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What Can Crystal Structures of Aminergic Receptors Tell Us about Designing Subtype-Selective Ligands?

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Abstract—G protein-coupled receptors (GPCRs) are integral membrane proteins that represent an important class of drug targets. In particular, aminergic GPCRs interact with a significant portion of drugs currently on the market. However, most drugs that target these receptors are associated with undesirable side effects, which are due in part to promiscuous interactions with close homologs of the intended target receptors. Here, based on a systematic analysis of all 37 of the currently available high-resolution crystal structures of aminergic

GPCRs, we review structural elements that contribute to and can be exploited for designing subtype-selective compounds. We describe the roles of secondary binding pockets (SBPs), as well as differences in ligand entry pathways to the orthosteric binding site, in determining selectivity. In addition, using the available crystal structures, we have identified conformational changes in the SBPs that are associated with receptor activation and explore the implications of these changes for the rational development of selective ligands with tailored efficacy.

I. Introduction

G protein-coupled receptors (GPCRs) are a large superfamily of receptors with seven transmembrane (TM) segments. They are critical in signal transduction of a wide variety of molecules, including hormones and

neurotransmitters, and consequently are pharmacological targets for many diseases (Overington et al., 2006). Aminergic GPCRs, which belong to class A rhodopsin-like GPCRs, are of critical importance as they are the targets for ~25% of current drugs (Rask-Andersen et al.,

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2011). However, most drugs that target these receptors have been associated with side effects, which in many cases are due to promiscuous interactions with proteins other than the intended therapeutic target(s) (Kola and Landis, 2004; Liebler and Guengerich, 2005; Besnard et al., 2012). Improving the pharmacological specificity profile of drugs, especially in the case of close receptor homologs of the primary target, is particularly challenging.

Based on their ability to recognize specific biogenic amines as their endogenous agonist, the 42 human aminergic GPCRs can be classified into subfamilies that include the adrenergic, muscarinic, dopaminergic, histaminergic, serotonergic, and trace amine receptors. Each subfamily can be further categorized into subgroups that consist of closely-related subtypes (Fig. 1). Receptors within a particular subfamily are often expressed in different brain regions and/or peripheral tissues and can also be coupled to divergent intracellular signaling transducers, only some of which are associated with the therapeutic effect of drugs. Therefore, selective targeting of a particular receptor subtype and/or a particular signal transduction pathway is critical for elucidating mechanistic underpinnings of disease states and may also lead to improved drug design and development (Keck et al., 2014).

In this review, based on our analysis of all the available high-resolution crystal structures of these receptors, we discuss progress in understanding the structural basis for subtype-selective compounds within the aminergic GPCRs. Building upon our understanding of selectivity among the receptor subfamilies that we discussed previously (Shi and Javitch, 2002), we focus on specificity within individual subgroups. We highlight the difficulty in developing selective compounds targeted solely to the orthosteric binding site (OBS), as well as the role of secondary binding pockets (SBPs) and ligand entry pathways in determining receptor subtype selectivity.

II. Ligand Binding Pockets Revealed by Crystal Structures of Aminergic Receptors

A. Orthosteric Binding Site

1. Residues Forming the Orthosteric Binding Site. Before the crystal structures of rhodopsin-like receptors became available, residues that were critical for ligand binding in aminergic GPCRs were indirectly

determined by extensive mutagenesis studies, and the substituted-cysteine accessibility method was used to identify systematically the surface of the binding site crevice (reviewed in Javitch et al., 2002). Several highly conserved residues in the TM domain were found to form critical receptor-ligand interactions in the endogenous ligand binding site—specifically, Asp^{3.32} [superscripts denote Ballesteros-Weinstein residue numbering (Ballesteros and Weinstein, 1995)] forms a hydrogen bond with the cationic amine of the ligand; Ser^{5.42} and Ser^{5.46} interact with the meta-OH and para-OH moieties of catecholamine agonists and a cluster of aromatic residues in TM6 (Trp^{6.48}, Phe/Tyr^{6.51}, and Phe^{6.52}) that interact with the aromatic moiety of the ligand (reviewed in Shi and Javitch, 2002, see references therein). In addition, residues in extracellular loop 2 (EL2) have been implicated in ligand specificity in aminergic receptors (Olah et al., 1994; Kim et al., 1996; Zhao et al., 1996; Wurch et al., 1998) and we found Ile184^{EL2.52}, the second residue after the conserved and disulfide-bonded Cys in EL2 (defined here as Cys^{EL2.50}), to be directly involved in ligand binding to the dopamine D2 receptor (D2R) (Shi and Javitch, 2004).

In light of recent progress in GPCR structure determination, we sought to further refine our understanding of the residues forming the OBS of all aminergic receptors and thus their role in ligand selectivity at closely related receptor homologs. Thirty-seven high-resolution crystal structures have been solved for 8 of the 42 aminergic receptors [the β_1 - and β_2 -adrenergic receptors (β_1 AR and β_2 AR), dopamine D3 receptor (D3R), histamine H1 receptor (H1R), serotonin 1B and 2B receptors (5HT_{1B}R and 5HT_{2B}R), and muscarinic acetylcholine M2 and M3 receptors (M2R and M3R)], including several structures in active conformations and one apo-structure without a bound ligand (see Fig. 2).

In the present study, we carried out a systematic comparative analysis of the ligand-contacting residues in these structures. Positions at which bound ligand decreased the solvent-accessible surface area (SASA) were defined as ligand-contacting residues (Ballesteros et al., 2001; Beuming et al., 2006). Overall, we found the number of contact residues to be correlated with ligand size, such that larger ligands have more contact residues (Fig. 3). Ligand contacting residues at 12 common positions were identified in 34 of 36 ligand-bound

ABBREVIATIONS: 77-LH-28-1, 1-[3-(4-butyl-1-piperidinyl)propyl]-3,4-dihydro-2(1*H*)-quinolinone; AR, adrenergic receptor; BI-167107, 8-[(1*R*)-2-[[1,1-dimethyl-2-(2-methylphenyl)ethyl]amino]-1-hydroxyethyl]-5-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one; D2R, dopamine D2 receptor; D3R, dopamine D3 receptor; D4R, dopamine D4 receptor; DR, dopamine receptor; EL2, extracellular loop 2; GPCR, G protein-coupled receptor; H1R, histamine H1 receptor; 5HT_{1B}R, serotonin 1B receptor; 5HT_{2B}R, serotonin 2B receptor; ICI-118551, (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol; JDTic, (3*R*)-1,2,3,4-tetrahydro-7-hydroxy-*N*-[(1*S*)-1-[(3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl]-2-methylpropyl]-3-isoquinolinecarboxamide; LY2119620, 3-amino-5-chloro-*N*-cyclopropyl-4-methyl-6-[2-(4-methylpiperazin-1-yl)-2-oxoethoxy]thieno[2,3-*b*]pyridine-2-carboxamide; M2R, muscarinic acetylcholine M2 receptor; M3R, muscarinic acetylcholine M3 receptor; MD, molecular dynamics; OBS, orthosteric binding site; PHNO, (+)-4-propyl-3,4,4*a*,5,6,10*b*-hexahydro-2*H*-naphtho[1,2-*b*][1,4]oxazin-9-ol; PP, primary pharmacophore; R-22, (*R*)-*N*-(4-(2,3-dichlorophenyl)piperazin-1-yl)-3-hydroxybutyl)-1*H*-indole-2-carboxamide; RMSD, root-mean-square deviation; SASA, solvent-accessible surface area; SB269652, 1*H*-indole-2-carboxylic acid {4-[2-(cyano-3,4-dihydro-1*H*-isoquinolin-2-yl)-ethyl]-cyclohexyl}-amide; SBP, secondary binding pocket; SP, secondary pharmacophore; TBPB, 1-(1'-(2-methylbenzyl)-1,4'-bipiperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one; TM, transmembrane; VHTS, virtual high-throughput screening.

