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Title page

G protein-coupled receptor pharmacology - insights from mass spectrometry

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Running title page

GPCR pharmacology captured by mass spectrometry

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Nonstandard abbreviations:

$A_{2A}R$	Adenosine A2A receptor
β_1AR	Beta-1 adrenergic receptor
β_2AR	Beta-2 adrenergic receptor
CTR	Calcitonin receptor
DESI	Desorption electrospray ionization
ECD	Extracellular domain
ECL1	Extracellular loop 1
ECL2	Extracellular loop 2
ECL3	Extracellular loop 3
ESI	Electrospray ionization
FFA1	Fatty acid receptor 1
GCGR	Glucagon receptor
GlcNAc	N-Acetylglucosamine
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
HDX	Hydrogen deuterium exchange
5-HT2B	5-Hydroxytryptamine receptor 2B
ICL3	Intracellular loop 3
M2R	Muscarinic acetylcholine receptor type 2
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
MD	Molecular dynamics
NAM	Negative allosteric modulator
NTR1	Neurotensin receptor 1
PAM	Positive allosteric modulator
PI	Phosphatidylinositol
PKA	Protein kinase A
PS	Phosphatidylserine
PTM	Post-translational modification
SBDD	Structure Based Drug Design
TM1	Transmembrane domain 1
TM3	Transmembrane domain 3
TM5	Transmembrane domain 5
TM6	Transmembrane domain 6
TM7	Transmembrane domain 7
7TM	7 transmembrane domains

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Abstract

G protein-coupled receptors (GPCRs) are key drug targets due to their involvement in many physiological processes. The complexity of receptor pharmacology however is influenced by multiple interactions with various types of ligands and protein transducers representing significant challenges for drug discovery. The ability of mass spectrometry to observe both the binding of ligand molecules such as lipids, ions or drugs and their impact on interaction with transducers provides an exciting opportunity to probe many aspects that are difficult to track directly in cell-based systems. From the early days, when hydrogen deuterium exchange (HDX) experiments were used to probe the different conformations of GPCRs, through to the most recent insights in which the intact receptor-G protein/arrestin complexes associated with small molecules can be preserved by mass spectrometry, this review highlights the potential of mass spectrometry techniques for in-depth investigations of GPCR biology. Herein, we will describe the utility of mass spectrometry (MS) including HDX-MS and native-MS, in investigating GPCR pharmacology. Specifically, we will include ligand/drug interactions and $G_{i/s}$ protein-coupling and illustrate how these techniques can lead to the discovery of endogenous allosteric ligands and thereby offer a new perspective for drug discovery of GPCRs.

Significance Statement

GPCRs are the largest and most diverse group of membrane receptors in eukaryotes. To carry out signalling GPCRs adopt a range of conformational to elicit G-protein coupling or arrestin binding. Because of their conformational dynamics GPCRs remain challenging to study, particular in the gas phase after release from their protective detergent micelles. Over the last decade great advances have been made however enabling direct measure of coupling and signalling across native membranes. In this review we highlight these advances and consider the future of this exciting and challenging area.

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Table of Content

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I. The challenges of G protein-coupled receptor pharmacology

GPCRs represent the largest family of druggable protein targets with receptors interacting with a diverse array of ligands, ranging from ions (Na^{2+}, Zn^{2+}, Ca^{2+}, H^{+}) and small molecules (hormones, vitamins, odorants, metabolites, etc.) through to peptides and proteins. These extracellular stimulations are translated allosterically into intracellular signals mediated by transducer proteins including trimeric G proteins and arrestins. Typically, the activation of GPCRs relies on the binding of endogenous or exogenous agonists to stabilize the receptors in active conformations. Agonists that achieve maximal activity with their cognate GPCRs are known as full agonists. Partial agonists do not induce full activation while inverse agonists act as antagonists that decrease basal/ constitutive activity and neutral antagonists suppress the agonist effect. Consequently, this range of agonist properties provides a means of fine-tuning receptor activity and suggests opportunities for intervention.

Accumulating structural and molecular studies of GPCRs have expanded our understanding of the fine-tuning of receptor pharmacology. Discrete conformations of receptors, stabilized by various ligands, have provided structural insights on how ligands achieve distinct pharmacological activity (Congreve et al., 2020). Moreover, the discovery of allosteric ligands, such as positive and negative allosteric modulators (PAMs and NAMs), unveiled promising avenues to fine-tune receptor activity for desired pharmacological outcomes (Gentry et al., 2015; Thal et al., 2018; Wootten et al., 2013). These allosteric ligands could interact with various regions of receptors and influence their functionality. For example, the binding of allosteric ligands adjacent to an extracellular binding pocket would influence the binding kinetics (association and dissociation rates) of orthosteric ligands (Haga et al., 2012; Kostenis and Mohr, 1996). The interaction of allosteric ligands on transmembrane helical bundles, or the cytoplasmic surface of a receptor, could shift the conformational equilibrium of the receptor towards either inactive or active states, and thereby impact the binding kinetics of the transducer proteins (Andrews et al., 2008; Liu et al., 2017; Nicholls et al., 2008; Salchow et al., 2010). These structural insights together offer new opportunities for GPCR drug discovery, in the design of biased ligands which selectively drive signaling toward specific G proteins or arrestins.

Receptor conformations are however often heterogeneous; as such their functional associations can lead to unforeseen challenges in designing drugs with desired efficacy and selectivity. Moreover, additional factors can potentially influence the equilibrium between GPCR conformations. These factors include regulatory proteins, pH and ions, as well as lipid molecules (Gentry et al., 2015; Rowe et al., 2021). This complex biology of GPCRs requires high-resolution techniques to inform the conformational heterogeneity and stoichiometry of receptor complexes which will in turn provide greater mechanistic understanding.

In this review, we will focus on applications of native-MS, cross-linking-MS and HDX-MS to contribute to the structural pharmacology of GPCRs. The progress made by these technologies to date demonstrates their ability to characterize ligand-binding, G protein-coupling and their associations with the native environment.

II. Structural mass spectrometry for the study of proteins and their interactions

Mass spectrometry has evolved in recent years to become a powerful technique for structural biology investigations. It provides an impressive array of information from protein primary structure and post-translational modifications (PTMs) to higher-order quaternary structure, protein dynamics and interactions. The principles of two techniques, native-MS (nMS) and HDX-MS, and their applications for protein structural study, are introduced in the following sections.

A. Probing conformational change with Hydrogen-deuterium mass spectrometry

The principle of HDX-MS is to measure the mass changes associated with isotopic exchange between the amide hydrogens of the protein backbone and the surrounding deuterated solvent (Englander and Kallenbach, 1983; Masson et al., 2019). The rate of deuterium exchange relies on the microenvironment of the folded protein and hence it can be applied to examine conformational changes associated with protein behaviours (Chalmers et al., 2011). Given the low sample requirement, compared with NMR for example, and the robustness of the workflow, HDX-MS has now been deployed in several research areas, including probing the dynamics of protein folding (Hamdi et al., 2017), epitope mapping of therapeutic antibodies (Adams et al., 2017), conformation of protein assemblies (Shukla et al., 2014), and the structural signatures associated with ligand-binding (de Vera et al., 2017). Overall, these experiments render HDX-MS a powerful tool to probe the conformational dynamics of proteins with links to their structure and function.

B. Interrogating protein interactions with native-MS

Investigation of protein-protein and protein-ligand interactions is important to elucidate the biological function of proteins. Capturing these interactions can be challenging however since the biophysical techniques employed can interfere with the system under study, particularly since many of these methods require extensive labelling and protein modification. nMS has been developed for protein complex characterization under non-denaturing conditions with no requirement for protein modification (Karch et al., 2022; Mehmood et al., 2015; Morgner et al., 2012; Robinson, 2019). Preserving non-covalent interactions allows direct interrogation of the composition and modification of protein assemblies, and their association with different small molecules can be defined through changes in mass (Fig. 1A). High-resolution nMS enables investigation of the interplay among various biological events, providing an exciting opportunity to study intractable biological systems, such as membrane proteins, and to provide a direct link between compound binding and functional outcomes.

A unique feature of nMS characterization of membrane proteins is the ability to interrogate these proteins away from detergent micelles in the gas phase. Although detergents typically represent suitable replacements for membrane lipids, it is accepted that many important non-covalent interactions can be lost following detergent solubilization and purification (Orwick-Rydmark et al., 2016; Seddon et al., 2004). Whilst most nMS analyses of membrane proteins have been performed using detergent purified proteins, (Laganowsky et al., 2013) (Fig. 1B) the ability of high-resolution MS to tolerate heterogeneity means that judicial application of detergents without over-purification from native tissues can be amenable to nMS (Abbas et al., 2020; Wang et al., 2020). Typically, the heterogeneity of lipid-bound states, glycoforms and small-molecule ligands leads to enriched datasets with novel insight on regulatory molecules and stoichiometry. A further extension of this tolerance to heterogeneity is the direct ejection of membrane proteins from bilayers without detergent solubilization (Fig. 1C) (Chorev et al., 2018). Such experiments have enabled endogenous lipids, small molecules and chaperones to be captured in contact with membrane protein complexes offering new opportunities for drug discovery in native membrane environments (Chen et al., 2022).

III. Ligand/drug-binding of GPCRs and links with PTMs

A. Capturing the effects of ligand/drug-binding via HDX-MS

Characterization of the structure and dynamics of a receptor by HDX-MS was first attempted in 2010. In this study recombinant beta-2 adrenergic receptor (β_2AR) co-purified with an inverse agonist (carazolol) was subjected to HDX-MS and results were correlated with thermal parameters derived from the x-ray structure (Zhang et al., 2010). The results were then used to probe differences and as such revealed a highly dynamic feature of the intracellular loop3 (ICL3) and a moderate exchange rate of the extracellular loop 2 (ECL2)
. This early demonstration highlighted the ability of HDX-MS to probe the dynamics of flexible/unstructured motifs that are often hard to define crystallographically.

To further investigate the structural dynamics of β_2AR bound to various ligands, HDX-MS was performed in the presence of the full agonist isoproterenol, partial agonist clenbuterol, antagonist alprenolol, and inverse agonists timolol and carazolol (West et al., 2011). Intriguingly, comparison with the apo receptor revealed decreased exchange rates for the extracellular loop 2 (ECL2) and extracellular loop 3 (ECL3) when the receptor is in complex with the antagonist alprenolol and inverse agonists timolol and carazolol (Fig. 2A). This can be rationalised by the hydrophobic interactions between these ligands and Phe193 of ECL2 (Cherezov et al., 2007; Hanson et al., 2008; Wacker et al., 2010). By contrast the full agonist isoproterenol only causes an slight inward shift (1-2 Å) of transmembrane domain 5 (TM5) of the receptor upon binding, for which did not translate into differential exchange rate. This could be due to the dynamic nature of the unstructured region between ECL2 and TM5 for which offsets the effect of the subtle structural changes caused by isoproterenol. Moreover, on the intracellular side of the receptor, the antagonist/inverse agonist induced protection of the intracellular loop 2 (ICL2) indicating a role for its helical structure in stabilizing the ionic lock of the receptor in its inactive state. By contrast the more dynamic feature of intracellular loop 3 (ICL3) was only observed in the presence of a full agonist, aligning with the structural signature of the outward movement of transmembrane domain 6 (TM6) during receptor activation.

In addition to these small molecule ligands, HDX-MS has also been applied to investigate the interactions between the glucagon receptor (GCGR), a secretin-like class B receptor consisting of 7 transmembrane domains (7TM) and an extracellular domain (ECD), together with its peptidic ligand (Josephs et al., 2021; Yang et al., 2015; Zhang et al., 2017). A comparative analysis was performed of receptor bound to Des-His1-[Nle9-Ala11-Ala16]-glucagon, a peptidic antagonist, and NNC2648, a small molecule targeting transmembrane domains of the receptor. Results revealed that the contacts of the peptidic ligand were located on the N-termini of the ECD, the stalk region between ECD and transmembrane domain 1 (TM1), and extracellular loop 1 (ECL1) (Fig. 2B). Given the dynamic feature of the ECD, which accommodates the receptor in open and closed conformations, the binding of the peptidic ligand is suggested to stabilize the open conformation of the receptor. Furthermore, crystal structures of GCGR indicate a critical role of the stalk region and its interactions with ECL1 in modulating ligand-binding and receptor activation. The increased HDX protection of the stalk and ECL1 align with the predicted structural changes for GCGR activation. Collectively, these studies demonstrate the potential of HDX-MS in delineating ligand contacts and structural changes associated with receptor activation and provide a dynamic dataset to complement and enhance structural studies.

