Mechanisms of signalling and biased agonism in G protein-coupled receptors

Denise Wootten[1,2], Arthur Christopoulos[1], Maria Marti-Solano[3], M. Madan Babu[3] and Patrick M. Sexton[1,2]**

Abstract | G protein-coupled receptors (GPCRs) are the largest group of cell surface receptors in humans that signal in response to diverse inputs and regulate a plethora of cellular processes. Hence, they constitute one of the primary drug target classes. Progress in our understanding of GPCR dynamics, activation and signalling has opened new possibilities for selective drug development. A key advancement has been provided by the concept of biased agonism, which describes the ability of ligands acting at the same GPCR to elicit distinct cellular signalling profiles by preferentially stabilizing different active conformational states of the receptor. Application of this concept raises the prospect of 'designer' biased agonists as optimized therapeutics with improved efficacy and/or reduced side-effect profiles. However, this application will require a detailed understanding of the spectrum of drug actions and a structural understanding of the drug–receptor interactions that drive distinct pharmacologies. The recent revolution in GPCR structural biology provides unprecedented insights into ligand binding, conformational dynamics and the control of signalling outcomes. These insights, together with new approaches to multi-dimensional analysis of drug action, are allowing refined classification of drugs according to their pharmacodynamic profiles, which can be linked to receptor structure and predictions of preclinical drug efficacy.

Rhodopsin
A light-sensitive G protein-coupled receptor involved in visual phototransduction.

[1]*Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia.*

[2]*School of Pharmacy, Fudan University, Shanghai, China.*

[3]*MRC Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge Biomedical Campus, Cambridge, UK.*

**e-mail: denise.wootten@monash.edu; patrick.sexton@monash.edu*

https://doi.org/10.1038/s41580-018-0049-3

G protein-coupled receptors (GPCRs) are the largest family of cell surface receptor proteins in eukaryotes. In humans, they are encoded by over 800 individual genes and are widely expressed in various tissues, where they control a broad range of physiological processes. The pervasive role of GPCRs in human physiology arises from evolutionary diversity in the sequence encoding the seven transmembrane domains (TMDs), which form the core of the receptor common to all GPCRs. On the basis of sequence and evolutionary conservation, these receptors are divided into subfamilies that include class A (rhodopsin-like), class B1 (secretin receptor-like), class B2 (adhesion receptors), class C (metabotropic glutamate receptor-like) and class F (frizzled-like) subfamilies as well as the taste 2 sensory receptor subfamily (GPCR Database). GPCR diversity is further elaborated by numerous mechanisms including alternative splicing, RNA editing, post-translational modifications and protein–protein interactions that alter both the repertoire of ligand interaction and the functional consequences of receptor activation[1]. This diversity allows these receptors to recognize and respond to an enormous variety of ligands that range from photons, odorants, ions, small neurotransmitters and small neuromodulatory peptides to large peptide hormones, glycoprotein hormones and other large protein domains, including those involved in direct cell–cell communication and viral entry[2]. Another key component of the versatility of GPCR signalling is the breadth of intracellular proteins that they can engage with (see next paragraph). These intracellular partners include numerous heterotrimeric G proteins, which serve as canonical transducer proteins, as well as regulatory and scaffolding proteins such as arrestins, PDZ-domain-containing scaffolds and non-PDZ scaffolds, such as A kinase anchor proteins (AKAPs) that initiate or control distinct patterns of signalling[3–7] (FIG. 1).

To elicit signalling, GPCRs need to couple with intracellular transducers such as heterotrimeric G proteins, which are formed by Gα, Gβ and Gγ subunits. In humans, there are 16 Gα, 5 Gβ and 13 Gγ subunits that can combine to form a wide range of heterotrimeric G proteins. Each Gα subunit can signal independently, whereas the Gβ subunits and Gγ subunits are obligate heterodimers that function as a single unit (Gβγ).

The 16 Gα subunits can be classified into 4 major Gα families (G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$) that regulate key effectors (for example, adenylyl cyclase, phospholipase C, etc.) and the generation of secondary messengers (for example, cAMP, Ca^{2+}, inositol 1,4,5-triphosphate ($Ins(1,4,5)P_3$), etc.), which in turn trigger distinct signalling cascades. It is now well established that numerous distinct receptors can couple to the same Gα protein and that the same receptor can also couple to more than one Gα protein. Gβγ subunits have both regulatory and signalling functions, including, for example, serving as scaffolds for receptor kinases and as modulators of ion channels[8]. More recently, our understanding of G protein activation has progressed to include ligand-dependent effects on G protein conformation that are linked to signalling efficacy[9,10]. Advances in cloning and sequencing and global approaches to identification of other GPCR–protein interactions[11,12] have further expanded the repertoire and/or complexity of potential consequences of GPCR activation. The most abundantly studied of these GPCR interacting partners are arrestins, which serve as negative regulatory proteins for signalling through G proteins (by blocking the activated receptors from binding to heterotrimeric G proteins (signalling desensitization) and by targeting ligand-occupied GPCRs for endocytosis). However, they can also function as scaffolds for initiation of additional signalling, prominently including activation of various MAPKs, such as ERK[13]. The latter is often termed 'arrestin-dependent, G protein-independent' signalling[14], although the extent to which such signalling may require initial G protein recruitment, or can be modulated by G protein-dependent signalling, is still under study[15]. For example, a thought-provoking recent study on the requirement for G protein for arrestin-mediated signalling has revealed that signalling (ERK phosphorylation), mediated by arrestin may require G protein, but that arrestin-dependent receptor internalization can be achieved in the absence of functional G protein[15]. Indeed, arrestin engagement by GPCRs may occur in the absence of receptor activation owing to heterologous phosphorylation by second messenger kinases[16], and these behaviours need to be understood when developing novel GPCR ligands.

Given the near universal importance of GPCRs in normal development and physiology, it is not surprising that perturbations in GPCRs and/or their transducers can have major roles in the initiation and progression of disease. Currently, ~30% of approved drugs target GPCRs, but these drugs act at only a small subset of the GPCR repertoire[17,18], and there is intense interest in further pharmaceutical exploitation of these proteins[18]. Nonetheless, lack of expected clinical efficacy remains a major cause of GPCR drug failure that is indicative of important gaps in our understanding of GPCR signalling and, in particular, their response to specific ligands. The current obstacles to successful pharmacological targeting of GPCRs include the disease-specific variability in drug efficacy that occurs in a signalling pathway-dependent manner, disease heterogeneity, the state of disease progression and the variation in receptor behaviour associated with polymorphisms in receptor sequences within the human population[18,19]. In the past decade, and particularly in recent years, there has been a leap in our understanding of the complexity of the mechanisms and dynamics of GPCR function that promises to provide new paths to both identification