subfamily	subgroup	subtype	2.61	2.64	2.65	EL1.50	3.28	3.29	3.32	3.33	3.36	3.37	3.40	4.57	EL2.48	EL2.49	EL2.50	EL2.51	EL2.52	EL2.53	EL2.54	EL2.55	5.38	5.39	5.42	5.43	5.46	5.47	6.44	6.48	6.51	6.52	6.55	6.56	6.58	6.59	7.32	7.35	7.36	7.39	7.40	7.42	7.43		
muscarinic		ACM1	Y	Y	L	W	L	D	Y	S	N	V	W	G	Q	C	Y	I	Q	F	L	I	T	T	A	A	F	F	W	Y	N	V	L	S	T	N	E	W	E	Y	W	C	Y		
		ACM2	Y	Y	T	T	W	L	D	Y	S	N	V	W	G	E	C	Y	I	Q	F	L	I	T	T	A	A	F	F	W	Y	N	V	L	N	T	N	K	W	N	Y	W	C	Y	
		ACM3	F	Y	I	T	W	L	D	Y	S	N	V	W	G	E	C	Y	I	Q	F	L	V	T	T	A	A	F	F	W	Y	N	V	L	N	T	N	K	W	N	Y	W	C	Y	
		ACM4	Y	Y	I	T	W	L	D	Y	S	N	V	W	Q	C	F	Y	I	Q	F	L	V	T	T	A	A	F	F	W	Y	N	V	L	N	T	D	W	S	Y	W	C	Y		
		ACM5	Y	Y	I	W	W	L	D	Y	S	N	V	W	D	E	C	Q	I	Q	F	L	I	T	T	A	A	F	F	W	Y	N	V	L	S	T	V	W	H	Y	W	C	Y		
adrenergic		ADA1A	S	F	E	W	W	A	D	V	C	T	I	S	T	I	C	Q	I	N	E	E	Y	V	S	A	S	F	F	W	F	F	M	P	G	S	E	F	K	F	W	G	Y		
		ADA1B	S	L	E	W	W	A	D	V	C	T	I	S	K	E	C	G	V	T	E	E	Y	A	S	S	S	F	F	W	F	F	L	P	G	S	D	E	F	K	F	W	G	Y	
		ADA1D	S	M	E	W	W	A	D	V	C	T	I	S	R	F	C	G	I	T	E	E	Y	A	S	S	S	F	F	W	F	F	L	P	G	S	E	E	F	K	F	W	G	Y	
		ADA2A	S	N	E	W	Y	L	D	V	C	T	I	S	P	R	C	E	I	N	D	Q	Y	V	S	C	S	F	F	W	F	F	Y	T	T	A	R	F	E	K	F	W	G	Y	
		ADA2B	S	N	E	W	Y	L	D	V	C	T	I	S	P	Q	C	K	L	N	Q	E	Y	I	S	S	S	F	F	W	F	F	Y	S	G	A	H	F	Q	F	W	G	Y		
		ADA2C	S	N	E	W	Y	L	D	V	C	T	I	S	P	Q	C	K	L	N	Q	E	Y	I	S	S	C	S	F	F	W	F	F	Y	S	Y	G	F	G	F	K	F	W	G	Y
		ADRB1	G	I	V	W	W	T	D	V	V	T	I	S	K	C	D	F	F	T	N	Y	A	S	S	S	S	F	F	W	F	F	N	V	K	A	D	F	V	N	W	G	Y		
		ADRB2	G	H	I	W	W	T	D	V	V	T	I	S	T	C	C	D	F	F	T	N	Y	A	S	S	S	S	F	F	W	F	F	N	I	H	V	K	Y	I	N	W	G	Y	
		ADRB3	A	L	A	W	W	T	D	V	V	T	I	S	R	C	C	A	F	A	S	N	Y	V	S	S	S	S	F	F	W	F	F	N	V	R	A	G	F	L	N	W	G	Y	
		DRD1	K	A	E	W	W	V	D	I	S	T	I	S	D	N	C	D	S	S	L	Y	A	S	S	S	S	S	F	F	W	F	F	N	C	L	P	S	F	D	V	W	G	Y	
DRD5	K	A	E	W	V	D	I	S	T	I	S	E	N	C	D	S	S	L	N	Y	A	S	S	S	S	S	F	F	W	F	F	N	C	V	P	E	F	D	V	W	G	Y			
dopaminergic		DRD2	V	L	E	W	F	D	V	C	T	I	S	N	E	C	I	A	N	P	F	V	S	S	S	S	S	F	F	W	F	F	H	I	N	I	P	Y	S	T	W	G	Y		
		DRD3	V	L	E	W	F	D	V	C	T	I	S	T	V	C	S	I	S	N	P	F	V	S	S	S	S	F	F	W	F	F	H	V	N	T	P	Y	S	T	W	G	Y		
		DRD4	F	S	E	W	L	M	D	V	C	T	I	A	A	V	C	R	L	E	D	R	Y	V	S	S	S	F	F	W	F	F	H	I	Q	A	P	V	S	T	W	G	Y		
		HRH1	N	Y	L	W	W	L	D	Y	S	T	I	W	D	K	C	E	T	D	F	Y	F	K	T	A	N	F	F	W	Y	F	F	M	I	A	N	H	I	W	G	Y			
		HRH2	S	Y	Q	W	Y	T	D	V	C	T	I	S	S	K	C	K	V	Q	V	N	Y	G	D	G	T	F	F	W	Y	F	F	V	R	G	E	A	L	F	W	G	Y		
		HRH3	Y	Y	V	W	W	L	D	Y	C	T	A	Y	G	H	C	A	B	F	F	L	A	S	E	F	F	W	Y	T	M	I	R	A	P	E	Y	E	F	W	G	Y			
		HRH4	Y	H	T	T	W	W	L	D	Y	C	T	V	N	S	E	C	P	G	F	F	I	L	T	S	E	F	F	W	Y	S	T	I	L	S	K	Y	R	F	W	G	Y		
		trace amine		TAAR1	S	R	S	W	H	T	D	I	S	I	A	G	G	C	S	V	F	F	S	S	G	T	F	S	F	F	W	F	F	T	D	V	P	P	N	D	I	W	G	Y	
				TAAR2	S	R	S	W	Y	D	L	S	I	A	I	A	S	C	P	V	M	F	N	W	G	L	F	G	F	F	W	C	F	I	L	D	P	P	F	D	T	W	G	Y	
				TAAR3	S	R	S	W	H	T	D	M	R	L	I	S	N	F	C	A	L	T	F	N	W	G	L	F	C	F	F	W	C	F	V	L	D	P	P	L	D	V	W	R	G
TAAR5	S			R	S	W	H	T	D	T	C	L	I	T	G	S	C	Q	L	L	L	N	W	G	N	F	L	F	Y	W	F	T	T	M	D	S	P	F	D	I	W	A	Y	G	Y
TAAR6	S			R	T	W	H	T	D	V	C	Y	L	S	G	G	C	Q	I	V	V	N	W	V	D	F	S	F	F	W	Y	S	L	D	A	A	Y	E	C	W	A	Y	G	Y	
TAAR8	S			R	T	W	H	S	D	V	C	Y	V	S	G	G	C	Q	I	I	V	S	W	V	D	F	L	F	F	W	Y	T	I	L	D	A	A	Y	E	C	W	A	Y	G	Y
TAAR9	S			R	S	W	H	T	D	T	C	F	L	S	G	G	C	Q	A	P	L	N	W	V	C	F	L	F	F	W	Y	L	A	V	D	A	P	Y	E	V	W	Y	G	Y	
serotonergic		5HT1A	A	Y	Q	W	F	I	D	V	C	T	I	S	D	A	C	T	I	S	K	D	Y	T	S	T	A	F	F	W	F	F	L	A	L	P	T	G	A	N	W	G	Y		
		5HT1B	S	Y	T	W	W	L	D	I	C	T	I	S	S	E	C	V	N	T	D	Y	T	S	T	A	F	F	W	F	F	S	L	M	P	L	F	D	T	W	G	Y			
		5HT1D	S	Y	T	W	W	L	D	I	C	T	I	S	S	D	C	L	V	N	T	S	Y	T	S	T	A	F	F	W	F	F	S	L	M	P	P	F	D	T	W	G	Y		
		5HT1E	S	Y	I	T	W	W	L	D	M	C	T	I	S	S	Q	C	T	I	Q	H	D	Y	T	S	T	A	F	F	W	F	F	L	V	G	S	A	D	T	W	G	Y		
		5HT1F	S	Y	I	T	W	W	L	D	I	C	T	I	S	D	E	C	I	K	H	D	S	T	S	T	A	F	F	W	F	F	E	L	V	N	E	S	N	A	W	G	Y		
		5HT2A	S	T	I	W	W	I	D	V	S	T	I	S	G	S	C	L	L	A	D	D	F	V	G	S	S	S	F	F	W	F	F	N	I	A	V	G	L	N	V	W	G	Y	
		5HT2B	A	T	I	W	W	L	D	V	S	T	I	A	I	T	C	V	L	T	K	E	F	M	G	S	A	F	F	W	F	F	N	I	L	V	Q	L	E	V	W	G	Y		
		5HT2C	S	A	I	W	W	I	D	V	S	T	I	S	T	C	V	L	N	D	P	E	V	G	S	A	F	F	W	F	F	N	I	S	V	E	L	N	V	W	G	Y			
		5HT4R	G	E	L	W	R	T	D	V	T	I	S	T	Y	C	V	F	M	V	N	Y	A	C	A	S	A	F	F	W	F	F	N	I	D	P	G	W	T	L	W	G	Y		
		5HT5A	S	H	E	W	W	I	D	V	C	T	I	S	E	E	C	Q	V	S	R	E	Y	A	S	T	A	F	F	W	F	F	E	L	S	P	A	K	S	L	W	G	Y		
5HT6R	A	N	A	W	W	T	D	V	C	T	I	S	G	Q	R	L	L	A	S	F	V	A	S	T	F	F	W	F	F	N	I	Q	A	P	F	D	T	W	G	Y					
5HT7R	V	T	D	W	F	I	D	V	C	T	I	T	K	V	C	L	I	S	O	D	T	S	T	A	F	F	W	F	F	S	T	R	P	L	E	R	L	W	G	Y					

