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Sequential receptor activation: A novel mechanism for stepwise engagement of a G protein-coupled receptor

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Unlocking a temporal dimension of GPCR signaling by a novel mechanism for stepwise receptor activation

A novel mechanism for stepwise activation of a G protein-coupled receptor

A novel mechanism for stepwise activation of a G protein-coupled receptor by a small molecule

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Summary

Ligands targeting G protein-coupled receptors (GPCRs) are currently classified as either orthosteric, allosteric or dualsteric/bitopic. Here, we report a new pharmacological concept for GPCR functional modulation: sequentially activating ligands. A hallmark feature of these is the initial contact with and transient activation of a first receptor site followed by sustained activation of a second topographically distinct site. We identify 4-CMTB, previously classified as a pure allosteric agonist of the free fatty acid receptor FFA2, as the first sequential activator and corroborate its two step activation in living cells by tracking integrated responses with innovative optical- and impedance-based label-free biosensors capable of visualizing multiple signaling inputs in real-time. We validate this unique pharmacology with traditional cellular readouts along with mutational and pharmacological perturbations including computational techniques, and propose a kinetic model applicable to analysis of sequential receptor activation. Our proof-of-concept study unveils 4-CMTB as the prototype ligand epitomizing a heretofore-unobserved molecular mechanism of receptor activation.

Introduction

GPCRs are involved in virtually every (patho)physiological process in mammals and therefore have been the most successful targets for drug development[1,2]. Most drugs are assumed to act via binding to the orthosteric site, thereby competing with the endogenous ligands that naturally regulate receptor function. During the past years allosteric modulation of GPCRs has received considerable interest, and significantly allosteric ligands (that is ligands that bind to a distinct location) are emerging as promising alternatives for therapeutic intervention because they may obviate several of the inherent challenges of orthosteric target-centered approaches[3–6]. First, allosteric ligands may achieve greater receptor subtype selectivity because allosteric epitopes are less well conserved than orthosteric recognition sites. Second, allosteric ligands show the advantage of "use-dependence", which entail lower propensity for receptor desensitization[7] and may also provide a means to fine-tune cellular signaling by favoring selected signaling routes over others[8]. Third, allosteric modulators are characterized by their saturability of effect on the orthosteric recognition site. This in turn allows preservation of a low-level tone of the endogenous ligand and thus safeguards physiological function even under conditions of full occupancy of the allosteric site[4,6].

To date, allosteric ligands are classified as inhibitors (negative allosteric modulators, NAMs), potentiators (positive allosteric modulators, PAMs) or allosteric agonists, as well as silent or neutral allosteric modulators (SAMs/NALs)[6,9,10]. Recently, dualsteric or bitopic ligands have become available as an additional class of pharmacological agents for modulating GPCR function[11,12]. These ligands harbor two pharmacophores connected by a linker to concomitantly engage both orthosteric and allosteric receptor binding pockets. Thus, dualsteric/bitopic ligands combine receptor subtype selectivity with the capacity to fine-tune the receptor's natural signaling pattern[13,14] (Bock NatComm).

This study introduces a novel mechanism of pharmacological intervention by bifunctional ligands to unlock a temporal dimension of GPCR modulation that is inaccessible by the conventional

understanding of orthosteric, allosteric and dualsteric/bitopic ligands: Sequential receptor activation. This mechanism is posited to explain the differences observed in investigations into the molecular modes of action of two agonists for the free fatty acid receptor 2 (FFA2, formerly GPR43)[15,16]: the short chain fatty acid propionic acid C3 and the small molecule 4-CMTB (2-(4-chlorophenyl)-3-methyl-*N*-(thiazol-2-yl)butanamide). Propionic acid is an endogenous agonist targeting the orthosteric pocket of FFA2[15,16]. 4-CMTB is a synthetic ligand that was identified in a high-throughput screening campaign in an effort to achieve selective activation of FFA2 over the closely related FFA3 receptor[17]. Interestingly, considerable optimization efforts have failed to provide ligands with significantly higher potency than the initial hit and attempts to map its binding site have been inconclusive[18,19]. Nevertheless, of the studies undertaken with 4-CMTB to date, all are indicative of a purely allosteric mode of action[17–22].

Herein, we confirm allosteric receptor engagement of FFA2 by 4-CMTB but additionally reveal a hitherto unappreciated orthosteric component in its mechanism of action. Intriguingly, this orthosteric activation is only temporary in nature but is followed by sustained activation via the allosteric site. We validate this stepwise mode of receptor activation using a broad array of signaling measurements under kinetic and equilibrium conditions in combination with pharmacological perturbations, receptor mutagenesis and structural analysis. We also develop a kinetic model applicable to the analysis of sequential receptor activation.

With the identification of 4-CMTB as bifunctional ortho-allosteric agonist we not only expand/enrich the pharmacological toolbox of GPCR modulators/enrich current pharmacological concepts but moreover add a new dimension to the repertoire of cellular communication via GPCRs in space and time.

Results

Label-free techniques unveil different activation modes of C3 versus 4-CMTB

Label-free assays based on the detection of dynamic mass redistribution (DMR) or bioimpedance capture integrated responses in living cells with high temporal resolution and broad signaling pathway coverage[23–26]. They have proven particularly well suited to visualize cellular activation profiles of signaling-competent proteins such as GPCRs[27–29]. We initially set out to compare the cellular reaction to either the endogenous, orthosteric agonist propionic acid (C3) or the synthetic allosteric agonist 4-CMTB in HEK293 cells engineered to stably express the human FFA2 wild-type receptor (hFFA2, hFFA2-wt). Label-free impedance sensing unraveled a striking temporal difference in the signaling patterns triggered by the two receptor ligands. C3 provoked a sharp transient negative peak rapidly after compound addition that reversed quickly toward baseline and that was followed by a second gradually descending phase. 4-CMTB largely lacked the first spike but preserved the second phase signal (**Figure 1A,B**, for magnification of the early timescale see **Figure S1A,B**). Comparable results were obtained in optical biosensor-based DMR recordings. C3 generated a uniform signature with a maximal DMR peak at about 1,500 sec, after which it decayed slowly (**Figure 1C**). 4-CMTB, in contrast, evoked a less pronounced initial increase with a delayed maximal response at approx. 3,000 sec. