold selectivity for 5-HT_{2A}Rs over all other targets except for 5-HT_{2C}Rs (~12-fold), where it also acts as an inverse agonist (78, 80). Pimavanserin also was found to bind L-type calcium channels with moderate to low affinity (K_i = 310 nM) (78). The off-target activity at 5-HT_{2C}Rs is especially noteworthy for researchers investigating mechanisms sensitive to midbrain dopamine release, which might be oppositely influenced by 5-HT_{2A} and 5-HT_{2C}Rs (67, 84–86); see refs. (87) and (88) for investigations into the effects of 5-HT_{2C}Rs on midbrain dopamine release.

Several studies report that pimavanserin may not impact motor function in humans (22, 83, 89, 90), likely due to its nil affinity at D_2 and H_1Rs (77, 91). In rodents, however, pimavanserin causes robust locomotor suppression (78, 80). This activity appears to be at odds with the role of 5-HT_2-type receptors in modulating rodent locomotor activity. For example, 5-HT_{2A}R activation enhances locomotor activity whereas 5-HT_{2C}R activation suppresses locomotor activity in mice (84). Conversely, antagonism of 5-HT_{2A}Rs normalizes the locomotor enhancing effects of 5-HT_{2C}R antagonists (85). Therefore, a potent and selective 5-HT_{2A}/5-HT_{2C}R inverse agonist such as pimavanserin might be expected to have neutral effects on locomotor activity in mice. A PET study in pigs also questions the selectivity of pimavanserin, which was not competitively displaced from the thalamus by unlabeled pimavanserin or ketanserin (92), suggesting the parent compound or a metabolite exhibits off-target binding, high non-specific binding, or a slow dissociation rate. Nevertheless, of the ligands discussed here, pimavanserin is the most thoroughly characterized putatively selective 5-HT_{2A}-preferring 5-HT_{2A}/5-HT_{2C}R antagonist/inverse agonist available.

2.5 Other 5-HT$_{2A}$R antagonists

In addition to spiperone and ketanserin (35, 93), scientists at Janssen Pharmaceuticals also discovered several other highly potent 5-HT$_{2A}$R antagonists with some selectivity for 5-HT$_{2A}$Rs, including altanserin, pirenperone, risperidone, and ritanserin (9, 94–97) (Figure 1). However, while these ligands demonstrate high affinity at, and some selectivity for, 5-HT$_{2A}$Rs, neither is likely to engage 5-HT$_{2A}$Rs selectively at behaviorally active doses due to their high affinity at off-target receptors (Table 1). Altanserin, a PET ligand for labeling 5-HT$_{2A}$Rs *in vivo* (98), is a possible exception to this shortcoming, although its *in vitro* characterization is sparse. Nevertheless, studies with altanserin (99, 100) and pirenperone (9–11, 101) have informed our understanding of 5-HT$_{2A}$R pharmacology, while structural biology studies with risperidone (79) and ritanserin (102) have begun clearing a path to the rational design of novel and selective 5-HT$_{2A}$R antagonists (described below).

3. Molecular determinants of polypharmacology and subtype selectivity at 5-HT$_{2A}$ GPCRs

3.1 Polypharmacology of aminergic ligands targeting 5-HT$_{2A}$Rs

The 5-HT$_{2A}$R orthosteric binding pocket—where the endogenous ligand 5-HT binds—shares significant homology with numerous aminergic GPCRs (receptors for neurotransmitters with basic amine moieties, e.g., acetylcholine, dopamine, histamine, norepinephrine, serotonin), and this overlap is critical to understanding ligand polypharmacology. Within transmembrane domain 3 (TM3) sits the amino acid residue D$^{3.32}$ (Ballesteros-Weinstein numbering system (103)), which is conserved in the binding pocket of all aminergic receptors. This conserved residue is negatively charged and serves to anchor the positively charged amine moiety present in most aminergic receptor ligands within the binding pocket via an ionic bond (Figure 3). Accordingly, point mutation of D$^{3.32}$ to uncharged residues drastically diminishes the binding affinity of most agonists and antagonists at aminergic GPCRs, including 5-HT$_{2A}$Rs (104–107).

The side chains of I$^{3.40}$, F$^{5.47}$, F$^{6.44}$, W$^{6.48}$, F$^{6.51}$, and F$^{6.52}$ form a highly conserved hydrophobic cleft at the base of the orthosteric binding pocket (Figure 3) and contribute to promiscuous antagonist binding at 5-HT$_{1B}$, 5-HT$_{2A}$, 5-HT$_{2C}$, D$_2$, and H$_1$Rs (79, 108). Interestingly, flexible-structured antagonists are more sensitive to manipulations in this region of the binding pocket, likely because they extend deeper than more rigid-structured antagonists such as those of the ergoline class (109, 110). For example, the affinity of flexible ligands such as risperidone and pimavanserin is attenuated by >900-fold at point mutated W336L$^{6.48}$ (tryptophan to leucine) and F339A$^{6.51}$ (phenylalanine to alanine) 5-HT$_{2A}$Rs (79). Similarly, the affinity of ritanserin at highly homologous 5-HT$_{2C}$Rs with W324L$^{6.48}$ or F327L$^{6.51}$ point mutations is attenuated by >10,000- and ~100-fold, respectively (102). In contrast, the affinity of the relatively structurally-rigid ergoline antagonist mesulergine (Figure 4) is attenuated by only 7-fold at W324L$^{6.48}$ 5-HT$_{2C}$Rs (102). Recently, the side chains of F243$^{5.47}$ and F340$^{6.52}$ within the 5-HT$_{2A}$R were found capable of expanding the binding pocket to accommodate various antagonist structural moieties (111). As such, mutation of either position to alanine or leucine attenuates the affinity (~5–46-fold) of many antagonists, including: spiperone, ketanserin, pimavanserin,

risperidone, aripiprazole, and cariprazine (79, 104, 111). Although the affinity of ketanserin at F340A$^{6.52}$ 5-HT$_{2A}$Rs was not different from WT 5-HT$_{2A}$Rs (111).

A subset of residues in the hydrophobic cleft forms the P$^{5.50}$–I$^{3.40}$–F$^{6.44}$ motif, a molecular ensemble wherein side-chain rearrangements are understood to mediate activation of class A GPCRs (112), along with other key, conserved motifs, including CWxP, E/DRY, and nPxxY (113). Accordingly, I$^{3.40}$ and F$^{6.44}$ are critical for agonist function and antagonist binding at 5-HT$_{2A}$ and 5-HT$_{2C}$Rs (79, 102, 104, 114). Indeed, the agonist activity of 5-HT is abolished at I163A$^{3.40}$ 5-HT$_{2A}$Rs, as is the affinity of mesulergine (79). Moreover, the affinity of pimavanserin and risperidone is attenuated at F332L$^{6.44}$ 5-HT$_{2A}$Rs (10- and 17-fold, respectively) (79), and of ritanserin at I142F$^{3.40}$ and F320L$^{6.44}$ 5-HT$_{2C}$Rs (20- and 9-fold, respectively) (102).

3.2 Molecular determinants of subtype-selectivity at 5-HT$_{2A}$Rs

Perhaps the most intuitive mechanism of achieving selectivity is the formation of ligand-receptor contacts with amino acid residues that differ across target and off-target receptors. Identifying such residues allows medicinal chemists to rationally design ligands that interact with these residues to realize a degree of selectivity. By combining site-directed mutagenesis, molecular modeling, and structural biology techniques, several non-conserved residues in the 5-HT$_{2A}$R have been identified and offer significant insight into the design of subtype-selective ligands.

3.2.1 S242$^{5.46}$—

The most thoroughly characterized 5-HT$_{2A}$R amino acid residue, S242$^{5.46}$ (A225$^{5.46}$ and A222$^{5.46}$ in 5-HT$_{2B}$ and 5-HT$_{2C}$Rs, respectively), has been implicated in species differences in ligand affinity (60, 61, 115), subtype-selective binding (60, 61, 115), binding kinetics (116, 117), and biased signaling (106). Importantly, S$^{5.46}$ diverges between humans (serine) and rodents (alanine) and is sufficient to explain species differences in the affinity of ergoline-based antagonists, tryptamine-based agonists (60, 115, 116, 118), as well as a 2-aminotetralin-based agonist (61). Such interspecies variations could be due to limited evolutionary pressure to conserve sequence identity for a non-deleterious mutation (119). Although, drug development programs designing ligands that engage interspecies variant residues (intentionally or not) would necessitate the development of humanized animal models.

The species-dependent affinity of 5-HT$_{2A}$R ligands was first shown by Kao et al. (60) using the N(1)-methyl ergoline antagonist mesulergine, which has a lower affinity at cloned human 5-HT$_{2A}$Rs (S242$^{5.46}$, K_d = 174 nM) compared to 5-HT$_{2A}$Rs in the rat cortex (A242$^{5.46}$, K_d = 8.52). Notably, the high affinity of mesulergine at human S242A$^{5.46}$ point mutated 5-HT$_{2A}$Rs (K_d = 3 nM) recapitulated its affinity at WT rat 5-HT$_{2A}$Rs (60). Johnson et al. (115) extended these findings to various N(1)-substituted ergolines. For example, LY86057, LY108742, and LY53857 differ only in their substitution of the N(1)-position (i.e., −H, −CH$_3$, and −CH[CH$_3$]$_2$, respectively; Figure 4), yet LY86057 (−H) had a higher affinity at human 5-HT$_{2A}$Rs than LY108742 (−CH$_3$) or LY53857 (−CH[CH$_3$]$_2$). In contrast, LY108742 (−CH$_3$) and LY53857 (−CH[CH$_3$]$_2$) had higher affinity at rat 5-HT$_{2A}$Rs than LY86057 (−H). However, these trends were reversed when rat 5-HT$_{2A}$Rs were point mutated to recapitulate

human 5-HT_{2A}Rs (A242S$^{5.46}$); e.g., the affinity of LY86057 (−H) better resembled its affinity at human 5-HT_{2A}Rs, whereas the affinity of LY108742 (−CH_3) and LY53857 (−$CH[CH_3]_2$) depreciated by ~4-fold. Such relationships likely stem from a combination of factors, including the availability of favorable electrostatic interactions between the indole N(1)-H and the side chain oxygen of S$^{5.46}$, as well as steric tolerance and opportunities for hydrophobic interactions between the indole N(1)-alkyl moieties and the side chain of A$^{5.46}$.