Fig. 1. Alignment of the ligand-contacting residues in the 42 human aminergic receptors. An analysis of ligand-contacting residues was carried out for all 36 available ligand-bound crystal structures of aminergic receptors. The residue positions are indicated by the Ballesteros-Weinstein numbers at the top of each column (for EL2, the residues are indexed relative to the conserved Cys residue, EL2.50, which makes a disulfide bond to Cys^{3.25}) and colored by the classification of the positions: OBS positions (see section II.A) in cyan, SBP positions (see section II.B) in yellow, and 7.42, which contacts ligand but is not in the OBS or any SBP, in gray (see Fig. 2). The OBS positions were defined as the common set of positions identified by the SASA analysis in all structures except for the agonist-bound M2R structures (PDB IDs **4MQS** and **4MQT**). The SASA values for each residue were calculated using the program Naccess (Hubbard and Thornton, 1993), with probe size of 1.4 Å, Z-slice of 0.001; we used a cutoff of 0.2% to identify the residues with different accessibility in the presence and absence of bound ligand. Note that the OBS includes one residue from EL2 approximately at position EL2.52—although in the H1R structure (PDB ID **3RZE**) or the antagonist-bound M2R and M3R structures (PDB IDs **3UON** and **4DAJ**) EL2.52 does not face the OBS, the residue at EL2.49 or EL2.55 contacts ligand in a manner similar to the residues at EL2.52 in other structures. The receptors are indicated by their UniProt entry names and colored by subfamily. We define a subgroup within a subfamily, indicated by brackets, to be a set of receptors in which all the pairwise sequence identities of the TM domain are >45%. The residues that contact a ligand in any of the structures for a particular receptor are shaded in cyan, yellow, or gray as described above. The residues interacting with the positive allosteric modulator LY2119620 in the M2R structure (PDB ID **4MQT**) are underlined, except for Glu172^{EL2.46} and Ala414^{EL3}, which are not shown. All the amino acid sequences are human; for β_1 AR and M3R, however, the contact residues were identified in the crystal structures of turkey β_1 AR and rat M3R.

structures. We define these positions—3.32, 3.33, 3.36, EL2.52, 5.42, 5.46, 6.48, 6.51, 6.52, 6.55, 7.39, and 7.43 (cyan in Figs. 1 and 4, A and B)—as the OBS residue positions. In the two M2R structures bound to the agonist iperoxo (PDB IDs [4MQS](#) and [4MQT](#), note [4MQT](#) is additionally bound with the allosteric modulator LY2119620), the residues at positions EL2.52, 5.42, and 6.55, which are slightly more peripheral, are not ligand-contacting, indicating that, at least for this rather small agonist, they are not essential as direct contacts. However, these residues still play a direct role for all other ligands in M2R and other receptors studied.

The OBS that we defined here is subtly different from that of Christopoulos et al. (2014) (“the binding site/s on a receptor macromolecule that is/are recognized by the endogenous agonist/s for that receptor”). Although the cavity enclosed by the OBS residues identified in this study is occupied by the endogenous agonists of aminergic receptors (e.g., all the OBS residues are in contact with the endogenous ligand adrenaline in β_2 AR based on our SASA analysis), our goal was to identify the entire set of residues critical for recognition of all orthosteric ligands, including full/partial agonists, antagonists, and inverse agonists.

Fig. 2. Identification of SBPs in the crystal structures of aminergic receptors. The numbered (“0” to “4”) positions for each complex indicate the residues outside of the OBS (non-OBS) that interact with a specific ligand moiety—the closest distance between any heavy atoms of the residue and a ligand moiety is within the van der Waals interaction distance, which we define in this study to be the sum of the van der Waals radii plus 0.8 Å. A SBP was identified as a cluster of ≥ 3 non-OBS residues that interact with a small ligand moiety, which we defined as a set of ligand heavy atoms with the largest pairwise distance between any pair < 4.2 Å (the largest pairwise distance within a hydroxyphenyl moiety). “0” indicates the residue is not within any SBP in that structure, “1” to “4” indicate the involvement of the residue in forming a particular SBP (“1,” “SBP₂₃₇,” “2,” “SBP₅₆₇,” “3,” “SBP₃₄₅₆,” “4,” “SBP₇”). These SBPs are classified according to their location and named according to the surrounding TMs (see Fig. 4 for the representatives of “1” to “3”); thus, in a particular complex, a ligand moiety does not necessarily interact with every surrounding TM. The structures are arranged according to the conformational state of the receptor (i.e., active, intermediate-active, and inactive, which are indicated by □, ◇, and Δ, respectively) and the ligand efficacy (i.e., agonist in red, partial or biased agonist in pink, and antagonist/inverse agonist in green). The residue sets from the structures for the same receptor bound with the same or highly similar ligand are combined, e.g., the structures with PDB IDs **3P0G**, **3SN6**, and **4LDE** are all β_2 AR in complex with BI-167107. The fragments bound to β_1 AR are categorized separately as their efficacies were unknown (colored in orange) (Christopher et al., 2013). The phosphate ion in the H1R structure (PDB ID **3RZE**) is counted as an extension of the orthosteric ligand (Shimamura et al., 2011). The positions in the top row are colored if they only interact with either agonists/partial agonists (red) or antagonists/inverse agonists (green).

Fig. 3. Statistics of ligand-contacting residues. The number of contact residues is plotted against ligand size for the 36 available ligand-receptor complexes. The color and shape of each point indicate the ligand efficacy and the receptor conformational state, respectively, as specified in Fig. 2. Although the number of contact residues is correlated with ligand size, the ligand efficacy is not correlated with ligand size.

Among the OBS residues, the triad consisting of Asp^{3.32}, Trp^{6.48}, and Tyr/Trp^{7.43} is conserved across the aminergic receptor family (Fig. 5). Whereas Trp^{6.48} is also conserved in many other rhodopsin-like receptors, Asp^{3.32}

and Tyr/Trp^{7.43} are likely critical for recognition of aminergic ligands. A hydrogen bond between Asp^{3.32} and Tyr/Trp^{7.43}, present in all available crystal structures of aminergic receptors, likely stabilizes the side chain of Asp^{3.32}, thereby facilitating its interaction with the amine group in the ligands (Fig. 6, A and B). Similar conservation is also observed in the opioid and somatostatin receptor subfamilies of the peptide receptor family, in which the presence of an Asp at position 3.32 is correlated with a Tyr at position 7.43. Indeed, in the available crystal structures of opioid receptors, Asp^{3.32} always interacts simultaneously with both Tyr^{7.43} and a charged amine group from the ligand (Granier et al., 2012; Manglik et al., 2012; Thompson et al., 2012; Fenalti et al., 2014) (Fig. 6C), with the exception of the structure of κ -opioid receptor in complex with JDTC [(3R)-1,2,3,4-tetrahydro-7-hydroxy-N-[(1S)-1-[(3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl]-2-methylpropyl]-3-isoquinolinecarboxamide] (PDB ID 4DJH), in which Asp^{3.32} interacts with two positively charged amine groups of the ligand (Wu et al., 2012).

2. Ligand Selectivity between the Subgroups of an Individual Subfamily. By analyzing the crystal structure of bovine rhodopsin in the context of experimental findings, we previously proposed a mechanism of structural mimicry in which the similar folds of rhodopsin and other rhodopsin-like GPCRs are formed by relatively divergent sequences (Ballesteros et al.,

Fig. 4. The orthosteric binding site and secondary binding pockets. (A) All the identified ligand-contacting positions for the aminergic receptors are mapped onto a β_2 AR structure (PDB ID 4LDO). The 12 OBS residues are in cyan, and the SBP residues are in yellow. Three representative SBPs shown in (C–E) are indicated by red dotted circles. (B) A zoomed-in view of the OBS in the β_2 AR bound with adrenaline (PDB ID 4LDO). The side chains of the three highly conserved residues within the aminergic family, Asp^{3.32}, Trp^{6.48}, and Tyr^{7.43}, and the conserved EL^{2.50}-Cys^{3.25} disulfide bond are shown as sticks; for all other residues, sticks were drawn from the α to the center of mass (represented as spheres) of the heavy atoms of the side chain. The zoomed-in views of the SBPs, “SBP₂₃₇” (C), “SBP₅₆₇” (D), “SBP₃₄₅₆” (E), are shown in representative structures [PDB IDs 4AMI (C), 4IB4 (D), 3UON (E)]. The SBPs are classified according to their locations and named according to the surrounding TMs (see Fig. 2). The ligands in the respective structures are in spheres with the carbon atoms of adrenaline (B) or the moieties occupying the SBPs (C–E) colored in orange.

	residue index	aminergic receptors													
			catecholamine receptors										histamine receptors	serotonin receptors	muscarinic receptors
				dopamine receptors				adrenergic receptors							
					D1-like	D2-like		β	α1	α2					
OBS	3.32	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00		
	3.33	0.28	0.87	0.81	1.00	1.00	1.00	1.00	1.00	1.00	0.68	0.47	1.00		
	3.36	0.33	0.42	0.65	1.00	1.00	0.68	1.00	1.00	1.00	0.67	0.43	1.00		
	5.42	0.24	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.41	0.33	1.00		
	5.46	0.25	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.41	0.53	1.00		
	6.48	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00		
	6.51	0.44	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00		
	6.52	0.35	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.40	1.00	1.00		
	6.55	0.11	0.25	0.69	1.00	1.00	0.29	1.00	0.83	1.00	0.39	0.30	1.00		
	7.39	0.16	0.29	0.69	1.00	1.00	0.66	1.00	1.00	1.00	0.43	0.26	1.00		
	7.43	0.78	0.77	0.72	1.00	1.00	1.00	1.00	1.00	1.00	0.71	1.00	1.00		
	Average	0.45	0.78	0.87	1.00	1.00	0.87	1.00	0.98	1.00	0.65	0.67	1.00		
SBP	2.61	0.23	0.24	0.39	1.00	0.71	0.47	0.76	1.00	1.00	0.40	0.34	0.73		
	2.64	0.15	0.18	0.41	1.00	0.67	0.22	0.38	0.43	1.00	0.69	0.24	1.00		
	2.65	0.18	0.39	1.00	1.00	1.00	0.34	0.42	1.00	1.00	0.28	0.22	0.42		
	3.28	0.29	0.32	0.40	1.00	0.79	0.72	1.00	1.00	1.00	0.73	0.44	1.00		
	3.29	0.22	0.24	0.73	1.00	0.70	0.40	1.00	1.00	1.00	0.68	0.41	1.00		
	3.37	0.31	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.78	1.00		
	3.40	0.39	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.42	1.00	1.00		
	4.57	0.32	0.81	0.78	1.00	0.77	1.00	1.00	1.00	1.00	0.28	0.51	1.00		
	5.38	0.26	0.74	0.71	1.00	0.72	1.00	1.00	1.00	1.00	0.40	0.42	0.79		
	5.39	0.21	0.44	0.72	1.00	1.00	0.43	0.74	0.74	0.79	0.40	0.31	1.00		
	5.43	0.25	0.47	1.00	1.00	1.00	0.44	1.00	0.77	0.66	0.44	0.72	1.00		
	5.47	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00		
	6.44	0.83	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00		
	6.56	0.21	0.21	0.40	1.00	0.79	0.25	0.82	1.00	0.74	0.43	0.43	1.00		
	6.58	0.09	0.14	0.29	0.77	0.71	0.24	0.39	1.00	0.38	0.40	0.17	0.70		
	6.59	0.20	0.21	0.29	1.00	0.40	0.31	0.74	1.00	0.80	0.45	0.28	1.00		
	7.32	0.11	0.17	0.42	0.68	1.00	0.21	0.39	0.77	0.38	0.28	0.16	0.23		
	7.35	0.17	0.45	0.40	1.00	0.67	0.78	0.72	1.00	1.00	0.39	0.18	1.00		
	7.36	0.11	0.21	0.72	1.00	1.00	0.28	0.45	1.00	0.73	0.28	0.21	0.23		
	7.40	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00		
	Average	0.33	0.51	0.68	0.97	0.85	0.60	0.79	0.93	0.87	0.55	0.49	0.86		
Other	7.42	0.33	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.39	1.00	1.00		

Fig. 5. Conservation of ligand-contacting residues. The conservation indices (CI) of ligand-contacting residues across the aminergic receptors and within the indicated subfamilies and subgroups are calculated by the ProperTM server (Shi et al., 2001; Beuming and Weinstein, 2004). The positions with low sequence conservation are indicated by filled colors (CI values ≤ 0.25 in light blue, and those between 0.25 and 0.50 in light green). The highly conserved positions among all amine receptors with CI > 0.75 are highlighted in red. Note, at the aminergic receptor family level, except for Asp^{3.32}, Trp^{6.48}, Tyr/Trp^{7.43}, other OBS positions are not more conserved than SBP positions.

2001). Indeed, using the same protocol used to identify OBS residues of aminergic receptors, we analyzed the crystal structures of opioid receptors and identified a set of OBS positions highly similar to that of aminergic receptors, with the exception of the 5.46 and EL2

positions (Figs. 1 and 7). Despite these similarities in the positions of the OBS residues, differences in sequence within the common fold also contribute to structural differences that determine the selectivity for different receptor subfamilies (Ballesteros et al., 2001),

Fig. 6. The highly conserved residues in the OBS. The highly conserved OBS residues (see Fig. 5) Asp^{3.32}, Trp^{6.48}, Tyr/Trp^{7.43} (cyan) are shown as sticks in the inactive (PDB ID 2RH1) (A) and active (PDB ID 4LDO) (B) structures of β_2 AR. Asp^{3.32} forms a hydrogen bond with Tyr^{7.43} in all available crystal structures of aminergic receptors, suggesting that this interaction is likely to be critical for ligand-recognition of this family. This triad is similarly conserved in the opioid receptor family, as exemplified in the structure of nociceptin opioid receptor bound to a peptide-mimetic compound (PDB ID 4EA3) (C). The ligands are shown as orange sticks; the hydrogen bonds between the nitrogen atoms of the positively charged amine groups and Asp^{3.32} or between Asp^{3.32} and Tyr^{7.43} are indicated by dotted lines.

and this is also likely the case for the selectivity between the subgroups of an individual subfamily.

For example, the adrenergic receptor (AR) subfamily consists of three subgroups, namely α_1 -, α_2 -, and β ARs, with three subtypes within each subgroup (Ahlquist, 1948; Bylund et al., 1994). These receptors are present in almost all peripheral tissues as well as the central and sympathetic nervous systems, are activated upon binding the endogenous ligands adrenaline and nor-adrenaline, and control heart and lung function, blood pressure, and a variety of metabolic and central nervous system functions (Ahlquist, 1948; Taira et al., 2008). The activation of α ARs and β ARs results in distinct, therapeutically relevant physiologic responses (vasoconstriction versus increased heart rate and contraction, respectively). Drugs have been developed that display significant selectivity between α_2 ARs and β ARs by taking advantage of the significant divergence within the OBS of these receptors (four OBS residues differ between these two subgroups). This is highlighted by the β_2 AR antagonist dihydroalprenolol, which is 23,000-fold selective for β_2 AR over α_{2A} AR. Mutation of just one of the four divergent OBS residues, Phe412^{7.39}, in the human α_{2A} AR to that in β_2 AR (Asn), resulted in a 3000-fold increase in binding affinity for this compound (Suryanarayana et al., 1991).

Dopamine receptors (DRs) are classified into two subgroups, D1-like and D2-like, based on sequence, G protein coupling, and pharmacology (Lachowicz and Sibley, 1997). D1-like DRs are quite divergent from the D2-like DRs, and in fact are closer in sequence to the β ARs (~43% versus ~39% sequence identity between D1-like DRs and β ARs and between D1-like and D2-like DRs, respectively) (Fredriksson et al., 2003). Although both D1-like and D2-like DRs share dopamine as their endogenous ligand, 6 of 12 OBS positions are divergent between these two subgroups (Fig. 1). Not surprisingly, agonists and antagonists that display a high degree of D1- or D2-like selectivity have been developed (Beaulieu and Gainetdinov, 2011).

Histamine H1R and H3R/H4R, which are classified into two subgroups, are relatively divergent (<~30%

sequence identity in TM domain and only 4 or 5 of 12 OBS residues conserved), and the development of subgroup-selective compounds may be of great clinical significance (Kiss et al., 2008). Structure-based virtual high-throughput screening (VHTS) of ~100,000 fragment-like compounds against the H1R crystal structure led to the identification of subgroup-selective compounds with up to 1000-fold selectivity for H1R over H3R/H4R (de Graaf et al., 2011), again illustrating effective differentiation between subgroups with divergent OBS residues.

3. The Difficulty of Developing Subtype-Selective Ligands Targeting the Orthosteric Binding Site. The development of subtype-selective drugs is one approach to optimizing therapeutic efficacy while diminishing untoward side effects. However, among the subtypes within a receptor subgroup, the sequence similarity in the OBS can be very high, and this has made the development of subtype-selective drugs for some aminergic GPCRs extremely challenging (Congreve et al., 2011). There are 11 subgroups of closely related aminergic receptors (defined as >45% sequence identity in the TM domain; see Fig. 1). Within these subgroups, the 12 OBS residues described above are completely conserved between two or more subtypes: M1,3,4,5Rs; $\alpha_{2B,C}$ ARs; β_{1-3} ARs; D1,5Rs; D2,3Rs; 5HT_{1B,D}Rs; and 5HT_{2B,C}Rs (Fig. 1).

Despite the high conservation of the OBS, subtype-specific compounds have been developed for some receptors through extensive medicinal chemistry campaigns. For example, within the D2-like dopamine receptor subgroup, D3R- or D4R-selective ligands (see below) and to a lesser degree D2R-preferential ligands have been discovered. However, within the D1-like dopamine receptor subgroup, separation of D1R- and D5R-selective ligands using traditional medicinal chemistry efforts has thus far been unsuccessful (Giorgioni et al., 2008), as has been the case with other subgroups as well.

