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THEMED ISSUE REVIEW

The path to the G protein-coupled receptor structural landscape: Major milestones and future directions

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G protein-coupled receptors (GPCRs) play a crucial role in cell function by transducing signals from the extracellular environment to the inside of the cell. They mediate the effects of various stimuli, including hormones, neurotransmitters, ions, photons, food tastants and odorants, and are renowned drug targets. Advancements in structural biology techniques, including X-ray crystallography and cryo-electron microscopy (cryo-EM), have driven the elucidation of an increasing number of GPCR structures. These structures reveal novel features that shed light on receptor activation, dimerization and oligomerization, dichotomy between orthosteric and allosteric modulation, and the intricate interactions underlying signal transduction, providing insights into diverse ligand-binding modes and signalling pathways. However, a substantial portion of the GPCR repertoire and their activation states remain structurally unexplored. Future efforts should prioritize capturing the full structural diversity of GPCRs across multiple dimensions. To do so, the integration of structural biology with biophysical and computational techniques will be essential. We describe in this review the progress of nuclear magnetic resonance (NMR) to examine GPCR plasticity and conformational dynamics, of atomic force microscopy (AFM) to explore the spatial–

Abbreviations: AF, AlphaFold; AFM, atomic force microscopy; AS, allosteric site; ECL, extracellular loop; FD-AFM, Force-Distance AFM; GPCR, G protein-coupled receptor; GRKs, G protein-coupled receptor kinases; ICL, intracellular loop; MD, Molecular Dynamics; NAM, negative allosteric modulator; NAL, neutral allosteric ligand; Nb, nanobody; OS, orthosteric site; PAR1, protease-activated receptor 1; PAM, positive allosteric modulator; TM, transmembrane.

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temporal dynamics and kinetic aspects of GPCRs, and the recent breakthroughs in artificial intelligence for protein structure prediction to characterize the structures of the entire GPCRome. In summary, the journey through GPCR structural biology provided in this review illustrates how far we have come in decoding these essential proteins architecture and function. Looking ahead, integrating cutting-edge biophysics and computational tools offers a path to navigating the GPCR structural landscape, ultimately advancing GPCR-based applications.

KEYWORDS
GPCR, structural biology, NMR, AFM, protein structure prediction

1 | INTRODUCTION

G protein-coupled receptors (GPCRs) are the most prominent family of cellular receptors in eukaryotes. Approximately 800 GPCRs are encoded by the human genome and mediate the effects of various stimuli, including hormones, neurotransmitters, ions, photons, food tastants, and odorants (Vass et al., 2018). GPCRs are involved in neurodegenerative disorders, cardiovascular ailments, cancer, obesity, diabetes, and mental health conditions (Alexander et al., 2023). Their key role has placed GPCRs at the forefront of pharmaceutical research, with 34% of FDA-approved drugs targeting them (Hauser et al., 2017; Yang et al., 2021), and even more applications could be explored by targeting orphan GPCRs (Scharf et al., 2024). However, GPCR structural complexity presents significant challenges for their determination. In this comprehensive review, we delve into the multifaceted world of GPCRs, with a focus on their evolving structural landscape. We highlight key milestones, starting with an examination of GPCR classification and key structural features, followed by an exploration of significant advances in structural biology. Additionally, we discuss the intricate dynamics of orthosteric and allosteric binding sites within GPCRs, complexes with downstream partners, and dimer formation. Finally, we outline the prospects offered by the use of biophysical and computational methods, which promise to extend our understanding of GPCR structures to new horizons.

What is already known?

- Structural biology advancements have revealed molecular details about GPCR activation, dimerization and ligand-binding modes.
- NMR and AFM techniques provide understanding of GPCR plasticity and spatial–temporal dynamics.

What does this study add?

- A critical overview of major milestones in GPCR structural biology.
- A perspective on integrating structural biology, biophysical, and computational tools for better understanding GPCR structures.

What is the clinical significance?

- Decoding GPCR structures promotes the development of GPCR-based therapies.

2 | THE GPCROME

GPCRs share a common structural architecture including seven transmembrane (TM) α-helices with an intracellular carboxyl tail and an extracellular amino terminus. TM helices are connected by three intracellular (ICL1, ICL2 and ICL3) and three extracellular loops (ECL1, ECL2 and ECL3). The A-F classification system divides GPCRs into class A (rhodopsin-like), consisting of over 80% of all GPCRs, class B (secretin-like), class C (metabotropic glutamate receptors), class D (pheromone receptors), class E (cAMP receptors) and class F (frizzled/smoothened family) (Davies et al., 2008; Harding et al., 2018; Pándy-Szekeres et al., 2018). Class D and class E are composed of non-mammalian GPCRs. The GRAFS system, based on their phylogenetic tree, divides mammalian GPCRs into glutamate (G), rhodopsin (R), adhesion (A), frizzled/taste2 (F), and secretin (S) families (Fredriksson et al., 2003). The main difference from A-F is that in the GRAFS system, the class B GPCR family is divided into two groups: the secretin family (B1) and the adhesion family (B2).

The Ballesteros–Weinstein (BW) numbering scheme is widely used to assign fast and immediate structural references to residues of class A GPCRs (Ballesteros & Weinstein, 1995). According to the BW system, TM residues are numbered with the highest conserved residue of each TM as the position X.50 (X indicates the helix number). The most conserved TM residues are $N^{1.50}$ (98%), $D^{2.50}$ (90%), $R^{3.50}$ (95%), $W^{4.50}$ (97%), $P^{5.50}$ (78%), $P^{6.50}$ (99%) and $P^{7.50}$ (88%) (Isberg et al., 2015). A similar numbering scheme has been implemented for classes B, C and F, but with different reference positions. Similar to the BW system, the Wootten numbering scheme is based on highly conserved residues

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in the transmembrane helices: the single most conserved residue is designated as X.50b, where X is a TM helix, and b stands for class B (Hollenstein et al., 2014; Wootten et al., 2013). This numbering scheme is predominantly used for the B1 subclass. However, the reference X.50 residues are also conserved in B2 adhesion receptors, except for the position 3.50 (frequency of E residue: 58%) and 4.50 (frequency of W residue: 42%). Class C GPCRs exist as obligate dimers, including homodimers such as metabotropic glutamate receptors (mGlu) and calcium-sensing receptor (CaS), and heterodimers such as $GABA_B$ receptors and Taste 1 receptors (TAS1Rs). The Pin numbering scheme, proposed by Jean-Philippe Pin in 2003, is specifically designed for class C receptors (Pin et al., 2003). Conserved residues in each TM (a G in the middle of TM1, a central F for TM2, a K near the end of TM3, a W at the end of TM4, a central L in TM5, a central W in TM6 and the conserved motif xPKxY in TM7) are indicated as X.50c numbers. The Wang scheme has been proposed for the class F GPCRs (Wang et al., 2014). However, as there are only 17 members of class F GPCRs in humans, the definition of conservation is challenging. Therefore, it has been proposed that if a helix has more than one fully conserved position, the reference position is chosen based on its proximity to the conserved position in class A. In 2015, Isberg and colleagues developed a generic GPCR numbering scheme based on conserved TM residues across all GPCR subtypes (Isberg et al., 2015). The generic GPCR numbering system allows structural comparisons across the different classes and is used in this article.

3 | GPCR STRUCTURAL BIOLOGY

Structural biology provides the tools to capture the essential molecular details of receptor structures. Until 2017, X-ray crystallography was the preferred approach for obtaining high-resolution structural insights into GPCRs, leading to the determination of numerous GPCR structures, mainly in their inactive or intermediate states (Figure 1). X-ray crystallography is a well-established technique based on the collision of an X-ray beam with atomic nuclei present in a crystal, producing a diffraction pattern that depends on the 3D coordinates of the macromolecular atoms. The application of Fourier transform algorithms provides a three-dimensional 3D map of the electron density.

Extracting GPCRs from their biological environment during purification can compromise the integrity, stability and function of the protein, which is why structural elucidation of GPCRs has been daunting for decades (Tate, 2010). Breakthroughs in GPCR crystallography were celebrated with the 2012 Nobel Prize in Chemistry awarded to Kobilka and Lefkowitz, and have led to the determination of several GPCR structures, revealing details of the orthosteric binding site and the interaction with different ligands and providing insights into the GPCR interaction with G protein (Kobilka, 2013; Zhang, Zhao, & Wu, 2015). Several technologies on protein expression, purifications, crystallization, and X-ray diffraction data collection have been developed (Xiang et al., 2016). For example, fusion proteins, such as T4