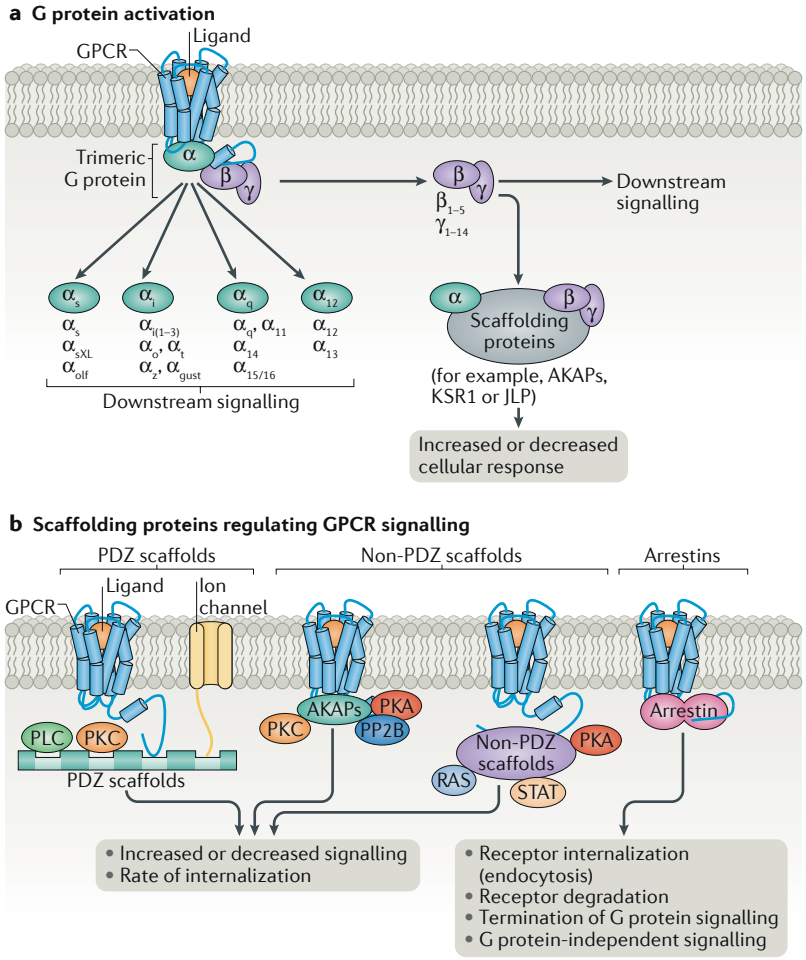

Fig. 1 | **Schematic illustration of GPCR signalling. a** | Canonical G protein-coupled receptor (GPCR) signalling occurs via coupling to heterotrimeric G proteins (Gα, Gβ and Gγ). Upon activation by a GPCR, the Gα and Gβγ subunits dissociate and can each activate downstream signalling. Gα proteins can be subdivided into four main families with different signalling properties. There are also multiple Gβ and Gγ subunits, which further diversifies signalling responses. Gα and Gβγ subunits can also associate with scaffolding proteins that regulate their signalling profiles. **b** | A schematic of GPCR scaffolding proteins that have key roles in the regulation of GPCR signalling and are also involved in forming higher order, tightly regulated signalling complexes, termed signalosomes. These scaffolds can be divided into three broad categories: PDZ scaffolds, which associate with the distal portions of GPCR carboxyl termini and can couple the GPCR to various signalling proteins such as kinases (for example, protein kinase C (PKC)), phospholipases (for example, phospholipase C (PLC)) and ion channels; non-PDZ scaffolds, such as A kinase anchor proteins (AKAPs), which bind to the cytoplasmic face of GPCRs and also associate with multiple signalling partners including kinases (for example, PKA and PKC), phosphatases (for example, serine/threonine-protein phosphatase 2B (PP2B)) and intracellularly localized receptors (such as inositol 1,4,5-triphosphate receptors ($InsP_3Rs$) in the endoplasmic reticulum; not shown); and arrestins, which associate with many GPCRs, disrupting G protein–GPCR interactions and driving GPCR internalization via endocytosis, and act as scaffolds to facilitate multiple interactions between GPCRs and cytoplasmic signalling proteins in a G protein-independent manner. Of note, GPCRs themselves can serve as scaffolding proteins for other membrane proteins, including other GPCRs and receptor modifying proteins, as exemplified by receptor activity-modifying proteins (RAMPs) (not shown). JLP, JNK-associated leucine-zipper protein (also known as SPAG9); KSR1, kinase suppressor of RAS1; STAT, signal transducer and activator of transcription.

and development of novel GPCR drugs and translational understanding of how these can be optimally used for therapeutic intervention. Among the foremost of these developments is recognition that GPCR ligands can exhibit biased agonism, the ability of individual ligands acting at the same receptor to initiate diverse cellular outcomes. In this Review, we outline recent advances in our understanding of the mechanistic basis for receptor activation and biased agonism.

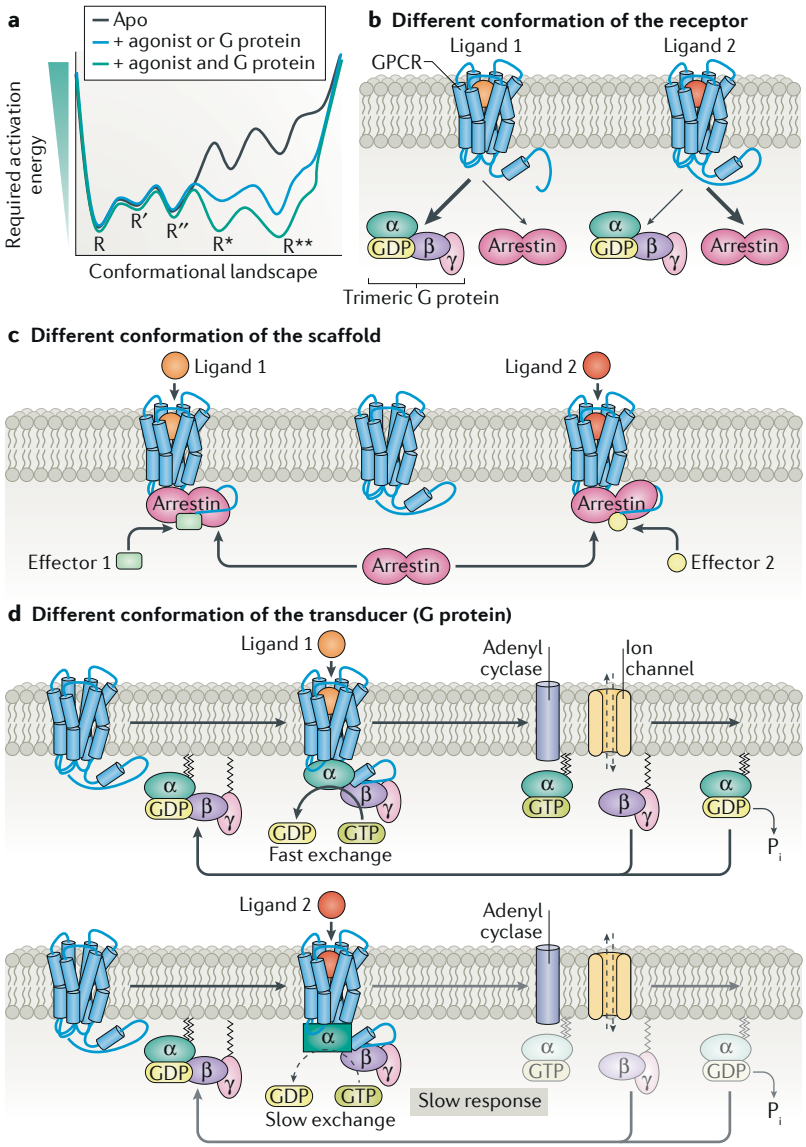

Fig. 2 | **Mechanisms of ligand-induced biased agonism. a** | A schematic illustrating conformational dynamics occurring in G protein-coupled receptors (GPCRs). GPCRs can move between various inactive-like (R, R′ and R′′) and active-like (R* and R**) states. This can occur in the absence of ligand (Apo, black line); however, the energy barrier to achieving these states makes their occurrence a low probability. Addition of agonist or G protein (blue line) can decrease the energy required to reach active states, but full conformational change is favoured by the addition of both agonist and G protein (green line). **b–d** | Biased agonism can arise via multiple mechanisms. Distinct ligands induce different conformations within the receptor, resulting in different recruitment profiles for effector proteins such as G proteins and arrestins (part **b**). Ligand-induced receptor conformations can promote different conformational changes within scaffolding proteins such as arrestins, which in turn promotes activation of different downstream signalling pathways (for example, different MAPKs) (part **c**). Different ligands can induce distinct conformational rearrangements within G proteins that result in differences in the rate of GTP–GDP exchange. Ligands that induce a faster rate of GTP association (and hydrolysis) (top panel) allow quantitatively more G protein and downstream signalling events per unit of time than ligands that induce a slow rate of exchange (bottom panel) (part **d**). P_i, inorganic phosphate.

Complexity of GPCR signalling

Historically, GPCRs were viewed as quiescent proteins that require activation by an agonist, which allows them to act as selective conduits between a physiological (or pharmacological) ligand and a specific G protein transducer pathway. Consequently, GPCR subtypes are still often classified according to both their activating ligand and their preferentially recognized subfamily of G proteins. Nonetheless, most, if not all, GPCRs can couple to multiple transducer and modulatory proteins as a consequence of the conformational dynamics intrinsic to all these proteins[5,20,21] (FIG. 2).

Conformational dynamics of GPCRs and diversity in GPCR signalling. GPCRs are allosteric proteins that allow communication from the outside to the inside of cells. To achieve this, they sample multiple conformations, even in the absence of activating ligands (the apo state) (FIG. 2a). Endogenous ligands and drugs alter these receptor conformational dynamics, affecting the temporal and spatial profile of transducer and regulatory protein engagement. There is now evidence that this conformational plasticity can profoundly influence responses that are elicited by a given ligand[4,5,22–24]. Ligand behaviour can be described by two key parameters: affinity (the ability to bind) and efficacy (the functional consequence of binding) (BOX 1). Our understanding of the complexity of GPCR responses has evolved from a simplistic, linear model of agonist efficacy — where all signalling is proportional — to a model that encompasses the pluridimensionality of receptor–transducer coupling and transducer activation as well as the concept of biased agonism — the ability of individual interacting ligands to differentially alter the pattern of the downstream cellular response[13,25] (BOX 2).

Furthermore, for some receptors, including viral chemokine receptor GPCR homologue US28 (REF.[26]), dopamine receptors[27], and 5-hydroxytryptamine receptor 2C (5HT2C)[28], ligand-independent signalling (constitutive activity) that can often be unmasked by overexpression or mutation of the GPCR can also be observed. This implies that GPCRs have been selected to exhibit a broad spectrum of intrinsic quiescence versus constitutive activity according to functional need. Biophysical and biochemical studies have confirmed that the level of constitutive activity exhibited by individual receptors is linked to their conformational dynamics and is modulated by the strength of interactions between amino acids within the transmembrane core of the receptor, particularly between the conserved polar amino acids that form interaction networks at the base of the receptor[5,21,29]. Constitutive activity of GPCRs can be modulated both physiologically, through changes to expression of receptor or transducer, RNA splicing or RNA editing (as noted for 5HT2C[27]), or through post-translational modifications, and in disease, when

Box 1 | **Understanding drug behaviour for clinical translation**

To contextualize new advances in understanding structure–function relationships in G protein-coupled receptors (GPCRs), we must first consider how we describe and classify drug behaviour and define those components that are intrinsic to the bi-molecular interaction between ligand and receptor relative to those that may be cell-type-specific, organ-specific or even disease-specific (so-called system-dependent parameters).