Crystal structures of receptors from the same subgroups are now available for two subfamilies of the aminergic receptors, thus enabling comparison of conformational differences in the OBS of these receptors.

subfamily	subgroup	subtype	2.53	2.56	2.60	2.63	EL1.50	3.28	3.29	3.32	3.33	3.36	3.37	EL2.50	EL2.51	EL2.52	5.35	5.39	5.42	5.46	6.48	6.51	6.52	6.55	6.58	7.35	7.39	7.42	7.43
OPR	PRD	OPRD	A	T	Q	K	W	V	L	D	Y	M	F	C	M	L	D	K	V	A	W	I	H	V	W	L	I	G	Y
		OPRK	V	T	Q	V	W	V	I	D	Y	M	F	C	S	L	D	K	V	A	W	I	H	I	E	Y	I	G	Y
		OPRM	A	T	Q	N	W	V	I	D	Y	M	F	C	T	L	E	K	V	A	W	I	H	V	K	W	I	G	Y
		OPRX	V	T	Q	D	W	V	I	D	Y	M	F	C	L	V	G	A	I	S	W	V	Q	V	Q	L	T	G	Y

Fig. 7. Alignment of the ligand-contacting residues in the four opioid receptors. The ligand-contacting residues were identified by SASA analysis for all five available crystal structures of opioid receptors. The residue positions are indicated by the Ballesteros-Weinstein numbers at the top of each column (for EL2, the residues are indexed relative to the conserved Cys residue, EL2.50, which makes a disulfide bond to Cys^{3.25}) and colored by the classification of the positions: OBS positions in cyan, SBP positions in yellow, and the remaining positions in gray. The OBS and SBP positions were identified similarly to those in the aminergic receptors. Note the identified ligand-contacting residues are similar to those previously summarized (Filizola and Devi, 2013). The bracket on the left indicates high pairwise sequence identities of >60% among all four receptors. The receptors are indicated by their UniProt entry names. All the amino acid sequences are human; for the μ -opioid receptor (OPRM), the contact residues are identified in the crystal structure of mouse OPRM.

The structures of β_1 AR and β_2 AR in inactive conformations reveal that, between the two subtypes, the size and shape of the OBS are almost identical ($\text{RMSD}_{\text{heavy atoms}}$ [root-mean-square deviation] $< 0.8 \text{ \AA}$). Similarly, the structures of 3-quinuclidinyl-benzilate-bound M2R (PDB ID [3UON](#)) and tiotropium-bound M3R (PDB ID [4DAJ](#)) show high structural similarity in the completely conserved OBS ($\text{RMSD}_{\text{heavy atoms}} < 0.9 \text{ \AA}$), although there are minor rearrangements of the Tyr^{7.39} side chains, likely caused by the different residues at position 2.61 (Haga et al., 2012; Kruse et al., 2012).

The difficulty of developing subtype-selective orthosteric ligands also has been reflected in VHTS efforts to identify subtype-selective compounds for closely related subtype receptors, such as the D3R and D2R. VHTS of over 3 million “lead-like” compounds from the ZINC library was performed against the D3R structure, and the identified hits were tested for binding at D3R and its close homolog D2R (Carlsson et al., 2011). This study was not designed to address specificity, and not surprisingly, the hit compounds showed little selectivity for D3R over D2R, despite having relatively high affinity for D3R (Carlsson et al., 2011).

In a separate study, a counter-screening strategy failed to identify M2R/M3R subtype-selective compounds (Kruse et al., 2013b). In this study, over 3 million “lead-like” and fragment compounds from the ZINC library were screened against the OBS of both the M2R and M3R structures, and the top-ranked M3R hits with the largest rank difference between the M3R and M2R were selected to identify novel M3R-selective compounds. The experimentally measured binding selectivity of the selected compounds, however, was only modest ($\text{M2R/M3R } K_i < 6\text{-fold}$) (Kruse et al., 2013b). This example highlights the challenge in targeting only the highly conserved OBS for subtype specificity. Curiously, although the virtual screening was against the antagonist bound M3R and M2R structures, the screening identified a M3R-selective partial agonist with low micromolar EC_{50} , which was devoid of activity at M2R. Intriguingly, this compound had extremely low binding affinity at both M2R and M3R ($K_i > 100 \text{ }\mu\text{M}$), highlighting the complexity of designing selective ligands with tailored efficacy (see below).

Thus, an approach that is focused solely on the near-identical OBS within a subfamily of aminergic GPCR is likely to fail in identifying highly selective compounds. In contrast, more recent efforts to develop subtype-selective drugs have focused on targeting concomitantly both the OBS and SBPs that are more divergent in sequence and structure than the OBS (Jones et al., 2008; Langmead et al., 2008; Chien et al., 2010; Newman et al., 2012a; Michino et al., 2013).

B. Secondary Binding Pockets Revealed by Crystal Structures of Aminergic Receptors

An SBP is a part of the ligand binding site that extends beyond the OBS. Because the residues at the

peripheral regions of the OBS are less conserved within individual subgroups (Fig. 5), these regions can be exploited to achieve subtype selectivity. In our analysis of the available aminergic receptor crystal structures, we define an SBP as a cavity consisting of three or more ligand-contacting residues outside of the OBS that are in direct contact with a defined ligand moiety (see Fig. 2). In this way, we avoid the complexity of identifying and defining cavities based solely on residue position and side chain orientation (Nayal and Honig, 2006). However, due to the difficulty in identifying such cavities with the SASA approach described above, we applied a distance-based approach to identify the residues that interact with a specific ligand moiety—the closest distance between any heavy atoms of the residue and a ligand moiety is within the van der Waals interaction distance, which we define in this study to be the sum of the van der Waals radii plus $0.8 \text{ \AA}$. We found ligand-contacting residues forming SBPs in 30 out of 36 ligand-bound crystal structures of aminergic receptors (Fig. 2). Except for position 7.42, all the other ligand-contacting positions outside the OBS are part of a SBP in at least one structure (Figs. 1 and 2). This method led us to identify several distinct SBPs, and we describe below several examples that appear to be of functional importance.

In the β_1 AR structure that is in complex with the agonist bucindolol (PDB ID [4AMI](#)), the aromatic substituent at the amine end of bucindolol occupies a SBP formed by TMs 2, 3, 7, EL1, and EL2 (SBP₂₃₇) and contacts residues Trp117^{3.28}, Cys199^{EL2.50}, Asp200^{EL2.51}, Phe325^{7.35}, and Val326^{7.36} (Fig. 4C). These contacts in the SBP, especially those with EL2 residues, have been proposed to result in biased agonism by increasing the probability of conformational changes that promote the binding of arrestin more efficiently than G protein (Warne et al., 2012).

In the structures of 5HT_{2B}R bound to ergotamine (PDB IDs [4IB4](#) and [4NC3](#)), because of an inward shift of TM5, the phenyl moiety of ergotamine occupies a SBP located near the top of TMs 5, 6, and 7 (SBP₅₆₇) and interacts with residues Thr210^{EL2.53}, Lys211^{EL2.54}, Met218^{5.39}, Leu347^{6.58}, Val348^{6.59}, and Leu362^{7.35} (Fig. 4D). In the structure of 5HT_{1B}R bound to ergotamine (PDB ID [4IAR](#)), the phenyl moiety does not interact with this SBP because of the lack of similar rearrangements at the extracellular tip of TM5. The additional interaction with the SBP at the tip of TM5 in 5HT_{2B}R has been proposed to contribute to β -arrestin biased signaling in 5HT_{2B}R by stabilizing a conformation of the receptor that selectively interferes with G protein signaling (Wacker et al., 2013; Wang et al., 2013).

In the inactive structure of M2R bound to the antagonist 3-quinuclidinyl-benzilate (PDB ID [3UON](#)), one of the phenyl moieties of 3-quinuclidinyl-benzilate points toward a SBP formed by TMs 3, 4, 5, 6 (SBP₃₄₅₆) and contacts residues Asn108^{3.37}, Val111^{3.40}, Trp155^{4.57},

and Phe195^{5,47} (Haga et al., 2012) (Fig. 4E). Similar occupancies of the SBP₃₄₅₆, which is located more intracellularly than the OBS, are also observed in the inactive M3R structure bound to the antagonist tiotropium (PDB ID 4DAJ) and the active M2R structures bound to the agonist iperoxo (PDB IDs 4MQS and 4MQT). In the two inactive M2R and M3R structures, the moieties occupying SBP₃₄₅₆ could help to stabilize the side chain of Trp^{6,48} in the OBS in a parallel orientation relative to lipid bilayer, in contrast to the relatively perpendicular orientation in the active M2R structures and other crystal structures of aminergic receptors.

As of yet, none of these SBPs have been targeted rationally for the development of selective compounds. However, in our recent studies (Newman et al., 2012a; Michino et al., 2013), we extensively characterized the role of the SBPs in dopamine D3R/D2R-subtype selectivity (see below).

Interestingly, using a similar analysis to identify the SBP residues in opioid receptors, we found the positions forming SBPs in opioid receptors to be very similar to those of aminergic receptors, except for the positions from TM2, i.e., positions 2.53, 2.56, 2.60, and 2.63 in opioid receptors versus 2.61, 2.64, and 2.65 in aminergic receptors (Figs. 1 and 7). Thus, it appears that there is a face shift in the helix orientations of TM2 in the opioid receptors compared with those in aminergic receptors. Although the functional significance of this divergence is not clear, it argues that structural mimicry is less conserved beyond the OBS for different rhodopsin-like receptor families.