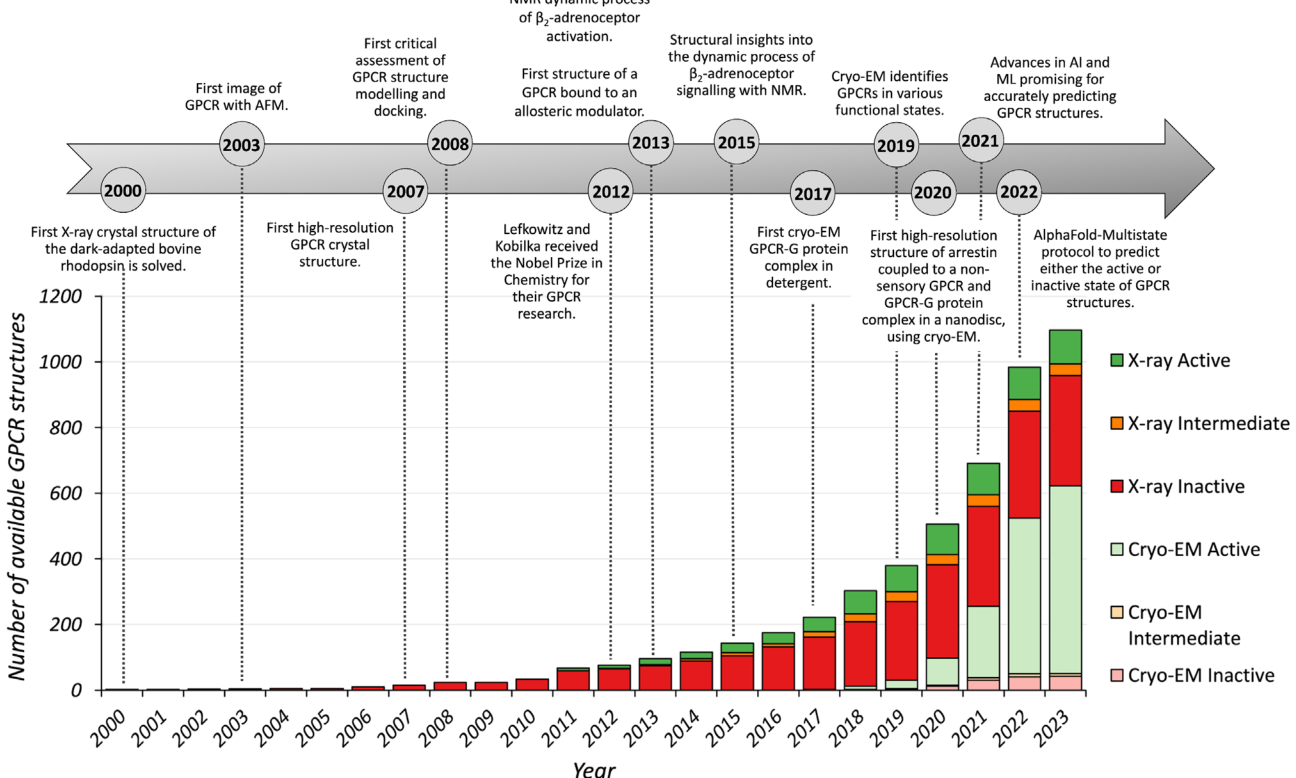

FIGURE 1 Main milestones in G protein-coupled receptor (GPCR) structural biology and their impact in determining GPCR structures in different conformational states with X-ray and cryo-EM. Literature to support the main milestones in chronological order: Palczewski et al. (2000), Fotiadis et al. (2003), Rosenbaum et al. (2007), Michino et al. (2009), Lefkowitz (2013), Nygaard et al. (2013), Kruse et al. (2013), Manglik et al. (2015), Liang et al. (2017), Garcia-Nafria and Tate (2019), Yin et al. (2020), Baek et al. (2021) and Heo and Feig (2022).

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lysosome and BRIL, are often used to stabilize ICLs (Chun et al., 2012; Rosenbaum et al., 2007). Another common approach is to introduce point mutations to produce detergent-resistant GPCRs with high thermostability (Heydenreich et al., 2015; Klenk et al., 2023; Robertson et al., 2011; Sarkar et al., 2008). Through this approach, thermostable **β_2-**, **β_1-**, **α_{1A}-**, and **α_{1B}**-adrenoceptors (Roth et al., 2008; Schuster et al., 2020; Serrano-Vega et al., 2008; Yong et al., 2018) and the **neurotensin receptor 1 (NTS_1)** (Schlinkmann et al., 2012; Shibata et al., 2009) were obtained. Furthermore, to reduce the flexibility of the receptors, high-affinity ligands or even covalent ligands can be employed (Weichert et al., 2014). Nanobodies (Nbs), derived from single-domain antibodies found in camelids, have emerged as a powerful tool to stabilize a specific receptor conformational state, taking advantage of their unique properties, such as small size and high affinity (Jin et al., 2023; Manglik et al., 2017). Nbs have been employed to obtain the X-ray structures of many GPCRs in the active state, like the **β_2-adrenoceptor**, **M_2 receptor**, **μ receptor**, **κ receptor**, and **angiotensin II type 1 (AT_1) receptors** (Che et al., 2018; Huang et al., 2015; Kruse et al., 2013; Rasmussen, Choi, et al., 2011; Rasmussen, DeVree, et al., 2011; Wingler et al., 2019). Moreover, as conformational biosensors, Nbs are used to monitor GPCR dynamics both in vitro and in vivo (Irannejad et al., 2013; Stoeber et al., 2018), and can also function as GPCR modulators (Staus et al., 2016; Yu et al., 2023).

The onset of high-resolution structure determination by cryo-electron microscopy (cryo-EM), celebrated with the 2017 Nobel Prize in Chemistry (Cressey & Callaway, 2017), has triggered a rapid growth in membrane protein structures. Since 2019, the number of protein structures determined by cryo-EM has increased and now exceeds those determined by X-ray crystallography (Figure 1). Cryo-EM is based on the flash-freezing of solutions containing proteins, which are analysed using an electron beam. This makes it possible to determine the 3D coordinates of macromolecules in their physiological environment. Rapid instrumental implementation is overcoming the main limitations of cryo-EM, and even atomic resolution can now be achieved (Danev et al., 2021; Nakane et al., 2020). Using cryo-EM, unstructured regions and post-translational modifications can be included (Lin et al., 2021). Cryo-EM has provided new insights into the biology of GPCRs (Ping et al., 2021; Xu et al., 2021) and is making a major contribution to revealing the structural details of many understudied GPCR subtypes (Scharf et al., 2024). Nevertheless, the alignment of particle projections, especially for individual GPCRs is challenging. Therefore, GPCR structures in the inactive state are particularly elusive for cryo-EM-based determinations, but much progress is being made in this direction. In 2020, Che and colleagues introduced Nb6, an antibody engaging the κ opioid receptor in the inactive state (Che et al., 2020). This approach then enabled high-resolution 3D reconstructions of several GPCRs, including the NTS_1, μ opioid receptor, **somatostatin receptor 2 (SST_2)**, and **histamine receptor 2 (H_2 receptor)** (Robertson et al., 2022; Uchański et al., 2021). More recently, Guo et al. have successfully solved the structures of the β_2-adrenoceptor bound to both antagonistic and agonistic ligands and the adhesion GPCR **ADGRL3** in its apo state, by tagging the receptors with a modified BRIL construct, termed mBRIL, anchored to the receptor helix 8 (Guo et al., 2024). This was proposed as a general approach for the cryo-EM-based determination of GPCR structures.

X-ray crystallography and cryo-EM are currently complementary techniques for understanding GPCR structure and function. According to data collected in the GPCRdb, most of the cryo-EM GPCR structures are captured in the active state, but overall, the number of cryo-EM structures (623) exceeds those obtained by X-ray (477) (Figure 1).

4 | ORTHOSTERIC AND ALLOSTERIC GPCR BINDING SITES

Understanding the shape and composition of ligand-binding sites and the differences between GPCR subtypes is crucial for designing ligands that selectively target specific classes of GPCRs. The orthosteric site (OS) of GPCRs is the site occupied by endogenous ligands. The OS of class A GPCRs is located in the extracellular region of the TM domain, between the highly conserved residue $W^{6.48}$ and the extracellular loop 2. Residues at BW position 3.32, 3.33, 3.36, 4.52, 6.48, 6.51, 6.55 and 7.39 are most frequently involved in ligand interaction (Chan et al., 2019; Di Pizio et al., 2016; Venkatakrishnan et al., 2013). Typically, the orthosteric binding site of class B receptors is deeper and wider than that of class A GPCRs. Residues in TM1 (1.47b) and TM2 (2.60b) were found to be important for peptide ligand binding (Hollenstein et al., 2014). The orthosteric binding site of class C GPCRs is in the extracellular Venus Fly Trap (VFT) domain. Binding of full agonists stabilizes a closed VFT conformation, antagonists bind to the open VFT conformation, and binding of partial agonists results in complete or partial, unstable closure of the VFT domain (Chun, Zhang, & Liu, 2012). The class F orthosteric binding site is located inside the 7TM, close to TM6, TM3 and TM7 (Huang et al., 2018). *Saccharomyces cerevisiae pheromone receptor* (Ste2), the only solved class D GPCR, has a large orthosteric binding pocket throughout the extracellular half of the receptor. Approximately 40% of the orthosteric binding residues are from TM5, TM6 and ECL3 and 35% from TM1, TM2, ECL1, TM3 and TM4 (Velazhahan et al., 2021). The depth of the orthosteric binding site is similar to that of class B receptors and deeper than that of class A receptors. In summary, the location of the GPCR orthosteric ligand binding site varies from class to class (OSs are schematically reported in Figure 2a).