The residue at position X$^{5.46}$ also diverges between 5-HT_{2A} (serine) and the highly homologous 5-HT_{2B} and 5-HT_{2C}Rs (alanine). In an elegant study, Almaula et al. (116) tested the hypothesis that S242$^{5.46}$ mediates subtype-selective binding to human 5-HT_{2A} over human 5-HT_{2C}Rs using exchange mutations to generate S242A$^{5.46}$ 5-HT_{2A}Rs and A222S$^{5.46}$ 5-HT_{2C}Rs. Ergolines with an N(1)-H moiety (i.e., ergonovine, LSD, and lisuride) that preferentially bound WT 5-HT_{2A}Rs over WT 5-HT_{2C}Rs, demonstrated lower affinity at S242A$^{5.46}$ 5-HT_{2A}Rs, and higher affinity at A222S$^{5.46}$ 5-HT_{2C}Rs. In contrast, ergolines with an N(1)-CH_3 moiety (i.e., mesulergine) that bound preferentially to 5-HT_{2C}Rs over 5-HT_{2A}Rs, demonstrated higher affinity at S242A$^{5.46}$ 5-HT_{2A}Rs, and lower affinity at A222S$^{5.46}$ 5-HT_{2C}Rs. These findings suggest that the affinity of ergolines at 5-HT_{2A} and 5-HT_{2C}Rs depends on hydrogen bonding as well as steric and hydrophobic interactions between the N(1)-substituent and the side chains of S/A$^{5.46}$.

While the side chain of S/A$^{5.46}$ explains differences in the structure-activity relationships (SAR) of ergolines between human and mouse or rat 5-HT_{2A}Rs, and between human 5-HT_{2A} and 5-HT_{2C}Rs, the effect of S$^{5.46}$ vs. A$^{5.46}$ does not reliably extend to other chemotypes. For example, tryptamine derivatives are not reliably impacted by the S/A$^{5.46}$ side chain (115, 120). Similarly, we and others have shown that the affinity of various 5-HT_{2A}R antagonists is not different at S242A$^{5.46}$ 5-HT_{2A}Rs, including, ketanserin, pimavanserin, risperidone, and certain 2-aminotetralins (79, 80, 115, 116, 121). Taken together, S242$^{5.46}$ is not a general determinant of subtype-selective antagonist binding at 5-HT_{2A}Rs over 5-HT_{2B} and 5-HT_{2C}Rs.

3.2.2 G238$^{5.42}$—The residue G$^{5.42}$ is unique to 5-HT_2-type receptors and is occupied by larger residues including alanine, cysteine, serine, or threonine in other aminergic GPCRs (4). The importance of this to drug design was highlighted in the crystal structure of a ritanserin-bound 5-HT_{2C}R, which indicates that one of the 4-fluorophenyl groups on ritanserin contacts the backbone of TM5 at G218$^{5.42}$ (102). Given that ritanserin demonstrates some selectivity to bind 5-HT_2-type receptors over other aminergic receptors, Peng and coworkers tested the hypothesis that G218$^{5.42}$ was in part responsible for the preferential binding of ritanserin to 5-HT_2-type receptors. They generated a G218S$^{5.42}$ 5-HT_{2C}R to mimic the corresponding S199$^{5.42}$ residue in 5-HT_{1A}Rs—for which ritanserin has low affinity (K_i = 309 nM, Table 1). Ritanserin showed 60-fold lesser affinity at G218S$^{5.42}$ 5-HT_{2C}Rs, supporting the hypothesis that G218$^{5.42}$ contributes to its preferential binding at 5-HT_2-type receptors. This hypothesis was further supported by a complementary experiment with clozapine, which exhibits appreciable affinity (K_i < 100 nM) for at least 26 receptors including the 5-HT_{1A}R and has an embedded 4-benzylidenepiperazine core like ritanserin. The affinity of clozapine, however, was attenuated by a mere 2–3-fold at G218S$^{5.42}$ 5-HT_{2C}Rs (102, 122, 123).

The role of $G^{5.42}$ in mediating selective binding at 5-HT_2-type receptors has since been extended to the 5-HT_{2A}R by Kimura et al. (79) and was recently replicated and expanded upon by our lab (80). Using a combination of molecular modeling and site-directed mutagenesis, this body of work suggests that the selectivity of pimavanserin to bind 5-HT_{2A}/5-HT_{2C}Rs stems from the isobutoxybenzyl moiety occupying a cavity between TM4 and TM5 afforded by the small side chain of $G^{5.42}$ (79, 80). Generation of a $G238S^{5.42}$ 5-HT_{2A}R confirmed this binding pose, as pimavanserin showed a near-complete loss in affinity and antagonist activity at the receptor variant (79, 80). Moreover, we used novel 2-aminotetralin derivatives to demonstrate that this effect was driven by the size of the ligand motif extending into the cavity between TM4 and TM5, and this was predictive of ligand affinity over several other aminergic GPCRs (80). In contrast, risperidone, which has high affinity at multiple aminergic receptors and binds to 5-HT_{2A}Rs in an extended conformation spatially distinct from the side cavity, was only modestly affected by the $G238S^{5.42}$ mutation (79, 80).

Certain lipids are known to potentiate 5-HT-mediated G protein activation through the 5-HT_{2A}R (124, 125). Very recently, the structural basis of this effect was delineated using crystal structures of 5-HT_{2A}Rs bound to the agonists 5-HT, psilocin, lisuride, or LSD (126). In each structure, the lipid monoolein snaked into the cavity adjacent to $G238^{5.42}$, between TM4 and TM5. The lipids monoolein, oleamide, oleoylethanolamide, and 2-oleoyl glycerol were all shown to modestly activate WT, but not $G238S^{5.42}$ 5-HT_{2A}Rs, suggesting that $G238^{5.42}$ also serves as a structural determinant of lipid-mediated G protein signaling through 5-HT_{2A}Rs. It remains unknown how the lipid environment impacts antagonist affinity or selectivity at 5-HT_{2A}Rs.

Taken together, published experimental and computational work supports the conclusion that $G^{5.42}$ contributes to the observed 5-HT_{2A}R selectivity of structurally distinct antagonists. However, this model incompletely describes 5-HT_{2A}R selectivity, as pimavanserin demonstrates high selectivity over 5-HT_{2B} and modest selectivity over 5-HT_{2C}Rs despite the presence of $G^{5.42}$ in both receptors. To better understand the molecular and structural features governing selective binding at 5-HT_{2A}Rs over other 5-HT_2-type receptors, a more detailed understanding of GPCR structure is required.

3.2.3 Amino acid ensembles governing 5-HT_{2A}R selectivity—Apart from single amino acid differences, amino acid ensembles can change the 3-dimensional (3D) architecture of binding pockets. A sophisticated model of this was recently put forth by Kimura et al. in detailing the first 5-HT_{2A}R crystal structures (79). Structural comparisons of a risperidone-bound 5-HT_{2A}R, ergotamine-bound 5-HT_{2B}R, and ritanserin-bound 5-HT_{2C}R indicated that the length and conformation of extracellular loop 2 (ECL2) was nearly identical between 5-HT_{2A} and 5-HT_{2C}Rs yet was 4–6 residues longer in the 5-HT_{2B}R due to a shortened TM4. In addition, divergent amino acid residues near the extracellular ends of TM4 and TM5 within the 5-HT_2-type receptors were proposed to contribute to the formation of a unique side chain rotamer of $F^{5.38}$, located one helical turn closer to ECL2 than $G^{5.42}$, in the 5-HT_{2A}R compared to that in 5-HT_{2B} and 5-HT_{2C}Rs.

In the risperidone-bound 5-HT_{2A}R crystal structure, a hydrophobic interaction between $F213^{4.63}$ and $F234^{5.38}$ appeared to orient the side chain rotamer of $F234^{5.38}$ toward the

extracellular end of TM4, leading Kimura et al. (79) to propose that a 5-HT_{2A}R-specific rotamer of F234$^{5.38}$ increases the volume of the side pocket formed by G238$^{5.42}$ to realize a “side-extended cavity” (Figure 5). In contrast, the side chains of F217$^{5.38}$ and F214$^{5.38}$ in 5-HT_{2B} and 5-HT_{2C}Rs, respectively, oriented toward the cytosol, effectively reducing the side-cavity’s volume. The orientation of F$^{5.38}$ within 5-HT_{2B} and 5-HT_{2C}Rs appeared to originate from steric restrictions with K193$^{4.63}$ in 5-HT_{2B}Rs, and I192$^{4.63}$ or D211$^{5.35}$ in 5-HT_{2C}Rs (79), potentially explaining the observed selectivity of pimavanserin to bind 5-HT_{2A}Rs over 5-HT_{2B} and 5-HT_{2C}Rs. Notably, although this work represents the most extensive mutagenesis investigation of the 5-HT_{2A}R to date, no mutations of 5-HT_{2A}R were made at positions X$^{4.63}$ or X$^{5.35}$ to suggest that a side chain rotamer of F$^{5.38}$ could be perturbed to impact the affinity of antagonists with subtype-selectivity at 5-HT_{2A}Rs.

To test the role of interactions between F213$^{4.63}$ and F234$^{5.38}$ in mediating subtype-selective antagonist binding at 5-HT_{2A}Rs, our lab point-mutated F213$^{4.63}$ within the 5-HT_{2A}R to the structurally equivalent residue in 5-HT_{2B}Rs (K$^{4.63}$) to mimic the side chain rotamer of F$^{5.38}$ found in 5-HT_{2B}R crystal structures. We then assessed the affinity of several 5-HT_{2A}R antagonists with varying selectivity, including risperidone and pimavanserin, at point-mutated F213K$^{4.63}$ 5-HT_{2A}Rs (80). However, no difference in antagonist potency (pK_b) was observed for any ligand compared to WT 5-HT_{2A}Rs. These results suggest that F213$^{4.63}$ alone does not meaningfully contribute to facilitating selective antagonist binding to 5-HT_{2A}Rs.

Ligand binding is a dynamic process wherein ligands and amino acid residues sample discreet interaction profiles and conformational states across a probability distribution (see ref. (112) for review). Accordingly, receptor crystal structures are static representations of a dynamic system that may not reliably indicate structurally or pharmacologically meaningful interactions. Therefore, we used molecular modeling to identify residues that could dynamically modulate the rotamer of F234$^{5.38}$ in 5-HT_{2A}Rs. Our simulations identified the side chain of D231$^{5.35}$ (F214$^{5.35}$ in 5-HT_{2B} and D211$^{5.35}$ in 5-HT_{2C}Rs), located one helical turn above F234$^{5.38}$, as a candidate for influencing the F234$^{5.38}$ rotamer (80). However, D231F$^{5.35}$ 5-HT_{2A}Rs were functionally null in response to 5-HT, and specific binding was not detected with [^{3}H]spiperone, [^{3}H]ketanserin, or [^{3}H]mesulergine (80). Notably, the absence of a fluorescent tag precluded us from confirming proper membrane localization of D231F$^{5.35}$ 5-HT_{2A}Rs.

Careful inspection of published 5-HT_2-type receptor crystal structures indicates that the D231F$^{5.35}$ mutation could have destabilized the conformation of ECL2, potentially leading to a misfolded receptor and, subsequently, inadequate membrane-trafficking. For example, the crystal structure of a risperidone-bound 5-HT_{2A}R shows the formation of an electrostatic cage around K220^{ECL2}, formed by the negatively charged side chains of D217$^{4.67}$ and D231$^{5.35}$, which may limit the conformational freedom of, and therefore stabilize, ECL2 (Figure 5). Similar interactions are apparent in the crystal structure of a ritanserin-bound 5-HT_{2C}R, where the positively charged side chain of K199^{ECL2} is enclosed by the negatively charged side chains of D196$^{4.67}$ and D211$^{5.35}$ (Figure 5). Thus, the absence of K^{ECL2} near TM4 in the 5-HT_{2B}R, and the presence of F214$^{5.35}$, may promote the conformational freedom observed in 5-HT_{2B}R ECL2 (127, 128), and contribute to the ability of some

antagonists to bind 5-HT_{2A} and 5-HT_{2C}Rs with higher affinity. Moreover, residues on the extracellular side of $D^{3.32}$ are less conserved across 5-HT_2-type receptors than those on the intracellular side of $D^{3.32}$, suggesting that antagonist selectivity could involve interactions with residues near ECL2. Although, steric interactions with $M218^{5.39}$ in the 5-HT_{2B}R do not appear to sufficiently explain the low affinity of pimavanserin since it exhibits ~2-fold higher affinity at $V235M^{5.39}$ 5-HT_{2A}Rs (80).

Concerning the modest selectivity of pimavanserin to bind 5-HT_{2A} over 5-HT_{2C}Rs, careful consideration of its affinity at, and selectivity over, 5-HT_{2C}Rs is warranted. Using molecular modeling techniques identical to those described elsewhere (80), we show that the binding pose of pimavanserin in 5-HT_{2C}Rs is nearly identical to that reported for 5-HT_{2A}Rs, even insofar as $F^{5.38}$ adopts a raised rotamer conformation (Figure 6). Notably, the only non-conserved residues present in the binding pockets of 5-HT_{2A} and 5-HT_{2C}Rs are $S/A^{5.46}$ and $I/V^{4.56}$ (80). Although mutagenesis studies do not support a role for $S/A^{5.46}$ in mediating subtype selectivity of pimavanserin (79, 80), pimavanserin does exhibit 4-fold increase in potency at $I206V^{4.56}$ 5-HT_{2A}Rs (79), suggesting that steric tolerance at this position may enhance affinity at 5-HT_{2C}Rs.

Insight into the ability of ligands to discriminate between 5-HT_{2A} and 5-HT_{2C}Rs comes from a recent report detailing the crystal structure of a spiperone-bound D_2R that proposes a mechanism for the ability of spiperone to bind D_2Rs and 5-HT_{2A}Rs selectively over 5-HT_{2C}Rs (129). Although the D_2R lacks a side-extended cavity, it does possess a spatially distinct extended binding pocket. Located between TM2 and TM3, the extended binding pocket—comprised of $V87^{2.57}$, $W90^{2.60}$, $V91^{2.61}$, $L94^{2.64}$, $W100^{23.50(ECL1)}$, $F110^{3.28}$, $V111^{3.29}$, and $C182^{45.50(ECL2)}$—accommodates the phenyl ring of spiperone. Notably, the side chains of $W90^{2.60}$ in D_2Rs and $V130^{2.60}$ in 5-HT_{2A}Rs allow the side chain rotamers of $F110^{3.28}$ and $W151^{3.28}$ to flip and expose the extended binding pocket. In 5-HT_{2C}Rs, however, the bulkier side chain of $L109^{2.60}$ may restrict $W130^{3.28}$ from flipping, thereby impeding the formation of an extended binding pocket (129). These findings were supported by the observation that the affinity of spiperone at $W90L^{2.60}$ D_2Rs was more severely attenuated (~20-fold) than at $F110W^{3.28}$ D_2Rs (~11-fold) (129), although the affinity of spiperone at $W90V^{2.60}$ D_2Rs was not reported. These findings are supported by medicinal chemistry work noting that replacing the phenyl ring on spiperone with smaller lipophilic substituents (e.g., −H, $-CH_3$, $-CH_2CH_3$, or $-CH[CH_3]_2$) negatively impacted affinity at 5-HT_{2A}, 5-HT_{2C}, and D_2Rs (130). In conclusion, these structural differences may contribute, in part, to the observed selectivity of spiperone to bind D_2 and 5-HT_{2A}Rs over 5-HT_{2C}Rs.

3.3 Alternative approaches for designing 5-HT_{2A}R selective antagonists

Most aminergic ligands incorporate a basic amine moiety that, upon protonation, can form a strong ionic bond with $D^{3.32}$ in the orthosteric binding pocket (105, 131). Such an anchoring strategy can be helpful in generating high affinity ligands, although protonated amines are also significant contributors to ligand promiscuity. In fact, screening studies have found that 20–50% of positively charged novel chemical entities bind to aminergic receptors with an IC_{50} < 10 μM, and serotonin receptors are among the most common targets (132, 133).

Therefore, the design of non-basic ligands can be an effective strategy for developing novel, selective ligands.

Non-basic ligands comprise the most subtype selective 5-HT_2-type receptor antagonists available. For example, RS-127445 is a 5-HT_{2B}R antagonist that does not possess a basic amine moiety and is not positively charged at physiological pH. Yet, it displays >1,000-fold selectivity over 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_3, 5-HT_5, 5-HT_6Rs, and 100 other receptors and ion channels, determined by Cerep screening (134). Successfully generating a selective 5-HT_{2B}R antagonist is an impressive accomplishment since ligand affinity at 5-HT_{2B}Rs may predict pharmacological promiscuity, with some surveys finding that ~50% of positively charged compounds interact with 5-HT_{2B}Rs (132, 133). Similarly, indoline urea derivatives such as SB 242084, SB 243213, and SB 206553 lack basic amines and are among the most selective antagonists targeting 5-HT_{2B} and/or 5-HT_{2C}Rs (135–137). Thus, rational strategies to identify non-basic 5-HT_{2A}R ligands hold promise for discovering novel subtype-selective ligands. One such approach was taken to discover 6-(1-ethyl-3-(quinolin-8-yl)-1*H*-pyrazol-5-yl)pyridazin-3-amine, a non-basic subtype-selective 5-HT_{2C}R agonist, identified through screening a compound library against $D134A^{3.32}$ 5-HT_{2C}Rs—an active site receptor variant that is functionally responsive to neutral, but not positively charged, agonists (138). Analogous point-mutated 5-HT_{2A}Rs might also be useful in identifying selective non-basic inverse agonists in platforms like R-SAT™ (68).

Results from recent molecular docking studies provide an alternative approach to achieving subtype-selective binding. Docking studies with SB 242084 suggest that it may interact with the same conserved amino acid residues in each of the 5-HT_2-type receptors, but that its selectivity stems from its ability to make closer, more numerous hydrophilic contacts in the 5-HT_{2C}R, e.g., with $D134^{3.32}$, $S138^{3.36}$, and $Y358^{7.43}$ (117). Moreover, ligand affinity is defined by the relationship between the association rate (k_{on}) of a free ligand to an unbound receptor, forming a binary complex, and the dissociation rate (k_{off}) of the bound ligand from the binary complex ($K_d = k_{off}/k_{on}$). Conceptually, it is helpful to consider k_{off} as reflecting the stability of a ligand-receptor complex, with more stable complexes having lower k_{off} values, and hence, higher affinity. Given this, it is interesting to speculate that closer hydrophilic contacts between SB 242084 and 5-HT_{2C}R residues may promote a uniquely stable ligand-receptor complex with a low k_{off} value. Further computational and experimental investigations to this end would provide a more textured understanding of how ligands are stabilized in 5-HT_2-type receptors.

4. Computational approaches to assist in drug design targeting 5-HT_{2A}Rs

4.1 Ligand-based drug discovery: QSAR

In the pre-structural biology era, 3D quantitative structure-activity relationships (3D-QSAR) were commonly used in drug discovery programs targeting 5-HT receptors. Among the most popular methods is comparative molecular field analysis (CoMFA), which correlates the biological activity (e.g., binding affinity) of a series of compounds with molecular features governing non-covalent ligand-receptor interactions, such as electrostatic and steric properties. Compared to conventional QSAR models based on the Hansch analysis, CoMFA

models are similarly capable of predicting biological activity yet can incorporate structurally diverse analogs typically omitted from traditional QSAR analyses (139).

The CoMFA method to predict the activity of novel ligands is based on a training set of experimental data. For example, 3D-QSAR studies on a series of 3-(1,2,5,6-tetrahydropyridin-4-yl)indole derivatives targeting 5-HT_{2A}Rs, yielded a model that accurately predicted the affinity of six novel ligands based on a training set composed of 45-compounds (139). Moreover, CoMFA models modified to account for hydrogen bonding and hydrophobic interactions have been generated to predict electrostatic, steric, and hydrophobic determinants of affinity for a series of conformationally restricted butyrophenones at rat 5-HT_{2A} and D_2Rs (140).

4.2 Use of homology modeling and molecular docking to delineate mechanisms of receptor inactivation and ligand affinity

Several GPCR crystal structures were solved in the early 2000s, including rhodopsin (141) and human β_2-adrenergic receptors (142). They were widely used to build homology models of 5-HT receptors to better understand the molecular mechanisms governing receptor function and ligand affinity and selectivity. Using a 5-HT_{2A}R model based on the crystal structure of bovine rhodopsin in combination with molecular modeling, site-directed mutagenesis, and functional assays, Shapiro et al. (143) investigated the molecular mechanism of 5-HT-mediated 5-HT_{2A}R activation. Their findings were consistent with the hypothesis that an ionic bond between R173$^{3.50}$ and E318$^{6.30}$, on the intracellular face of the receptor, serves as an essential interaction that is disrupted upon receptor activation (143). The side chain of R173$^{3.50}$ has also been implicated in resolving the inverse agonist activity of constitutively activated C322K$^{6.34}$ 5-HT_{2A}R variants, supported by molecular docking and molecular dynamics simulations with a model of the 5-HT_{2A}R based on metarhodopsin (144, 145).

A homology model of the 5-HT_{2A}R based on rhodopsin helped predict the relative contribution of S239$^{5.43}$ and S242$^{5.46}$ in the binding of tryptamine and phenylalkylamine-based agonists. Using a combination of site-directed mutagenesis and radioligand binding to confirm model predictions, Braden and Nichols demonstrated that a hydrogen bond could form between S239$^{5.43}$ and tryptamines substituted at the 4- or 5-position with hydrogen-bond acceptors, as well as phenylalkylamines substituted at the 4-position (121). Moreover, phenylalkylamine binding was predicted to involve a hydrogen bond between the 2- and 5-position substituents and S159$^{3.36}$ and S239$^{5.43}$, respectively. This prediction was supported over a decade later by the structure of a 5-HT_{2A}R bound to the phenylalkylamine derivative *N*-(2-hydroxybenzyl)-2,5-dimethoxy-4-cyanophenylethylamine, demonstrating the formation of a unique hydrogen bond between the C(2)-OH moiety and the side chain of S159$^{3.36}$ (106). In contrast, the side chain of S159$^{3.36}$ does not appear to be involved in the binding of LSD or ketanserin at 5-HT_{2A}Rs (106, 120).

4.3 Virtual high-throughput screening

Traditional drug discovery programs often rely on experimental HTS with large compound libraries in biological assays, and known active ligands, to identify leads for SAR and

guide drug development (146). However, while these methods have proved fruitful, they barely scratch the surface of exploring the chemical space of drug-like molecules—routinely cited to be $>10^{20}$—thus limiting the pace of medication development (147). Compared to experimental HTS, virtual HTS, pairing large computational libraries of compounds and molecular docking, is a more economical screening approach to drug discovery (148–151). Several studies have used virtual HTS with models of 5-HT_2-type receptors to identify possible novel ligands (152, 153), with some predicted to demonstrate subtype-selective binding (154). Importantly, these investigations have led to the discovery of experimentally validated novel ligands with high affinity at (155), or selectivity for (156, 157), particular 5-HT_2-type receptors.

With the increasing abundance of experimental 5-HT_{2A}R structures, 13 at the time of writing (79, 106, 111, 126), coupled with the availability of free ultra-large ligand libraries, including the ZINC database (http://zinc20.docking.org)—an expanding library of over a billion compounds—the discovery of novel ligands with new scaffolds is increasingly accessible. Virtual HTS and ultra-large library docking have proven fruitful in identifying novel and potent receptor modulators (158–161). It seems increasingly likely that these methods will assume a more prominent role in drug discovery campaigns searching for novel 5-HT_{2A}R ligands as we advance. To predict the functional activity of novel ligands as agonists or antagonists, and especially functionally selective (biased) agonists, however, requires additional computational methods capable of tracking global changes in receptor structure and receptor interactions with signaling proteins.

4.4 Molecular dynamics as a method to predict functionally relevant receptor conformations

Both structural and dynamic factors determine protein function. To better understand the dynamic and temporal aspects of protein function, molecular dynamic (MD) simulations are an integral and powerful computational approach. For example, one microsecond-long MD simulations using a 5-HT_{2A}R homology model bound to psychedelic or non-psychedelic 5-HT_{2A}R agonists identified distinct conformations of intracellular loop 2 (ICL2) characteristic of psychedelic agonists (162). These findings suggest that psychedelic 5-HT_{2A}R agonists could stabilize discreet receptor conformational states relevant to intracellular signaling cascades, as suggested by experimental work (163). Moreover, MD simulations have been used to predict that the side chains of $S242^{5.46}$ and $N343^{6.55}$ in the 5-HT_{2A}R contribute to biased agonism, a prediction later supported by experiment (164).

When designed to encompass a sufficient temporal window, MD simulations may also distinguish agonist from antagonist ligands at the 5-HT_{2A}R. This was shown by Shan et al., using 350 nanosecond MD simulations of a 5-HT_{2A}R model, based on rhodopsin and β_2AR crystal structures, in complex with a full agonist, a partial agonist, and an antagonist of canonical 5-HT_{2A}R signaling (i.e., 5-HT, LSD, and ketanserin, respectively) (165). The simulations indicated that ligands of varying functional activity differentially influenced well-documented microswitches of GPCR activation and stabilized conformational states of the receptor that lead to distinct deformations in the surrounding plasma membrane. Using a complementary approach, we have aimed to expand the work described elsewhere

(143) to characterize 5-HT_{2A}R dynamics with one microsecond MD simulations of an apo-5-HT_{2A}R, methiothepin-bound 5-HT_{2A}R (antagonist-state), and an ergotamine-bound 5-HT_{2A}R (agonist-state). We confirmed that a stable ionic bond (~2 Å) between $R173^{3.50}$ and $E318^{6.30}$ locks the apo-5-HT_{2A}R in an inactive state. This bond is also present in the inactive methiothepin-bound 5-HT_{2A}R, and broken (~4 Å) upon conformational transitions to the active state in the ergotamine-bound 5-HT_{2A}R between 300–700 nanoseconds (Figure 7). Similar findings have been presented independently for the 5-HT_{2B}R (166).

5. Considerations and conclusions

The development of selective antagonists for clinical and preclinical purposes will likely continue, although the methods used to discover them are destined to evolve. Historically, the discovery of putatively selective 5-HT_{2A}R antagonists relied on experimental screening and lead optimization. Yet, sparingly few compounds have withstood the test of rigorous receptor profiling *in vitro*, and the number of chemically diverse antagonists with robust selectivity for 5-HT_{2A}Rs remains low. Moving forward, it would be incumbent for medicinal chemists and pharmacologists reporting on selective ligands to clearly describe the context used to define a ligand as "selective". As an example, a ligand may be defined as selective if it has >100-fold higher affinity at its target GPCR, based on *in vitro* affinity tests at a pre-defined set of off-target GPCRs within the target's receptor family, e.g., "compound X is selective for 5-HT_{2A} relative to 5-HT_{2B}, and 5-HT_{2C}Rs based on *in vitro* affinity assays using antagonist radiolabels." Moreover, novel compounds should be characterized alongside reference ligands in orthogonal assays to account for affinity differences due to assay conditions and facilitate fair comparison across literature reports (80, 167). Authors should then note that the compound may only be useful—without further characterization—as an *in vitro* tool.

Discerning ligand selectivity with confidence *in vivo* poses a distinct challenge, due to a need to quantify ligand occupancy at on- and off-target binding sites across multiple tissues, doses, and time points following administration. In addition, the pharmacokinetic parameters used to determine occupancy will likely vary between routes of administration (i.e., intraperitoneal, subcutaneous, oral, or intravenous), and thus would require further experimentation to validate selectivity following dosing across routes. These experiments may be seen as cost-prohibitive, especially for academic labs developing medication candidates, but these data ultimately would help prevent misinterpretation of results and provide the broader scientific community (e.g., behavioral pharmacologists, neurophysiologists, etc.) critical information to guide the selection, and dosing, of tool compounds for *in vivo* use.

In the absence of detailed pharmacokinetic profiling, there is the modus operandi of titrating antagonist dose to prevent behaviorally disruptive or off-target effects while achieving a specific behavioral outcome. However, there can be problems with this approach if the behavioral outcome under evaluation is affected by multiple targets. For example, the head-twitch response in mice is associated with activation of the 5-HT_{2A}R, yet is also modulated through numerous other mechanisms (163, 168, 169). Without the pharmacodynamic and pharmacokinetic information described above, we cannot make firm conclusions about

selective receptor engagement *in vivo* or about the role of the GPCR target in the measured outcome. For example, we have shown that M100,907—which has ~160-fold *in vitro* selectivity for mouse 5-HT_{2A} over 5-HT_{2C}Rs—significantly suppresses the head-twitch response in adult, male C57BL/6J mice elicited by 1 mg/kg (±)-2,5-dimethoxy-4-iodo-amphetamine (s.c.), when administered at 0.0025 mg/kg (s.c.) (71), though, at this dose, only ~35% of the head-twitch response is blocked. Meanwhile at 0.025 mg/kg and 0.25 mg/kg M100,907, ~85% and 100% of the head-twitch response is blocked, respectively. Without evaluating receptor occupancy at these doses, it is not possible to conclude what dose (if any) of M100,907 selectively blocks central 5-HT_{2A}Rs that elicit the head-twitch response.