C. Allosteric Ligand Binding Pockets

The significant sequence divergence in the SBPs of aminergic GPCRs (Fig. 5) makes these cavities an enticing target for the development of more specific pharmacological agents. It is important, therefore, to consider the relationship between the SBPs that we identified for extended ligands and allosteric sites in aminergic GPCRs that have been targeted to achieve improved selectivity profiles (Valant et al., 2012; Wootten et al., 2013). The first and only high-resolution structure of a GPCR in complex with an allosteric ligand was recently reported; in M2R, the M2R/M4R-selective positive allosteric modulator LY2119620 (3-amino-5-chloro-*N*-cyclopropyl-4-methyl-6-[2-(4-methylpiperazin-1-yl)-2-oxoethoxy]thieno[2,3-*b*]pyridine-2-carboxamide), cocrystallized with an agonist bound in the OBS, was bound in a more extracellular location >10 Å away from the OBS (Kruse et al., 2013a). Intriguingly, except for Glu172^{EL2,46} and Ala414^{EL3}, all residues in contact with LY2119620 have already been shown to interact with the extended portions of orthosteric ligands in various receptors (Fig. 1). Thus, the binding site of LY2119620 consists of two OBS residues Tyr426^{7,39} and Phe181^{EL2,55} and spans two SBPs in the extracellular

vestibule identified by our analysis (Fig. 2), including one at the interface of TMs 2 and 7 and another enclosed by TM6, TM7, and EL2. Furthermore, the binding site overlaps significantly with those identified for several allosteric modulators of M2R by long-timescale molecular dynamics (MD) simulations (Dror et al., 2013). Similarly in this region of M1R, Tyr179^{EL2,51}, and Trp400^{7,35} were found to be critical for the binding of the selective positive allosteric modulator benzylquinolone carboxylic acid, as well as its modulation of the efficacy of carbachol (Ma et al., 2009; Abdul-Ridha et al., 2014). The interactions of receptor with these allosteric modulators involve several nonconserved SBP residues, which may account for the high selectivity of some allosteric compounds for specific subtypes of muscarinic receptors.

For example, the presence of positively charged Lys523^{7,32} in the extracellular vestibule of M3R was implicated as the critical determinant of M2R over M3R selectivity for currently available allosteric modulators of these receptors (Dror et al., 2013), and the introduction of acidic or basic residues at this position increases or decreases, respectively, the affinity of the allosteric modulator gallamine for several muscarinic receptors (Gnagey et al., 1999). Recent characterization of two M1R-selective agonists, TBPB (1-(1'-(2-methylbenzyl)-1,4'-bipiperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one) and 77-LH-28-1 (1-[3-(4-butyl-1-piperidinyl)propyl]-3,4-dihydro-2(1*H*)-quinolinone), indicates that the crevice formed by TMs 2, 3, and 7 is likely involved in binding the allosteric components of these compounds and may contribute to the selectivity of these agonists (Keov et al., 2014).

III. Mechanisms for Achieving Subtype Selectivity

A. Improving Selectivity by Targeting Secondary Binding Pockets with Extended Ligands.

Because allosteric compounds do not bind the OBS, developing such compounds with high affinity has been challenging, especially for certain aminergic receptors, such as D2-like receptors (Hoare et al., 2000; Raghavan et al., 2009). Another strategy to improve subtype-selectivity is to extend the orthosteric or primary pharmacophore (PP) of a ligand to take advantage of the divergence in the SBPs. In particular, a novel strategy is to link orthosteric and allosteric pharmacophores to create so-called “bitopic” ligands (Lane et al., 2013), which has been successfully applied to improve ligand affinity and selectivity in muscarinic acetylcholine receptors (Leach et al., 2012). Moreover, a SBP that has not yet been successfully targeted by an allosteric ligand can still be targeted by an extended moiety from the PP to gain selectivity, such as in the case of selective compounds for individual D2-like receptors described below. Although such compounds may not be formally

bitopic as currently defined, because their secondary pharmacophore (SP) is not in itself a documented allosteric compound, they nonetheless bind and function in a bitopic manner (Christopoulos et al., 2014).

The subtypes within the D2-like subgroup are highly homologous. All 12 OBS residues are identical between D2R and D3R and only one differs in D4R. Overall, D2R and D3R are more closely related to each other than either is to D4R (78% identity between D2R and D3R and ~50% identity between D4R and D2R/D3R in the TM domain). Although it is generally thought that antagonism of the D2R is essential for therapeutic efficacy of all antipsychotic drugs, these agents block both D3R and D2R. A significant difficulty in determining the relevance for antipsychotic efficacy of signaling processes downstream of specific dopamine receptors stems not only from limitations in modeling the diseases, e.g., in rodents, but just as importantly from a lack of appropriate pharmacological tools to selectively target the different receptors (Beaulieu and Gainetdinov, 2011). Furthermore, the finding of increased D3R expression in response to drugs of abuse, e.g., in the brains of cocaine-associated fatalities (Staley and Mash, 1996) and more recently in cocaine and methamphetamine abusers (Boileau et al., 2012; Matuskey et al., 2014; Payer et al., 2014) using the D3-preferential PET ligand [^{11}C]PHNO [(+)-4-propyl-3,4,4a,5,6,10b-hexahydro-2H-naphtho[1,2-b][1,4]oxazin-9-ol], has prompted efforts toward the development of selective D3R antagonists for the treatment of drug addiction (Heidbreder and Newman, 2010; Newman et al., 2012b). The D4R has lower levels of expression in the brain compared with D2R and D3R but also was implicated recently in relapse to stimulant abuse (Di Ciano et al., 2014).

Early studies aimed at understanding ligand binding specificity at the D2-like subgroup focused on the selectivity between the relatively more divergent D2R and D4R (Simpson et al., 1999; Kortagere et al., 2004). From the accessible positions identified in D2R using substituted-cysteine accessibility method (Javitch et al., 2002), nonconserved residues in D2R were mutated either individually or in combination with the aligned residues in the D4R. A combined substitution of 4 to 6 residues in TMs 2, 3, and 7 was found to be sufficient to switch the affinity of the receptors for several chemically distinct D4R-selective antagonists. For example, the D2R affinity of the D4R-selective compound, CPPMA (chlorophenylpiperazinyl methylazaindole) (D2R/D4R $K_i > 1000$ -fold), could be increased by three orders of magnitude to D4R-like affinity with a combination of mutations at residues Val91^{2.61}, Phe110^{3.28}, Val111^{3.29}, and Tyr408^{7.35}, and it was hypothesized, prior to the availability of any high resolution GPCR structure, that these residues form a divergent pocket into which the ligand extends (Simpson et al., 1999).

The high-resolution structure of D3R (Chien et al., 2010) has invigorated interest in understanding the

structural basis of subtype-selectivity between the closely related D3R and D2R. The D3R structure revealed a binding site located in the upper-half of the TM domain, in which the nonselective D2-like dopamine receptors antagonist eticlopride is bound. Of the 18 eticlopride contact residues in the D3R structure, 17 are identical in the D2R and one is similar (Fig. 1), highlighting the challenge in creating subtype-selective agents.

Previously, extensive medicinal chemistry efforts have led to the discovery of a class of 4-phenylpiperazine derivatives that is highly selective for D3R over D2R (e.g., compound R-22 [(*R*)-*N*-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)-3-hydroxybutyl)-1*H*-indole-2-carboxamide] has a >100-fold selectivity for D3R over D2R) (Boeckler and Gmeiner, 2006; Newman et al., 2009). These D3R-selective compounds are characterized by a 4-phenylpiperazine scaffold and an aryl amide moiety connected by a four-carbon linking chain (Heidbreder and Newman, 2010). Based on previous studies (Ehrlich et al., 2009; Newman et al., 2009), we hypothesized that the 4-phenylpiperazine, the PP of R-22, would bind within the OBS of both D3R and D2R (Chien et al., 2010). This orientation of the PP positions the arylamide, the SP of R-22, in the SBP₂₃₇ (see section II.B), which can accommodate the SP in several distinct binding modes. Given that the OBS is completely conserved between D3R and D2R, the substantial difference between the SBP in these receptors suggests that the different interactions of ligands with the SBP is responsible for D3R over D2R selectivity.

Indeed, our results from docking and subsequent MD simulations of D3R and D2R in complex with the D3R-selective 4-phenylpiperazine compound, R-22, showed that the PP moiety makes extensive interactions with the conserved OBS in both D3R and D2R. Although the SP moiety of R-22 in D3R packs against a hydrophobic pocket formed by residues Val86^{2.61}, Leu89^{2.64}, Gly94^{EL1.48}, Phe106^{3.28}, and Cys181^{EL2.50} [we define this part of the SBP₂₃₇ to be Ptm23, to indicate the TMs (2 and 3) that form contact with the SP], the SP in D2R binds at the interface of TMs 1, 2, and 7 (Newman et al., 2012a). Interestingly, the Ptm23 residues overlap with the positions of the residues identified previously as responsible for D2R versus D4R selectivity (see above) (Simpson et al., 1999; Ehrlich et al., 2009).

Based on our simulation results, we hypothesized that the ability of the SP to adopt a favorable binding pose in Ptm23 of D3R is dependent on local receptor conformational flexibility. In particular, the flexibility of TM2 is likely determined by both the conserved Pro^{2.59}, which acts as a hinge, and by the length and configuration of EL1, which modulates the degree of bending at the proline kink. EL1 in D3R is one residue longer than in D2R: D3R has two Gly residues in EL1 (93GGV95), whereas D2R has only one Gly residue (98GE99). The extra Gly94 in D3R both interacts directly with the SP of R-22 and renders EL1 sufficiently flexible so as to allow

the TM2 rearrangement necessary to accommodate optimally the SP of R-22 (Michino et al., 2013). This is consistent with the dramatic loss of affinity of R-22 for D3R when this residue is deleted and the large increase of affinity of R-22 when a Gly is inserted into EL1 of D2R (Michino et al., 2013). In addition, computational hydration site predictions suggest that the Ptm23 sub-pocket has higher binding affinity in D3R than in D2R, which is optimally exploited by the R-22 binding mode in the SBP of D3R.

Thus, our combined computational and experimental results point to the difference in the conformations of the SBP between D3R and D2R and identify TM2 and EL1 as the critical determinants for D3R over D2R selectivity (Michino et al., 2013). The D3R-selective ligands exploit interactions at amino acid residues in both the OBS and SBP in a bivalent manner, resulting in subtype selectivity in binding.

A recent study of SB269652 (1*H*-indole-2-carboxylic acid {4-[2-(cyano-3,4-dihydro-1*H*-isoquinolin-2-yl)-ethyl]-cyclohexyl}-amide), an allosteric modulator of D2R, used molecular modeling and simulations in combination with site-directed mutagenesis to show that a SBP between TM2 and TM7 extensively interacts with the linker and indole moieties of SB269652 that extend from the PP bound in the OBS (Lane et al., 2014). Functional studies suggested that the allosteric effect is manifest through a dimeric interface between two D2R protomers. Interestingly, the TM1-TM1 dimer interface of D2R (Guo et al., 2008), which is close to the binding cavity for the indole moiety of SB269652, includes the extracellular portion of TM1, which is divergent even between highly homologous D3R and D2R (Shi et al., 2001; Chien et al., 2010). Thus, such a novel mechanism of allostery opens a new venue to achieve selectivity by taking advantage of the divergence in dimer interfaces, which very likely exist in other subfamilies of aminergic receptors.

To our knowledge, no other studies of aminergic receptors have specifically addressed the structural basis of ligand selectivity as being due to interactions with a divergent SBP. Nevertheless, several studies have implied that interactions with allosteric sites contribute to the selectivity of bitopic ligands (see section II.C), such as the M1R-selective agonists, TBPB, and 77-LH-28-1, mentioned above—their interactions with the crevice formed by TMs 2, 3, and 7 may contribute to their selectivity (Keov et al., 2014). Given the overall similar shapes and sizes of binding pockets among the aminergic receptors, we propose that the extension of ligands into the divergent SBP may be a common exploitable scheme to achieve subtype selectivity for other subfamilies of aminergic receptors.

Interestingly, and conversely, modifications to the SP (aryl amide) that binds in the SBP (as well as the PP that binds to the OBS) in the 4-phenylpiperazine class of drugs have been used to develop multifunctional atypical antipsychotics (Butini et al., 2009; Brindisi

et al., 2014). In these studies, using structure-based drug design, compounds with a defined “balance” of affinities and efficacies (e.g., potent D3R antagonism with 5HT_{1A}R partial agonism and 5HT_{2A}R antagonism and low affinities for both D2R and 5HT_{2C}R) have been discovered. The lead compound 5bb showed promising antipsychotic actions in animal models, devoid of D2R side effects that render classic and atypical D2R antagonists therapeutically suboptimal. Hence, clearly defining the structural components of the SBP, in particular, can be used to design novel therapeutically superior drug molecules, with multiple and defined activities.

B. The Role of Extracellular Loop 2 and Ligand Entry/Exit Pathways in Ligand Selectivity.

Whereas the sides and the bottom of the OBS are formed by positions in the TM domain, EL2 forms a “lid” enclosing the OBS with at least one residue in direct contact with the ligands in all the structures of amine receptors (Fig. 1). Despite the conserved role of this lid, the overall structure of EL2 varies greatly between receptors due to differences in the length and physicochemical properties of the composite residues, especially in the region N-terminal to Cys^{EL2.50}, which is highly divergent even among the most homologous subtypes. For example, EL2 was suggested to play a role in D2R/D3R-selectivity based on receptor chimera experiments, in which replacing EL2 in the D2R with that of D3R improved binding affinity of a D3R-selective 4-phenylpiperazine compound (Newman et al., 2009; Banala et al., 2011).

The divergence of EL2 may affect its dynamics as well, and consequently the access of ligand to the OBS and the egress of the bound ligand from the OBS. In a recent study, it was found that the compound tiotropium more easily dissociates from the OBS of M2R than M3R due to higher mobility of EL2 in M2R (Kruse et al., 2012). This may explain the clinically important “kinetic selectivity” of this drug (Barnes, 2000) for M3R over M2R, despite its similar equilibrium binding affinities for both receptors.

β ARs are also significantly divergent in their extracellular loops—how may the divergency play a role in ligand selectivity? Several β_1 AR and β_2 AR structures are now available that are bound with a variety of ligands (Kobilka, 2011; Katritch et al., 2012; Venkatakrishnan et al., 2013), and although there are no currently available structures of β_1 AR bound with β_1 AR-selective ligands, there are several β_2 AR structures bound with β_2 AR-selective ligands [including ICI-118551 [(±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol], salbutamol, and timolol; 500-, 30-, and 20-fold selective for β_2 AR over β_1 AR, respectively (Baker, 2005)]. Despite the availability of these structures, the mechanistic basis of selectivity of currently

available subtype-selective β AR compounds remains poorly understood.

To investigate the contribution of static ligand binding modes to the selectivity of β AR compounds, we can make use of predicted ligand poses. Retrospective docking benchmarks (Beuming and Sherman, 2012) have shown that docking of most β AR ligands into the crystal structures can replicate the binding modes identified by these structures with very high confidence (typical RMSD values <1.0 Å), even when crystal structures with other cocrystallized ligands are used. Thus, the availability of accurately predicted binding modes for several specific ligands for which no crystal structure is available makes it possible to assess the basis of β_1 AR/ β_2 AR selectivity through an analysis of predicted contacts formed in the binding site.

Based on both the crystal structures and docking studies, in very few cases, selective binding to either β_1 AR or β_2 AR can be rationalized by specific interactions formed between ligands and a divergent binding residue. One such case is that of the compound salmeterol, which is bound with ~ 1500 higher affinity by β_2 AR than by β_1 AR. In its predicted binding mode, salmeterol can interact with β_2 AR via aromatic interactions with the side chains of Tyr308^{7.35} and His296^{6.58}, which are Phe and Lys, respectively, in β_1 AR. Indeed, it has been shown that a mutation of Tyr308^{7.35} to Ala reduces binding of this compound to β_2 AR by more than a 100-fold (Isogaya et al., 1998), and the H296K mutation of β_2 AR reduces the affinity by 18-fold (Baker et al., 2015). Conversely, chimeras of β_1 AR with either TM7 alone or both TM7 and TM2 replaced by those of β_2 AR showed 8- and 88-fold increased affinities for this compound, respectively; interestingly, the chimera with only TM2 replaced showed a 5-fold decreased affinity (Isogaya et al., 1998). Thus, it is likely that the orientation of position 7.35 is affected by TM2, similar to our finding in studying D3R versus D2R selectivity that divergence in a peripheral region can affect the functional role of residues that are directly involved in ligand binding (see above). However, unlike salmeterol and the extended D3R-selective compounds discussed above, most existing β AR-selective compounds are relatively small and do not appear to possess two distinct pharmacophores.

In a number of β_2 AR structures, Tyr308^{7.35} forms a hydrogen bond with the conserved Asn293^{6.55}. It is likely that the presence or absence of the 7.35–6.55 hydrogen bond has a strong effect on the rotameric preferences of both residues and that this in part mediates the selectivity of a number of selective compounds. Indeed, structures of the β_1 AR and β_2 AR reveal a spectrum of conformations of these residues depending on the ligand bound, suggesting that the conformational properties of these residues may affect the specificity of a subset of compounds, including the β_2 AR-specific compound timolol, which forms a direct hydrogen bond with Asn293^{6.55} (but not Tyr308^{7.35}) (PDB ID 3D4S).

However, in addition to these examples of roles for the divergence of 7.35 or its interaction with 6.55, there are also several examples of highly selective compounds that only interact with residues that are identical in both β_1 AR and β_2 AR. For example, the highly β_2 AR-selective compound ICI-118551 (Bilski et al., 1983) forms neither aromatic nor hydrogen bond interactions with the Tyr^{7.35}–Asn^{6.55} pair. Comparing the crystal structures of β_2 AR complexed with ICI-118551 (PDB ID 3NY8) and β_1 AR complexed with cyanopindolol (PDB ID 2VT4), ICI-118551 and cyanopindolol have very similar binding modes, but ICI-118551 is significantly more selective than pindolol (500- versus 2-fold selectivity, respectively) for β_2 AR over β_1 AR (Hoffmann et al., 2004; Baker, 2005, 2010). Thus, additional elements beyond the completely conserved contact residues must also play an important role in determining selectivity.