When the binding pocket of a ligand differs from the site of endogenous ligand binding, the ligand is classified as an allosteric modulator (Nussinov & Tsai, 2013). Binding of ligand(s) to any pocket(s) has the potential to disrupt the free energy landscape of the receptor and consequently affect downstream signalling (Latorraca et al., 2017). An allosteric ligand that enhances an agonist-mediated receptor response is termed a positive allosteric modulator (PAM), whereas one that diminishes such a response is termed a negative allosteric modulator (NAM). Alternatively, an allosteric ligand is classified as a neutral allosteric ligand (NAL) if it exerts no influence on either the receptor or the orthosteric ligand activity (Ballante et al., 2021). Moreover, a subclass of PAMs called ago-PAMs can act

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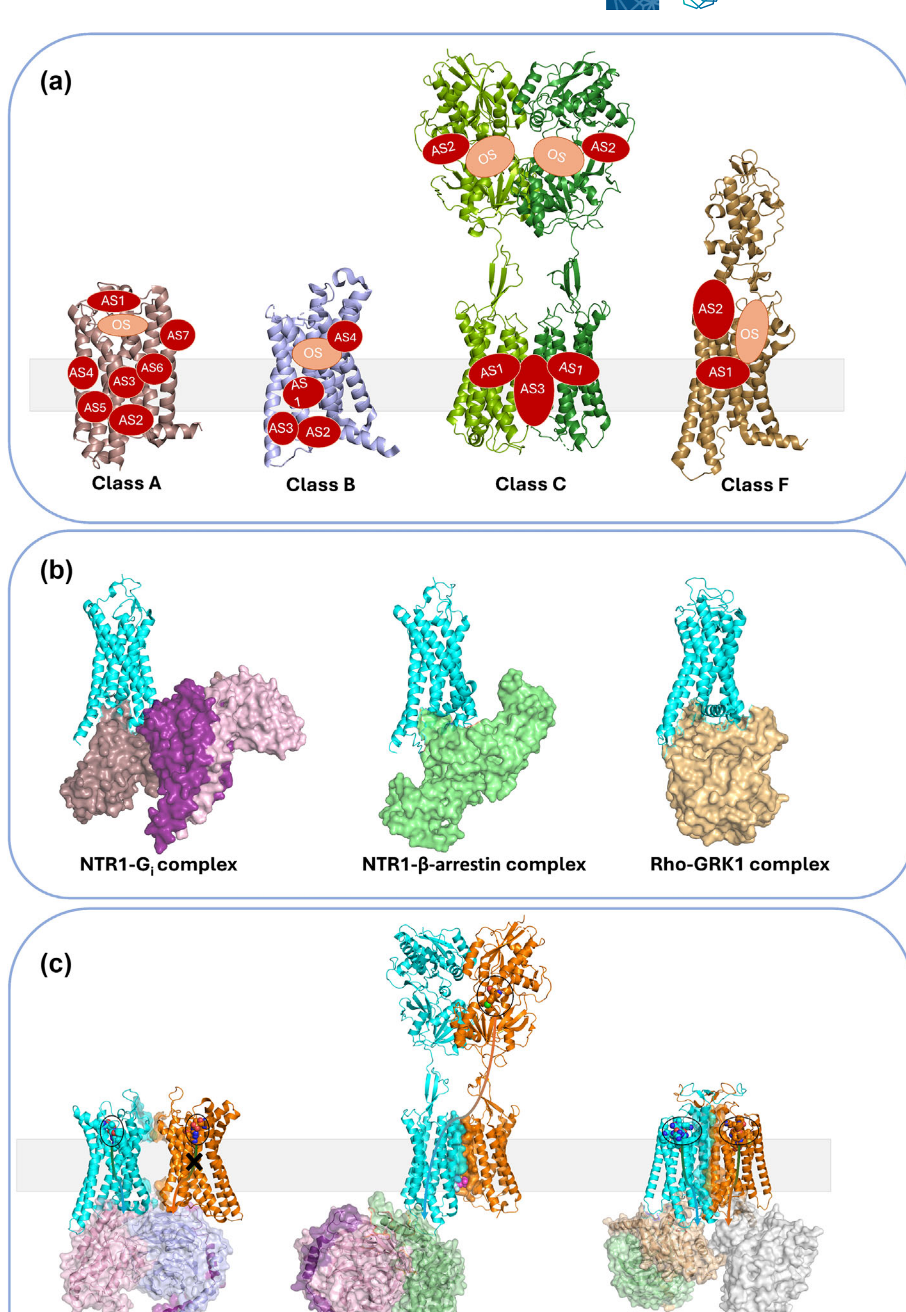

FIGURE 2 (a) Schematic location of allosteric (AS) and orthosteric sites (OS). Proteins are represented as dark salmon, light blue, split pea and forest and sand cartoons for Classes A, B, C, and D (PDB IDs: 6KQI, 5EE7, 6UO8 and 6O3C), respectively. (b) G protein-coupled receptor (GPCR) (NTR1, shown as cyan cartoons) in complex with G_i protein α, β and γ subunits (dirty violet, deep purple and light pink surfaces) (PDB ID: 6OS9), β-arrestin (green surface) (PDB ID: 6UP7) and Rho (cyan cartoons) with rhodopsin kinase GRK1 (yellow surface) (PDB ID: 7MTA). (c) Schematic locations of dimer interfaces for Classes A, C and D GPCRs (PDB IDs: 7W0L, 7EB2, 7AD3). Small molecule and peptide agonists are shown in spheres with carbon atoms coloured according to the chain to which they are bound. The $GABA_B$ PAM is represented in spheres with magenta carbon atoms. The G protein is depicted as a transparent solid surface and a cartoon representation coloured according to the chain. GPCR protomers are represented as cartoons and colour-coded by chain, with the protomer coupling to the G protein shown in cyan and the other in orange. The dimer interfaces (TM3/ICL2 in [a], TM6/TM6 in C-Terminus and N-Terminus, TM1, TM2, TM7, ECL3 in [d]) are highlighted as surfaces matching the colour of the chain to which the residues forming the interface belong.

as agonists independently of orthosteric ligands (Liu et al., 2012; May et al., 2007). In GPCR allosteric systems, ligands binding at spatially distinct sites can reciprocally affect each other's affinity and potency. The interplay between affinity and potency is complex. For example, a PAM can potentiate downstream signalling by various mechanisms: (1) enhancing orthosteric agonist binding affinity without directly affecting signalling, (2) directly enhancing signalling without affecting orthosteric agonist binding, (3) simultaneously enhancing both orthosteric ligand binding affinity and signalling or (4) decreasing orthosteric ligand binding affinity while independently enhancing signalling. A NAM may use analogous combinations to decrease downstream signalling. Since ASs differ between subtypes, targeting GPCR allosteric sites opens new opportunities for drug selectivity (Congreve et al., 2017; DeVree et al., 2016; Roth et al., 2017; Wodak et al., 2019).

The increasing number of experimental structures of GPCRs has revealed several allosteric binding sites in GPCRs, which have been reviewed by Persechino and colleagues (Persechino et al., 2022). In Table 1 and Figure 2a, we list and schematically report the allosteric sites (ASs) for different GPCR classes identified by structural studies. Allosteric binding sites of class A GPCRs can be found on the extracellular side above the orthosteric site (AS1), on the intracellular side (AS2) or at the interface of TM helices (AS3, AS4, AS5, AS6 and AS7). For class B GPCRs, allosteric binding pockets were found in an

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TABLE 1 GPCR allosteric binding sites.

Class	Site	Receptor	Ligand	Location	PDB ID	Reference
A	AS1	M_2	LY2119620	Extracellular side, near ECL2	4MQT	(Kruse et al., 2013)
		$P2Y_1$	MRS2500		4XNW	(Zhang et al., 2015)
		FFA1	MK-8666 MK-8666 TAK-875	Extracellular side, protruding out of the TM bundle	5TZR, 5TZY 4PHU	(Lu et al., 2017), (Srivastava et al., 2014)
	AS2	CCR2	CCR2-RA-[R]	Intracellular side	5T1A	(Zheng et al., 2016)
		CCR9	Vercirnon	Intracellular side	5LWE	(Oswald et al., 2016)
		β_2-adrenoceptor	Cmp-15PA	Intracellular side	5X7D	(Liu et al., 2019)
	AS3	CB_1	ORG27569	Extrahelical site, between TM2, TM3 and TM4	6KQI	(Shao et al., 2019)
		PAR2	AZ3451	Extrahelical site, between TM2, TM3 and TM4	5NDZ	(Cheng et al., 2017)
	AS4	FFA1	AP8	Extrahelical site, between TM3, TM4 and TM5	5TZY	(Lu et al., 2017)
		$C5a_1$	NDT9513727	Extrahelical site, between TM3, TM4 and TM5	5O9H	(Robertson et al., 2018)
	AS5	GPR88	(1R,2R)-2-PCCA	Extrahelical site, cytoplasmic ends of TM5 and TM6	7EJX	(Chen et al., 2022)
	AS6	A_1	MIPS521	Extrahelical site, between TM1, TM6 and TM7	7LD3	(Draper-Joyce et al., 2021)
	AS7	$P2Y_1$	BPTU	Extrahelical site, between TM1, TM2 and TM3	4XNV	(Zhang et al., 2015)
B	AS1	CRF_1	CP-376395	In the TM bundle, between TM3, TM4 and TM5	4Z9G	(Doré et al., 2014)
	AS2	PTH1	PCO371	Intracellular side	8JR9	(Zhao et al., 2023)
	AS3	Glucagon	MK-0893	Extrahelical site, between TM6 and TM7	5EE7	(Jazayeri et al., 2016)
	AS4	GLP-1	LSN3160440	Extrahelical site, between TM1 and TM2	6VCB	(Bueno et al., 2020)
C	AS1	$mGlu_1$	FITM	TM domain, extracellular TM bundle	4OR2	(Wu et al., 2014)
		$mGlu_5$	AFQ-056	TM domain, extracellular TM bundle	4OO9	(Doré et al., 2014)
		$mGlu_5$	MCN-3377-98	TM domain, extracellular TM bundle	6FFH	(Christopher et al., 2018)
	AS2	$GABA_B$	GS39783 Rac-BHFF	Between monomers, TM6-TM6	6UO8 7C7Q	(Shaye et al., 2020)
	AS3	$mGlu_2$	LY354740	VFT domain	4XAQ	(Monn et al., 2015)
		$mGlu_1$	LY341495	VFT domain	3KS9	(Wang & Yang, 2009)
F	AS1	SMO	cholesterol SANT-1	Deep in the TM bundle	6O3C 4N4W	(Deshpande et al., 2019) (Wang et al.,2014)
	AS2	SMO	Cholesterol	Extracellular cysteine-rich domain (CRD)	6O3C	(Deshpande et al., 2019)

induced-fit allosteric site within the TM bundle, between TM3, TM4 and TM5 (AS1), in extrahelical sites (AS2) or in the intracellular side (AS3). The best characterized allosteric sites of class C GPCRs are those located in the TM domain (AS1). Interestingly, allosteric sites in the TM domain have also been found between the GPCR monomers (AS2). Furthermore, class C GPCRs can be modulated by ligands that bind to allosteric pockets in the VFT domain (AS3). The solved structures of SMO, class F GPCRs, also reveal multiple ligand-binding pockets and a hydrophobic tunnel connecting them, allowing a dynamic interplay between allosteric and orthosteric sites and a sophisticated modulation of the receptor (Bansal et al., 2023; Huang et al., 2018; Radhakrishnan et al., 2020).