Modelling signalling output

In its simplest form, the action of a drug can be separated into two key parameters: the ability of a drug to bind to the receptor (affinity) and its ability to trigger a cellular response upon binding (efficacy). A common pharmacological model for describing drug action at the receptor and cellular level is the operational model of agonism[178], which ascribes a functional affinity and efficacy value to an observed response. This was originally applied to quantification of physiological measures, such as changes in whole organ responses, with the functional affinity value (K_A) representing a macroscopic composite of all true microscopic affinities for each physical ligand–receptor–transducer complex making up the conformational ensemble. The efficacy parameter (τ) in the operational model is a composite measure subsuming: the strength of interaction between each ligand–receptor complex that determines receptor coupling to a transducer protein to initiate a cellular signalling stimulus; the efficiency with which the stimulus is processed by cellular signalling pathways; and the total number of receptors mediating the observed response. By contrast, clinical efficacy is the observed, whole body outcome of integrated cellular responses across all target tissues upon administration of any pharmacological agent. At the most fundamental level, differences in drug behaviour are driven by the distinct chemical interactions between ligand and receptor, which determine the conformational sampling of the ligand–receptor complex to influence both transducer and regulatory protein interaction and transducer activation. This behaviour is independent of cellular context, and quantification of individual pathway efficacies in a chosen cell type can allow phenotypic clustering of drug chemotypes (see also below). Nonetheless, such surrogate measures of drug behaviour will be distinct from those displayed in native context, as each cell type will exhibit a unique level of expression, repertoire of receptors and composition of transducer, scaffolding and regulatory proteins that combine to determine cellular and tissue response. Moreover, this response can be further diversified by disease-specific changes to the proteins and the cellular environment. Successful drug discovery and development requires both an understanding of the full spectrum of drug behaviour and the ability to predict those properties in a manner that can bridge preclinical and clinical efficacy.

Overcoming translational barriers

Knowing that we have an incomplete understanding of the cellular consequence of GPCR drug action, how can we overcome the barrier of their pharmacological diversity? Observed bias can, and does, change with time, and it varies according to the breadth of end points used to analyse drug action. An increasingly popular approach is broad assaying of drug behaviour to cluster compounds into functional chemotypes[66,151,179–182]. The most advanced of these types of approach can interrogate over 30 end points of GPCR function, including kinetic measurement of effects on G protein recruitment and activation, signalling via second messengers and regulatory kinases and alterations to receptor trafficking. The functional chemotypes determined with these methods[180,183] can now be linked to clinical drug behaviour (M. Bouvier, personal communication). Incorporating a minimum panel of diverse measures that can define a meaningful functional chemotype during compound validation, hit-to-lead and candidate nomination pathways that occur within a drug discovery pipeline may improve preclinical to clinical translation through greater pharmacological understanding of the drug leads entering into trials. Most importantly, though, insights from structural studies are now leading to the design of compounds with specific efficacies[72], and solution of new structures of these designer ligands in complex with target receptors, combined with broad cellular assessment of receptor function, will provide increasing insight into the interactions that selectively alter receptor dynamics, leading to desired signalling profiles.

alterations to these mechanisms, such as by mutation of active-conformation-stabilizing amino acid networks or via alterations to the cellular environment (changes to membrane environment, pH, etc.), can occur[30,31]. Activity of GPCRs may also be intrinsically regulated, with examples of receptors that require induced expression (or chaperones)[32–34] to reach the cell surface, and receptors that undergo very rapid turnover, being dynamically endocytosed and recycled from and to the membrane[35]. Pathological constitutive activity can be disease causing, as observed for the role of the constitutively active mutant of the parathyroid hormone/parathyroid hormone-related peptide receptor (PTH1R) in the pathology of Jansen's metaphyseal chondrodysplasia[36] or by the impact of constitutively active mutants of the extracellular Ca^{2+}-sensing receptor CASR on the loss of Ca^{2+} homeostasis[37]. Aberrant constitutive activity can also modify disease, as illustrated by the role of constitutively active metabotropic glutamate receptor 5 (mGluR5) in brain alterations observed in autism spectrum disorder[38].

It is now clear that GPCRs exist in multiple inactive and active conformations, even in the apo state (FIG. 2a). Studies on rhodopsin have provided clear delineation of multiple meta-stable conformational states of varying half-life that are altered during photon-driven isomerization of retinal and receptor activation[39]. Multiple ligand-independent and ligand-specific states have also been observed for other GPCRs[5,21].

An evolving view of ligand pharmacology. The goal of the vast majority of pharmaceutical discovery and development programmes is to identify drugs that either block or promote receptor activation. For a given readout of activation, an individual ligand can be typically classified as a full agonist, partial agonist, neutral antagonist or inverse (full or partial) agonist (BOX 2). Differences in both the type and the strength of chemical interactions between distinct ligands and an individual GPCR can affect ligand residence times, drive changes in receptor conformation and determine

Arrestins
A family of intracellular transducers that can act as G protein-coupled receptor modulators by blocking G protein-mediated signalling, promoting receptor internalization and activating G protein-independent signalling pathways.

Agonist
A molecule that binds to and stabilizes the receptor in an active conformation, thereby resulting in an intracellular response.

Box 2 | **Ligand classification, pluridimensionality of signalling and biased agonism**

Ligands can be classified according to their binding site on the receptor, with the binding site of the canonical endogenous agonist termed the orthosteric site and binding sites that are topographically distinct from the orthosteric site termed allosteric sites. In some cases, orthosteric binding sites and allosteric binding sites can reside in close proximity, with ligands that can concomitantly bridge these two sites termed bitopic ligands[184]. Major outcomes from the increasing number of G protein-coupled receptor (GPCR) structures is recognition of the diversity of location of orthosteric and allosteric binding sites (with the location of these sites interchangeable across some receptor classes) and the observation that most, if not all, GPCRs possess at least one allosteric site[185]. Furthermore, allosteric ligands can be highly diverse, including Na^+, which is a negative allosteric modulator of many class A GPCRs, classic small molecule compounds, antibodies, lipids and lipidated peptides (for example, pepducins)[185,186]. When used as drugs, allosteric ligands can possess the same spectrum of pharmacological activity as orthosteric ligands, but they establish distinct chemical interactions with the receptor. As allosteric ligands can bind simultaneously with orthosteric ligands, they can also alter the pharmacology of the latter, providing novel therapeutic opportunities (reviewed extensively elsewhere[184–186]). In effect, when bound to an allosteric ligand, the receptor–ligand complex can be viewed as a novel receptor with respect to how an orthosteric agonist propagates activation-associated conformational changes, and not surprisingly, alteration to the profile of transducer engagement is a common feature of many allosteric modulators.

An individual ligand can be typically classified as a full agonist, partial agonist, neutral antagonist or inverse (full or partial) agonist. The changes in receptor function arise from the specific chemical interactions that each ligand establishes with its target receptor, which impact the conformational ensemble of the GPCR, shifting the states sampled and the rate of interchange between conformational states. The efficiency with which the resulting conformational ensemble subsequently engages with or disengages from transducer–effector pathways thus leads to the aforementioned traditional phenotypic drug classifications. However, hitherto unappreciated differences in both the nature and the strength of chemical interaction between distinct ligands and an individual receptor can also drive changes in ligand residency and in transducer and/or regulator engagement that can vary for every downstream pathway. This is often referred to as 'pluridimensional signalling' and has led to the discovery of biased agonists as drugs that differentially promote this phenomenon. Although such differences in receptor–ligand complex conformations can be biophysically measured, these measurements are technically difficult and are often restricted to measurement of distance changes between a single pair of residues within the receptor.

In practice, the identification of biased agonism occurs via functional measures in cells but can be determined only when multiple (at least two) signalling end points are measured. Moreover, changes to signalling profiles can be subtle, requiring both depth of cellular interrogation and quantitative methods for measurement of relative agonist efficacy across all pathways. Because assessment of signalling bias is based on phenomenological readouts, the result can vary greatly according to cellular background and the nature of the response. Furthermore, biased agonism is always a relative term that is meaningful only when described relative to any differences from a reference agonist that has been assessed in parallel in the same assay and cell type. Terms such as unbiased ligand or balanced ligand, when used to describe a ligand with equivalent potency and/or efficacy in two different assays, can be problematic when system-dependent end points are measured (for example, cAMP accumulation versus arrestin recruitment), as alterations to the system background can change such relative potencies (for example, increased expression of G protein or altered expression of a GPCR kinase, which is linked to efficiency of arrestin recruitment). Thus, while biased agonism can be demonstrated from only two measures of cellular response, for example, G protein-mediated signalling versus arrestin-mediated signalling, this can provide only a very limited understanding of the potential pharmacologies of studied drugs. Consequently, if the two end points, even if different, are not the only (or worse, are not directly related to) therapeutically relevant signalling outputs, clinical translation will remain problematic.

transducer and/or regulator engagement, resulting in biased agonism.

While there has been general agreement that the fundamental basis for biased agonism is ligand-specific changes to GPCR conformation[40–42], until recently, the classical view has been that differential signalling by a particular ligand (and alterations to drug efficacy) arises from the differences in efficiency of recruitment of the different transducers to the specific ligand–receptor complex conformation (FIG. 2). New studies that used bioluminescence resonance energy transfer (BRET)-based and fluorescence resonance energy transfer (FRET)-based conformational biosensors embedded within subdomains of specific G proteins[9,10,43] or arrestins[44,45] have now revealed that the conformational differences in ligand–receptor complexes are also propagated to the transducers. For arrestins, such conformational changes influence the interaction with potential scaffolded partners. For G proteins, these changes influence the rate of GTP binding as well as G protein residence times in GPCR-bound complexes and, thus, G protein turnover, thereby modulating the activation of their downstream signalling targets (FIG. 2). Furthermore, the nature of the GPCR–arrestin interaction is at least partially dependent upon phosphorylation of conserved motifs on the receptor by either GPCR kinases (GRKs) or second messenger kinases[46]. There is accumulating evidence that individual ligands can induce specific patterns of receptor phosphorylation (termed phosphorylation barcodes[47]) linked to specific GRK recruitment[13,48] that control both the strength of arrestin interaction with the GPCR and the subsequent recruitment of downstream effectors[13,46–52].