One such element in the extracellular region is the salt bridge formed between Asp192^{EL2.51} and Lys305^{7.32} in β_2 AR (Bokoch et al., 2010) that is absent in β_1 AR or β_3 AR, in which the corresponding residues are two aspartic acids in β_1 AR and alanine and glycine in β_3 AR. It has been found that K305D mutation of β_2 AR reduces the affinity of salmeterol by more than 30-fold, possibly due to an effect on the overall size of and access to the binding site in the absence of the salt bridge (Baker et al., 2015). Interestingly, in β_1 AR there is a salt bridge formed between Asp184/Asp186 in EL2 and Arg317 in EL3 that may play a functionally similar role but result in divergent loop conformations and dynamics.

In addition, it has been postulated that selectivity at β ARs can also arise from different initial transient interactions between the ligand and receptor in an extracellular vestibule, which influences ligand association rate. However, it has been difficult using direct experimental approaches to identify transient ligand binding sites involved in the specificity of β AR ligands. Thus, several groups have sought to characterize these interactions along the ligand entrance pathway using MD simulations (Wang and Duan, 2009; Dror et al., 2011; Gonzalez et al., 2011; Selvam et al., 2012), and a number of common transient binding sites near EL2, EL3, and TM7 have been identified. The most computationally rigorous study, carried out by Dror and colleagues (2011), implicated a pocket proximal to the OBS in an extracellular vestibule enclosed by EL2, EL3, and TMs 5–7 and proposed that transient interactions with divergent residues in this pocket could affect the specific binding of ligands. In particular, the authors proposed that these interactions are associated with an energetic barrier related to the partial dehydration of both the ligand and the extracellular vestibule. Selvam and colleagues (2012) further characterized this entrance pathway and identified specificity-determining interactions from the simulated unbinding of the selective ligands esmolol in β_1 AR and ICI-118551 in

β_2 AR. They suggested that the nonconserved residue Lys347^{6,58} in the β_1 AR was a key transient interaction for esmolol, whereas ICI-118551 formed mostly hydrophobic and aromatic interactions along the entrance pathway in β_2 AR. Interestingly, other groups have computationally identified a second ligand entrance site closer to TM2 (Wang and Duan, 2009; Gonzalez et al., 2011)—the existence of multiple entry routes suggests that the preference for one route over another for different ligands in different receptors may also contribute to ligand selectivity.

C. Conformational Changes within Secondary Binding Pockets during Receptor Activation.

The development of superior subtype-selective drugs will require a better understanding of the molecular determinants not only of selectivity but also of efficacy. Among the available crystal structures of aminergic receptors, an active conformational state stabilized by G protein or a G protein-mimetic nanobody has been captured for β_2 AR (Rasmussen et al., 2011a,b; Ring et al., 2013) and M2R (Kruse et al., 2013a). Relative to the inactive states of these receptors, the largest conformational changes in the active state are on the intracellular side, including a >10 Å outward movement of the intracellular end of TM6 that results in dissociation of the TM3–TM6 ionic lock as well as smaller rearrangements of TM5 and the NPXXY motif of TM7 that form the Tyr^{5.58}–Tyr^{7.53} interaction (Rasmussen et al., 2011b; Kruse et al., 2013a).

Smaller conformational changes, characterized by various degrees of contraction of the ligand binding crevice (including both the OBS and SBP) have been observed in active state structures of M2R and β_2 AR, as well as in agonist-bound structures of β_1 AR (Warne et al., 2011; Kruse et al., 2013a; Ring et al., 2013). Although larger conformational changes in this region are seen in the M2R active structure compared with that of β_2 AR, both show similar trends: the extracellular portions of TM6 and TM7 move toward the center of the OBS, and a bulge near position 5.46 of TM5 protrudes into the OBS (Fig. 8, A and B). Compared with the OBS residues, some SBP residues in β_2 AR and M2R display only subtle changes in the active structures, e.g., those in TM2 and TM3. However, substantially larger conformational changes are seen in SBP residues 5.38, 5.39, 6.58, 6.59, 7.32, 7.35, and 7.36 (Fig. 8, C and D). Interestingly, based on our analysis of the crystal structures of aminergic receptors available so far, some SBP residues appear to uniquely contact agonists/partial agonists (see top row of Fig. 2), which include the aforementioned positions 6.58, 6.59, 7.32, and 7.36. Given the sequence divergence of these SBP residues (e.g., positions 6.58, 6.59, 7.32, 7.35, and 7.36 are KADFV in β_1 AR and HVKYI in β_2 AR), agonist-induced conformational rearrangements in the SBP may differ among receptor

subtypes, creating an opportunity for the rational development of both subtype-selective agonists and inverse agonists by taking advantage of the divergence at these positions between active and inactive states.

Interestingly, different agonists can stabilize divergent configurations of the SBP residues of β_2 AR, e.g., the side chain of His296^{6,58} is oriented more toward the OBS in the adrenaline-bound structure (PDB ID 4LDO) than in the BI-167107 (8-[(1R)-2-[[1,1-dimethyl-2-(2-methylphenyl)ethyl]amino]-1-hydroxyethyl]-5-hydroxy-2H-1,4-benzoxazin-3(4H)-one)-bound structure (PDB ID 4LDE) and forms an extended polar interaction network that is propagated up to EL3 (shown by two arrows for position 6.58 in Fig. 8C). Thus, in addition to the divergence in residue identity, the conformational dynamics around the SBP residues may also be exploited in developing subtype-selective agents with tailored efficacy.

IV. Conclusions

Unlike for receptors from different subfamilies or subgroups, the OBS of receptors within the same subgroup can be highly homologous or even identical, and thus the development of subtype-selective compounds has been extremely challenging. With recent progress in aminergic GPCR structure determination and molecular modeling, the basis of subtype selectivity is beginning to be elucidated. Divergent extracellular regions can be exploited for the development of 1) selective compounds that bind concomitantly to the OBS and SBP, 2) allosteric modulators that bind only to SBPs, or 3) compounds that differ in their interactions along entry/exit pathways to the OBS of different receptor subtypes. Although there are opportunities to achieve selectivity with all three strategies, practically, it would be easier to achieve rationalized optimizations of existing lead compounds for strategy 1 and possibly strategy 2; to fully understand the structural basis for the ligand selectivity that results from strategy 3 will require significant effort from both the experimental and computational perspectives, but it nonetheless has the potential to address more difficult selectivity issues.

Significant challenges remain, however, in the development of subtype-selective compounds. Although currently available subtype-selective compounds (e.g., the D3R over D2R-selective compound R-22) can be used as tools to identify targetable SBPs, the identification of SBPs in receptors for which there are no available leads to selective compounds remains challenging. In addition, although characterization of binding modes of SBP-targeting compounds can reveal the identity and conformational spectrum of SBPs, further efforts are required for the development of subtype-selective allosteric modulators with sufficient affinity and in vivo activity for therapeutic development.

Fig. 8. Rearrangements of binding site residues in the conformational transition from the inactive state to the active state. The rearrangements in the OBS (A and B) and SBPs (C and D) between the inactive and active structures of β_2 AR (PDB IDs [2RH1](#) and [4LDE/4LDO](#)) (A and C) and M2R (PDB IDs [3UON](#) and [4MQT](#)) (B and D) are shown. The color coding of residues is the same as in Fig. 1. For simplicity, EL2 is not shown in (C and D). The agonists bound in the active-state structures are represented as cyan surfaces to indicate the space occupied by the OBS. For clarity, only the backbones of the inactive-state structures are shown in gray cartoon representation, whereas the active-state structures are superimposed to the corresponding inactive-state structures by the α atoms of 70 TM residue positions that undergo the smallest changes between the inactive and active states. To select these positions, we ranked the distance changes of the corresponding α atoms in the inactive and active structures, after aligning them by TM residues using the iterative-fit “align” command in PyMOL (version 1.3r1; Schrödinger LLC, New York, NY). This ranking has been carried out for both β_2 AR and M2R structures pairs; the 70 highest ranked positions were selected after averaging the ranks from both receptor comparisons and ensuring that at least two positions from each TM were included. The ligand-binding site residues are represented as sticks, drawn from the α atom to the COM of the side chain heavy atoms of the residue (for Gly, only the α atom is shown). The red arrows indicate the conformational changes from the inactive to the active state for the COM of each residue. In (C), two arrows are drawn for each of the positions 6.58 and 6.59, showing the differing conformational changes to the BI-167107-bound active-state (PDB ID [4LDE](#)) compared with the adrenaline-bound active-state (PDB ID [4LDO](#)) for β_2 AR.

Given that crystal structures provide information on the binding modes of relatively rigid compounds, characterization of binding modes of selective compounds that are bivalent and flexible, determination of ligand entry/exit pathways of existing selective compounds, and in silico screening for selective compounds that take advantage of these features must rely heavily on computational analysis, in particular MD simulations that take protein dynamics into consideration.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Michino, Beuming, Donthamsetti, Newman, Javitch, Shi.

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