5 | GPCRs IN COMPLEX WITH DOWNSTREAM PARTNERS

The primary mode of GPCR signalling involves the activation of G proteins, which are heterotrimeric proteins consisting of α, β and γ subunits. Agonist binding to a GPCR leads to the recruitment of the heterotrimeric G protein, nucleotide exchange in G_α, and the subsequent dissociation of the G protein subunits α and $\beta\gamma$. While there are hundreds of GPCRs known to bind a wide spectrum of natural and synthetic ligands, only a limited number of G proteins are available for coupling. G_α proteins are encoded by 16 human genes and grouped into four major families (G_S, $G_{i/O}$, $G_{q/11}$, and $G_{12/13}$) based on their homology and downstream signalling pathways. The discrepancy in the number of GPCRs and G_s proteins leads to a promiscuous coupling, where different receptors can activate the same G_α protein. The efficiency of coupling can vary, and the most efficient interaction is termed 'primary coupling', while less efficient interactions are known as 'secondary coupling' (Harding et al., 2018; Inoue et al., 2019). The complexity and versatility of GPCR-mediated signalling is further enhanced by the ability of GPCRs to couple with multiple G_α proteins and activate different downstream signalling pathways. For example, the β_2-adrenoceptor primarily binds to the stimulatory G_s but also to the inhibitory G_i to regulate different signalling pathways and modulate different cellular responses (Xiao, 2001). The GproteinDb

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database compiles information on G protein couplings from currently available datasets and available structural data (Hauser et al., 2022; Pándy-Szekeres et al., 2024). Currently, a total of 122 structures are available for non-complexed G proteins and 584 for receptor: G protein complexes, of which there are 208 distinct complexes (Pándy-Szekeres et al., 2024). Although the structural coverage of the complexes is limited, it is increasing over time, suggesting specific molecular features and interactions that drive subtype selectivity (Bernhard et al., 2023; Huang, Xu, et al., 2022) or primary vs. secondary coupling (Kim et al., 2020) for certain receptors.

The classical model of homologous desensitization proposes that the active receptor is ultimately phosphorylated by **G protein-coupled receptor kinases (GRKs)** (Gurevich et al., 2012). This phosphorylation leads to the recruitment of arrestins, which bind to the phosphorylated receptors with high affinity (Lohse et al., 1990; Seyedabadi et al., 2021). By outcompeting G proteins, arrestins effectively terminate G protein-mediated signalling. There are four arrestin subtypes: two visual arrestins (arrestin 1, or S antigen, and arrestin 4, or C-arrestin) and two β-arrestins (arrestin 2 and 3, also called β-arrestin 1 and β-arrestin 2). A certain specificity exists between arrestin family members and GPCRs. Visual arrestin binds rhodopsin, class A GPCRs do not interact with visual arrestin and show a preference for a β-arrestin subtype, while class B GPCRs do not appear to have such selectivity (Macey et al., 2004; Oakley et al., 2000; Sanni et al., 2010). Arrestins not only terminate G protein signalling through receptor desensitization but also initiate alternative signalling pathways leading to sustained cellular responses (Daaka et al., 1998; Reiter et al., 2012). This is known as 'β-arrestin-biased signalling', which is the ligand-dependent activation of the arrestin signalling pathways over others, which can lead to a 'functionally selective' response (Kolb et al., 2022). In recent years, there has been remarkable progress in understanding of the structural details of GPCR:arrestin interaction. The ArrestinDb database contains 48 structures of non-complexed arrestins and 10 receptor:arrestin complexes (https://arrestindb.org/), including arrestin 1 in complex with rhodopsin, and β-arrestin 1 in complex with the neurotensin receptor NTS_1, M_2 receptor, β_1-adrenoceptor, **5-hydroxytryptamine receptor 2B ($5\text{-}HT_{2B}$ receptor)**, and the **glucagon receptor**, and the highly phosphorylated tail and the full-length of the **V_2 receptor**. These structures revealed a biphasic mechanism of GPCR:arrestin interaction involving the phosphorylated receptor tail (i.e., the C-terminus) and the receptor core (Ranjan et al., 2017). Currently, only a crystal structure of β-arrestin2 in complex with a phosphopeptide (C7pp) derived from the carboxyl terminus of CXCR7 is available and revealed a slightly different conformation of the C7pp compared to the GPCR C-tail in β-arrestin1 complexes (Min et al., 2020).

Currently, structures of GPCR:GRK complexes are also available. Chen et al. conducted cryo-EM single-particle reconstructions of the light-activated rhodopsin bound to **rhodopsin kinase (GRK1)** (Chen, Plasencia, et al., 2021). Duan et al. reported a complex structure involving **NTS_1** bound to **β-adrenergic receptor kinase 1 (GRK2)**, G_q, and the arrestin-biased ligand SBI-553 (Duan et al., 2023).

Taken together, the dynamic interplay between GPCRs, G proteins, arrestins and GRKs (Figure 2b) underscores the complexity and versatility of cellular signalling mechanisms orchestrated by GPCRs and the structural landscape of these complexes provides the basis for the design of biased ligands that could modulate the receptor profile towards the downstream partner or G protein family or G protein subtype.

6 | GPCR OLIGOMERISATION

The increasing number of experimental structures of GPCR homo- and hetero-dimers in different receptor states and complexes with ligands and signalling partners is providing insights into GPCR oligomerisation and its relationship to receptor activation and G protein coupling (Table 2). With the exception of class C GPCRs, which operate as obligate homo- and hetero-dimers, receptor dimerization in other classes of GPCRs has long been debated. In the last decade, increasing evidence has shown that class A GPCRs can also exist as homo-, hetero-dimers and larger oligomers in native tissues and animal models (Albizu et al., 2010; González-Maeso, 2014). Moreover, GPCR dimers have recently been linked to receptor constitutive activity (Zhang et al., 2022) and biased signalling (Liu et al., 2022). Conversely, the monomeric β_2-adrenoceptor has been proven to efficiently activate G_s and exhibits GTP-sensitive allosteric ligand binding properties (Whorton et al., 2007), thus suggesting that the monomeric form represents the minimal functional unit essential for signalling for this GPCR. However, it remains crucial to consider the dynamic nature of these receptors and potential variations in cellular contexts (Table 2).

Understanding the interplay between G protein cooperativity and oligomer: G protein stoichiometry can provide insights into how GPCRs function as dynamic molecular complexes in the cellular context. G protein cooperativity refers to the phenomenon whereby the activation of one GPCR within a dimer or oligomer influences the activation of an adjacent GPCR, potentially enhancing and/or shifting the resulting signalling response (Lazim et al., 2021). In the context of dimeric GPCR:G protein complexes, stoichiometry plays a role in determining how many G protein subunits can associate with the receptor and the cooperativity of the resulting complex in G protein signalling (Kamal et al., 2011). Activation of one protomer in a dimer may indeed allosterically affect the ability of the neighbouring protomer to couple to a G protein, thus leading to cooperative effects and altered signalling with respect to monomer activation.

In Figure 2c, we compare the dimer interfaces and dimer: G protein stoichiometry as identified by structural investigations of selected GPCRs in fully active state complexed with small molecule (a and b) or peptide (c) agonists.

The dimer interface observed in the fully active **apelin receptor** structure bound to a potent small molecule agonist (PDB ID: 7W0L) has a head-to-head arrangement with a twofold symmetry axis (Figure 2c) (Yue et al., 2022). In the dimer, both orthosteric binding sites are occupied by a copy of the small molecule agonist (spheres with cyan and orange carbon atoms in Figure 2c, left panel). The dimer

TABLE 2 GPCR dimer structures solved to date. If not otherwise specified, the listed receptors are GPCR homodimers solved by cryo-EM.