B. Preservation of ligand/drug interactions in the gas phase

The binding pocket for orthosteric ligands in GPCRs is structurally diverse to accommodate various types of ligands. Often fluorescently-labelled or radiolabelled ligands are used to monitor indirectly the ligand-binding activity of the receptor. While these approaches provide sensitive measurements for ligand interactions, the chemical conjugation of fluorophores to ligands may influence their binding affinity or kinetics. Moreover, it is not always possible to generate a functional ligand with an attached fluorophore. Competition assays are also often employed using high affinity radiolabelled ligands, but it is not always possible to assess binding of low affinity ligands using this approach. Gaining stoichiometric information of ligand-binding can also be challenging with these labelled-ligand techniques since readouts are typically of averaged signals.

One key feature of nMS is its ability to interrogate stoichiometry through its ability to preserve non-covalent interactions between proteins and other molecules in a label-free manner. This attribute provides a potential avenue for investigating interactions of GPCRs and their ligands. An early demonstration was a nMS study of the human purinergic receptor ($P2Y_1R$) a class A receptor for extracellular adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP). This study demonstrated that the receptor in a ligand bound state could be maintained in the gas phase

following release from its detergent micelle (Yen et al., 2017). The binding of endogenous ADP and a potent antagonist, MRS2500, were captured in an Orbitrap mass spectrometer under non-denaturing conditions (Fig. 3A). The high dynamic range of detection allowed the receptor in both *apo* and ligand-bound states to be captured simultaneously, providing a measure of ligand occupancy and binding stoichiometry. Furthermore, competitive binding between ATP and MRS2500, two ligands with similar masses (507 Da vs 560 Da), was observed directly (Fig. 3B). This MS data agrees well with the competitive binding of MRS2500 to the same pocket responsible for nucleotide binding (Zhang et al., 2015)

The high-resolution feature of this mass spectrometer (Rose et al., 2012) inspired the idea for its application in screening receptors against multiple compounds simultaneously. The proof-of-principle study was employed with a desorption electrospray ionization (DESI) set-up to analyse the binding of $P2Y_1R$ with a drug cocktail (Ambrose et al., 2017). The purified receptor was deposited on the surface and desorbed in a buffer containing a mixture of antagonists and agonists designed to related GPCRs including MRS2500 ($P2Y_1R$), MRS2211 ($P2Y_{13}R$), PSB0739 and Ticlopidine ($P2Y_{12}R$), and PSB1115 ($A_2\beta$). The DESI-MS spectrum revealed a discrete mass increase (560.03 Da) in exact agreement with the mass of the $P2Y_1R$-MRS2500 complex, highlighting the potential utility of MS for screening a specific binder to a GPCR from a multicomponent mixture of ligands (Fig. 3C). It is noteworthy that only ~50% occupancy of MRS2500 was detected after a very short time of ligand exposure during desorption electrospray, rather than the full complex formation that was observed after longer incubation times.

Turning to peptidic ligands, binding of glucagon to its receptor GCGR was also characterized by nMS in the presence of a high concentration of sodium ions (Agasid et al., 2021) (Fig. 4). Moreover, the binding of a small molecule (NNC0666), a negative allosteric modulator (NAM) which interacts with TM6 and transmembrane domain 7 (TM7) motifs of GCGR, was detected with high occupancy under these elevated salt conditions (Fig. 4). Taken together, these studies highlight the applicability of mass spectrometry to interrogate the interactions between GPCRs and different types of ligands in high definition under a variety of solution conditions.

C. Linking receptor PTMs with their functional impact

Phosphorylation of GPCRs has proven critical to modulate receptor signalling (Kim et al., 2005). Typically, complex formation between a receptor and arrestin requires receptor phosphorylation of ICL3 and the C-terminal tail, with the latter playing a critical role in arrestin recruitment and signalling (Homan and Tesmer, 2014; Kristiansen, 2004; Pitcher et al., 1998). In the study of $P2Y_1R$ by nMS, a satellite signal was detected with a molecular weight corresponding to phosphorylation of the receptor (Fig. 5A) (Yen et al., 2017). While the extent of phosphorylation observed for the intact receptor on average equated to the occupancy of only one site, three phosphoserine residues (S346, S352, and S354), located at the C-terminus of the receptor, were identified using phosphoproteomics. These results collectively revealed that while the stoichiometry appears to be only a single phosphorylation, three different sites are partially occupied, highlighting the complementarity of nMS and conventional phosphoproteomics.

An interesting phenomenon was observed however with respect to the level of phosphorylation of the receptor-MRS2500 complex which was significantly decreased when compared to the receptor in its *apo* or ADP-bound state (Fig. 5A and 5B). This observation implies that MRS2500 supresses receptor phosphorylation via GPCR kinases (GRKs) at the C-terminal motif. It is established that GRKs rely on their activation through direct docking with active GPCRs (Homan and Tesmer, 2014; Pitcher et al., 1998). Hence the restrained conformation of the MRS2500-$P2Y_1R$ complex may restrict access of GRKs and thereby inhibit receptor phosphorylation. Furthermore, incubating MRS2500 with an equimolar solution of unmodified and phosphorylated receptor in its *apo* state

revealed that drug binding occurred preferentially to the non-phosphorylated receptor. A reduced population (~20%) of MRS2500 bound to the phosphorylated receptor. Together these results suggest inhibition of phosphorylation in the presence of the drug and an allosteric effect of C-terminal phosphorylation that hinders access to the drug-binding site (Yen et al., 2017).

A further critical PTM for GPCRs is that of glycosylation. A class B GPCR, the calcitonin receptor (CTR), possesses a glycan at asparagine 130 (N130), a modification that enhances its binding affinity to the calcitonin hormone (Lee et al., 2020). However, the GlcNAc residue at N130 does not cause significant structural changes of the receptor ECD based on the crystallographic structures reported (Lee et al., 2020). Speculating that the glycan might impact structural dynamics of the receptor, HDX-MS was applied to the receptor, with or without the GlcNAc modification. Results revealed that the glycan stabilized the helical motif (41-52) adjacent to the calcitonin-binding site. By contrast the flexibility of the turret loop (119-127) at the ligand-binding site was increased. Structurally these changes in dynamics were proposed to increase the on-rate and decrease the off-rate for calcitonin-binding (Lee et al., 2020).

IV. Interrogation of interactions between receptors and transducers

A. Investigation of the dynamics of G protein-coupling

The signal transduction of GPCRs is primarily mediated by their association with transducer proteins, including G proteins and arrestins. Active receptors recruit transducers to elicit distinct signalling pathways which are responsible for specific physiological responses. Therefore, understanding the mechanisms of receptor complexation with transducers (such as G protein-coupling selectivity, and their spatial and temporal regulation) and how ligands/drugs modulate this coupling will expand our knowledge of the pharmacological regulation of GPCRs.

To gain insight into the temporal control of G protein-coupling, time-resolved HDX-MS was applied to investigate complex formation between β_2AR and trimeric G_s proteins (Du et al., 2019). Deuterium uptake of the ICL2 of the receptor decreased rapidly, within 10 seconds of incubation, supporting the hypothesis of its structural transition, from an unstructured loop to a helical structure, during complex formation with G proteins. However, deuterium exchange of the N terminus of ICL3 was decreased much more gradually (>110 mins), in-line with the extension of the α-helix of TM5 according to the β_2AR-Gs structure. The results from HDX-MS suggest that ICL2 undergoes faster conformational changes than the N-terminus of ICL3, in accord with ICL2 being critical for the initial engagement of β_2AR and G_s proteins (Fig. 6).

Considering next the structural changes of the G_s protein upon complex formation, deuterium uptake of the nucleotide-binding pocket (P loop and β6/α5 loop) was increased significantly within 10 s, consistent with rapid GDP-release during complex formation. Intriguingly, the HDX rate of the α-helix 5 (α5) motif, the most critical motif inserting into the cytoplasmic core of the receptor, was consistent with a bimodal mode of structural change. The uptake of the C-terminus of α5 is decreased gradually, aligning with the timescale of changes observed in TM5/ICL3. By contrast, the deuterium exchange of the N-terminus of α5 is increased within 3 mins. The faster timescale of structural change of the N-terminus of α5 suggests its interaction with F139 of ICL2 of β_2AR (Fig. 6). Interestingly, mutation of F139 abolished the GDP-release from G_s proteins. This observation implies a role for the N-terminus of α5 in modulating the rate of nucleotide release from G proteins. Moreover, the slower exchange observed for the C-terminus of G_s α5 and receptor ICL3 may indicate that there are serial structural transitions between receptors and G proteins after the GDP-release step. Overall, HDX-MS provides critical information about protein dynamics, which complements static protein structures, and offers detailed insights into the structural transitions of complex formation between GPCRs and G proteins (Ahn et al., 2021; Liu et al., 2019).

B. Recapitulation of receptor pharmacology

Preserving non-covalent interactions by nMS presents an opportunity to interrogate the interactions between GPCRs and G proteins in high definition. Previous studies have demonstrated that the receptor-G protein complex can be maintained in the gas phase of a mass spectrometer (Yen et al., 2018) (Gavriilidou et al., 2019). Specifically, an engineered G_α subunit (mini-G_s,) was utilized to study complex formation between β_1AR and G proteins due to the slow dissociation rate of the complex. Various ligands, such as full agonists (norepinephrine, carmoterol and isoprenaline); partial agonists (dobutamine and salbutamol) and antagonists (cyanopindolol, carazolol and carvedilol) were included and complex formation in response to various ligands was investigated. Complete complex formation was observed for all full agonists, while two partial agonists elicited limited responses. No significant complex formation was detected for the antagonists carazolol and carvedilol whereas a very small population of complex was observed for the weak agonist cyanopindolol (Yen et al., 2022). Overall, the degree of complex formation was well correlated with the ligand efficacy at a saturating concentration (Fig. 7).

The sensitivity of MS detection, to low populations of complexed receptor, highlights the utility of the MS method in obtaining compound pharmacology directly through G protein-coupling. To explore this potential further, the dose-responsiveness of each agonist was measured. The resulting curves clearly represent their differential efficacy, with EC_{50} values comparable to previous cell-based studies (Baker et al., 2011) (Fig. 7). In addition, an antagonistic effect was examined by measuring isoprenaline-induced G protein complex formation in the presence of carazolol at different concentrations. These MS results indicate a dose-dependent inhibition of carozolol for receptor-mini-G_s complex formation without changing the maximal levels of complex formation, confirming the competitive binding between isoprenaline and carazolol for the same orthosteric ligand-binding site. Collectively, the capability of nMS to recapitulate and quantify complex formation between GPCRs and G proteins offers a highly sensitive approach to interrogate receptor pharmacology *in vitro*.

C. Application of native-MS for the study of a pharmacophore

Crucial to pharmaceutical development is the ability to identify the chemical signatures of drug-like molecules that are necessary for their pharmacological activity. However, it is often a complex and time-consuming process involving chemical synthesis, assay development, molecular modelling and validation. With the breakthroughs in solving high-resolution structures of GPCRs the concept of Structure Based Drug Design (SBDD) became feasible. However systematic and rational approaches to guide the design of chemical modifications, to improve compound affinity and efficacy now need to be realised (Congreve et al., 2020). An example of such an approach involving native MS is described here.

The crystal structures of β_1AR bound to isoprenaline revealed the chemical moieties which provide the main contacts to the receptor. These include the catecholamine meta-hydroxyl groups, forming the main hydrogen bonds to Ser211, Ser215 and Asn310 of β_1AR, and the secondary amine and β-hydroxyl group interacting with Asp121 and Asn329 (Warne et al., 2011) (Fig. 8). To explore the application of nMS for pharmacophore optimization, six derivatives of isoprenaline were investigated and their effects on G protein-coupling assessed. Frist orciprenaline and 1-phenyl-2[(propan-2-yl)amino]ethan-1-ol, were examined, both of which are expected to abolish the interactions between the catecholamine meta-hydroxyl and the receptor. The ESI-MS spectra revealed a 60% reduction in complex formation when the meta-hydroxyl group was changed from position 3 to position 4. With the removal of two meta-hydroxyl groups 90% attenuation was observed. These results indicate the crucial role of meta-hydroxyl groups for agonism, consistent with the structure of β_1AR-isoprenaline complex (Fig. 8).