Selective knockdown of 5-HT_{2A}R with genetic tools in combination with autoradiography would also clarify the *in vivo* selectivity of a ligand following administration at a defined dose, route, timestep. For example, [^{3}H]M100,907 could be administered at various doses to 5-HT_{2A}R knockout mice together with another, structurally unique 5-HT_{2A}R antagonist, and receptor occupancy could be used to determine the dose at which it binds off-targets, considering potential issues of non-specific binding.

There is also the option of combining ligands in an experiment to interrogate mechanism. For example, SB 242084, a putatively selective neutral antagonist of 5-HT_{2C}Rs might be co-administered with pimavanserin to mask the potent inverse agonist activity of pimavanserin at 5-HT_{2C}Rs. However, SB 242084 would block activity-dependent signaling through 5-HT_{2C}Rs, like pimavanserin, without preserving its native signaling capacity in response to synaptic release of 5-HT. Moreover, ligand combinations increase the possibility of confounding pharmacokinetic and pharmacodynamic interactions *in vivo*, e.g., competition for common metabolic enzymes might increase the plasma concentration of each ligand, and parent compound metabolites, and their pharmacological activities, are often unknown.

Here, we conclude that M100,907 and pimavanserin, on balance, are the most authenticated putatively selective 5-HT_{2A}R antagonists available. Nevertheless, M100,907 and pimavanserin, as well as all other 5-HT_{2A}R-selective antagonists, have off-target activities that need to be considered when used to elucidate biological mechanisms. Thus, we echo a recommendation made nearly forty-years ago (8) that mechanistic investigation of 5-HT_{2A}Rs by pharmacological antagonism should utilize two structurally distinct antagonists with different off-target activities, albeit, feasibility of conducting the same experiments twice with different antagonists is limited by increased costs.

For the reasons detailed above, we define “selective” empirically within a context, meaning that if a ligand can be administered such that it acts on its intended target without acting on pre-defined off-targets, then it is selective. We acknowledge the shortcomings of this definition, because of the “unknown unknowns” phenomenon, i.e., there are tens of thousands of proteins, and ~800 GPCRs, expressed in mammalian tissue, and a ligand could have activity at any one of them. Because this is a critical issue, we believe that medicinal chemists and pharmacologists should convene to establish parameters for defining a ligand as “selective” with the goal of providing the general scientific community information to choose dose ranges for *in vivo* studies in particular species.

The design of novel unambiguously selective 5-HT_{2A}R antagonists will require expertise spanning various fields. Structural biology studies could help by delineating the structure of 5-HT_2-type receptors bound to subtype-selective antagonists from multiple chemotypes to identify points of contact reliably involved in subtype-selective binding. Notably, structural biology studies often detail a single receptor conformational state, leaving unanswered questions about the dynamic nature of the receptor. Therefore, a library of diverse ligand-receptor structures could improve the efficiency of ultra-large library docking and prediction of ligand function *in silico*, which will likely lead to the next generation of selective 5-HT_{2A}R antagonists. Nevertheless, the proper implementation of MD simulations in the context of drug discovery is still a challenge, and computational approaches supported by experimental evidence will provide the most compelling models for ligand-receptor interactions.

Funding and acknowledgments:

Some of the original research described in this review was funded by the National Institute on Drug Abuse (NIDA) grants R01DA047130 and R01DA030989 (R.G.B.), National Institute of Neurological Disorders and Stroke (NINDS) grant R15 NS118352-01 (C.E.C), U.S. Department of Defense grants CDMRP W81XWH-17-1-0322 (R.G.B.) and CDMRP W81XWH-17-1-0329 (C.E.C), and the Northeastern University David Bear Scholar Fund (A.B.C.). The computational work shown in figures 6 and 7 was completed using the Discovery cluster, supported by Northeastern University's Research Computing team.

Abbreviations

LSD	lysergic acid diethylamide
R-SAT™	receptor selection and amplification technology™
TM	transmembrane domain
ECL	extracellular loop
ICL	intracellular loop
Q-SAR	Quantitative structure-activity relationships
CoMFA	comparative molecular field analysis
MD	molecular dynamics
HTS	high throughput screening

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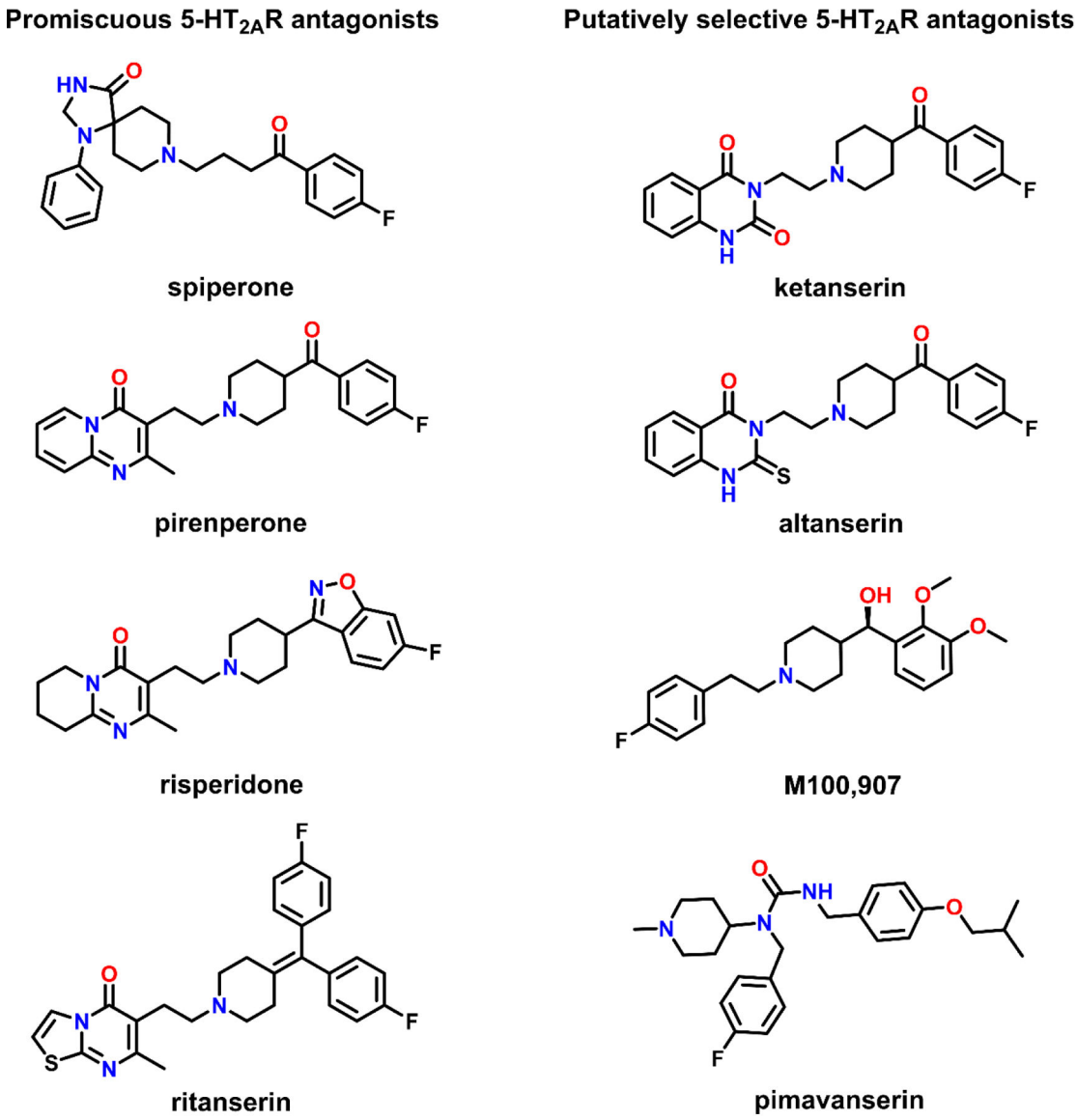

Figure 1:

Structures of antagonists, with varying degrees of selectivity, commonly employed to probe the 5-HT_{2A}R.

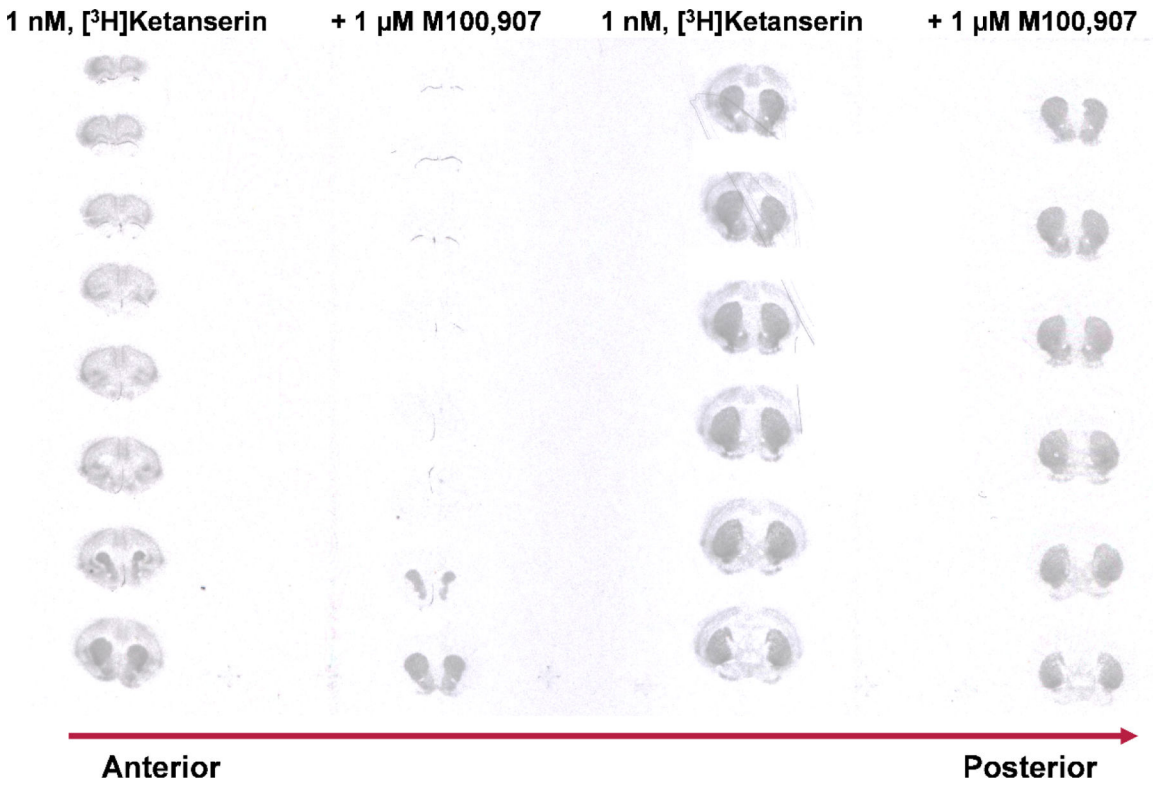

Figure 2:

[^{3}H]Ketanserin binds with high affinity (1 nM) a large population of targets in the striatum that are not 5-HT$_2$-type receptors. Note that co-incubation with the putatively selective 5-HT$_{2A}$R inverse agonist, M100,907 (1 μM, a concentration chosen to occupy and block the vast majority of 5-HT$_{2A}$Rs, in order to reveal off-target binding of [^{3}H]ketanserin), blocked binding of [^{3}H]ketanserin in the cortex, but not the striatum. [^{3}H]ketanserin (42.5 Ci/mmol) autoradiography (10-week film exposure) of coronal brain sections from FVB.129P2-*Pde6b*$^+$ *Tyr*$^{c\text{-}ch}$/AntJ (sighted FVB) mice.

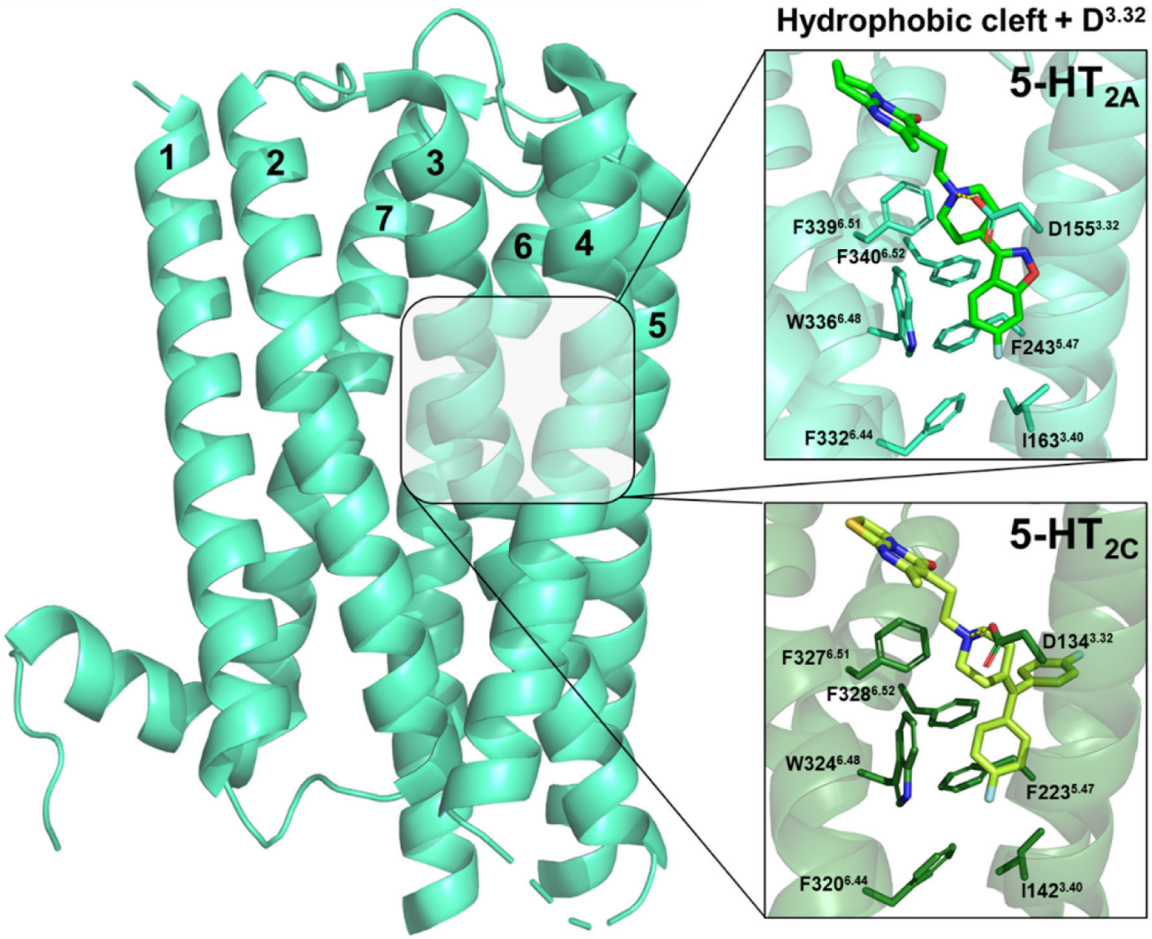

Figure 3: Molecular determinants of nonselective binding at aminergic receptors.

Structures of a risperidone-bound 5-HT_{2A}R (light green, PDB: 6A93) and ritanserin-bound 5-HT_{2C}R (dark green, PDB: 6BQH) show aromatic and hydrophobic interactions with conserved residues forming a hydrophobic cleft deep in the binding pocket, and an ionic bond between the ligand's protonated amine and the negatively charged side chain of $D^{3.32}$. The whole 5-HT_{2A}R is shown (left) for perspective, with each transmembrane domain numbered.

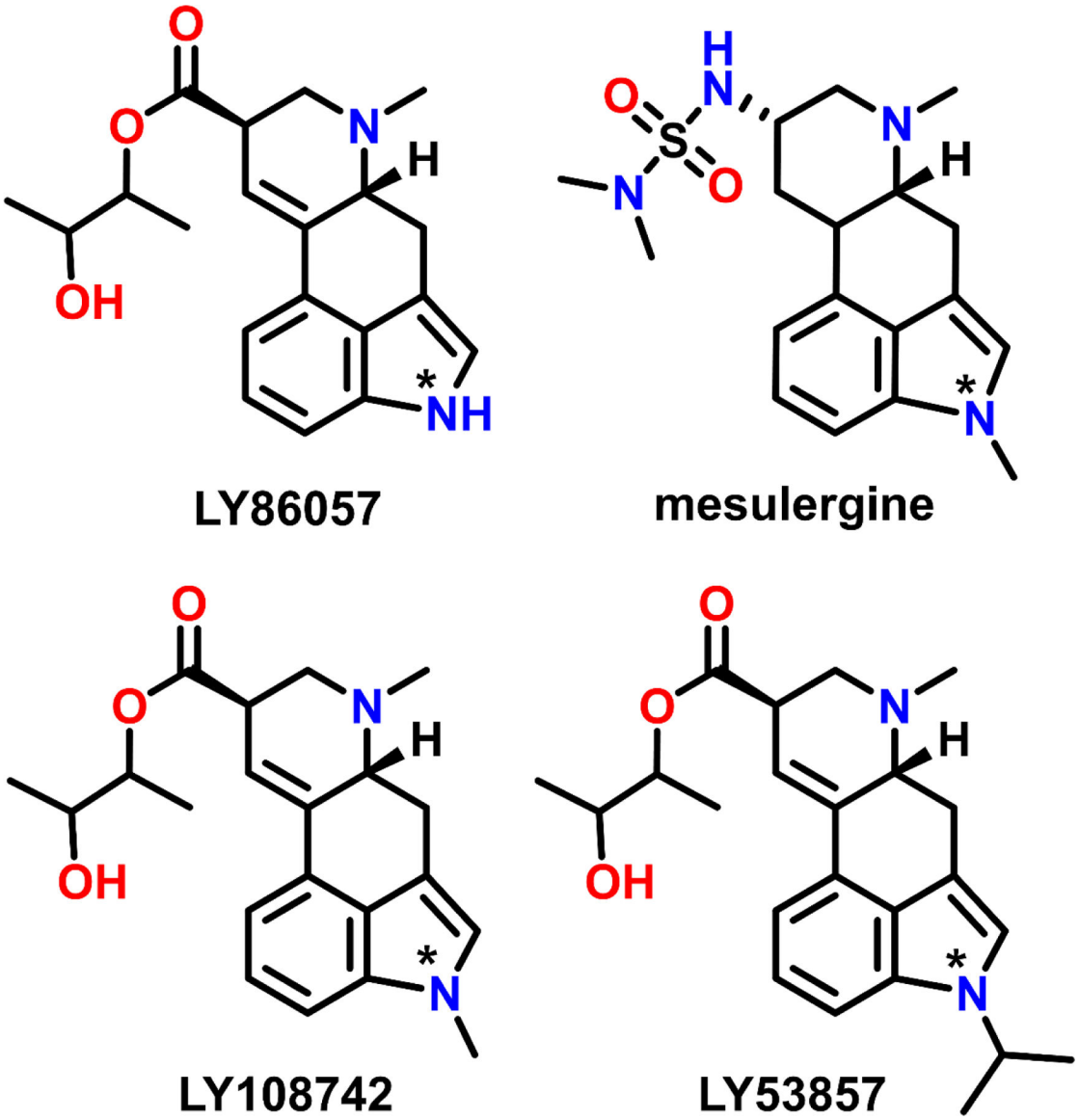

Figure 4:
Structures of ergoline-based 5-HT_{2A}R antagonists variably substituted at the N(1) position (denoted by *).