Class	Receptor	State (G protein)	Ligand	PDB IDs	Reference
A	Rhodopsin	Inactive	None	6OFJ	(Zhao et al., 2019)
	CXCR4	Inactive	Antagonist	3ODU; 3OE0; 3OE6; 3OE8; 3OE9[a] 4RWS[a]	(Wu et al., 2010) (Qin et al., 2015)
	Apelin	Active (Gi)	Agonist	7W0N; 7W0L	(Yue et al., 2022)
C	$GABA_B$[(b)]	Active (Gi)	Agonist, PAM	7 EB2	(Shen et al., 2021)
		Active	Agonist, PAM	6UO8 7C7Q	(Shaye et al., 2020) (Mao et al., 2020)
		Active	PAM	7CA3	(Kim, Jeong, et al., 2020)
		Intermediate I	Agonist	6UO9	(Shaye et al., 2020)
		Intermediate II	None	6UOA	(Shaye et al., 2020)
		Inactive	Antagonist, NAM	7CUM	(Kim, Jeong, et al., 2020)
		Inactive	Antagonist	7C7S	(Mao et al., 2020)
		Inactive	Inverse agonist, NAM	6W2Y; 6W2X	(Papasergi-Scott et al., 2020)
		Inactive	NAM	6WIV	(Park et al., 2020)
		Inactive	None	7CA5 6VJM	(Kim, Jeong, et al., 2020) (Shaye et al., 2020)
	$mGlu_1$	Inactive	Apo	7DGD	(Zhang et al., 2021)
		Intermediate	Agonist	7DGE	(Zhang et al., 2021)
	$mGlu_2$	Active (Gi)	Agonist, Ago-PAM	7MTS	(Seven et al., 2021)
		Active	Agonist	7EBP	(Du et al., 2021)
		Active	Agonist, Ago-PAM	7MTR	(Seven et al., 2021)
		Intermediate (Gi)	Agonist, PAM	7E9G	(Lin et al., 2021)
		Intermediate	None	7EPA	(Du et al., 2021)
		Inactive	Antagonist	7MTQ	(Seven et al., 2021)
	$mGlu_7$	Inactive	None	7EPC	(Du et al., 2021)
	$mGlu_2$-$mGlu_7$[b]	Inactive	None	7EPD	(Du et al., 2021)
	$mGlu_5$	Inactive	None	6 N52	(Koehl et al., 2019)
		Intermediate	Agonist	6 N51	(Koehl et al., 2019)
	$mGlu_5$-5 M	Active	Agonist	7FD8	(Nasrallah et al., 2021)
		Inactive	Antagonist	7FD9	(Nasrallah et al., 2021)
	$mGlu_3$	Inactive	Antagonist	7WI8; 7WI6	(Fang et al., 2022)
		Active	Agonist	7WIH	(Fang et al., 2022)
	$mGlu_4$	Intermediate (Gi)	Agonist	7E9H	(Lin et al., 2021)
	CaS	Active	Agonist	7DTT 7SIL; 7SIM	(Ling et al., 2021) (Park et al., 2021)
		Active	Agonist, PAM	7DTV 7E6T 7MF3; 7M3G	(Ling et al., 2021) (Chen et al., 2021) (Gao et al., 2021)
		Intermediate	PAM	7DTU	(Ling et al., 2021)
		Inactive	Agonist, PAM, NAM	7M3E	(Gao et al., 2021)
		Inactive	Antagonist	7SIN	(Park et al., 2021)
		Inactive	NAM	7M3J	(Gao et al., 2021)
		Inactive	None	7E6U 7DTW	(Chen et al., 2021) (Ling et al., 2021)
D	Ste2	Active (Gpa1)	Agonist	7 AD3	(Velazhahan et al., 2021)
		Active	Agonist	7QBI	(Velazhahan et al., 2022)
		Inactive	Agonist	7QBC	(Velazhahan et al., 2022)
		Inactive	Antagonist	7QA8	(Velazhahan et al., 2022)

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TABLE 2 (Continued)

Class	Receptor	State (G protein)	Ligand	PDB IDs	Reference
		Inactive	None	7QB9	(Velazhahan et al., 2022)

[a]Structure solved by X-ray.
[b]Heterodimer.

interface includes the extracellular tip of TM2 and TM3, including ECL1, with a surface area of ~445 Å^2 (cyan and orange surface in Figure 2c, left panel). It is stabilized by aromatic interactions and hydrophobic contacts established by residues at positions 2.54 and 23.52 to 3.24 on the EC and at position 1.58, 12.48 and 12.49 on the IC of each protomer. As per the oligomer: G protein stoichiometry, mixed species were observed in both 2:1 and 1:1 ratios. In the dimer structure, only one protomer (Prot A, cyan cartoon in Figure 2c, left panel) couples to the G protein and is therefore deputed to signal transduction. Superimposing the G protein-bound protomer (Prot A) on the G protein-free protomer (Prot B, orange cartoon in Figure 2c, left panel) in the dimer structure reveals that it is not possible to accommodate two G proteins due to steric constraints imposed by both the dimer interface and the orientation of Prot B with respect to Prot A.

An asymmetric activation model has also been proposed for the metabotropic $GABA_B$ GPCR (Mao et al., 2020; Shen et al., 2021). This class C receptor functions as an obligate heterodimer of two subunits, namely **$GABA_{B1}$** and **$GABA_{B2}$**, whose TMs share ~70% sequence homology. In the cryo-EM structure of the fully active state in complex with a small molecule agonist and a positive allosteric modulator (PAM, PDB ID: 7W0L), $GABA_{B1}$ (orange cartoon in Figure 2c, central panel) is responsible for ligand recognition with the agonist binding its VFT domain, whereas $GABA_{B2}$ (cyan cartoon in Figure 2c, central panel) is responsible for G protein coupling through a shallow binding site at the TM intracellular side. Several conformations have been observed for the G protein-coupled complex (Mao et al., 2020), the most populated of which shows the ICLs and the IC tip of TM3 interacting with the α5-helix of the G_α subunit. The dimer interface is composed of a symmetric TM6/TM6 interface, similar to other class C GPCRs (Koehl et al., 2019), and involves residues in TM6, ECL3 and the EC tip of TM7 with a surface area of ~1190 Å^2. The interface is stabilized on the EC side by aromatic interactions and hydrophobic contacts established by residues at positions 6.48, 6.52, 6.56, 6.59, 6.60, 7.27 and 7.31 in GB1 and 6.41, 6.48, 6.49, 6.52, 6.55, 6.56, 6.60, 7.24 and 7.27 in GB2. In addition, an H-bond network in the middle of TM6 is established by residues 6.44 and 6.45 in both subunits, while further stabilization is provided by the PAM (spheres with magenta carbon atoms in Figure 2c, middle panel) anchored in a hydrophobic cavity formed by the IC tip of TM5 and TM6 in $GABA_{B1}$ and TM6 in $GABA_{B2}$. As per the oligomer: G protein stoichiometry, only species with a 1:1 ratio were observed. Superimposing the isolated G protein-bound $GABA_{B1}$ monomer on the heterodimer shows that it is impossible to accommodate two G proteins due to steric hindrance. This suggests that only one G protein can bind to the $GABA_B$ receptor at a time.

The cryo-EM structure of a class D GPCR homodimer, namely *Ste2* (PDB ID: 7 AD3) (Velazhahan et al., 2021), in complex with a peptide agonist revealed a unique dimer interface and G protein coupling, not previously observed in other GPCR classes. The dimer interface extends from the N-terminus involving TM1, TM2, TM7 and ECL1 with a surface area of ~2310 Å^2 (in the TM domain only). The interface is stabilized mainly by hydrophobic contacts, with a few H-bonds established by residues bearing polar side chains in both monomers. The entire TM1 (cyan and orange surface in Figure 2c, right panel) is involved in the interface stabilization via a highly conserved motif (glycine zipper) ranging from positions 1.43 to 1.51. A copy of the peptide agonist (spheres with cyan and orange carbon atoms in Figure 2c, right panel) is bound to the orthosteric binding site in each protomer, which, in turn, seems to couple to the engineered mini-G protein (continuous arrows in Figure 2c). The mini-G protein shows an orientation of the alpha subunit to the TM bundle that is remarkably different from the canonical orientation previously observed for other GPCR classes. However, only one mini-G protein was well ordered in the cryo-EM structure, while the other (grey surface in Figure 2c, left panel) showed low density. Therefore, only the C-terminal α5 helix was modelled in the final construct. MD analysis of the dimer model as coupled to two mini-G proteins revealed alternate stability of the beta subunit in one G protein as the result of stabilizing the alpha subunit in the other. The authors ascribed this alternate dynamism to a deletion in the G protein alpha subunit in the engineered mini-G protein. Further structural biological evidence is needed to support the 1:2 dimer: G protein stoichiometry and the relative G protein cooperativity in such complexes.

7 | NMR AS A TOOL TO PROBE GPCR PLASTICITY

Understanding the dynamic nature of GPCRs is essential for drug discovery and the development of novel therapeutic strategies (Lee et al., 2019). NMR spectroscopy is an effective method for investigating the flexibility of GPCRs, as it offers detailed insights into the atomic-level dynamics of proteins over a wide range of time scales (Chao & Byrd, 2018). However, performing NMR studies on GPCRs is a challenging task due to several factors. One of the main obstacles is obtaining stable samples in sufficient amounts. Moreover, the high molecular weight of GPCRs reconstituted in membrane mimetics leads to long rotational correlation times and, therefore, reduced NMR sensitivity and resolution. However, advances in instrumentation, such as ultra-high-field NMR spectrometers and cryogenic probes, have made it possible to study naturally stable GPCRs such as rhodopsin (Ahuja

et al., 2009; Stehle et al., 2014), the **chemokine receptor (CXCR1)** (Park et al., 2006, 2011) and the adenosine **A_{2A} receptor** (Eddy, Lee, et al., 2018; Ye et al., 2016).

The first NMR studies of GPCRs were carried out alongside efforts to obtain their X-ray structures. As discussed above, the structural biology of GPCRs has made significant progress with the development of stable receptors. The majority of GPCR structures solved to date have been obtained from baculovirus-infected insect cells (Milić & Veprintsev, 2015; Saarenpää et al., 2015). These cells are easier to maintain than mammalian cells, but still have the required machinery for protein translation, folding, membrane insertion, and post-translational modification that is not present in lower cellular systems such as yeast and *Escherichia coli*. Native expression of GPCRs in *E. coli* usually results in low yields, but the engineering of fusion constructs that comprise periplasmic proteins attached to the receptor N-terminus and cytoplasmic proteins at the receptor C-terminus has allowed for the successful high-yield expression of some GPCRs (Abiko et al., 2021; Dodevski & Plückthun, 2011; Schuster et al., 2020; Tucker & Grisshammer, 1996; Yeliseev et al., 2005; Yong et al., 2018). The preparation of GPCR samples for NMR presents additional challenges due to the need for isotope labelling. Uniform ^{15}N and ^{13}C labelling and (partial) deuteration (^{2}H) have been accomplished in *E. coli* (Abiko et al., 2021; Mohamadi et al., 2023; Park et al., 2011; Thomas et al., 2015), yeast (Eddy, Gao, et al., 2018), and insect cells (Joedicke et al., 2018; Opitz et al., 2015). The large signal overlap in the spectra of uniformly labelled receptors, the low sensitivity of triple resonance experiments, and the ambiguity of the NOE (Nuclear Overhauser Effect) make it difficult to achieve the complete assignment of GPCR resonances. In addition, the high levels of deuteration required for high-resolution NMR experiments lead to the disappearance of resonances in the transmembrane helices due to limited amide proton back-exchange (Mohamadi et al., 2023). Therefore, most studies using solution-state NMR have focused on employing selective labelling strategies such as side-chain methyl ^{13}C (Baumann et al., 2023; Bokoch et al., 2010; Bumbak et al., 2023; Casiraghi et al., 2016; Goba et al., 2021; Kaneko et al., 2022; Kleist et al., 2022; Kofuku et al., 2014; Rößler et al., 2020; Solt et al., 2017; Sounier et al., 2015; Wu et al., 2020), amino acid-specific backbone ^{15}N (Eddy, Gao, et al., 2018; Eddy, Lee, et al., 2018; Imai et al., 2020; Isogai et al., 2016; Wu et al., 2022), or the incorporation of single probes containing ^{19}F (Dixon et al., 2022; Huang et al., 2021; Kim et al., 2013; Liu et al., 2012; Sušac et al., 2018). Each of these labelling methods presents unique advantages and disadvantages. Assignments of side-chain methyl ^{13}C and backbone ^{15}N are typically obtained by mutagenesis. However, not all amino acids can be mutated, as this may impair protein functionality, limiting the mutagenesis approach. Automated methyl group assignments based on an available GPCR structure and 3D and 4D NOESY experiments might be a solution, but this requires NMR spectra of sufficient resolution and additional data (Kooijman et al., 2020). A new method to assign GPCR resonances has been proposed based on pseudocontact shifts obtained from bound nanobodies tagged with lanthanoid DOTA chelators (Wu et al., 2022). This method provides multiple resonance assignments in one experiment but requires engineered antibodies that recognize specific receptor functional states.