The impact of the secondary amine and β-hydroxyl group were examined next using two derivatives: 3,4 dihydroxypropiophenone and isopropyldopamin, respectively. The results indicate 75% attenuation of coupling for 3,4 dihydroxypropiophenone, and surprisingly, no activity was detected for isopropyldopamin (Fig. 8). Although the secondary amine and β-hydroxyl group share common interactions with Asp121 and Asn329, the MS data suggest their differential contribution in stimulating G protein-coupling. Overall, these results exemplify the utility of nMS in guiding pharmacophore optimization and are complementary to high-resolution structural studies of GPCRs.

D. Investigation of G protein-coupling selectivity and biased effects

The selectivity of GPCRs toward specific G proteins plays a critical role in triggering the appropriate physiological responses. In general, receptors can couple to either one or more G_α subunits that result in the activation of different downstream effectors. For example, β_2 adrenergic receptor (β_2AR) primarily couples to the $G_{\alpha s}$ subunit to trigger activation of protein kinase A (PKA) but also secondarily interacts with $G_{\alpha i}$ subunit to inhibit the activity of adenylyl cyclase (Daaka et al., 1997; Lefkowitz et al., 2002). Fatty acid receptor 1 (FFA1) shows a broad specificity to couple to G_s, G_i, G_q and G_{12} subunits to modulate the activity of PKA, PKC and the level of secondary messengers Ca^{2+} and cAMP (Kimura et al., 2020). The nature of GPCRs coupled to different G proteins reflects the complexity of the receptor-G protein signalling pathways, hence it is important to investigate the coupling selectivity of receptors to gain deeper insights into their pharmacological regulation.

Previous studies have shown that many different receptors and G proteins can be co-expressed simultaneously in various cell types (Malbon, 2005; Regard et al., 2008), suggesting alternative mechanisms to modulate the selectivity of G protein-coupling rather than just differential tissue expression of G proteins. To assess the coupling selectivity of β_1AR in a native-like scenario, a competition experiment was performed by incubating β_1AR with mini-G_s and mini-G_i at equimolar ratios (Fig. 9A) (Yen et al., 2022). The receptor coupled to both G proteins in a competitive manner. However, the percentage of receptor-mini-G_i complex was significantly decreased (by 30%) indicating a strong preference for the receptor towards the G_s protein (Fig. 9B). Comparing the ability of agonists to stimulate G_i protein-coupling norepinephrine, isoprenaline and carmotorol were investigated individually. The propensity of isoprenaline to stimulate G_i protein-coupling over the other two agonists was revealed clearly by nMS (Fig.7D). Together these results recapitulated the preference of the receptor, which couples primarily to G_s, with G_i and G_o proteins serving as secondary transducers.

Using an alternative approach to nMS, a highly sensitive matrix-assisted laser desorption/ionization (MALDI-MS) method was developed to interrogate the selectivity of GPCRs for different mini-G proteins (Fig. 9C) [50]. Protein cross-linking was employed to target lysine residues present at the G protein–interacting interfaces of GPCRs. A bifunctional amine-reactive reagent with a spacer arm length of 38.5 Å was used to cross-link the GPCR-G protein interface. Critical to the approach is the fact that protein–protein complexes can be captured in their solution phase equilibrium state, preventing their dissociation during the ionization process. After reaction, intramolecular cross-linked protein, monolinks, and intermolecular cross-links were captured. Using an optimized experimental procedure, all Gα-proteins, or their truncated versions, were found to form approximately two intermolecular cross-links in each complex. More than 70 ligand-receptor-transducer combinations were examined in this way to profile the selectivity of three GPCRs (rhodopsin, β_1AR and the angiotensin receptor). Results were found to be consistent with their pharmacological activity (Wong, 2003). The quantitative manner of this method using β-galactosidase as an internal standard allowed the binding affinity of various mini-G proteins to be determined. The results further supported the universal G_o-coupling activity of the three receptors

studied. This observation is therefore consistent with the view that G_o-coupling is independent of the main selectivity determinants located on H5 motif of G proteins (Flock et al., 2017). Moreover, the propensity of β_1AR toward the G_i protein and β-arrestin 1 was examined. The results revealed the biased effect of carvedilol toward the β-arrestin pathway, in accord with previous results (Wisler et al., 2007).

While it is established that one receptor can couple to more than one G protein subtype, and the coupling efficiency is known to vary depending on the GPCR-G protein complex in question, the precise mechanism underlying different coupling efficiencies is unknown. Comparative HDX-MS analysis for the muscarinic acetylcholine receptor type 2 (M2R) and β_2AR suggested the structural determinants for the efficiency of $G_{i/o}$ protein-coupling[51]. HDX-MS and mutagenesis reveal that the engagement of the distal C-terminus of $G\alpha_{i/o}$ with the receptor differentiates primary and secondary $G_{i/o}$ couplings. Specifically, a conserved hydrophobic residue within ICL2 of the receptor is not critical for primary $G_{i/o}$-coupling; but rather, it might be important for secondary $G_{i/o}$-coupling.

In general, these MS approaches recapitulate the selectivity of receptors and provide a quantitative measurement of receptor propensity in multi-component environments, similar to physiological conditions.

V. Identification of endogenous modulators of G protein-coupling

Modulation of GPCR activity by various allosteric ligands implies a dynamic conformational equilibrium that can be manipulated through protein-protein or protein-ligand interactions. Accumulating evidence has suggested that the activity of receptors can be influenced through their interactions with their native environments. The lipids from plasma membranes, small ions, such as sodium, calcium and zinc, nucleotides and other endogenous ligands are thought to play important roles in modulating the kinetics of receptor activation (Jones et al., 2020; Zarzycka et al., 2019). How the native environment impacts the pharmacological properties of GPCRs is however often difficult to elucidate.

The utility of nMS in deciphering interactions of receptors with endogenous lipids and ions, and their functional roles in GPCR activation, is described in the following sections.

A. Identification of PIP_2 as a positive allosteric modulator for G protein-coupling

The observation of lipid interactions in high-resolution structures of various GPCRs has implied potential functions for lipids in stabilising complexes. For instance, cholesterol has been identified in several class A receptors including β_2AR, Adenosine A2A receptor (A_{2A}R), 5-Hydroxytryptamine receptor 2B (5-HT2B), and the μ-opioid receptor and, in some cases, cholesterol was suggested to modulate receptor dimerization and structural stability (Gimpl, 2016). However, the superposition of cholesterol-bound structures of various receptors indicates versatile binding modes of cholesterol, leading to a fundamental question as to whether the lipid can function as a specific modulator. Moreover, the versatility of lipid-binding leads to low-resolution electron density seen in structures from X-ray crystallography or cryo-EM, making it challenging to investigate the identity of bound lipids.

Native-MS has proved powerful in interrogating the interactions between GPCRs and lipids (Yen et al., 2018). The lipid interactions of purified β_1AR and A_{2A}R-trimeric G_s protein were probed by nMS and two classes of endogenous lipids, phosphatidylinositol (PI) and phosphatidylserine (PS), were revealed to contact the receptors directly (Fig. 10A). The resolution and sensitivity of MS allows the interactions of these receptors and various lipids to be quantified, in either a non-competitive or competitive manner. Preferential interactions of PIP_2 toward the receptors, β_1AR and the neurotensin receptor 1 (NTR1), were identified (Fig. 10B).

The preferential binding of PIP_2 observed from nMS analysis coincides with the results from molecular dynamics (MD) simulations (Song et al., 2019). Binding hotspots for PIP_2 locate on the cytoplasmic interfaces of TM1-TM2, ICL2-TM4 and TM7-H8 of β_1AR, A_{2A}R and NTR1. It is intriguing that PIP_2 contacts are conserved across various receptors including β_1AR, β_2AR, A_{2A}R, NTR1, histamine H1 receptor, CB1 cannabinoid receptor, M4 muscarinic acetylcholine receptor, dopamine D3 receptor, Sphingosine 1-phosphate receptor and rhodopsin (Yen et al., 2018). This conservation suggests the potential of PIP_2 to act as a specific modulator for class A GPCRs.

To investigate the functionality of PIP_2 for receptor activation, the extent of β_1AR in complex with mini-G_s protein was examined by nMS in the presence of PIP_2 or PS. The results revealed a remarkable enhancement of complex formation between β_1AR and mini-G_s when two or three PIP_2 molecules are bound the complex (2.7- or 4.5-fold compared to the receptor without lipid, respectively) (Fig. 11B). The combinatorial approach of nMS and MD simulations further unveiled a unique "bridging" mechanism of PIP_2 in stabilizing the receptor-G protein complex. The polyanionic lipid headgroups of PIP_2 interacts with both the residues of mini-G_s proximal to the lipid contacts in TM3, TM4 and TM5 of GPCR (Fig. 11A), suggesting the electrostatic interactions between the lipid headgroups and the basic sidechains on both receptor and G protein are necessary for the allosteric effect of PIP_2. This hypothesis is further supported by the results that lipids lacking polyanionic headgroups, such as PS, do not significantly improve the coupling. Moreover, a G protein-mimetic nanobody, which does not contain the PIP_2 contact residues of mini-G_s, shows no coupling enhancement in the presence of PIP_2 (Fig. 11C).

B. Investigation of ion interactions and their functional impact

The impact of endogenous ions on the functional regulation of GPCRs has been described previously (Zarzycka et al., 2019) with various monovalent and divalent ions (Na^+, Zn^{2+}, Hg^+, PO_4^{3-} and SO_4^{2-}) interacting with different receptors to achieve regulatory effects. For example, sodium interacts allosterically with the opioid receptor, A_{2A}R, β_1AR and the D4 dopamine receptors to stabilize their inactive conformations (Fenalti et al., 2014; Liu et al., 2012; Miller-Gallacher et al., 2014; Wang et al., 2017). The transmembrane binding pocket for sodium is highly conserved among various class A receptors, suggesting its universal impact on modulating receptor activity. In addition, the proximity of the sodium-binding pocket to the orthosteric ligand-binding site presents the possibility that sodium may also directly influence the kinetics of ligand/drug interactions.

Although high-resolution structures of receptors provide detailed structural information on the mechanism of ion interactions, it remains challenging to predict interactions and functional consequences of different ions on specific receptors due to the structural diversity of ion-binding sites. Therefore, a high-resolution method is required to probe receptor interactions of diminutive ions. The development of a high-resolution Orbitrap mass spectrometer for nMS analysis, offers an approach for interrogating ion interactions with GPCRs (Gault et al., 2016; Rose et al., 2012). A technique using nanoscale ESI emitter tips (~100 nm) (nanoemitters) enabled the MS analysis of proteins in non-volatile salt solutions (Agasid et al., 2021; Susa et al., 2017). Interestingly sodium binding to A_{2A}R revealed its relationship to distinct conformational states of the receptor induced by agonists or antagonists (Figure 12A) ESI-MS spectra were obtained in NaCl/Tris buffer with antagonists XAC and ZM241385 and consistently showed greater binding of sodium ions (stoichiometrically from 1 to 7). The presence of agonists NECA or CGS21680, by contrast, significantly attenuated the intensities of sodium bound states. These results support the structural evidence of a specific sodium-binding pocket present in antagonist-bound receptor complexes (Hu et al., 2019; Massink et al., 2015). To validate whether the difference in sodium-binding is due to ligand-induced structural changes of the receptor, a competition assay between antagonist ZM241385 and agonist NECA was carried out. While adding 40-fold excess of NECA to the ZM241385 pre-treated receptor, sodium binding of the receptor was reduced significantly (Fig.

12A). This result suggested collapse of the allosteric binding pocket for sodium and prompted the conclusion that the structural transition of the receptor from inactive to active state is maintained.

The ability of native-MS to capture both ion-binding and G protein-coupling of GPCRs highlights its great potential to interrogate the functional associations between receptors and diminutive ions (Yen et al., 2022) . While interrogating the mini-G_s-coupling of β_1AR, a small adduct, with mass of 62-65 Da, was observed bound to the receptor-mini-G_s complex with high occupancy (Fig. 12B). The binding stoichiometry of this adduct (1 or 2 ions) was found to vary with different agonists, implying a potential role of this endogenous ligand in modulating G protein-coupling.
The significant attenuation of the binding of the observed adduct by EDTA further confirmed its identity as a divalent cation. Most intriguingly, a significant population of uncomplexed receptor was observed in EDTA-treated solutions. The reduced coupling activity is correlated with the degree of ion binding, suggesting the allosteric effect of this divalent cation in stabilizing receptor-mini-G_s complex (Fig. 12B). Moreover, the addition of exogenous Zn^{2+} recovered the coupling activity of EDTA-treated β_1AR and mini-G_s, whereas Cu^{2+} did not improve coupling activity significantly. Collectively, the identity of the endogenous ligand was likely a zinc ion, an observation later confirmed using ICP-MS. As a result a positive allosteric effect was proposed for efficient receptor coupling induced by Zn^{2+} (Yen et al., 2022).

Given that zinc binding sites are less conserved in comparison to the sodium binding pocket, MD simulation was used to investigate the structural mechanism of zinc ion binding. Two interaction hotspots were observed from the model of the β_1AR-mini-G_s complex. In addition to the residues surrounding the orthosteric ligand-binding pocket, zinc interacts with the cytoplasmic interface (TM3, TM5 and TM6 motifs) of the receptor. Intriguingly, the residues of mini-G_s, Asp381, Gln384, Glu392 on the H5 motif and Asp354 on the α4-β6 loop, were also found to contribute to zinc binding, suggesting collaborative actions between the receptor and G protein for zinc ion interactions (Fig. 13). By introducing a nanobody and using site-directed mutagenesis, the TM3-TM5-TM6 interface of the β_1AR-G_s protein complex was further implicated as a positive allosteric site for the zinc effect (Yen et al., 2022). A structural mechanism was proposed in which Zn^{2+} facilitates stable complex formation between the receptor and G_s protein.

C. Capturing GPCR signalling across native membrane environments

A further recent breakthrough in the field of n-MS has been to decipher the interactome of proteins in their native bilayer environments (Chorev et al., 2018). Using a sonicated lipid vesicle method (Chorev et al., 2020) involving the direct ejection of intact protein assemblies from native membranes into the mass spectrometer, multiple complexes were assigned. The results implied the importance of the lipid bilayer environment for retaining small-molecule binding in solute carriers, for maintaining subunit interactions, and defining associated chaperones (Chorev et al., 2018). Further progress, with important consequences for GPCR pharmacology, enabled the capture of endogenous rhodopsin in fragments of bovine native disc membranes (Chen et al., 2022). The rod disc membrane fragments, when exposed to light, signalled via conversion of *cis* to *trans* retinal, with eventual hydrolysis of retinal to form opsin (Fig. 14). Conjugation of all-*trans*-retinal with phosphatidylethanolamine, which can be light activated to release cis-retinal and thereby interact with opsin to form rhodopsin, is part of a regeneration mechanism uncovered during this work.

Supplementing the rod disc membrane with the soluble fraction, containing G_t and PDE6, and illuminating with light, enables the activated states of rhodopsin to engage with transducin (G_t.GDP) and exchange GDP for GTP. G_t then dissociates to form $G_t\alpha$·GTP and G$\beta\gamma$, a reaction that can also be monitored due to the change in mass. Next $G_t\alpha$·GTP interacts with PDE6 initiating the hydrolysis of cGMP which then dissociates from the complex. Interestingly, the results indicated a slower

reaction rate in the native membrane than in detergent micelles which may be assisted by long-chain phosphatidylcholine (Chen et al., 2022) (Fig. 14).

The fact that all reactions can be monitored through changes in mass enables ligands to be assessed both for their ability to accelerate or decelerate retinal hydrolysis or to perturb the transducin GDP hydrolysis reaction. From a high throughput screen of a diverse library of 50,000 small molecules (Getter et al., 2019) nine compounds were selected. Results showed that these ligands divide into two groups, capable of either accelerating with a marginal effect or, more commonly, slowing retinal hydrolysis. **1** and **6** show that clear differences emerge post illumination (3 min) since rhodopsin and opsin predominate in the presence of **1** and **6** respectively (Fig. 15). Since there no evidence for displacement of retinal by these compounds, or changes in the conformation of rhodopsin, **1** and **6** were proposed to act as allosteric modulators (Chen et al., 2022).

VI. Outlook and conclusions

The critical roles of GPCRs in a wide range of physiological and pathological processes has led to long-term interest in understanding the molecular regulation of these complex systems. Although many breakthroughs in GPCR structural biology have provided unprecedent structural insights into the mechanism of receptor activation, how ligand interactions modulate the conformations of receptors remains challenging to investigate. The discovery of allosteric effect of endogenous lipids and ions exemplifies the combinatorial aspects of nMS with other structural biology techniques. Both PIP_2 and Zn^{2+} regulate the complexation between receptors and G proteins through modulating the conformations at the interaction interface, suggesting a potential strategy for targeting the receptor cytoplasmic surface for drug discovery. Intriguingly, several novel allosteric antagonists targeting the receptor intracellular domains have been described previously (Andrews et al., 2008; Gentry et al., 2015; Liu et al., 2017; Nicholls et al., 2008; Salchow et al., 2010), such as CCR2-RA-[R] which interacts with the intracellular tips of TM1-3 and TM6-7 to block the access of the H5 motif of $G_{\alpha s}$ subunit (Zheng et al., 2016). Although intracellular allosteric agonists have not yet been identified, the observation that G_s protein itself is involved in the allostery of these endogenous ligands may inform new strategies for receptor positive allostery. Designing a molecule to mimic the mechanism of endogenous allostery, or regulate their binding, may inform new approaches for modulating the kinetics of specific receptor/G protein signalling for therapeutics purposes.

Despite the challenges in defining new molecules to regulate receptor activity it is now known that receptor interactions are modulated via interactions with various small molecules such as orthosteric/ allosteric ligands, as well as lipids and ions from the endogenous environment. In this review, we have showcased the ability of native-MS and HDX-MS to probe the stoichiometry, structural dynamics, and regulation of receptor interactions with different small molecules. Collectively these results have led to a better understanding of function, G-protein selectivity and drug targeting of GPCRs.

Most importantly, we believe that it is the interplay among cofactors, modulators and the lipid bilayer that will help inform a more complete picture of GPCR signalling in cellular environments. To this end recent developments that include the ability to identify small molecules/ metabolites within membrane protein assemblies (Gault et al., 2020) together with an ability to sequence proteins directly from native bilayers, will ensure that MS continues to play a critical role in future studies of GPCR pharmacology.

Authorship Contributions
Participated in research design: Yen, Jazayeri, Robinson.
Performed data analysis: Yen, Robinson.

Wrote or contributed to the writing of the manuscript: all authors

Competing Interests

Yen was an employee, and Jazayeri is a current employee of OMass Therapeutics. Robinson and Yen are founders of OMass Therapeutics.

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Figure legends

Fig. 1. The principles of membrane protein native-MS analysis. (A) Applications of nMS can define the composition and stoichiometry of soluble protein-protein/-ligand assemblies and complement the structural insights from established structural biology approaches (EM and crystallography. (B) The principal idea of characterizing membrane proteins from a detergent micelle involves the desolvation of the electrospray droplet followed by 'stripping' of the detergent micelle in the gas phase prior to analysis in the MS. (C) When proteins are ejected from a native membrane, including bacterial, mitochondrial (purple) or rod disc membranes, the heterogeneity is captured with lipids, modifications and co-factors in complex (blue). By contrast in a standard

proteomics or lipidomics experiment, wherein proteins are digested to peptides and lipids are extracted, it is often challenging to infer the cellular context and interaction partners.

Fig. 2. The conformational dynamics of GPCRs associated with ligand interactions. (A) The differential deuterium uptake of β_2AR incurred by the ligands with different pharmacological profiles (dark grey no change, blue and dark blue a reduction in dynamics, yellow and green enhanced dynamics compared to the *apo* receptor; light grey the regions cannot be resolved) (West et al., 2011). (B) The structural motifs of the glucagon receptor, including the N-terminus of the ECD, the stalk region between the ECD and TM1, and ECL1, showed differential rates of deuterium uptake upon peptidic ligand treatment (Yang et al., 2015) (grey no change, blue a reduction in dynamics and yellow enhanced dynamics compared to the *apo* receptor; black related to regions without sequence coverage. Adapted from (West et al., 2011) (Yang et al., 2015) with permission.

Fig. 3. Preservation of $P2Y_1R$ in complex with its natural ligand and in competition with a drug. (A) The ESI-MS spectra of the $P2Y_1$ receptor bound to an endogenous ligand ADP (yellow circle; upper ESI-MS spectrum) or a high potent drug MRS2500 (orange pentagon; lower ESI-MS spectrum). The mass changes correspond to protein phosphorylation (green circle) and zinc ion-binding (magentas) as well as 100% drug binding. (B) ESI-MS spectra recorded during a competition experiment in which the concentration ratios of ATP and MRS2500 are varied. With a two-fold excess of ATP (bottom) minimal drug binding is observed. With excess MRS2500 both nucleotide and drug-bound receptor are observed (middle). In the presence of the drug alone (top) Zn^{2+}-binding is clearly observed. (C) DESI-MS characterization of $P2Y_1$ receptor desorbed in a compound cocktail and the interaction between MRS2500 and receptor was captured specifically.

Fig. 4. Preservation of peptidic and non-peptidic ligand binding to the glucagon receptor. A. Glucagon binding to GCGR is preserved using nanoemitters to enable the use of NaCl-containing buffers during nESI-MS. The charge states of GCGR are highlighted (blue) and glucagon-bound GCGR (orange). A low population of two glucagon binding events is observed, likely due to peptide aggregation (red). Extensive peak splitting of the charge states reveals up to five different glycoforms. B. Preservation of lipophilic drug binding to the glucagon receptor in NaCl-containing electrospray buffers. Structure of the full-length glucagon receptor (PDB 5XEZ) showing the allosteric binding pocket binding of NNC0666, situated between TM6 and TM7. GCGR was released into the gas phase from micelles composed of G1(a tailored detergent (Urner et al., 2020)) and cholesteryl hemisuccinate in 50 mM NaCl, 5 mM Tris, pH 7.5 electrospray buffers. Inset shows expansion of the 13+ charge state (highlighted green) with peaks assigned to GCGR and binding of NNC0666 (a variant of NNC0640) to the various glycoforms highlighted in blue and pink respectively.

Fig. 5 Comparison of mass spectra recorded for MRS2500 binding to phosphorylated and unmodified forms of $P2Y_1R$. (A) ESI-MS spectra recorded following addition of MRS2500 to $P2Y_1R$ assigned as follows: unmodified $P2Y_1R$ (blue background/square) with phosphorylation (green circle), endogenous ADP binding (red circle), and MRS2500 binding to $P2Y_1R$ (orange background/hexagon). (i) Comparison of the relative peak intensities of the 11+ charge state normalized to the phosphorylated form of $P2Y_1R$. (ii) Upon addition of MRS2500, the population of free $P2Y_1R$ is reduced relative to the phosphorylated form ($-21 \pm 4\%$) and (iii) concomitant binding to the apo receptor is enhanced ($17 \pm 1.4\%$). (B) Schematic representation of ADP and drug binding to the phosphorylated receptor with phosphosites identified at the C terminus [serine residues (green)]. ADP binding takes place with equal probability to phosphorylated and non-phosphorylated forms. By contrast, drug binding occurs preferentially to the nonphosphorylated form. Adapted from (Yen et al., 2017).

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Fig. 6. Investigation of the mechanism of G protein-coupling via HDX-MS measurements. Upper: A multi-step model was proposed to illustrate the initial engagement between GDP-bound G protein and receptor (step I), and the release of GDP (step II) when the stable receptor-G protein complex forms (step III). Lower: HDX-MS results for β_2AR and A_{2A}R, co-incubated with G-protein:GDP in different time-course in each case. Deuterium uptake is plotted against time for peptides derived from the individual protein or the complex (peptides are colour-coded according to the location on the structure).

Fig. 7. Pharmacological characterization of β_1AR in complex with engineered G$\alpha_{i/s}$ subunits. (A) ESI-MS spectra of β_1AR in complex with mini-G_s in the presence of compounds shown (orange and blue peaks are charge states of the native mass spectra (raw data) assigned to complexes and free receptor respectively). (B) Dose-response curves for mini-G_s coupling to β_1AR agonists (ISO: isoprenaline, CARM: carmoterol, DOB: dobutamine, SALB: salbutamol) with annotated log EC_{50} values. (C) Response curves for mini-G_s-coupling to isoprenaline in the presence of carazolol at different concentrations. (D) ESI-MS spectra of β_1AR in complex with mini-G_i in the presence of various compounds (β_1AR-G_i complex peaks shown in pink).

Fig. 8. Employing a native-MS platform for pharmacophore optimization. Isoprenaline and its derivatives were employed to exemplify the utility of nMS to delineate the chemical moieties crucial for coupling efficacy. Colterol and isoprenaline show the highest efficiency of coupling, intermediate levels are observed for orciprenaline, 1-(4-chlorophenyl)-3-(dimetylamino)propan-1, 3,4 dihydrox-propiophenone and 1-phenyl-2-[(propan-2-yl)amino]ethan-1-ol while no complex formation was observed for isopropyldopamine. The peaks assigned to receptor-mini-G_s complex are highlighted in orange whereas receptor monomer is denoted in blue.

Fig. 9. Interrogation of G protein-coupling selectivity of β_1AR by mass spectrometry. (A) Native-MS characterization of complex formation between β_1AR and various engineered Gα proteins revealed the selectivity of receptor toward G_s and G_i proteins. (B) The propensity of β_1AR in complex with G_s and G_i proteins in a competitive manner. (C) A methodology combining protein cross-linking and MALDI-MS was applied to delineate the propensity of three GPCRs to form complexes with four engineered Gα proteins (G_s, G_i, G_o and G_q) and a nanobody Nb80 (Wu et al., 2021). The extent of cross-linked complex formation is plotted as function of the components and the GPCR where apo designate the ligand-free condition and ligands were as follows: atr, all trans-retinal; iso, isoprenaline; pro, propranolol; nad, nadolol; car, carvedilol; angII, angiotensin II; azi, azilsartan.

Fig. 10. Identification of endogenous lipids associated with the A_{2A}R-trimeric G_s protein complex. (A) Tandem MS characterization of the A_{2A}R-trimeric G_s protein complex. Isolation of the complex at 26+ charge state in the mass spectrometer (top left) for collisional dissociation lead to the products consistent with the assembly stoichiometry and binding of the receptor to two phospholipids, phosphatidylserine and phosphatidylinositol (top right). (B) MS investigation of β_1AR and NTSR1 binding to various phosphatidylinositol derivatives (PI, PIP, PIP_2 and PIP_3) in equimolar concentrations in the same receptor solution unveiled a preferential interaction toward PIP_2 for both receptors. *Insert* The relative ratio (y axis) represents the peak intensities of the apo receptor (β_1AR or NTSR1) to the PI bound species as a function of the concentration of the different PI derivatives.

Fig. 11. Structural mechanism of PIP_2 in stabilizing G_s protein-coupling. (A) MD simulation of $A_{2A}R$ identified binding hotspots for PIP_2 located on the intracellular interfaces, including TM1-TM2, ICL2-TM4 and TM7-H8. (B) The binding equilibrium of PIP_2 with β_1AR shows 1, 2, and 3 binding sites occupied while when β_1AR forms a complex with mini-G_s protein the 2-PIP_2 bound state predominates. (C) A proposed model of PIP_2 acting as a "staple" to stabilize the interface of a GPCR- mini-G_s protein complex.

Figure 12. Ligand dependent effects on sodium and zinc bound states of $A_{2A}R$ and β_1AR. (A) Schematic illustrating the sodium binding pocket of $A_{2A}R$ in an inactive conformation (PDB 4EIY) and the collapse of the pocket upon adopting an active conformation (PDB 3QAK). The purified $A_{2A}R$ was electrosprayed from a buffer containing non-volatile salts in the absence or presence of ligands (10 μM) and its 13+ charge state was highlighted. Up to 7 Na peaks are observed in the presence of antagonists while very few are discerned with agonists. (B) Endogenous metal adducts were detected in the tβ_1AR-mini-G_s complex under stimulation with different agonists. The stoichiometry of metal-binding is denoted on the individual peak (1x: one adduct, 2x: two adducts, xyl: xylosylation). The impact of EDTA on tβ_1AR-mini-G_s complex formation and its association with the endogenous metal was investigated (right hand panel). The binding stoichiometry of the metal ligand was denoted (1x-4x, one to four adducts). A supplement of exogenous $ZnCl_2$ at 25 μM into the EDTA pre-treated receptor recovered tβ_1AR: mini-G_s complex formation (bottom ESI-MS spectrum). Peaks assigned to the receptor-mini-G_s complex, receptor monomer and mini-G_s are highlighted in orange, blue and grey respectively**.**

Figure 13. Binding hotspots for zinc ions and a potential mechanism for stabilizing G_s protein-coupling. The contacts for zinc ions (green) are highlighted on β_1AR (blue) and mini-G_s protein (orange) from the MD simulations. The contacts located in the interface between the receptor and mini-G_s protein are proposed as a positive allosteric site for G_s protein-coupling.

Figure 14. Schematic showing the rhodopsin signalling cascade. Activated rhodopsin interacts with G_t consisting of Gtα . GDPβγ and exchanges GDP for GTP. Gt then dissociates to form Gtα . GTP and Gtβγ; loss of retinal from rhodopsin leads to the formation opsin. α-subunits of Gt interact with the γ subunits in the PDE6 enzyme, with γ-subunits undergoing a conformational change, relieving inhibition thereby activating PDE6 to cause hydrolysis of cGMP. The rates for the hydrolysis and isomerisation reactions measured in membrane fragments are given. A) Change in ESI-MS spectra over time showing the faster rate of conversion of rhodopsin to opsin in LMNG (upper) than in the membrane (lower). B) Mass spectrometry analysis of the intact PDE6 following light activation. PDE6 . cGMP complex show that it has undergone hydrolysis to form GMP, which then dissociates from the enzyme.

Figure 15. Chemical structures of rho targeting compounds and their effects on k_{hyd} of rhodopsin. A. Charge deconvoluted ESI-MS spectra of rhodopsin/opsin ejected from bovine outer segments prior to illumination (t=0) and 3 min after light in the presence of compound 6 upper and 1 lower. B. Chemical structures of the compounds 1 - 9. C. Bar chart to compare the rate of hydrolysis of rhodopsin in the presence of 1 - 9 and a control comprising rod outer segment vesicles in 200 mM ammonium acetate with 0.1% DMSO at pH 7.0. **1 - 9** were solubilised in DMSO and diluted to give a final concentration of 90 μM in ROS vesicles in 0.1% DMSO, 200 mM ammonium acetate, pH 7.0. Data are presented as mean values +/- SE (n=3).

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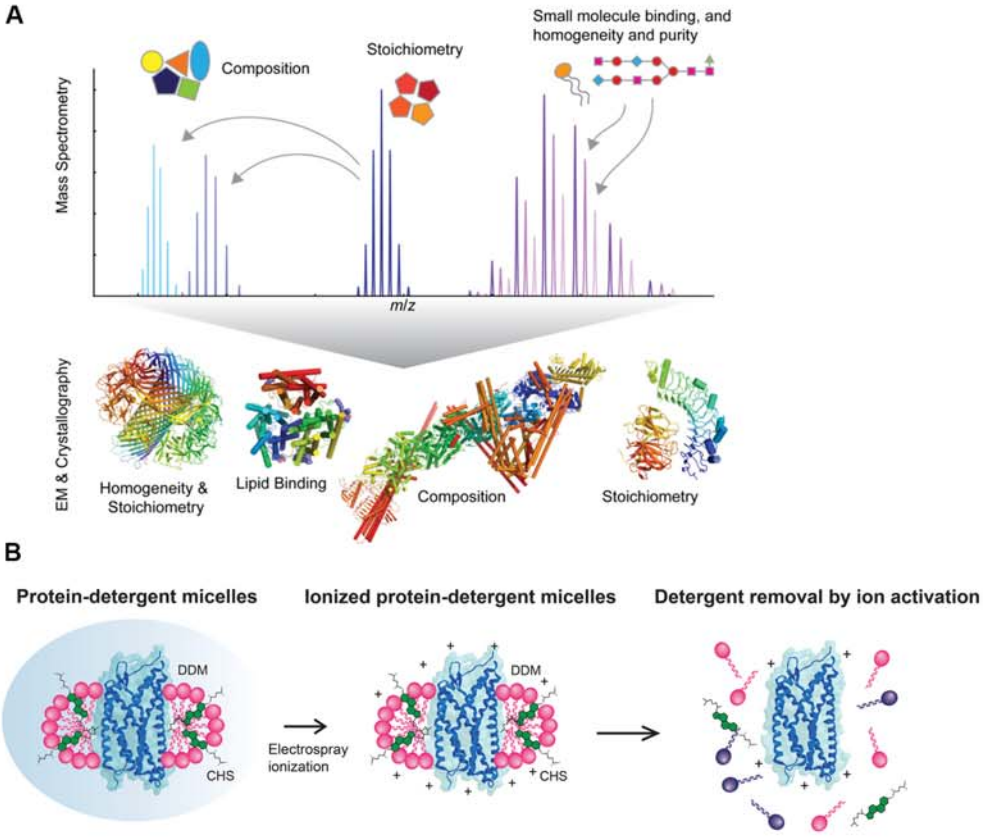