Bioluminescence resonance energy transfer
(BRET). A biophysical technique combining a photon-emitting bioluminescent luciferase and an acceptor fluorescent protein, which is used to monitor changes in intramolecular and intermolecular proximity.

Fluorescence resonance energy transfer
(FRET). A biophysical technique combining a donor chromophore and an acceptor chromophore, which is used to monitor changes in intramolecular and intermolecular proximity.

Structural basis of GPCR signalling

Advances in GPCR structure determination and biophysical measures of GPCR dynamics are now providing key insights into how GPCRs are activated by both cognate ligands and pharmacological agents. While details vary between GPCR classes and even between closely related receptors, the propagation of conformational

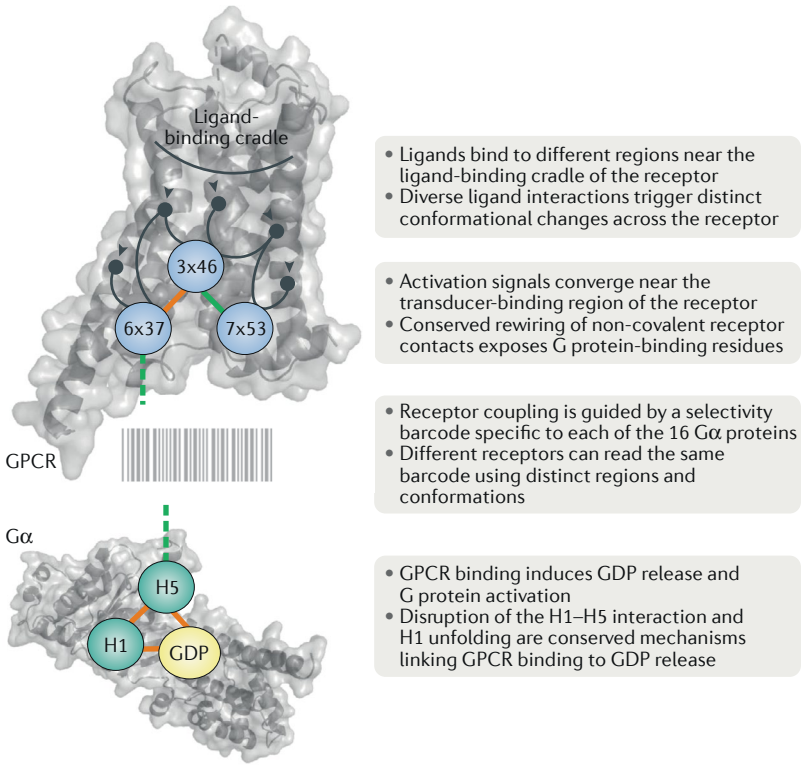

Fig. 3 | **Conserved residue contact networks between class A GPCRs and G proteins.** The G protein-coupled receptor (GPCR) and the G protein have been depicted in grey in cartoon and surface representations. Binding of ligands near the ligand-binding cradle in the receptor triggers distinct conformational changes in different receptor regions (schematically represented as black circles). These changes converge near the effector-binding region of the receptor through a conserved rewiring of non-covalent contacts among key receptor residues (represented as circles and labelled according to GPCR Database nomenclature. Engagement of these residues exposes receptor regions capable of recognizing a conserved G protein selectivity barcode (a pattern of amino acids specific to a given G protein; shown as a barcode logo). Binding to the receptor triggers a universal G protein allosteric mechanism that uncouples interactions between the α1 helix (H1), the α5 helix (H5) and the GDP-binding site of the Gα subunit and leads to GDP release. This allows GDP exchange for GTP and activation of the G protein. Connections between residues reflect inactivating (orange lines) and activating (green lines) contacts between them. Green dashed lines indicate that activation signals do not occur through direct contact.

changes arising from agonist engagement converges at the intracellular face of the receptor and leads to a conserved outward movement of transmembrane helix 6 (TM6) that allows transducer recruitment and activation. Nevertheless, despite conserved mechanisms of transducer engagement, downstream signalling from GPCRs can vary considerably depending on the context. Here, we analyse the three best studied classes of GPCR, focusing on structural details that determine their signalling and contribute to biased agonism.

Class A GPCRs. Class A GPCRs are the most abundant GPCR subfamily, and this is reflected in the number of unique receptor structures solved for the members of this class. Despite the vast diversity in their size and architecture[53], a detailed analysis of several GPCR structures has revealed common, conserved, non-covalent contacts between equivalent receptor residues in the TMD, termed molecular signatures, which are linked to receptor quiescence and transition to activation (FIG. 3). The existence of a ligand-binding cradle has also been determined[54]. Although the structures of numerous receptors have been solved, there are very few receptors for which structures of both the agonist–receptor–transducer (or transducer mimetic) complex and the inactive, antagonist-bound or inverse agonist-bound state are available. Receptors solved in these two states include rhodopsin, β2-adrenergic receptor (β2-AR), muscarinic acetylcholine receptor M2 (mAChR), adenosine receptor A2A, μ-type opioid receptor (μ-OR) and κ-type opioid receptor (κ-OR)[5]. There are common general features that can be drawn from these structures. For example, the extracellular face of the receptor undergoes contraction upon agonist binding, and this contraction is allosterically linked to the opening of the transducer-binding site. Moreover, this allosteric mechanism is reciprocal, also working in the opposite direction, whereby the binding of the intracellular transducer fosters ligand-binding site closure[55].

An analysis of non-covalent contacts between equivalent residues of the available structures in the active and inactive conformations has also revealed that, despite the diversity in the conformational changes near the ligand-binding region between receptors, the changes converge near the G protein-coupling region (FIG. 3). This convergence is mediated by a highly conserved structural rearrangement of residue contacts between TM3, TM6 and TM7 that exposes G protein-contacting residues[56]. This permits interaction with subdomains of the Gα subunit, including the carboxy-terminal α5 helix that is inserted deep within the receptor, as seen in the solved active state structures. These interactions promote nucleotide exchange and enable G protein activation. This convergence may possibly explain how the conformational changes initiated by ligands that are very structurally diverse enable GPCRs to bind a common repertoire of transducers.

It is clear that reorganization of receptors to a fully active state is not related to agonist binding alone but rather reflects changes to receptor dynamics that are driven by both the agonist and the transducer[5,21]. This reorganization is sometimes referred to as a 'loose allosteric coupling' and is based on the concept that the receptor can explore multiple inactive, intermediate and active-like states in the absence of a bound ligand (FIG. 2a). This loose allosteric coupling can be visualized in molecular dynamic simulations that examine the stability of the active receptor–agonist complex following G protein removal, as illustrated for the β2-AR–G_s structure. These simulations revealed that the receptor could revert to an inactive-like conformation, even in the presence of agonist[57]. Nonetheless, multiple potential meta-stable conformations were sampled during the simulation, in particular, conformations associated with changes to TM7, where TM6 was still retained in an active-like conformation. This is consistent with NMR studies implicating relative differences in the positioning of TM5 and TM7, but not necessarily TM6, in states of the β2-AR favoured by agonists[58,59] and with the idea that interactions with the transducers are required for the receptor to acquire a fully active state[58,59]. Recent data suggest that G proteins can also form non-functional

GPCR kinases
(GRKs). G protein-coupled receptor (GPCR)-regulating protein kinases that phosphorylate intracellular receptor sites and modulate the ability of GPCRs to interact with G proteins and other intracellular transducers.

Transducer mimetic
A non-functional protein such as a camelid nanobody that binds within the transducer-binding cleft of an activated receptor to induce structural reorganization of the receptor similar to that induced by functional transducers (for example, G proteins).

interactions with receptors that can prime the receptor for activation[60,61]. Such data also provide evidence that, besides the conserved deep binding of the α5 helix of the Gα subunit into the receptor core, other important interactions between the receptor and the G protein can be established. Nonetheless, this conserved, deep interaction between the GPCR and the G protein is likely required for G protein activation, as it contributes to the allosteric conformational changes required for full nucleotide exchange[62,63].