The developments in biochemical and spectroscopic methods described above have allowed for NMR studies of various receptors. These studies revealed considerable conformational diversity in all functional states, which cannot be detected in static structures (Figure 3a).

Studying GPCRs in their apo form by NMR is challenging due to their high flexibility and instability, particularly for insensitive experiments such as 2D ^{1}H-^{15}N NMR spectroscopy. Nevertheless, this was feasible for a thermostabilised mutant of ^{15}N-valine labelled β_1-adrenoceptor (Grahl et al., 2020; Isogai et al., 2016). The more sensitive 1D ^{19}F and 2D ^{1}H-^{13}C NMR experiments have successfully captured the dynamic nature of apo receptors, as demonstrated by studies on A_{2A} receptor (Ye et al., 2016), β_2-adrenoceptor (Horst et al., 2013; Liu et al., 2012; Manglik et al., 2015; Nygaard et al., 2013) and β_1-adrenoceptor (Solt et al., 2017).

A study on the **β_1-adrenoceptor** using ^{19}F NMR relaxation experiments and double electron–electron resonance EPR spectroscopy (Manglik et al., 2015) showed that apo and antagonist-bound states have two conformations (S1 and S2) that are interconverting within hundreds of microseconds and which differ by the presence or absence of a salt bridge between TM3 and TM6. The addition of an agonist to the apo β_1-adrenoceptor increased the receptor heterogeneity, and an additional long-lived active state (S3) was observed in slow exchange with S1 and S2. By adding a G protein-mimicking nanobody to the agonist-bound receptor, a distinct, fully active state (S4) was observed (Figure 3a). As S4 was not observed in the agonist-bound state, it was suggested that the orthosteric- and effector-binding sites are only weakly coupled.

A ^{19}F NMR study conducted on the A_{2A} receptor (Ye et al., 2016) revealed that the ligand-free receptor populates four different conformations (Figure 3b). Of these states, two are inactive conformations (S1 and S2) similar to those observed in the β_2-adrenoceptor (Figure 3a). The other two conformations are associated with active states (S3 and S3′) as identified by the binding of G protein-derived peptides. It was observed that the addition of ligands or G protein mimics did not lead to the observation of other conformations but instead shifted the conformational equilibrium between the already existing four states, according to their pharmacological profile. This suggested that the binding of ligands to the A_{2A} receptor occurs through conformational selection instead of an induced fit process.

While several studies provided important information based on single ^{19}F probes located on the intracellular side of TM6 (Horst et al., 2013; Kim et al., 2013; Liu et al., 2012; Manglik et al., 2015; Ye et al., 2016), ^{15}N resonances from specifically labelled amino acids (Abiko et al., 2022; Eddy, Gao, et al., 2018; Eddy, Lee, et al., 2018; Grahl et al., 2020; Imai et al., 2020; Isogai et al., 2016; Wu et al., 2022), as well as ^{13}CH$_3$-methyl resonances (Baumann et al., 2023; Goba et al., 2021; Imai et al., 2020; Nygaard et al., 2013; Okude et al., 2015; Solt et al., 2017) have revealed additional details about the GPCR activation process.

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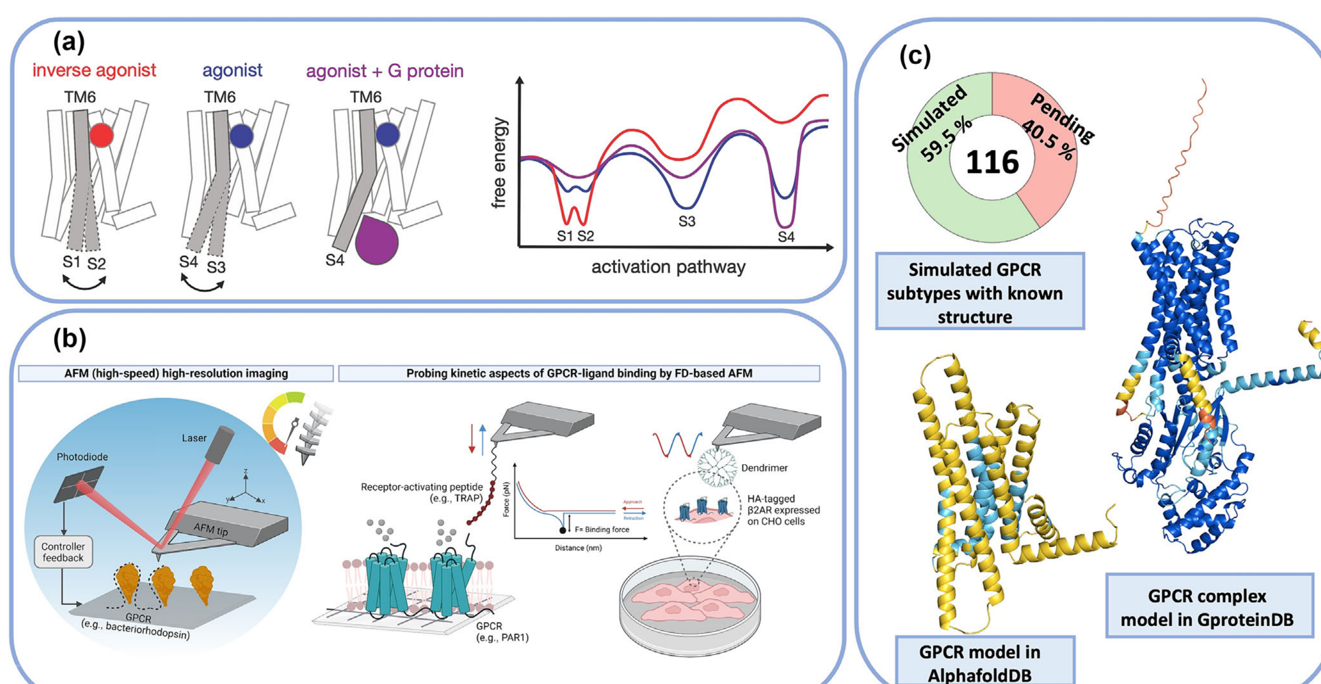

FIGURE 3 (a) GPCR dynamics captured by NMR. Schematic diagrams and free energy landscapes derived from solution state NMR studies of the β_2-adrenoceptor($_2$, A_{2A} receptor and β_1-adrenoceptor. The colour code of the free energy landscapes follows the ligand binding states from the schemes on the left. Diagrams inspired from: Grahl et al., 2020; Manglik et al., 2015; Nygaard et al., 2013; Ye et al., 2016. B: AFM and GPCRs (Dumitru & Koehler, 2023; Müller et al., 2020). Left: Dynamic mode (DM) AFM, which oscillates the AFM tip to reduce friction while contouring the sample (GPCRs) for high-resolution topography imaging (Fotiadis et al., 2003). High-speed AFM, which speeds up the image acquisition time by a factor of $\sim$1000, can provide access to dynamic processes in GPCR structural biology (Perrino et al., 2021). Right: Imaging of GPCRs while quantifying their ligand binding free energy landscape at single molecule and cell level. To characterize ligand binding to GPCRs (e.g., PAR1), the receptor-activating peptide (e.g., thrombin receptor-activating peptide, TRAP) is covalently linked to a poly (ethylene glycol) spacer that was chemically attached to the AFM tip (Alsteens et al., 2015). Alternatively, dendrimers, for instance, can be also directly attached to an amino-functionalized AFM tip (Dague et al., 2022). Using the functionalised AFM tip, GPCR containing proteoliposomes (deposited on a substrate, e.g., mica) or cells overexpressing the GPCR of interest were imaged and FD curves recorded pixel by pixel, from which sample topographs and adhesion maps can be reconstructed (not shown). (c) Top left: current coverage of MD simulations in GPCRmd. Bottom left: Example of a GPCR model, TAS2R1, in AlphaFoldDB (coloured by per-residue model confidence score, pLDDT). Right: Example of a complex model, the muscarinic M_5 receptor:$G_{q/11}$, in GproteinDB (coloured by per-residue model confidence score, pLDDT).

Studies on the $^{13}CH_3$-methionine labelled β_2-adrenoceptor showed that agonist binding increases the receptor intracellular heterogeneity compared to apo or antagonist-bound states (Nygaard et al., 2013). However, the various states resulting from agonist binding do not include the fully active conformation observed when the receptor is in a ternary complex with G protein. The study suggests that the conformational heterogeneity caused by high-efficacy agonists may be crucial in enabling the β_2-adrenoceptor to interact with different downstream signalling proteins.

^{15}N relaxation experiments and line shape fitting of ^{15}N-valine β_1-adrenoceptor resonances near the orthosteric binding site indicated a fast exchange equilibrium (microseconds to milliseconds) between an inactive and a preactive state for the antagonist-bound receptor (Grahl et al., 2020). These states are equivalent to S1 and S2 states observed for the β_2-adrenoceptor and the A_{2A} receptor. Additionally, for agonist-bound β_1-adrenoceptors, a slow exchange (>5 ms) between the preactive and an active state was detected (Abiko et al., 2019; Grahl et al., 2020). This active state largely corresponds to the conformation in ternary complexes with G protein and was fully populated by the addition of a G protein-mimicking nanobody, indicating conformational selection.

The dynamic equilibrium of GPCRs is influenced by various factors such as ligands, mutations, lipids, and pressure. In many cases, the effect of such factors cannot be evaluated by X-ray crystallography or cryo-EM due to their specific requirements for crystal formation or preparation of vitrified samples. NMR, on the other hand, allows for a wider range of sample conditions, for example, the effect of pressure (up to 2500 bar) on the conformational equilibria of the β_1-adrenoceptor (Abiko et al., 2022). Surprisingly, pressure alone can completely shift the agonist-bound β_1-adrenoceptor to its fully active state in the absence of intracellular binding partners. By analysing peak intensities in ^1H-^{15}N NMR spectra, the relative proportions of the different conformations were estimated at increasing pressure, giving a quantitative measure of the volume variation between the preactive and active states.

Since stabilization of GPCRs might lead to altered functional properties, NMR is a well-suited tool to probe the conformational landscape and the change in the population of the structural substates when bound to different orthosteric ligands. Like ^{19}F labelling described above, solvent-exposed cysteine residues can be chemically modified with ^{13}C-labelled methyl groups using the reagent methyl methanethiosulfonate (MMTS). Using the NTS_1 receptor as a model system, this readout enabled the restoration of the conformational switching functionality of a highly stabilized NTS_1 variant by just a few back-mutations to the wild-type amino acid type (Goba et al., 2021).

The high sensitivity of NMR for probing small changes in the conformational landscape of a GPCR makes this method suitable for detecting the impact of specific lipids as allosteric modulators (Jones et al., 2020). For such studies, lipids can be simply added to the detergent-solubilized GPCR, or the NMR experiments can be performed in a defined lipid environment using lipid nanodiscs as a membrane mimetic. With the help of nanodiscs, it is possible to create a detergent-free and lipid-based environment and capture the protein in the most native state possible while increasing the stability or moderate protein dynamics of GPCRs in purified systems. Therefore, they are now used also for GPCR structural analysis by cryo-EM (Günsel & Hagn, 2021; Zhang, Wu, et al., 2022; Zhang, Gui, et al., 2021). Nanodiscs that facilitate solution NMR experiments are available (Daniilidis et al., 2022; Hagn et al., 2013; Miehling et al., 2018). Using ^{19}F NMR, it has been shown that cholesterol has a rather indirect activating effect on the adenosine A_{2A} receptor by changing the properties of the membrane (Huang, Almurad, et al., 2022), contradicting the observation of cholesterol molecules in GPCR crystal structures and previous NMR binding experiments (Gater et al., 2014). Using methyl groups as probes, the ability of cholesterol to modulate the conformational landscape of GPCRs has been shown (Casiraghi et al., 2016). In contrast, cholesterol stabilized the inactive state of the β_1-adrenoceptor probed by 2D-[^{1}H,^{15}N]-NMR (Abiko et al., 2022). Thus, it appears that the response to cholesterol may vary with different GPCRs. In addition to cholesterol, the presence of anionic lipids has been reported to enhance the GPCR active state of A_{2A} receptor (Thakur et al., 2023), and the addition of lipids containing long chain fatty acids, such as docosahexaenoic acid, has been shown to activate the same GPCR (Mizumura et al., 2020). Recently, phosphatidylinositol-4,5-bisphosphate has been shown to tune GPCR motions without strong effects on the structural ensemble (Bumbak et al., 2023).

The combination of NMR data and structural information from X-ray crystallography and cryo-EM has provided a detailed picture of GPCR conformational switching upon activation and G protein or β-arrestin binding (Hilger et al., 2018; Lee et al., 2020; Rasmussen, DeVree, et al., 2011). NMR studies with μ-opioid receptor using ^{13}C-ε-methyl groups in methionine residues and ^{13}C-methylated lysine side chains as probes show that specific receptor conformations are induced by biased agonists (Cong et al., 2021). However, a broader picture is still missing and will require more systematic investigations to extract common principles that will be essential to design more specific biased agonists as novel drugs with improved efficacy and reduced side effects.

Another important area where NMR can provide new insights is the identification of allosteric binding sites. A recent study combining high pressure NMR and X-ray crystallography on the β_1-adrenoceptor demonstrated that empty cavities detected by xenon may serve as allosteric pockets (Abiko et al., 2022). Kaneko and colleagues used solution NMR analysis of the μ opioid receptor to investigate the conformational equilibrium between conformational states and were able to monitor the equilibrium shift by an allosteric modulator, which shifted the equilibrium to a level that could not be reached by orthosteric ligands alone. The binding site of the allosteric modulator and the residues contributing to the regulation of the equilibrium could be identified (Kaneko et al., 2022). The ability of NMR to probe and map the binding of allosteric modulators is crucial for guiding their chemical optimisation, making NMR an important method for the design of GPCR allosteric ligands.

8 | ATOMIC FORCE MICROSCOPY AND GPCRs

Information obtained from static GPCR structures is not sufficient to provide a complete picture on the inter- and intra-molecular behaviour of GPCRs under physiological conditions. The interaction dynamics of a transmembrane protein depend on its surrounding microenvironment. While there is evidence for physical changes in GPCRs during signal transduction, inferring such nanoscale kinetics is challenging. Atomic force microscopy (AFM) is one such tool used to study and understand the behavioural dynamics of GPCRs in their native-like state with (sub)-nanometre resolution and in real time.

Invented more than three decades ago, AFM has evolved from a robust, multifunctional tool to a nanoscopic analytical laboratory due to critical improvements in recent years (in terms of force sensitivity, thermal stability, lateral and temporal resolution and imaging modes) (Dumitru & Koehler, 2023; Müller et al., 2020). Therefore, it is now readily applicable to unravel the GPCR structural landscape in multiple dimensions. AFM uses a small probe to visualize biological structural surface topography and mechanical properties, while providing valuable information about their chemical composition and interactions, enabling a comprehensive understanding of biological systems, such as GPCRs.

The first GPCR imaged by high-resolution contact mode AFM in a physiological buffer was rhodopsin in the native membrane of rod outer segments (ROS), enabling structural insights into the arrangement of rhodopsin (Fotiadis et al., 2003). This study elucidated not only the physical attributes of the protein (e.g., total protein height), but also the dimerization of rhodopsin arranged as paracrystalline arrays. Technological advances in AFM have also made it possible to obtain spatial–temporal protein dynamics. Using High-Speed AFM (HS-AFM), different conformational states of the protein (open vs. closed) have been reported for bacteriorhodopsin (Perrino et al., 2021).

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An essential model of AFM, called FD-AFM (Force-Distance AFM), can be used to probe the kinetic aspects of proteins, such as their folding and/or ligand (un)binding. This can enable mapping of the energy landscape, thereby explaining the existence of different transition states between bound and unbound GPCRs, as extensively reviewed by Zocher and colleagues (Zocher et al., 2013). An example is human **proteinase-activated receptor 1 (PAR1)**, where its binding strength in the absence and presence of an antagonist, for example, thrombin receptor-activating peptide (TRAP), has been quantified (Alsteens et al., 2015). By extrapolating the data and applying the Bell-Evans model, the authors were able to extract the kinetic and thermodynamic parameters of these interactions, allowing them to accurately describe the PAR1 ligand binding free energy landscape. Dumitru and colleagues, by combining AFM with steered MD simulations, elucidated the ligand-binding mechanism of the human G protein-coupled **$C5a_1$ receptor** and discovered a cooperativity between two orthosteric binding sites (Dumitru et al., 2020). The ligand-binding free energy was measured for an overall $\Delta G \sim -13.6 \pm 4.1$ kcal·mol^{-1}, which is higher than the sum of individual binding and effector sites, that is, $\Delta G \sim -8.6 \pm 2.3$ kcal·mol^{-1}.

The cell membrane matrix that houses the GPCRs also influences their overall functionality. Any change in the microenvironment has been shown to alter the role of GPCRs. Cholesterol, an essential component of the mammalian cell membrane, maintains membrane fluidity and integrity of the membrane and its ability to form microdomains (called lipid rafts), which are suggested to affect the overall organization of phospholipids (Karanth et al., 2021). In 2012, Zocher and colleagues reported that cholesterol increases the kinetic, energetic and mechanical stability of the β_2-adrenoceptor compared to the receptor reconstituted in proteoliposomes lacking this steroid. Around the same time, several laboratories showed a remarkable dependence of rhodopsin activation on curvature-induced elastic stress in lipid monolayers (Zocher et al., 2012). Importantly, phospholipids display key processes such as lateral and rotational diffusion and can influence the overall organization of proteins in the membrane. The quantification of these biophysical activities is still poorly understood. It remains an open question whether transmembrane proteins always act in concert in the phospholipid bilayer. Therefore, understanding GPCRs at the cellular level can redefine binding kinetics. Recently, Dague and colleagues made the first attempt, where the HA-tagged β_2-adrenoceptors expressed on CHO cells were probed by FD-AFM, revealing different oligomerization states on the cell membrane along with the unfolding of the GPCR on the cell surface (Dague et al., 2022). Such experiments help obtain the overall spatial organization of the protein and add complexity to the GPCR pharmacology.