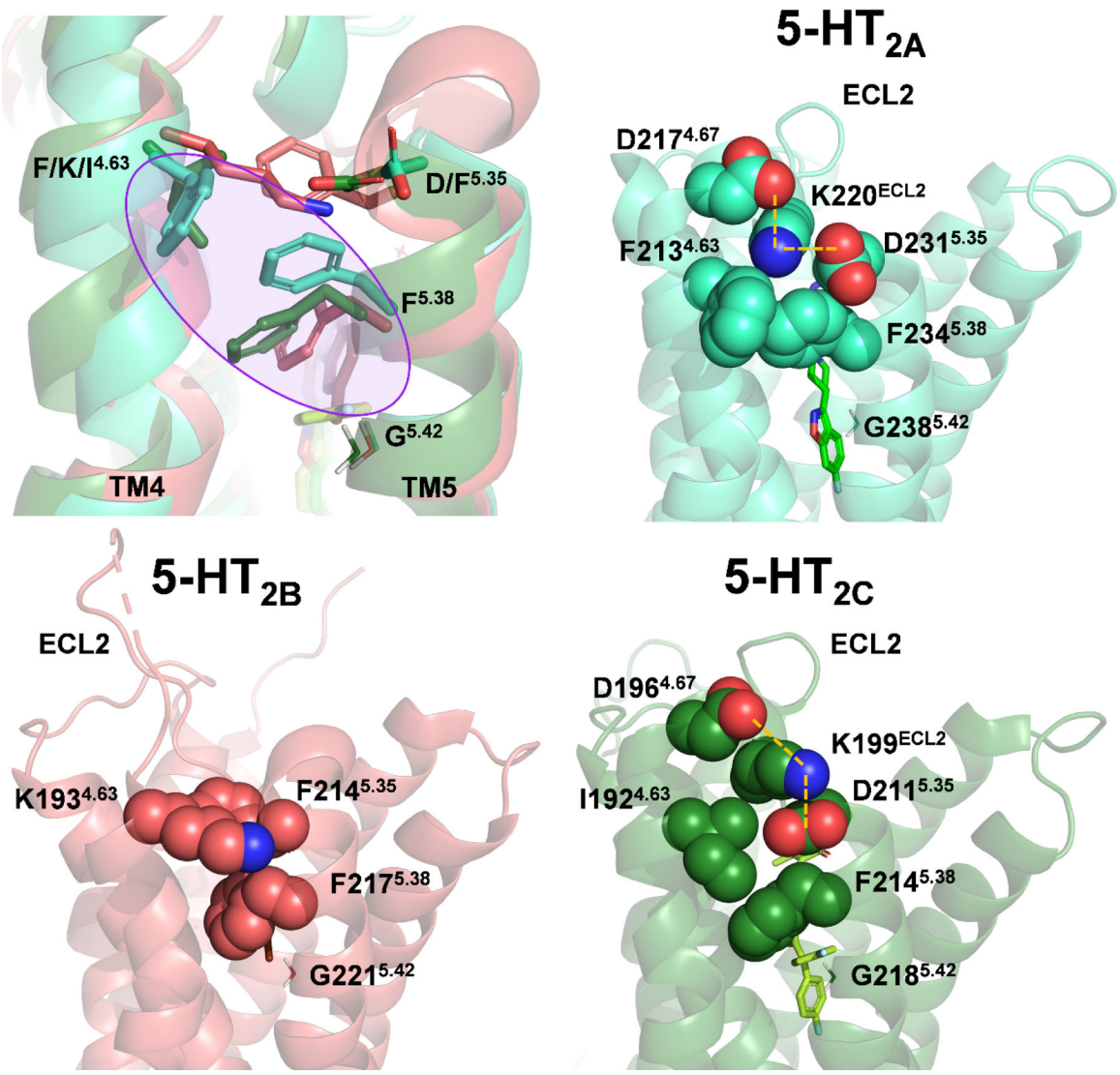

Figure 5: Putative molecular determinants of subtype-selective binding at 5-HT$_2$-type receptors.

Superimposition of 5-HT$_2$-type receptors suggests that a "side extended cavity" is formed in the 5-HT$_{2A}$R (light blue color) by a unique rotamer of F$^{5.38}$, making hydrophobic interactions with the non-conserved residue F$^{4.63}$ (purple circle, top left). Structures of the 5-HT$_{2A}$R (PDB: 6A93, top right), 5-HT$_{2B}$R (PDB: 6DS0, bottom left), and 5-HT$_{2C}$R (PDB: 6BQH, bottom right) indicate that ECL2 of the 5-HT$_{2B}$R exhibits greater conformational freedom compared 5-HT$_{2A}$ and 5-HT$_{2C}$Rs, wherein K^{ECL2} is electrostatically constrained (orange dashed lines). The side chain of G$^{5.42}$ is shown for reference to the side cavity.

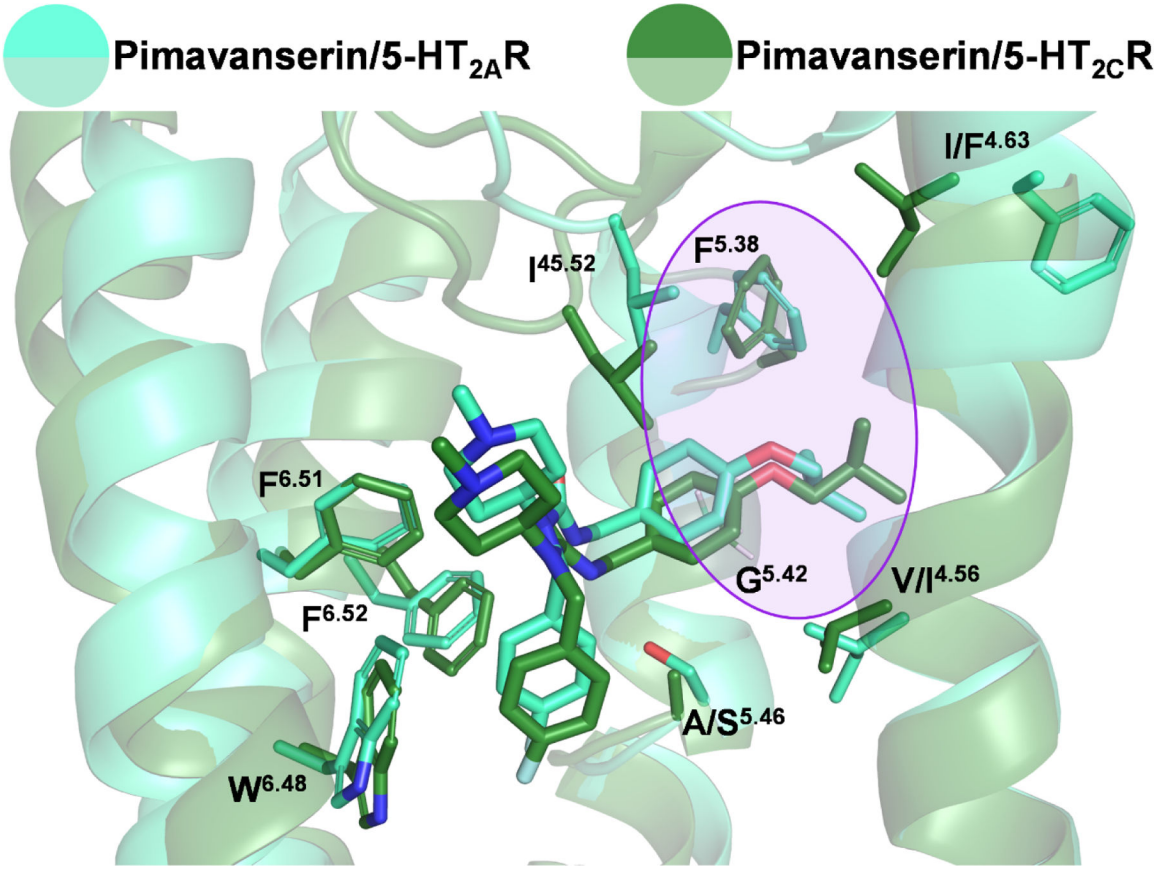

Figure 6: Proposed binding mode of pimavanserin at 5-HT$_{2A}$ (light green) and 5-HT$_{2C}$Rs (dark green).

Molecular docking and molecular dynamics simulations indicate that pimavanserin engages nearly identical amino acid residues within the binding pocket of 5-HT$_{2A}$ and 5-HT$_{2C}$Rs. The primary determinant of selectivity, the extension of the isobutoxybenzyl moiety into the side-extended cavity between TM4 and TM5 (purple circle), as allowed by the small side chain of G$^{5.42}$, is conserved in both receptors and is accompanied by a raised rotamer conformation of F$^{5.38}$ in both receptor subtypes.