The expanding availability of solved receptor structures and their complexes with signal transducers has also allowed a more systematic analysis of receptor–transducer coupling and of how downstream G proteins are activated. Analysis of receptor-bound and unbound G proteins, together with a detailed investigation of sequence conservation among Gα subunits, has helped elucidate a universal allosteric mechanism for Gα activation by GPCRs[62] (FIG. 3). This was further facilitated by the development of a common Gα subunit numbering (CGN) system that allows identification of equivalent residues across the different G proteins[62]. Gα proteins contain a RAS domain (named for its homology to the small G protein RAS). Short segments within this domain undergo disorder-to-order transitions as part of the G protein activation mechanism (initially described for the activation of RAS), and these changes could provide a mechanism that is compatible with conserved allosteric activation pathways and the selective binding of the G protein to specific receptor types. In more detail, following recruitment to the receptor, the carboxy-terminal part of the α5 helix in the Gα subunit adopts a more ordered helical structure, which is required to expose residues responsible for the tight and specific binding of different Gα subtypes to the GPCR. Molecular dynamics simulations indicate that removal of GDP from Gα subunits is sufficient to allow this helical ordering of the α5 helix[63] and suggest that nucleotide release precedes full engagement of the G protein by the receptor. Indeed, in the inactive G protein, GDP is in direct contact with the α5 helix and also with the α1 helix. The α1 helix is allosterically linked to nucleotide release via an increase in flexibility resulting from the disorder-to-order transition of the α5 helix described above. Thus, recruitment of the G protein to the GPCR could drive GDP release mediated by allosteric effects from the receptor, and this in turn could allow subsequent full engagement of receptor and G protein through the rearrangement of α5 helix. This might potentially explain how the GPCR–Gα system can be so diverse while conserving a common allosteric activation mechanism across the different G proteins[62]. Recent studies have also sought a structural explanation for why particular receptors have the ability to couple to some G protein types and not others, a question that until recently had remained elusive. A detailed sequence and structural analysis of human GPCRs and G proteins revealed the existence of patterns of amino acids, termed G protein selectivity barcodes, on each of the 16 human Gα proteins that can be recognized by distinct regions on the approximately 800 human receptors. Importantly, some of the positions in the barcode are highly conserved, whereas others are unique to the individual G proteins. While the highly conserved positions in the selectivity barcode allow the receptors to bind and activate G proteins in a similar manner, the unique positions are recognized by specific receptors through distinct residues, and only some barcodes can be recognized by any given receptor. This situation could be compared with a scenario of having multiple keys (receptors) opening the same lock (G protein) using non-identical patterns. Furthermore, it was shown that studying the evolutionary history of GPCRs allows the identification of these selectivity-determining residues on the receptor[20].

Beyond G protein engagement, there is increasing evidence, at least for class A GPCRs, that the conformation of TM7 and, in particular, that of its conserved NPXXY (in single letter amino acid code, where X is any amino acid) motif can contribute to the efficiency of arrestin coupling and activation and consequently to the observed signalling bias between agonists[21,64–66]. This is supported by spectroscopy studies of β2-AR in complex with agonists with divergent pharmacology, particularly those that activate both G protein and arrestin signalling and those biased towards arrestin recruitment[67]. In that study, in the absence of transducer, G protein-competent and arrestin-competent ligands altered conformations of both TM6 and TM7, whereas those that exhibited limited G protein engagement and favoured arrestin-mediated signalling primarily altered only the TM7 conformation. Similarly, the differential recruitment of arrestin to κ-OR versus μ-OR by the ligand 3-iodobenzoyl naltrexamine (IBNtxA) could be altered by mutation of a key residue in TM7 (Y/W7.35; single amino acid code numbered according to the Ballesteros and Weinstein class A numbering scheme[68]). This mutation induced subtle changes in ligand orientation across the two receptors, suggesting that this difference in ligand-binding pose, and in the strength of chemical bonds between ligand and receptor, is sufficient to alter key components of the intramolecular allosteric networks within the receptor that govern selective signalling[69]. A role for TM7 conformational changes has also been proposed for the differential engagement of transducers by 5HT1B and 5HT2B stimulated by a promiscuous agonist ergotamine. This ligand activated both G protein and arrestin pathways downstream from 5HT1B and primarily activated arrestin-mediated signalling downstream from 5HT2B. In this case, a key difference was the extent of conformational change to TM7 (REF.[65]). Additional insights into mechanisms governing arrestin recruitment arise from the crystal structure of 5HT2B bound to lysergic acid diethylamide (LSD), whereby a single point mutation in extracellular loop 2 (ECL2; connecting TM4 and TM5) that reduced interaction with LSD and increased ligand off-rate markedly attenuated arrestin recruitment[70,71]. Interestingly, combining this knowledge with previous information on biased agonism at dopaminergic receptors allowed the identification of a set of polypharmacological compounds exhibiting β-arrestin bias[72].

It is known that differentially phosphorylated carboxy-terminal peptides that mimic phosphorylated receptor carboxy-terminal tails can induce distinct arrestin conformations in regions responsible for protein scaffolding[73,74]. Thus, an additional mechanism

Inhibitory antibody
An antibody directed against a G protein-coupled receptor that inhibits receptor activation.

for bias may be related to ligand-specific differences in coupling of the receptor–ligand complex with different kinases, which in turn alter patterns of receptor carboxy-terminal phosphorylation (phosphorylation barcode). Very recent work has shed additional light on the complex relationship between phosphorylation of the receptor carboxy-terminal tail and arrestin recruitment, with evidence for allosteric modulation of the receptor core by the phospho-carboxy-terminal tail, which regulated arrestin binding to the core of β2-AR[52]. Many GRKs require interaction with the βγ subunit of the G protein, providing a potential explanation as to why arrestin signalling requires G protein binding to the receptor. Notably, ligating the same phosphopeptide to different class A GPCRs has been shown to elicit distinct effects on arrestin engagement with the receptor[75]. These differences in arrestin engagement by phosphorylated receptors could be explained by the fact that at least two major conformations exist for arrestin–GPCR interactions: a tail conformation and a core conformation, which differ for different arrestin–GPCR pairs. The tail conformation arises from interaction of arrestin with the phosphorylated carboxy-terminal tail of the GPCR[76] and is compatible with concomitant GPCR–G protein interaction[77]. In the core conformation, in addition to the interaction with the tail, arrestin establishes interactions with the receptor core through a finger–loop domain, as illustrated by the rhodopsin–arrestin crystal structure[76]. These two conformations have been linked to specific functions of arrestins (arrestin in tail conformation was shown to be functional in GPCR internalization and some forms of signalling, whereas receptor desensitization was attributed to the core conformation)[49]. It will be important to understand the extent to which the receptor carboxyl tail (or intracellular loops) undergoes structural reorganization upon phosphorylation to influence both receptor and transducer conformations. Currently, these regions are often excluded from structural analyses to limit receptor flexibility, although recent work with minimally modified class B GPCRs[43,78,79] indicates that the receptor carboxyl tail is conformationally dynamic, at least in a G protein-bound state.

Most studies that link protein structure to biased agonism have been limited to the primary G protein transducer of the target receptor and arrestin recruitment, and this limits our understanding of the spectrum of ligand-mediated conformational switching that contributes to biased signalling. As a consequence, there has recently been substantial interest in developing multiplexed approaches to interrogate changes to GPCR function (BOX 1). The power of using a broader range of measures of GPCR function is illustrated by a recent study that combined evolutionary trace analysis to predict, and subsequently mutate, 28 amino acids in the β2-AR that may be involved in the distinct conformational networks that govern biased signalling[66]. Through assessment of constitutive and agonist-induced G protein recruitment, G protein activation, arrestin recruitment and receptor endocytosis, this work provided additional evidence for the predominant importance of conserved motifs in TM7 in the differential ability of β2-AR to engage different transducers.

Class B GPCRs. Class A GPCRs display considerable diversity in the size and architecture of their amino-terminal extracellular domains (ECDs). By contrast, class B1 peptide hormone GPCRs share ECDs that are approximately 100–150 amino acids in length and form a conserved 3D fold that binds the carboxy-terminal segment of activating peptide ligands[80]. Recent structural studies have provided major advances in our understanding of the activation of class B1 GPCRs and first insights into the mechanisms that can contribute to biased agonism. These receptors are maintained in inactive states by a series of conserved, polar, hydrogen-bonded amino acid networks at the base of the receptor, which are observed in inactive state structures of the isolated TMD[81–84] (FIG. 4a). Additionally, there is evidence that domain–domain interaction between the amino-terminal ECD and the transmembrane receptor core may support receptor quiescence[85,86].

In the past year, multiple structures containing both the ECD and the transmembrane core have been solved, including receptor complexes with an inhibitory antibody[87] and partial agonists[88,89] as well as three structures of full-length receptors in complex with both peptide agonists and the heterotrimeric G_s protein[43,78,79], making this the richest receptor class for fully active, G protein-bound structures. Comparison of TMDs of the inactive state structures with the agonist–receptor–G protein-bound structures of the calcitonin receptor (CTR)[78] and glucagon-like peptide 1 (GLP-1) receptor (GLP-1R)[43,79] revealed common large-scale changes in the transmembrane core architecture upon binding of peptide agonists. These included marked kinking and an outward movement of the extracellular ends of TM6 and TM7 and an inward movement of the extracellular top of TM1. These changes are unique to class B GPCRs, but as observed for class A GPCRs, they are translated into the outward movement of TM6 at the base of the receptor. This event is conserved across all GPCR classes and allows accommodation of the α5 helix of Gα. These large-scale changes occur around conserved class B1 motifs, including the $Pro^{6.47b}$–X–X–$Gly^{6.50b}$ (superscript numbers refer to the class B GPCR transmembrane core numbering system[90]) in TM6, $Gly^{7.50b}$ in TM7 and $Gly^{1.46b}$ in TM1.

Comparison of fully active, G protein-bound GLP-1R with the recent structure of the related glucagon receptor (GCGR) bound to a partial agonist, in the absence of G protein[88], may provide clues to the sequence of conformational changes that are required for G protein engagement for class B GPCRs. In the partial agonist–GCGR structure, the receptor displays only a subset of the transmembrane core changes observed in the G protein-bound structures. Specifically, while there is a reorganization of ECL1 and ECL2 and an outward movement of TM6 to accommodate peptide binding, TM1 and TM7 are not kinked, and the base of the receptor overlaps with the structure observed for inactive receptors. While we do not yet understand the sequence of events that drive full receptor activation, structures of GLP-1R bound to an 11-mer peptide agonist[89] and an allosteric inhibitor[84] provide additional clues as to the minimal conformational changes that may allow adoption of a

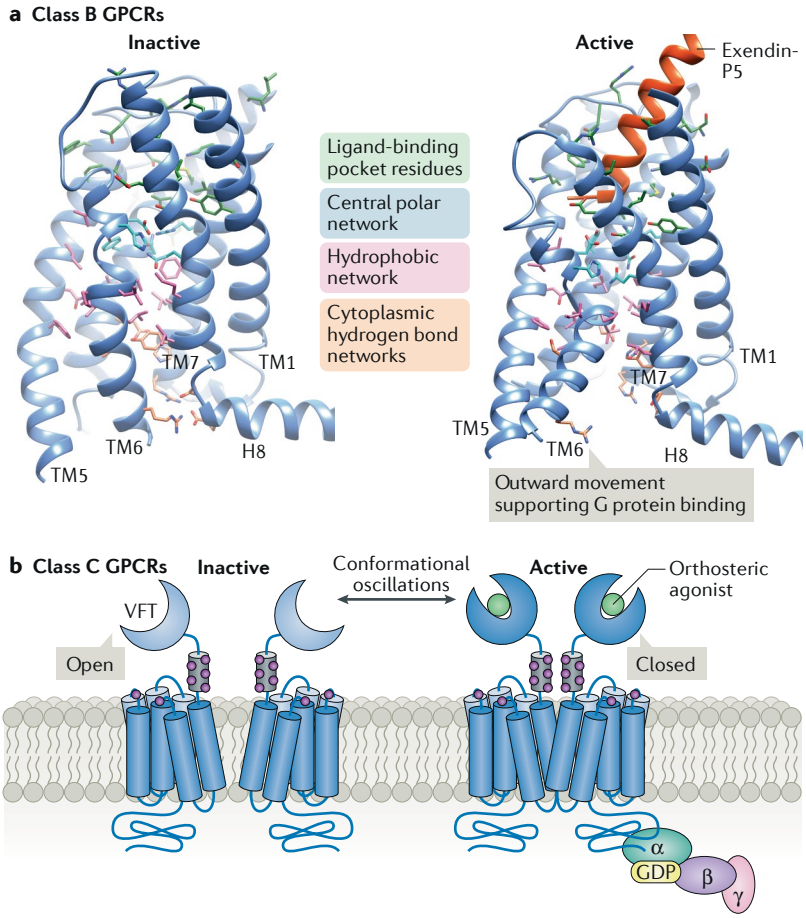

Fig. 4 | **Conformational changes in class B and class C GPCRs required for G protein coupling. a** | The inactive homology model of glucagon-like peptide 1 receptor (GLP-1R), based on the inactive structure of the related glucagon receptor (left) and active, exendin-P5-bound GLP-1R structure[40] (right), highlighting key amino acid side chains that undergo reorganization during activation transition. These are grouped according to their function and displayed in distinct colours. Prominently, polar, hydrogen-bonded interactions at the base of the receptor are broken or reorganized in the active state. Clear differences in the organization of the hydrophobic network can also be observed between the inactive and active receptor structure. Class B G protein-coupled receptor (GPCR) activation is also associated with conserved changes in the orientation of transmembrane (TM) helices, an outward movement of the extracellular ends of TM6 and TM7 and an inward movement of the extracellular end of TM1. These changes culminate in an outward movement of TM6 at the cytosolic side, which supports accommodation of the transducer. **b** | A schematic of class C GPCR activation. These receptors are functionally dimeric (homodimer form shown) and bind ligand (green circle) via the large extracellular 'venus fly trap' (VFT) domain. Notably, the VFT domain spontaneously oscillates between open and closed conformations. Agonist binding leads to conformational changes in the VFT domain that support the closed conformation of the VFT, and these alterations are linked to repositioning of the TM domain bundles from the two receptor subunits towards each other. As a result, these transitions induce an asymmetric conformational change in one protomer that enables G protein binding. The structural details behind these transitions are not known. The purple circles indicate cysteines in the TM bundle or cysteine-rich linker domain. H8, helix 8.

fully active state. Each of these latter structures exhibits key features of the fully active state in the organization of the extracellular segment of the receptor core, including reorganization of ECL2, outward movement of TM6 and TM7 and inward movement of TM1. Both structures were modified in key transmembrane segments to allow receptor stabilization and crystallization. In the GLP-1 R inhibitor-bound structure, a disulfide bond was introduced between TM6 and TM5, which resulted in the breaking of interactions that stabilize the inactive state, implying that this breakage may be sufficient to drive structural change in the absence of other constraints. In the 11-mer-agonist-bound structure, the Pro–X–X–Gly motif in TM6 had been mutated, and this may also contribute to mimicry of the fully active state of the upper half of the receptor core. It is likely that there is a coordinated series of interactions between the peptide and receptor and between the receptor and the G protein (or other transducer) that are allosterically transmitted between the intracellular and extracellular domains and allow adoption of the fully active state. These transitions are consistent with the loose allosteric coupling described above for class A GPCRs and correspond well with biophysical observations of conformational changes associated with agonist–receptor, receptor–G protein and full agonist–receptor–G protein complexes in class A GPCRs[5,21].

The current general model for class B GPCR activation can be summarized as follows: after initial binding of peptide agonists, there is reorganization of receptor ECLs, which accommodates peptide binding to the receptor core and contributes to propagation of conformational change that is linked to both receptor activation and biased agonism[43,91,92]. Within the core of the receptor, there is reorganization of a conserved central network of polar amino acids, and mutational data support a subtle role for this network in peptide-specific signal bias[93]. Below this network, towards the intracellular face of the receptor, are hydrophobic residues that stabilize both the inactive and the active (G protein-complexed) states, although these processes occur via distinct interactions. Receptor activation is associated with breakage of key polar networks at the base of the receptor; this is required for TM6 movement and G protein interaction[43,78,79,94,95] (FIG. 4a). The dynamics underlying these conformational changes are not currently understood. However, mutational data support a role for redistribution of conserved, polar or tightly packed small amino acids in this transition[90]. The recent structures of the GLP-1R bound to its primary physiological ligand, GLP-1 (REF.[79]), or the biased agonist exendin-P5 (REF.[43]) have also provided new insight into the structural basis of efficacy and biased signalling at this receptor, revealing key differences in the conformation of ECL3 and the top of TM1 of the receptor, when bound by the different ligands.

Intriguingly, all G protein-complexed structures solved to date exist in monomeric forms. However, there is robust evidence that class B1 GPCRs undergo both homodimerization and heterodimerization and that this is functionally important, as disruption of dimerization is associated with reduced agonist potency for canonical G_s coupling with the receptor[80]. Dimerization may also contribute to the biased profiles of agonists[96] and to alteration of cellular response to agonists in select class B receptor heterodimers[97,98], though this has not yet been extensively investigated. The limited data suggest that the receptors are dimeric upon initial interaction with the ligand, which contributes to high-affinity binding, but that these dimers are destabilized upon activation and G protein interaction, which may favour preferential

isolation of the monomeric receptor–G protein complex in structural studies[9,80,99].

Class C GPCRs. Unlike many other GPCR families, class C GPCRs are obligate dimers, and this is critically linked to their activation[100–102]. For example, the GABA type B receptor is an obligate heterodimer with one subunit providing the agonist-binding site and the other providing the G protein-binding domain[103]. Class C receptors contain very large extracellular domains that comprise a structurally conserved 'venus fly trap' (VFT) module, which acts as the endogenous ligand-binding site, and an interconnecting domain that is contiguous with the TMD. VFT modules exhibit varying complexity depending on receptor subtype. Orthosteric agonist binding to the VFT domain engenders a conformational shift of the VFT module from an open to closed state, which in turn provides marked alteration to the position and orientation of the TMDs within the receptor dimer that trigger transducer binding[104] (FIG. 4b). FRET studies of conformational dynamics within mGluRs have indicated that the VFT domains can spontaneously oscillate between the resting (open) and active (closed) orientations and that agonists of different efficacies distinctly alter this equilibrium[104], which serves as a mechanism driving receptor activation. To date, no structures of full-length receptors are available, and the nature of the interaction between TMDs of the dimer that contribute to receptor activation is still unclear. However, for mGluR2, cysteine disulfide bridging of the TM4–TM5 interface of the dimer subunits prevents agonist-mediated activation, while TM6–TM6 crosslinking engenders a constitutively active receptor[101], which is consistent with reorganization of the dimeric interface as part of the activation mechanism. Of note, both positive and negative modulators that bind in the TMD have been identified for most class C receptors[105]. In the full-length receptor, most positive modulators lack intrinsic efficacy, though they can activate amino-terminally truncated receptors lacking the ECD, implying that the ECD contributes to maintenance of an inactive state. Nonetheless, such modulators can modify the profile of orthosteric agonist signalling, as has been observed for CASR[106]. This observation indicates that, in class C receptors, similar to other classes of GPCR, the allosteric modulators alter conformational sampling of the receptor during activation transition[80,107].

Local control of GPCR signalling

Our understanding of GPCR signalling has also benefited from an evolution in appreciation of the importance of the receptor location within the cell and the role of the environmental context within each location (FIG. 5). This local control can include 'lateral' allostery via interaction with membrane lipids and changes to lipid composition[108–113] and recruitment of the receptor into protein signalosome complexes that restrict or alter effector and regulator interactions with the receptor and thereby the signalling effect[4,114]. Moreover, receptor location can also influence the functional selectivity of agonists of differing physicochemical properties, whereby hydrophilic compounds require cell surface expression of receptors to act but lipophilic compounds may access spatially restricted receptors located within the cell, as has been demonstrated for β1-AR[23].