Fig. 1

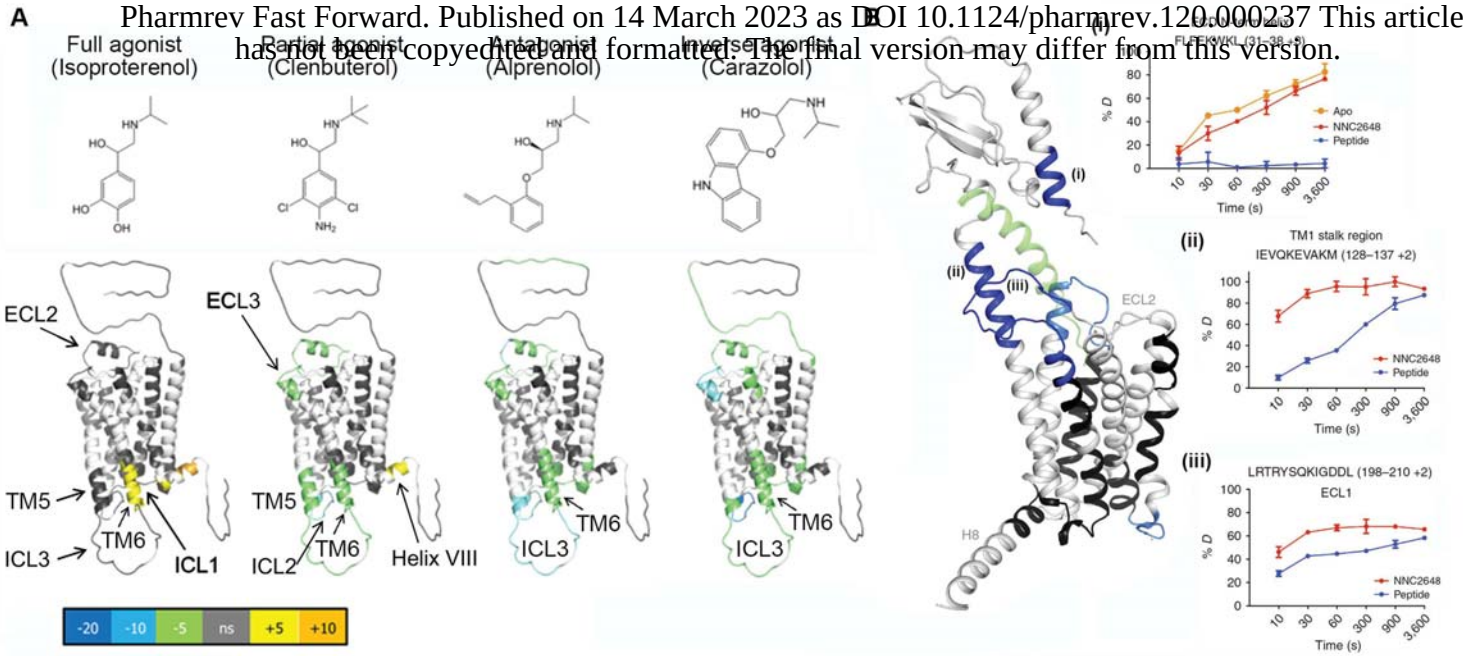


Fig. 2

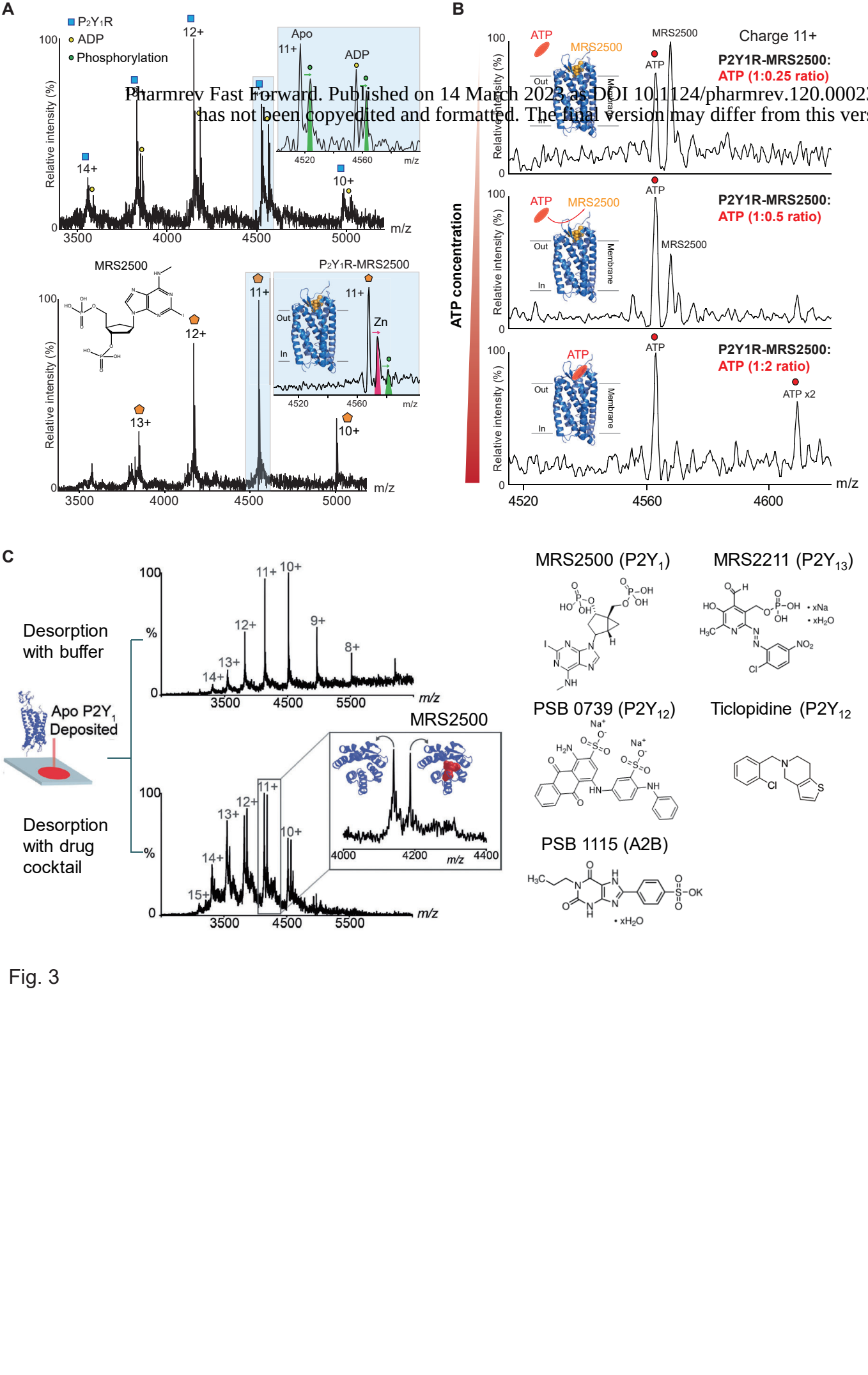


Fig. 3

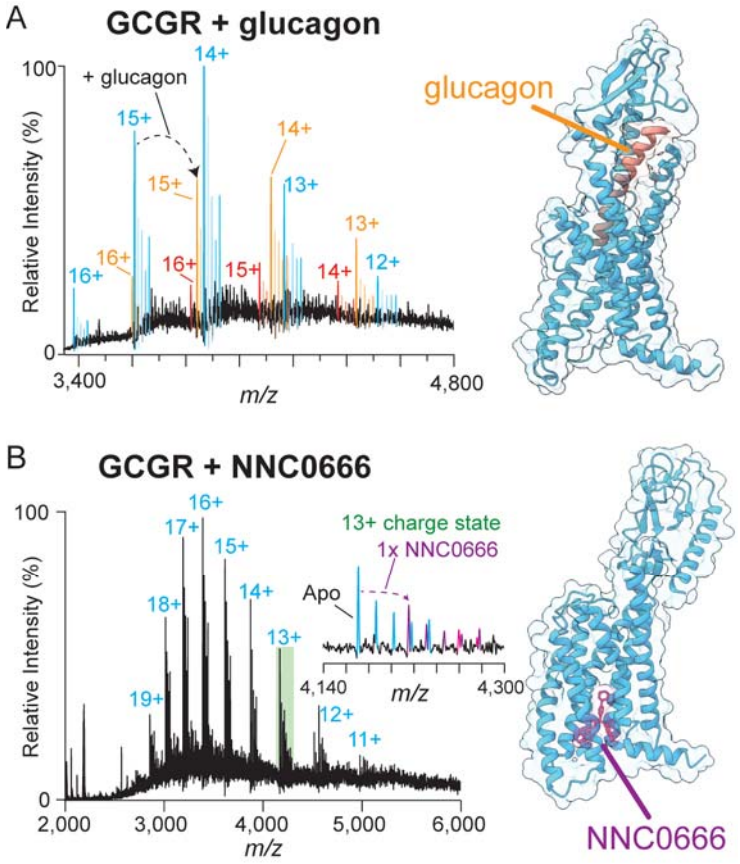
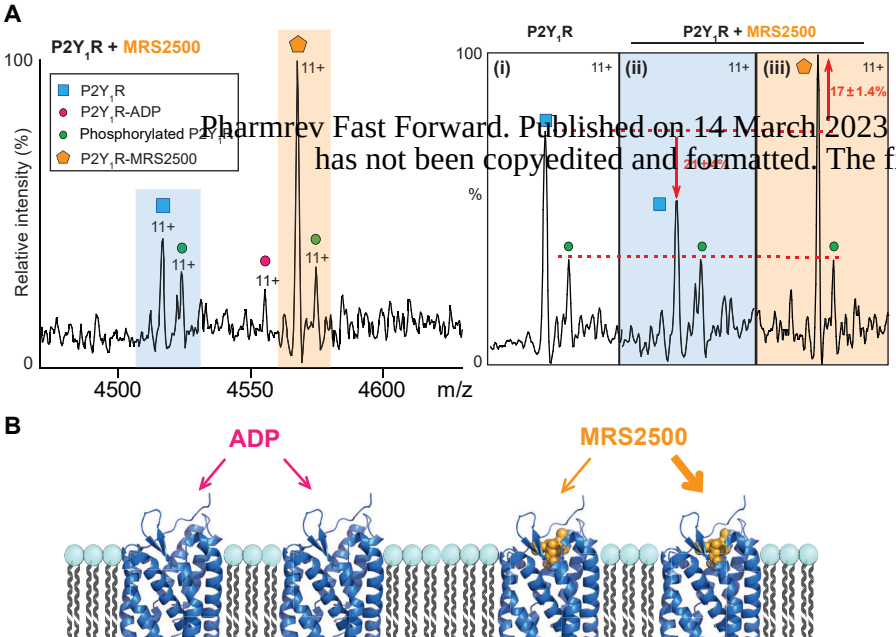
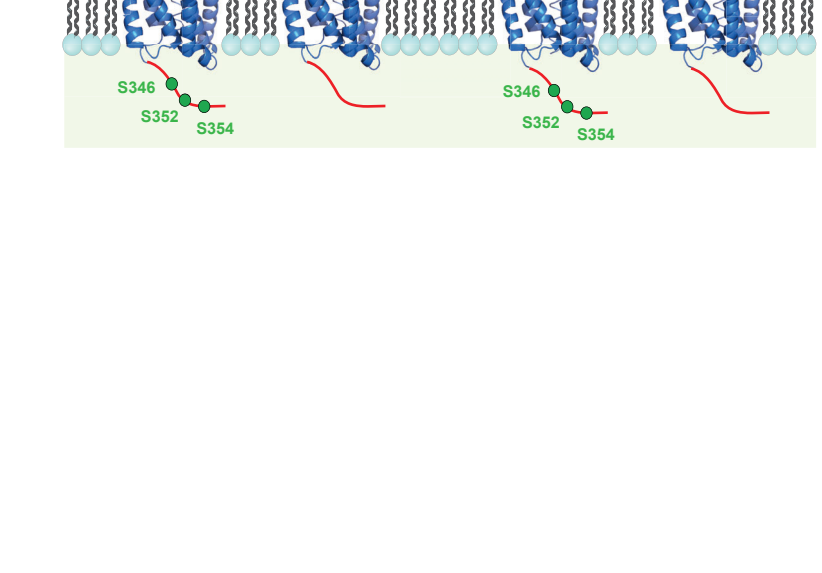