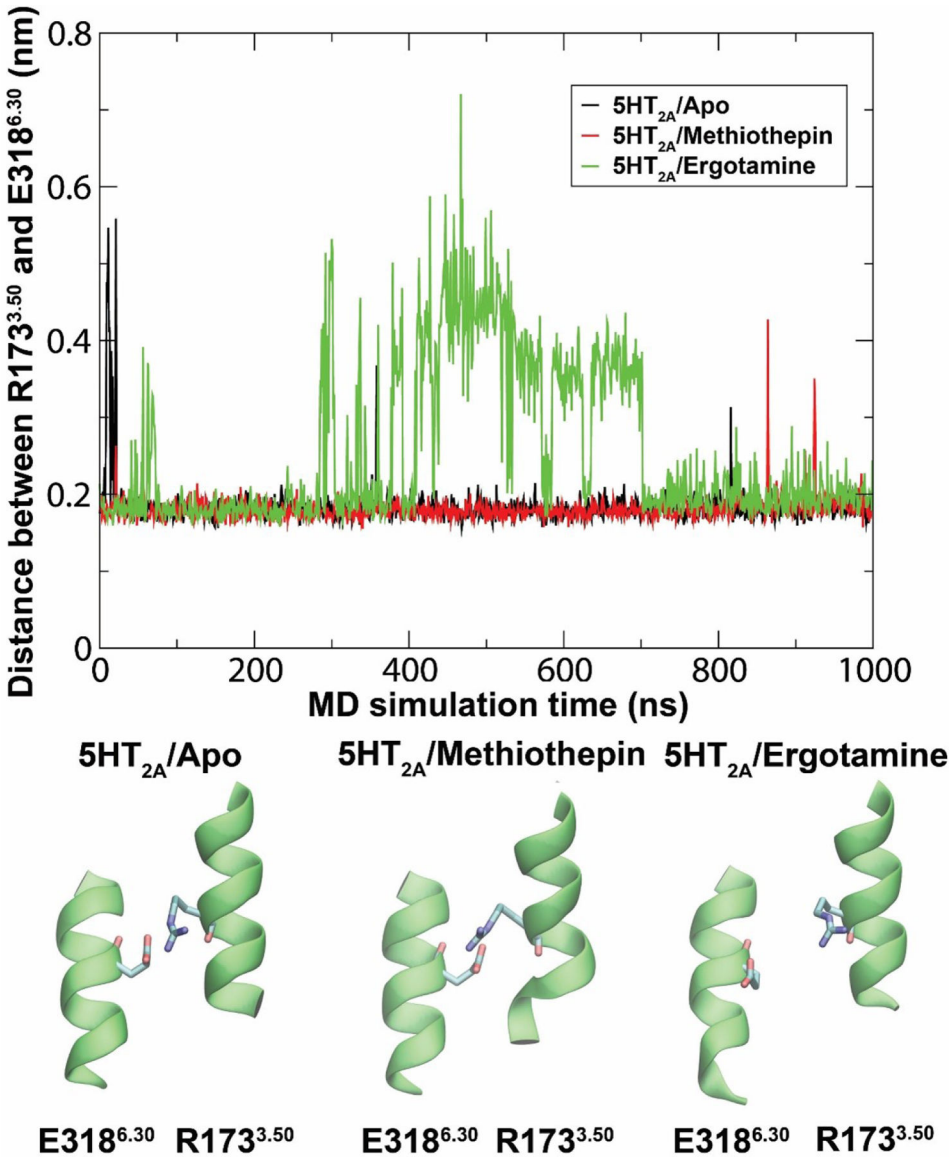

Figure 7:

Molecular dynamics simulation results of an unbound, antagonist-, and agonist-bound 5-HT_{2A}R (APO, methiothepin, and ergotamine, respectively). Ionic lock ($E318^{6.30}$—$R173^{3.50}$) distances are shown as a function of simulation time.

Table 1:

Binding affinities of various 5-HT_{2A}R antagonists at relevant off-target GPCRs.

	Spiperone	Ketanserin	M100,907	Pimavanserin	Altanserin	Ritanserin	Pirenperone	Risperidone
GPCR	**Affinity (K_i, nM)**							
5-HT 2A	**1.2** [a,e,g]	**1.13** [a,e,g]	**0.47** [a,e,g]	**0.24** [a,e,g,(80)]	**0.3** [b,e,g,(170)]	**0.22** [a,e,g]	**1.1** [a,e,h,(171)]	**0.3** [a,e,g,(80)]
5-HT 2B	1,114 [a,e,g]	234 [a,e,g,(172)]	261 [a,e,h]	437 [a,e,g,(80)]	-	0.14 [a,e,h]	61 [a,e,h,(171)]	15.5 [a,e,g,(80)]
5-HT 2C	923 [a,e,g]	88 [a,e,g]	100 [a,e,g]	2.8 [a,e,g,(80)]	6 [b,e,g,(170)]	0.24 [a,e,g]	77 [a,e,h,(171)]	19 [a,e,g,(80)]
5-HT 1A	17 [a,e,g]	>1,000 [a,e,h]	>1,000 [a,e,h]	>1,000 [a,e,g,(78)]	>1,000 [b,f,h,(173)]	309 [a,e,g]	485 [b,f,g]	296 [a,e,g]
5-HT 1B	>1,000 [b,f,g]	>1,000 [a,e,g]	>1,000 [a,e,g]	>1,000 [a,e,g,(78)]	>1,000 [b,f,h,(173)]	194 [a,e,h]	>1,000 [b,f,g]	53.6 [a,e,g]
5-HT 1D	>1,000 [a,e,h]	111 [a,e,g,(174)]	>850 [d,f,h,(58)]	>1,000 [a,e,g,(78)]	-	36.8 [a,e,h]	-	29 [a,e,g]
5-HT 1E	>1,000 [a,e,h,(175)]	>1,000 [a,e,h,(175)]	>850 [h,j,(58)]	>1,000 [a,e,g,(78)]	-	>1,000 [a,e,h]	-	>1,000 [a,e,h]
5-HT 1F	>1,000 [a,e,h,(176)]	>1,000 [a,e,h,(176)]	>850 [h,j,(58)]	>1,000 [a,e,g,(78)]	-	-	-	>1,000 [a,e,h,(177)]
5-HT 3	>1,000 [c,f,g]	>1,000 [c,f,g]	>1,000 [a,e,g]	>1,000 [a,e,g,(78)]	-	>1,000 [b,f,g,(178)]	-	>1,000 [b,e,g,(177)]
5-HT 4	-	>1,000 [e,(179)]	>1,000 [b,f,g,(58)]	>1,000 [a,e,g,(78)]	-	>1,000 [a,e,g]	-	-
5-HT 5A	>1,000 [a,e,h]	>1,000 [b,e,h]	>1,000 [a,e,h]	-	-	77 [a,e,h]	-	206 [a,e,h]
5-HT 6	>1,000 [b,f,g]	>1,000 [a,e,h]	>1,000 [a,e,h]	>1,000 [a,e,g,(78)]	>1,000 [a,e,h,(170)]	67 [a,e,h]	-	>1,000 [a,e,h]
5-HT 7	110 [a,e,h]	794 [a,e,h,(180)]	226 [b,f,g,(58)]	>1,000 [a,e,g,(78)]	15 [a,e,g,(170)]	29.6 [a,e,g]	6.5 [a,e,h,(181)]	4.8 [a,e,g]
H 1	272 [c,f,g,(43)]	1.8 [c,f,g,(43)]	>1,000 [a,b,e,g,(57, 68)]	>1,000 [a,e,g,(80)]	20 [b,f,g,(173)]	<40 [b,f,g,(95)]	-	18.2 [a,e,g,(80)]
H 2	-	-	-	>1,000 [i,j,(78)]	-	4.5 [a,e,g]	-	120 [a,e,g]
α 1A	20 [a,e,g,(182)]		128 [a,e,g,(67)]	>1,000 [i,j,(78)]		80.7 [a,e,g]		5 [a,e,g]
α 1B	3 [a,e,g,(182)]	28 [b,f,g,k,(35)]	425 [a,e,g,(67)]	>1,000 [i,j,(78)]	4.55 [b,f,g,k,(173)]	223 [a,e,g]	20 [b,f,g,k,(183)]	9 [a,e,g]
α 1D	8 [a,e,g,(182)]		-	>1,000 [i,j,(78)]		86.5 [a,e,g]		-
α 2A		-	>1,000 [a,e,g,(67)]	>1,000 [i,j,(78)]		95.8 [a,e,g]		16.5 [a,e,g]
α 2B	135 [b,f,g,k,(182)]	199 [a,e,g,(184)]	271 [a,e,h]	>1,000 [i,j,(78)]	99 [b,f,g,k,(96)]	>1,000 [a,e,g]	20 [b,f,g,k,(183)]	8.5 [a,e,g,(177)]
α 2C		-	>1,000 [a,e,g,(67)]	-		269 [a,e,g]		1.3 [a,e,h]
D 1	220 [a,e,g,(185)]	190 [a,e,g,(185)]	>1,000 [a,e,g]	>1,000 [i,j,(78)]	-	344 [a,e,g]	-	249 [a,e,g]
D 2	0.28 [a,e,g,(80)]	>1,000 [b,f,g]	>1,000 [a,e,g]	>1,000 [a,e,g,(78)]	62 [b,f,g,(173)]	>1,000 [a,e,g]	-	4.35 [a,e,g]
D 3	0.14 [a,e,g,(80)]	-	>1,000 [b,e,g,(58)]	>1,000 [i,j,(78)]	-	57.7 [a,e,g]	-	12.2 [a,e,g]
D 4	1.39 [a,e,g]	-	540 [a,e,g,(58)]	>1,000 [i,j,(78)]	-	51.5 [a,e,g]	-	4.7 [a,e,g]

	Spiperone	Ketanserin	M100,907	Pimavanserin	Altanserin	Ritanserin	Pirenperone	Risperidone
GPCR	**Affinity (K_i, nM)**							
D 5	>1,000[a,e,g,(185)]	>1,000[a,e,g,(185)]	>1,000[a,e,g]	-	-	163[a,e,g]	-	290[a,e,g]
sigma	632[b,f,g,k,(186)]	-	87[b,f,g,k,(58)]	>1,000[i,j,(78)]	-	-	-	-

Note: Unless otherwise noted by reference (in parenthesis), K_i values represent PDSP certified values or an average obtained from the NIMH Psychoactive Drug Screening Program database, https://pdspdb.unc.edu/pdspWeb/; search conducted 01/18/2022. K_i values were derived from [a]human, (when available), [b]rat/mouse, [c]guinea pig, or [d]cow receptors expressed in [e]heterologous cell systems or [f]native tissue, using *g*antagonist or *h*agonist radioligands. Some values have undefined [i]radioligands [j]species or [k]receptor subtypes. '-' denotes ligand affinities that are, to the best knowledge of the authors, not reported in the literature.