Membrane composition. While we commonly refer to the receptor environment as a lipid bilayer and have presumed that the plasma membrane is the key signalling domain, physiological membranes can be very complex, containing up to 1,000 different types of lipid[115,116] that can interact directly with receptors and can also be assembled into microdomains through interactions with membrane proteins (the most common being caveolins to form caveolae). Such microdomains can be enriched with specific transducers, including G protein subtypes[117]. Differences in lipid composition also alter membrane curvature, which in turn may influence GPCR conformational dynamics[118]. Notably, GPCRs can dynamically partition between such microdomains, leading to alterations in signalling, and this can contribute to observed differences in drug behaviour. An important example is the μ-OR, as compartmentalization of this receptor within distinct plasma membrane subdomains has a key role in the distinct pharmacological response of drugs[24].

Receptor trafficking. GPCRs undergo cycles of dynamic trafficking through different cellular compartments. This aspect is also regulated by ligand-dependent interactions as part of desensitization of specific signalling transducers and adoption of alternative signalling engagement, as well as downregulation of receptor response, which is accomplished by targeting receptors to degradative pathways. The rates of spontaneous and ligand-dependent trafficking of receptors between different membrane compartments are coordinated by the selective interaction of receptors with the repertoire of sorting and transporting machineries. Notably, receptor trafficking rates show great variability among GPCRs and can be differentially altered with distinct ligands[119–123]. These trafficking events are controlled by conformational sampling of receptor states (and subsequent events including post-translational modifications such as phosphorylation, palmitoylation and ubiquitylation) and have been shown to be specific to ligand concentration, chemotype and residency time[119–123]. Not surprisingly, even in the absence of intracellular signalling, agonist-selective differences in the rate and extent of receptor trafficking can alter the pattern of cellular response and constitute a component of the pharmacological profile of drugs that contributes to observed cellular efficacy.

Assembly of signalosomes. Although we have understood for many years that GPCRs and other proteins can assemble into multi-protein complexes and that this can alter observed receptor function and cellular response (explicitly exemplified by the signalling at the neuronal synapse), our appreciation of the importance of these events for concepts such as biased agonism is only recently being realized[4]. The simplest assembly unit comprises receptor dimers or oligomers, and this has been discussed extensively elsewhere[124,125]. It is

Protein signalosome
A spatially restricted group of transducers and/or regulatory proteins that jointly produce a specific signalling output.

Chemotype
A chemical description of a molecule that allows identification of the similarities and differences in chemical structure compared with other molecules.

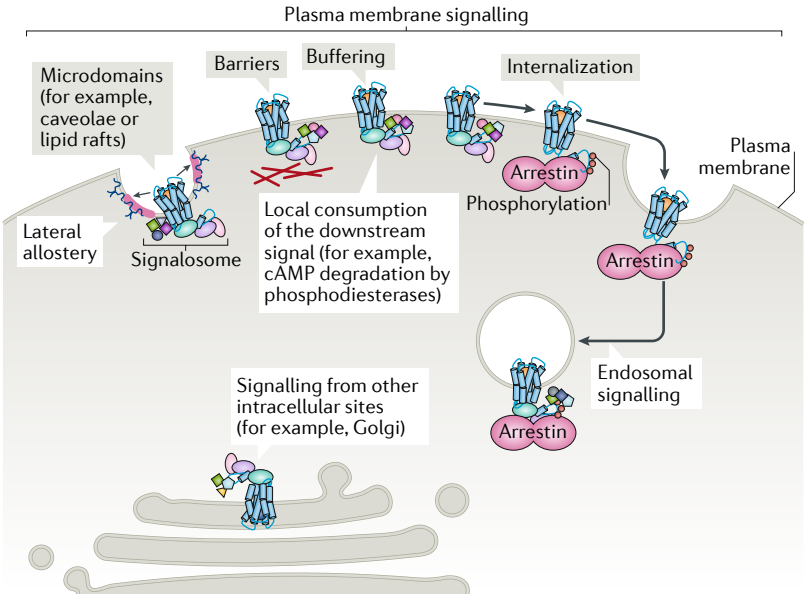

Fig. 5 | **Compartmentalization of signalling by GPCRs.** Signalling by G protein-coupled receptors (GPCRs) can be spatially (and temporally) compartmentalized to encode unique responses at the cellular level. This is facilitated by the formation of higher order protein complexes (signalosomes) around GPCRs and through the signalling of GPCRs at multiple locations within the cell — apart from the canonical signalling initiated at the plasma membrane, GPCRs can signal from endosomes after receptor internalization. They can also be activated by membrane-permeable ligands from intracellular organelles (including endosomes and the Golgi apparatus). The composition of the lipid bilayer can also influence GPCR signalling. For example, localization of receptors and their signalling partners into microdomains (such as caveolae or lipid rafts) can physically influence their conformation and signalling output through interactions with lipids and membrane proteins that are enriched in these domains (lateral allostery). Spatial and temporal dynamics can also be regulated by physical barriers, such as the cytoskeleton, and by biochemical buffering. An example of this latter process is the buffering of GPCR-dependent G_s-mediated cAMP production, whereby discretely positioned phosphodiesterases degrade cAMP, acting as local cAMP sinks. This buffering can occur at various sites within the cell, including at the plasma membrane and the endosomes, to control cAMP diffusion and to create discrete cAMP gradients within a cell.

worth noting that heteromeric assembly of receptors can alter transducer engagement or receptor trafficking in response to physiological ligands[125,126] and that dynamic changes of one of the receptor partners could contribute to either disease or disease treatment. GPCRs can also interact with non-GPCR partners to alter ligand recognition, transducer engagement and activation, or receptor trafficking. This includes the interaction of GPCRs with scaffolding proteins such as AKAPs and other proteins[3–5,7,103], which are recognized scaffolds for multi-protein assembly that provide fine control of signalling (see below). GPCRs can also interact with membrane proteins such as receptor activity-modifying proteins (RAMPs)[33], melanocortin receptor-accessory proteins (MRAPs)[34] and low-density lipoprotein (LDL) receptor-related proteins (LRP)[127]. RAMPs, a family of three single-pass transmembrane proteins, exemplify the spectrum of effects that can be engendered by such interacting proteins. RAMPs were originally recognized for their prerequisite role in trafficking of functional calcitonin receptor-like receptor (CALCRL)–RAMP1 heterodimers (CGRP) or adrenomedullin receptors (CALCRL–RAMP2 or CALCRL–RAMP3 heterodimers) to the cell surface, contributing to both escape of CALCRL from the Golgi apparatus and/or endoplasmic reticulum and the formation of the ligand-binding pocket of these receptors[128]. RAMPs are now recognized as broad partners of GPCRs, including those from all major subfamilies[32,33]. While not widely required for cell surface expression of GPCRs, RAMPs can alter receptor regulation, ligand specificity and the pattern of transducer engagement[33]. As RAMPs, and other interacting proteins, are dynamically regulated in normal physiology and in disease, the impact of these GPCR partners on receptor function must be considered in drug screening and development strategies.

Overall, assembly of GPCRs into signalosomes can provide the cell with very fine control of signalling events and has been correlated with spatially restricted signalling[114,129–132], very high sensitivity of response[133] and control of specificity of transducer coupling[103,134,135] (FIG. 5). This latter property in particular should be considered in the translation of biased agonist profiles measured in receptors expressed via recombinant protein expression systems versus those exhibited in target cells in whole organs (healthy and diseased). Moreover, the assembly of GPCRs into signalosome complexes is likely to alter their conformational sampling, with the potential for such interactions to allosterically regulate ligand binding, downstream signalling and other regulatory mechanisms, as has been observed with RAMP–GPCR interactions[33].

Physiological consequences of compartmentalized signalling. The potential for GPCRs to signal from intracellular compartments has been traditionally associated with arrestin-dependent receptor internalization and recruitment of G protein-independent transducers to endocytosed receptors. However, phenomena such as persistent activation of G protein-dependent pathways have alluded to a role for G protein signalling from sites beyond the plasma membrane[136]. Advances in tools to localize components of signalling complexes have led to the detection of transducer activation within different cellular subdomains, and there is evidence of signalling, apart from endosomes after endocytosis, also from sites including secretory compartments (the endoplasmic reticulum and the Golgi apparatus), the nucleus and mitochondria[137–142]. Moreover, experiments to selectively intervene with signalling from intracellular compartments have demonstrated that this signalling is physiologically important and can be potentially targeted therapeutically, with prominent examples for the β2-AR[141], NK-1 receptor (NK-1R; also known as TACR1)[143], CGRP[144] and PTH1R[136,142]. Intriguingly, crosstalk between receptors can drive changes in compartmentalized signalling, with a recent example of transient activation of β2-AR, via Gβγ subunits, modulating prolonged endosomal PTH1R signalling[145].

Alterations to physiologically important compartmentalization of signalling are likely to be disease causing or disease modifying, as has been demonstrated for β-ARs in heart failure[129,130,146] and melanocortin receptor 4 (MC4-R) in some genetic forms of obesity[134]. For example, the spatial restriction of β2-AR (and consequent

Chemogenetically modified receptors
Genetically engineered receptors that can be chemically modified to be able to alter receptor signalling properties. These include receptors selected for their capacity to interact with previously unrecognized ligands.