Fig. 4

Fig. 5





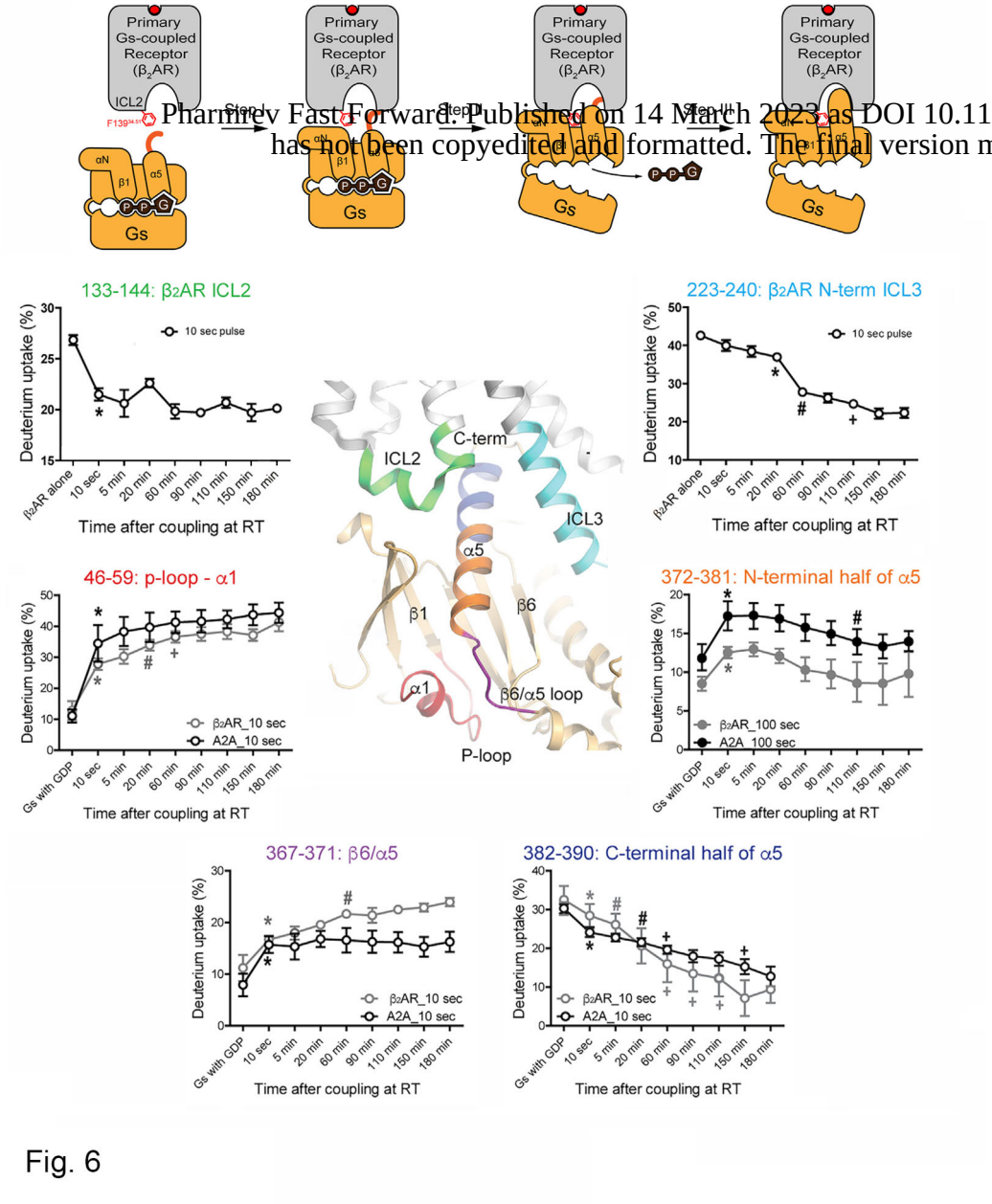


Fig. 6

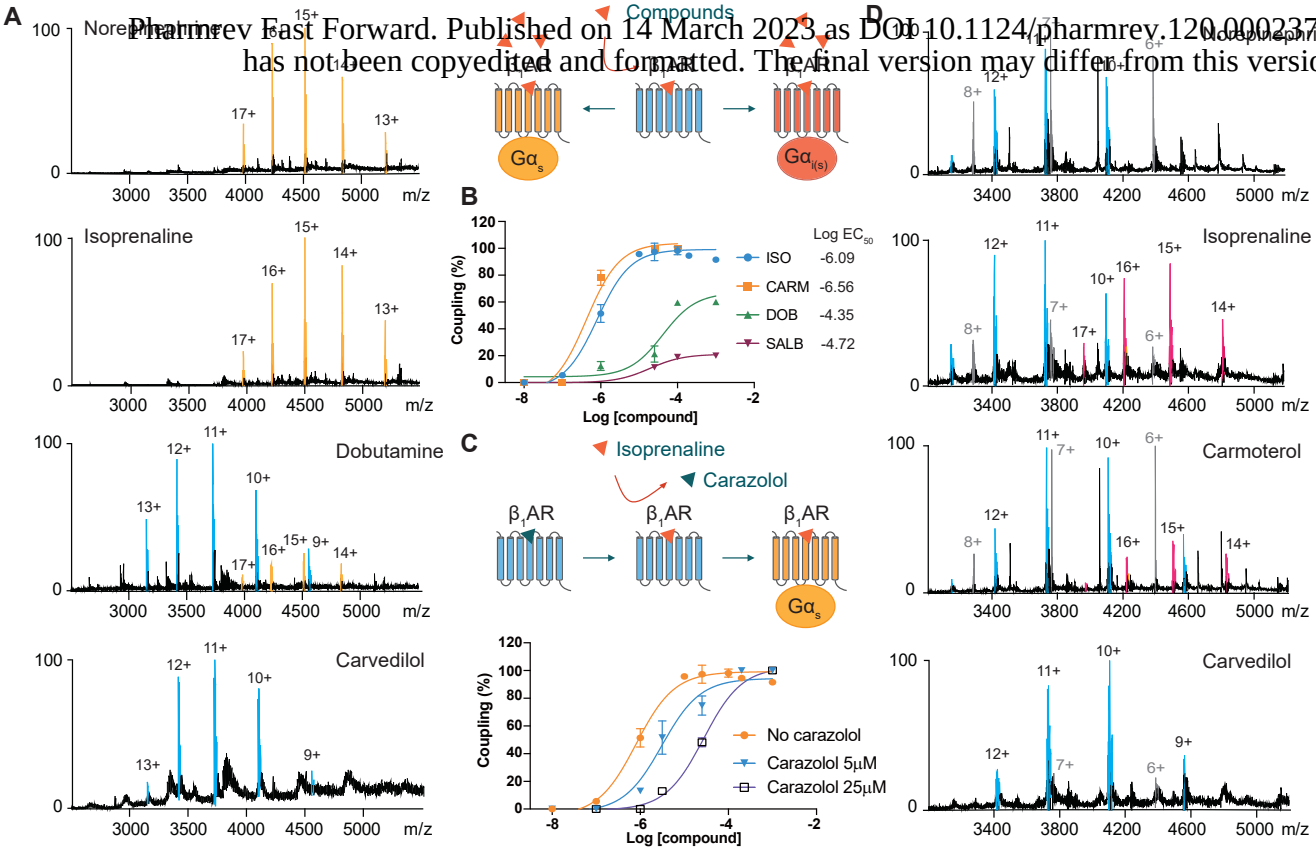


Fig. 7

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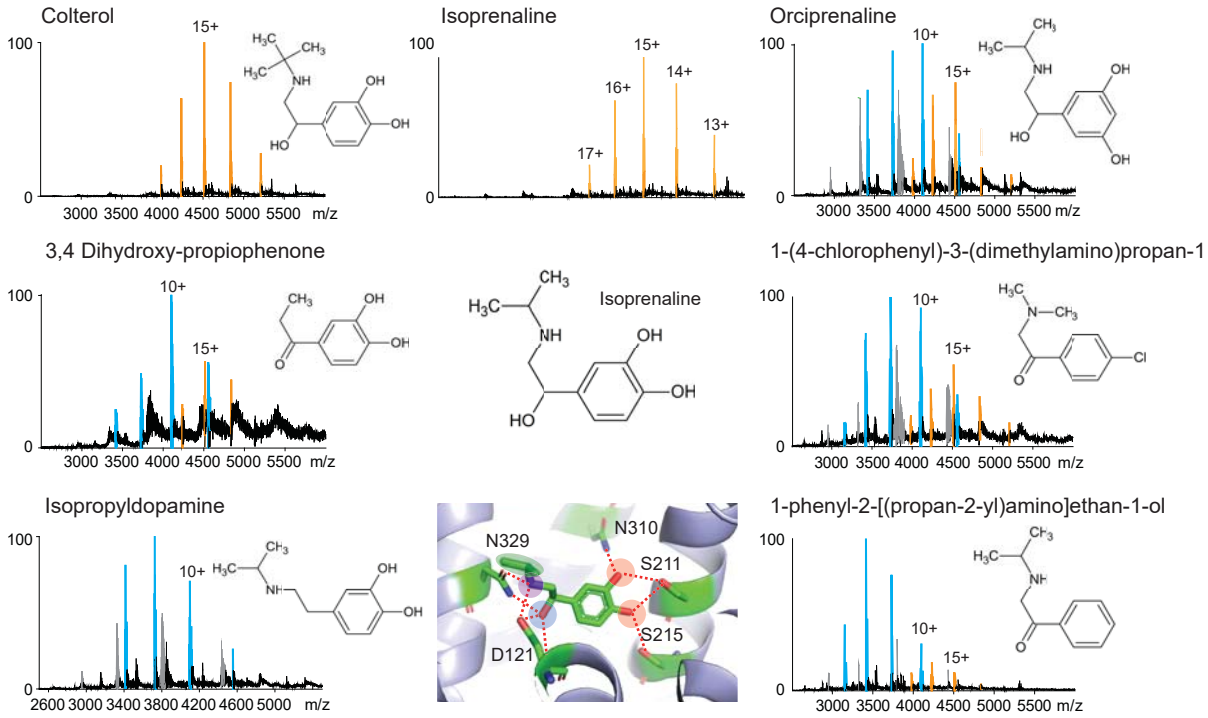