The main advantage of AFM over other techniques is its adaptability to observe individual GPCRs down to their organization at the cellular level and to sense their interaction with ligands under physiological conditions. Such obtained adhesive or binding force can be further extrapolated to broader applications (e.g., screening of drug molecules). The easy integration of AFM with other microscopy methods makes it a lynchpin to answer many biological questions and provide more insights into how cellular states modulate GPCRs and vice versa (Dumitru & Koehler, 2023). Correlating this information with functional assays may provide a more reliable basis for controlling GPCR activity with pharmacological chaperones in health and disease.

9 | GPCR STRUCTURE PREDICTION

For quite some time, understanding GPCR activation mechanisms and advancing structure-based drug design has posed significant challenges due to the limited availability of high-resolution structures. As a result, specialized protein structure prediction methods for GPCRs have emerged, allowing the generation of structural models even in the absence of experimental structures. Available resources are summarized in Table 3. Despite advances in GPCR structural biology, currently, only 23% of unique GPCR structures are available, and we know even less about complexes with downstream partners and dimer formation, highlighting the continued need for structure prediction methods in GPCR research.

Two major approaches dominate modern protein modelling: template-based modelling (TBM) and neural network (NN)-based modelling. TBM, which includes techniques like threading and homology modelling, relies on existing protein structures as templates to generate models. However, TBM struggles when no known structure closely resembles the target protein domain, leading to potential inaccuracies. On the other hand, since the last Critical Assessment of Structure Prediction (CASP) 14 round, CASP14, NN-based algorithms have emerged as convincing tools and are being used more and more. AlphaFold 2 (AF2), a programme developed by DeepMind, the arm of Google AI, was able to determine protein structures with an accuracy level previously obtained only with experimental methodologies (Callaway, 2020; Service R, 2020). In 2021, Baker's group developed RosettaFold, able to predict the protein structure with accuracies near to AlphaFold (Baek et al., 2021). Artificial intelligence (AI)-based protocols, exemplified by AF2 (Jumper et al., 2021) and RoseTTAFold (Baek et al., 2021), deploy NN models trained on co-evolutionary coupling and high-resolution structure data from known experimental structures. In a recent study by Lee et al., the novel NN-based modelling methods, AF2 and RoseTTAFold, were thoroughly tested on a set of solved GPCRs obtained from the Protein Data Bank, and compared with models generated by Modeller, a TBM approach (Lee et al., 2022). They found that AF2 and RoseTTAFold outperformed Modeller in cases where no good templates were available, but TBM performed better when good templates were available. AF2 outperformed RoseTTAFold in terms of the accuracy of the top-ranked models, while RoseTTAFold showed less variance, ie generated more similar models, than AF2. However, it was found that NN-based models often only represented the inactive state. To address this, Heo and Feig introduced a multi-state prediction protocol that extends the capabilities of AF2 to predict both active and inactive states with remarkable accuracy (Heo & Feig, 2022). This protocol uses state-annotated GPCR template databases to accurately capture state-specific structural changes. GPCRdb now contains

TABLE 3 Webservers for modelling GPCRs.

Tool	Description	URLs	References
GOMoDo, pyGOMoDo	An automated homology modelling protocol based on MODELLER. Homology modelling is followed by small molecule ligand docking (using AutoDock VINA or HADDOCK) if binding site information is available. pyGOMoDo is a python library with the updated functionality of the GOMoDo web server, specifically designed for human GPCRs.	https://github.com/rribeiro-sci/pygomodo	(Sandal et al., 2013; Riberio et al., 2023)
GPCR-I-TASSER	A specialized version of I-TASSER designed for GPCRs. Based on the use of GPCR structure-specific features with the I-TASSER template-based and template-free modelling pipeline.	https://zhanggroup.org/GPCR-I-TASSER/	(Yang et al. 2015)
GPCR-ModSim	Dedicated web server for GPCR modelling using homology modelling and molecular dynamics simulation (GPCR structure prediction and refinement).	https://modsim-pharma.com/index.php/gpcrmodsim/	(Esguerra et al. 2016)
MODELLER	A widely used homology modelling software.	https://salilab.org/modeller/	Web and Sali 2016
GPCR-SSFE2.0	It selects templates for each α-helix by using a database of sequence fingerprint features correlated with experimental GPCR structural data.	http://www.ssfa-7tmr.de/ssfe2/ https://proteinformatics.uni-leipzig.de/sl2/	(Worth et al., 2011)
GPCRM	A web service designed to predict GPCR structures, using advanced homology modelling techniques. It uses profile-profile alignment, multiple structural templates, and Z coordinate-based filtering to refine the models. Two different loop modelling techniques—Modeller and Rosetta—are used to further improve model accuracy. The final ranking of GPCR models is determined using the BCL::Score, a knowledge-based energy function tailored for membrane-protein structures.	https://gpcrm.biomodellab.eu/	(Miszta et al., 2018)
GPCR-AIM	This approach is entirely template-free (ab initio method). It consists of a four-step protocol including 2D helix topology optimisation, 3D helix topology optimisation, full-length optimisation and a refinement step.	https://zhanggroup.org/GPCR-AIM/	(Hongjie Wu, 2018)
RosettaGPCR	A dedicated version of the Rosetta modelling protocol for GPCRs. It uses a combination of sequence- and structure-based alignment. It considers also the structural conservation in the extracellular loops and then builds the model with the best possible templates for each region of the target GPCR.	https://github.com/benderb1/rosettagpcr Link to GPCR structural models	(Bender et al., 2020)
AF2	A deep-learning based method. **AlphaFold Multimer**: AF extension to model complexes. **AlphaFold MultiState**: AF extension to model specific GPCR conformational states.	https://github.com/google-deepmind/alphafold Available also via Google Colab: https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb	(Heo & Feig, 2022; Jumper et al., 2021; Mirdita et al., 2022)
RosettaFold	A deep-learning based method.	https://github.com/RosettaCommons/RoseTTAFold	(Baek et al. 2021)

state-specific structural models of all human non-olfactory GPCRs, generated using AlphaFold2-MultiState (Pandy-Szekeres et al., 2023).

AlphaFold Multimer is an extension of AlphaFold specifically designed to predict the 3D structures of protein complexes and can now be used to generate models of GPCR oligomers or GPCR in complex with downstream partners (He et al., 2022; Lee et al., 2023; Paradis et al., 2022). AF2 structure models of GPCR:G protein complexes are now also incorporated in the GproteinDb, significantly expanding the database structural templates and enhancing interactive analysis tools for interface studies, crucial in understanding coupling selectivity (Pandy-Szekeres et al., 2024).

A main challenge in GPCR structure prediction results from the ECL2, the longest and most diverse loop among class A GPCRs (Nicoli et al., 2022). Due to its pivotal role in ligand binding and subsequent drug design, various computational groups have committed their research efforts to understanding and modelling ECL2 (Blaszczyk et al., 2013; Gervasoni et al., 2023; Goldfeld et al., 2011; Kmiecik et al., 2014; Nicoli et al., 2022; Wink et al., 2019; Won et al., 2018). Indeed, the loop conformational space increases exponentially with the length, posing a great challenge for the loop structure prediction (Wink et al., 2019; Won et al., 2018). Even in the models built with AF2, the ECL2 is often indicated as a low or very low confidence region (Varadi et al., 2022), and various studies have shown as

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AlphaFold performs poorly on loop regions (Burley et al., 2021; Lee et al., 2022). Impressively, the recently solved first structure of an odorant GPCR, the OR51E2, has the same folding for the ECL2/N-terminus of the AF2 model (Billesbølle et al., 2023; Nicoli et al., 2023). However, in this case, as in others (Callaway, 2022; He et al., 2022; Terwilliger et al., 2022), it has been observed that the residue arrangements in the binding sites of GPCR AF models are not optimal for structure-based investigations. Addressing the flexibility of residue side chains with sampling optimization, molecular dynamics simulations, as well as flexible docking tools could improve the applicability of GPCR structural models in drug design campaigns (Kapla et al., 2021). MD simulations are often integrated with experimental investigations (X-ray, cryo-EM, AFM, NMR, single-molecule spectroscopy) to investigate the structural changes and interactions of GPCRs with ligands and signalling partners over time, providing important insights into the mechanisms underlying receptor activation (Albizu et al., 2010), dimerization (Di Marino et al., 2023), G protein selectivity (Sandhu et al., 2022), or β-arrestin binding to the membrane and receptor (Grimes et al., 2023). Docking and MD simulations are also used to map and predict allosteric pockets (Ciancetta et al., 2021; Hedderich et al., 2022). These are just a few examples, and others are discussed in other sections of this review. We will not go into further detail on the application of simulations to structural data, as this is outside the scope of this review. However, MD data are precious structural data and efforts are underway to make them accessible to the GPCR community. The GPCRmd web-based platform (https://submission.gpcrmd.org/home/) has recently been developed to collect MD simulations of GPCRs and currently contains simulations of 116 GPCR subtypes (Figure 3c) (Rodriguez-Espigares et al., 2020).

10 | CONCLUSIONS

This review has highlighted the remarkable progress in understanding the GPCR structural landscape. From the early pioneering work that provided the first GPCR structures to the more recent breakthroughs in cryo-EM, the complexity of receptor activation, ligand binding, oligomerisation and signalling bias has been unveiled. These findings underscore the need for sophisticated methods to probe GPCR plasticity. The integration of biophysics and innovative computational tools could play a pivotal role in addressing the issues raised. The future of GPCR research paves the way for uncovering the complete structural landscape, including the dynamic nature of these receptors and understanding them in multiple dimensions, ultimately driving advances in drug development by improving our ability to target GPCRs precisely.

10.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24 (Alexander Christopoulos et al., 2023; Alexander Fabbro et al., 2023).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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