Optogenetics
A biophysical technique that uses modified, light-activated G protein-coupled receptors or channels to control cells in living tissue.

cAMP signalling) to the deep transverse tubules of healthy cardiomyocytes is disrupted in a rat model of chronic heart failure, leading to diffuse pathological cAMP signalling[13]. Moreover, the selective ability of individual ligands of a receptor to regulate receptor transitions between compartments (for example, between different cellular compartments or between plasma membrane microdomains) may contribute to the observed biased agonist profiles[24,147].

Translational considerations

Taken together, the aforementioned advances in GPCR structural biology have contributed to a more nuanced picture of the influence of ligand, receptor and signal transducer structural determinants on signalling and ligand efficacy. Importantly, these structural insights have pointed to novel ways to modulate GPCRs in a more refined manner, thus paving the way for the development of new chemical biology tools to interrogate receptor function and, most importantly, for the development of a new generation of drug candidates.

Understanding the impact of ligand–receptor kinetics on receptor signalling. Although not explicitly elaborated in this Review, many of the receptor–transducer interaction and transducer activation properties described above are a consequence of the kinetics of ligand–receptor interaction, ligand residency and the consequent probability of transducer or regulatory protein engagement. This is a component of the selective, bi-molecular interaction between individual ligands and target receptors and has become an increasing focus of some therapeutic drug development programmes. Not surprisingly, the classification of observed biased agonism can change depending on the temporal resolution of the functional readout of signalling, and this also provides potential rationalization for discrepancy in ligand classification between different laboratories[22]. Ligand residency, in particular, has become an area of focus in understanding pharmacological differentiation of compounds, and kinetics of ligand–receptor interaction have been linked to signalling efficacy, pleiotropy (including arrestin recruitment) and clinical efficacy of compounds[22,71,148–150].

Identification and progression of biased agonists in drug pipelines. A key lesson from the increasingly detailed molecular insights into GPCR behaviour and consequences of their interaction with drugs is that GPCR signalling is complex and that, consequently, drug discovery and development for these receptors is challenging. Biased agonism can be detected with, at a minimum, two measures of cellular function (that is, by monitoring the activity of two independent signalling outputs), but understanding the nature of the signalling bias of a drug and its contribution to disease modulation requires an understanding of the pathways linked to both the desired therapeutic efficacy and potential side effects[151]. At best, we currently have only partial understanding of therapeutic signalling. So how do we progress? The answer is multi-pronged. There are increasingly sophisticated studies using mice with chemogenetically modified receptors or light-modulated receptors (optogenetics) to specifically alter receptor signalling and to try to dissect key physiological and pathophysiological responses to such altered signalling[142–155]. These types of study can determine key signalling components that may be selectively targeted (either activated or deactivated) and they can be further adopted to models of disease progression. In fact, in vivo studies using different disease models have already started to shed light on the relative contribution of selected receptor pathways to therapeutic effects. Some efforts in this direction include the characterization of β-arrestin 2-biased ligands for type 1 angiotensin II receptor in mouse models of cardiomyopathy[156,157] and the in vivo analysis of analgesic and side-effect profiles of G protein biased compounds in κ-OR[158] and μ-OR[159,160]. Importantly, a study systematically analysing a collection of differentially biased ligands of μ-OR recently demonstrated a positive correlation between their degree of G protein-mediated signalling over arrestin recruitment (G protein bias) and the width of their therapeutic window (the balance between therapeutic efficacy and side effects), providing a framework for the development of therapeutics with improved clinical effect for this class of drugs[161]. At the level of drug discovery, we can now use our knowledge of receptor structure and dynamics, the capacity to chemically modify the type of biased signalling and the diversity of drug response to incorporate multiplexed and kinetic measurements of cellular function to improve pharmacological clustering of chemotypes (BOX 1). Distinct pharmacological chemotypes can then be empirically linked to desired in vivo effects. Such studies can be incorporated into decision points for drug candidate selection.

Understanding the impact of disease context on drug action. Pathological states can modify cellular function, including through alterations to the local environment of receptors. One example is changes in membrane composition, which can be observed in metabolic diseases such as hyperlipidaemia, obesity and diabetes[162] and in disorders of the central nervous system including Alzheimer disease and schizophrenia[163]. Events such as hypoxia, pH changes and redox alterations and generalized inflammatory responses can all change the local cellular environment and thus a potential drug response[164–168]. One example where such changes occur is the tumour microenvironment, which is frequently acidic. Notably, GPCRs contain networks of residues that can be protonated and are sensitive to altered pH, with alteration to these networks being consequential for receptor activation. In particular, select GPCRs are believed to function as proton sensors via alteration of the protonation state of conserved negatively charged residues (such as D3.32 and D2.50 (using the class A GPCR numbering scheme[68])) as well as the charge of buried histidines[168,169] that could lead to altered receptor signalling in low pH environments. Alterations to the levels of other ions should also be considered when assessing GPCR signalling. Importantly, cations, such as Na^+, are among the most ubiquitous allosteric

modulators of GPCR function and contribute to receptor activity (quiescence versus constitutive activation) and to the nature of biased signalling[69,170,171]. As such, the nature of GPCR responses to drugs could be altered dramatically in diverse disease contexts, resulting, for example, in differences in transducer and regulatory protein engagement (that is, changing the observed bias profile of ligands). Moreover, most diseases are heterogeneous and evolve as they progress. Therefore, using drug interventions optimized for disease stage is an important component for translational success. More complex model systems that mimic the disease context are thus important for understanding efficacy translation and for establishing the extent to which clustering of drug behaviour in routine recombinant cell studies remains predictive of their behaviour under conditions of altered cellular environment.

Considering the impact of natural receptor variation on GPCR–drug interaction. Other key considerations that can influence drug discovery, development and eventually translational success include the genetic variation of receptor sequences (polymorphisms) and the variability in receptor expression between individuals in the human population. Several independent studies have characterized the functional impact, including differential drug response, of a number of polymorphisms in a collection of receptor types[172–174]. Importantly, a recent study that comprehensively analysed genome sequence data from 68,496 individuals showed that currently drugged GPCRs present extensive genetic variation within functional regions such as drug-binding and transducer-binding sites[19]. Experimental follow-up of μ-OR revealed that individuals with certain polymorphisms had unexpected response to agonists, partial agonists and antagonists. For instance, polymorphisms at positions near the drug-binding pocket rendered a gain of function effect, whereby an antagonist or partial agonist could elicit a full agonist response. Studies on cholecystokinin receptor type A further revealed that certain polymorphisms near the G protein-binding region of this receptor completely altered the selectivity profile of transducer coupling[19]. Similarly, genetic variation can alter receptor engagement with arrestins to impact receptor regulation and/or downstream signalling as noted for CXC-chemokine receptor 4 (REF.[175]) and vasopressin receptors[176]. Such altered or gain of function effects can result in severe side effects of drugs and possibly life-threatening consequences. These findings highlight the need to understand and characterize receptor variants that are prevalent in the human population and consider them in the drug discovery pipeline. Such consideration should help limit failure of drugs during clinical trials, which can be both very expensive and time consuming and may put patients at unnecessary risk. Characterizing variants of the current GPCR drug targets also has the potential to improve the precision of drug prescription by patient stratification into defined genotypic groups with predicted drug responses. Such stratification would increase drug safety and efficacy and in consequence would improve the quality of life of patients and relieve the economic and societal burden resulting from non-optimal and/or ineffective drug action.

Conclusions and perspectives

Biased agonism is a fundamental property of GPCR ligands that can be used — serendipitously or constructively — for improved therapeutic targeting of these receptors. Consideration of this aspect of GPCR pharmacology will be key to achieving clinical drug efficacy and safety. Our understanding of drug action is limited to what we measure as a drug response, and high-resolution structural insights into the control of GPCR conformation are still at an early stage, providing limitations to conscious application of the principle of biased agonism in drug design. Advances in structural approaches to understand drug–receptor interaction and the increasing ability of researchers to analyse multiple signalling end points and to measure these events in real-time are progressively enabling integration of a breadth of cellular signalling outcomes, and of the kinetic and spatial elements of GPCR signalling, into structure-based drug discovery. This progress is expected to improve drug candidate selection. Nonetheless, applying the biased agonist properties of ligands for improvement of the therapeutic effect of new drugs will require an understanding of the expression of receptors and components of their signalling pathways in different tissues and of the tissue-specific and context-specific, integrated signalling that differentiates beneficial from detrimental effects. Given this complexity, rational application of biased agonism is expected to have the greatest impact on the treatment of diseases for which pathological mechanisms are well characterized[177].

Published online 13 August 2018

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Acknowledgements
P.M.S., A.C. and D.W. are Principal, Senior Principal and Career Development Fellows of the National Health and Medical Research Council of Australia, respectively. M.M.B. and M.M.-S. acknowledge the UK Medical Research Council (MC_U105185859) for support. M.M.-S. is supported by a Federation of European Biochemical Societies Long-Term Fellowship, and M.M.B. is a Lister Institute Fellow and is also supported by the European Research Council (ERC-COG-2015-682414).

Author contributions
D.W., A.C., M.M.-S., M.M.B. and P.M.S. researched data for the article. D.W., A.C., M.M.B. and P.M.S. substantially contributed to discussion of content. D.W., M.M.B., M.M.-S. and P.M.S. wrote the article. D.W., A.C., M.M.-S., M.M.B. and P.M.S. reviewed and edited the manuscript before submission.

Competing interests
The authors declare no competing interests.

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Reviewer information
Nature Reviews Molecular Cell Biology thanks T. Hebert, V. Katritch, S. Rajagopal and the other anonymous reviewer(s) for their contribution to the peer review of this work.

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