Fig. 8

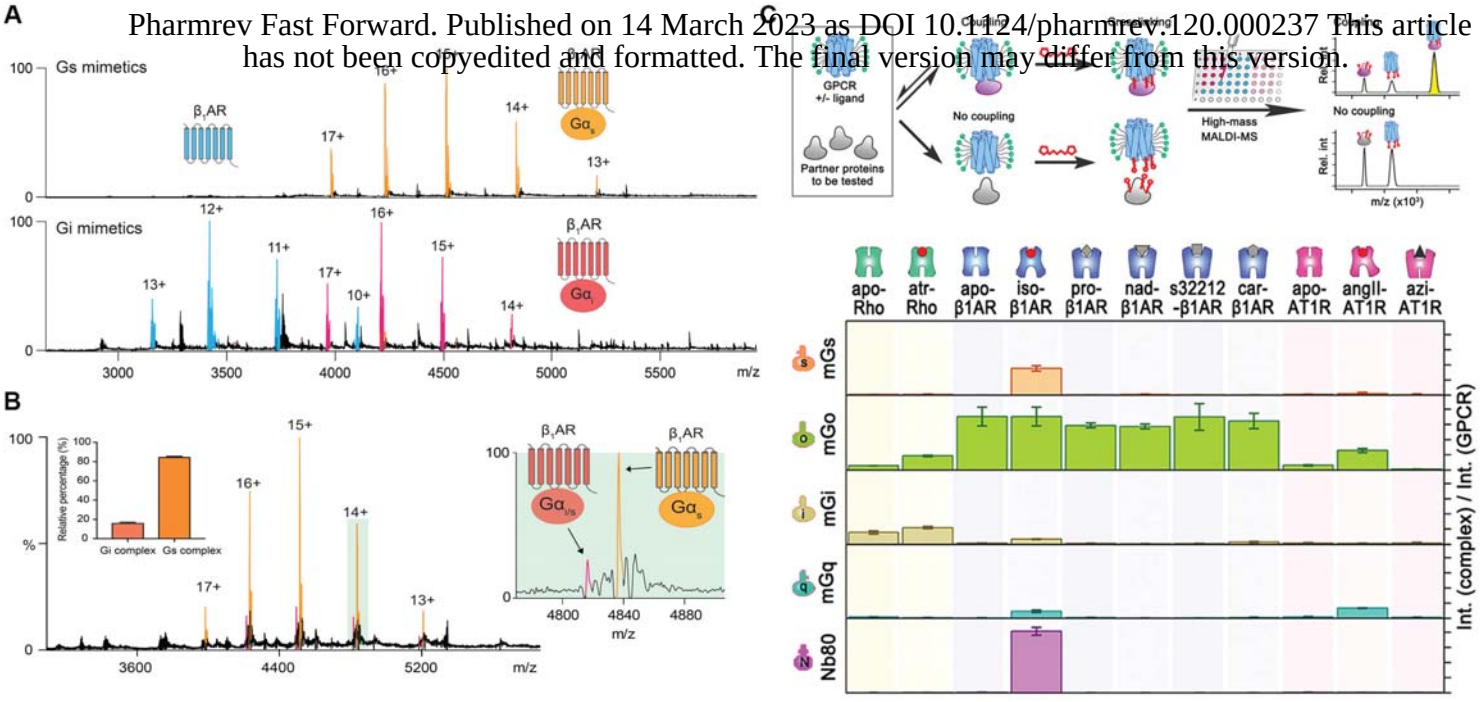

Fig. 9

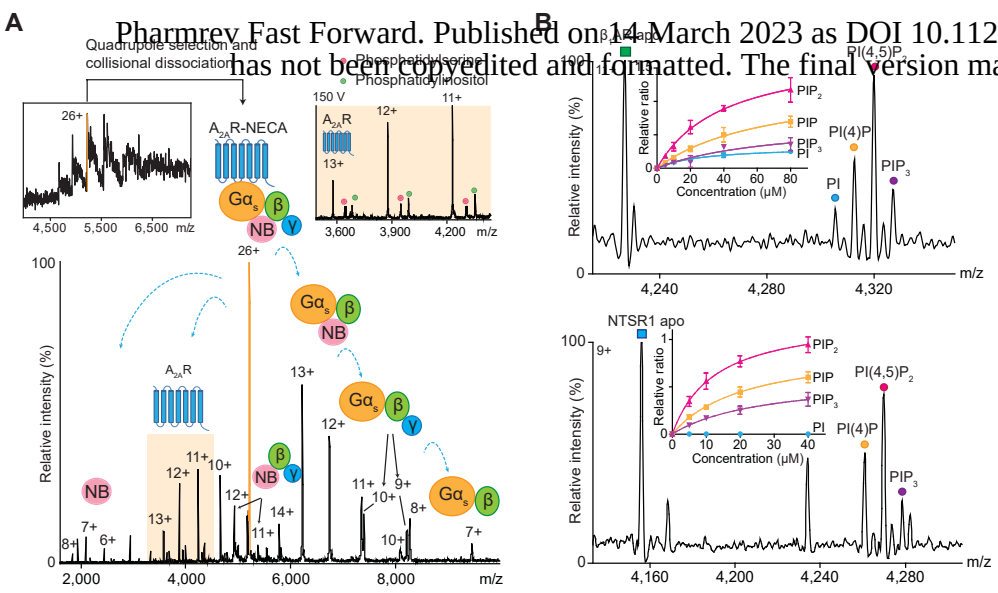

Fig. 10

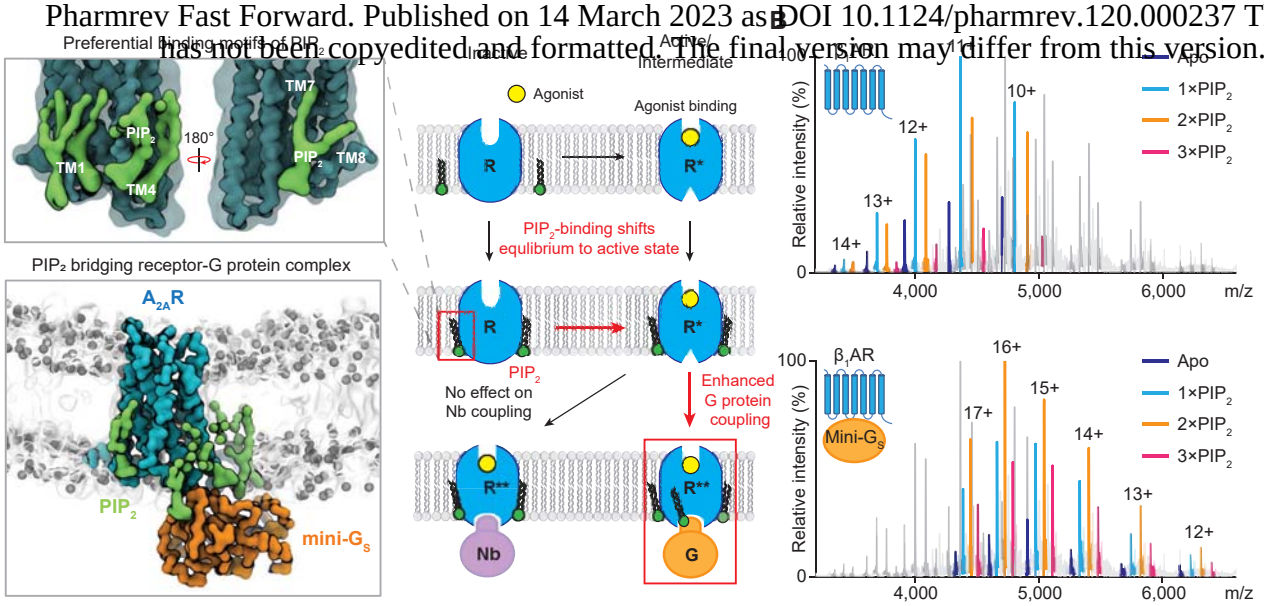

Fig. 11

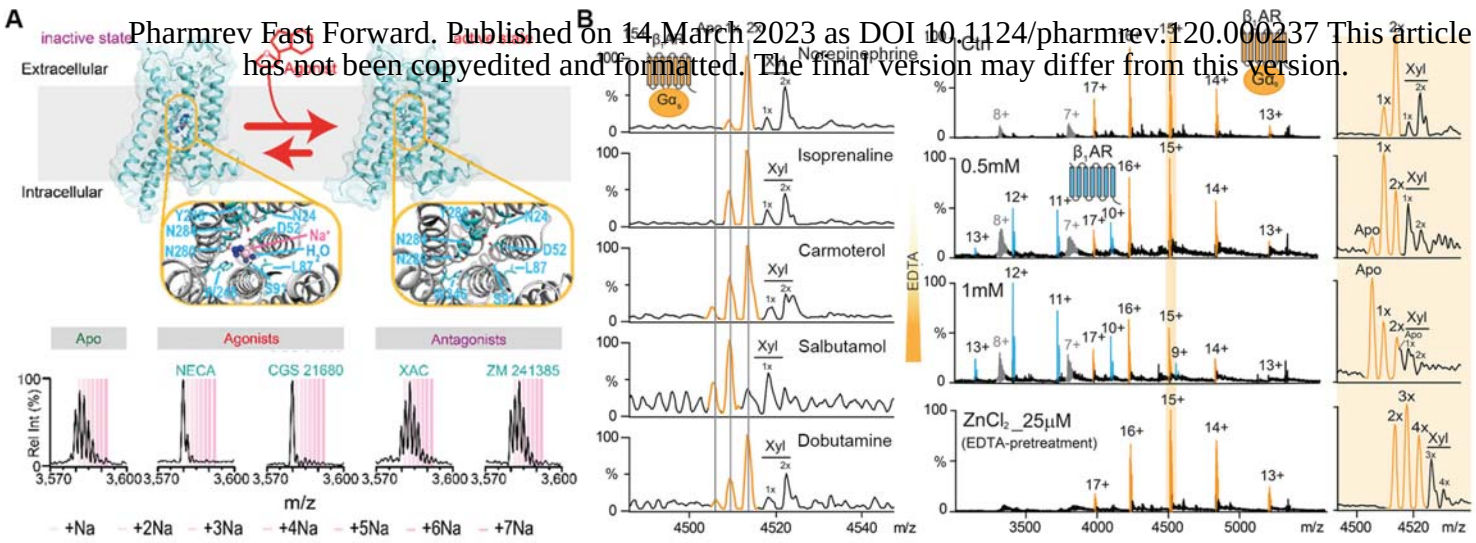

Fig. 12

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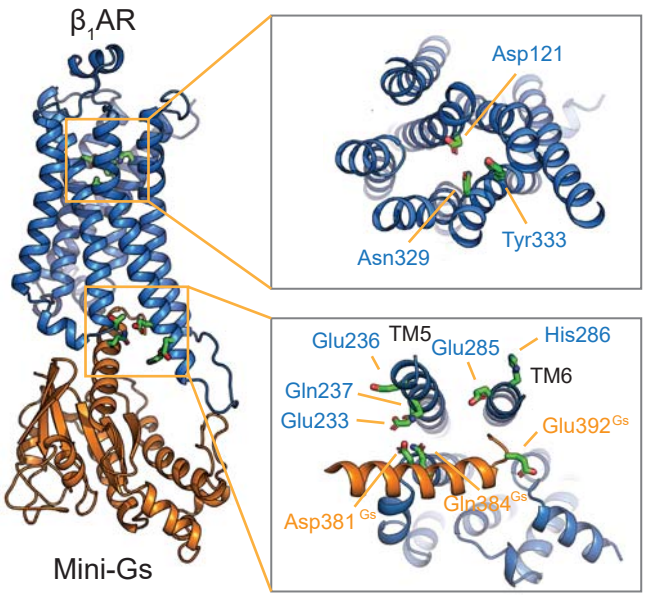

Fig. 13

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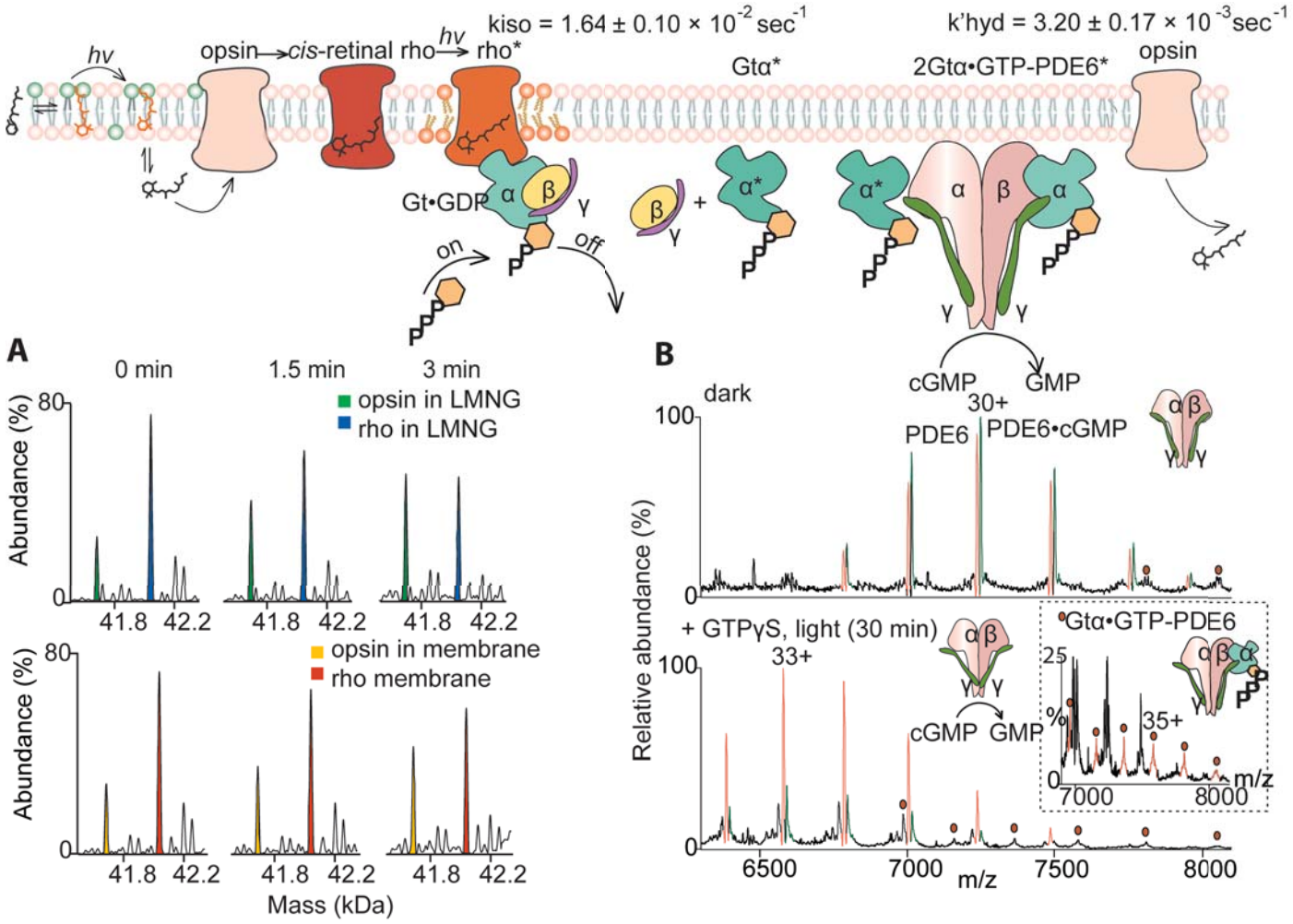

Fig. 14

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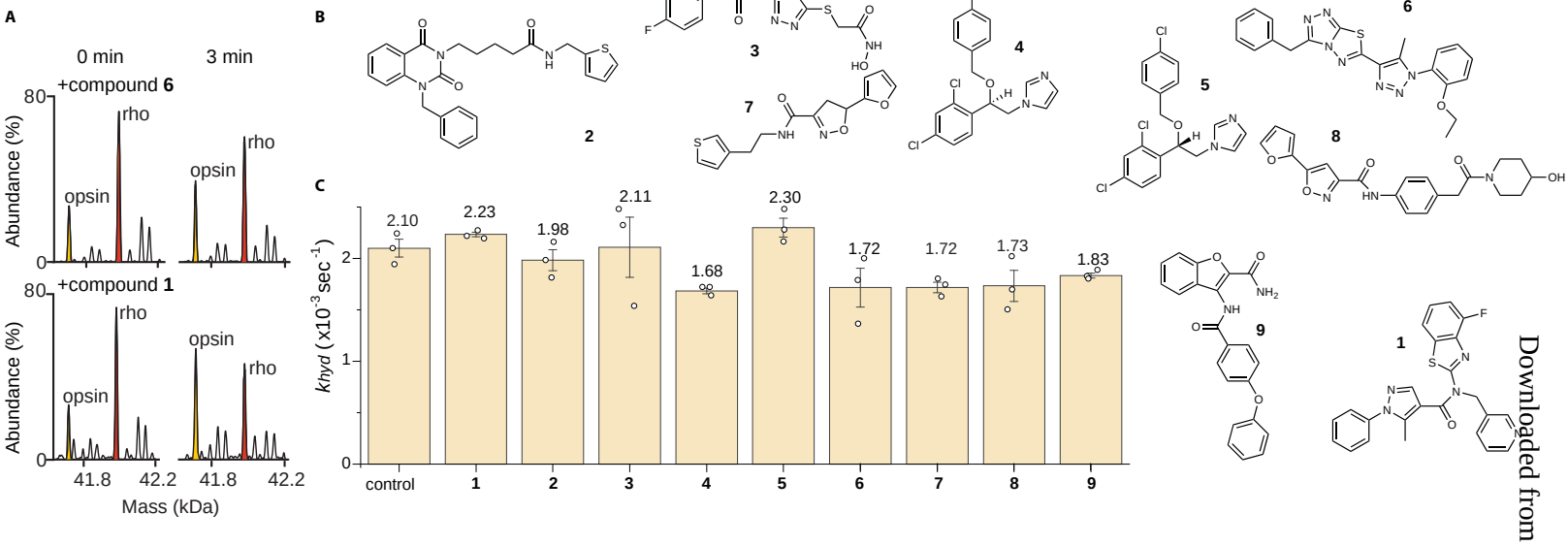

Figure 15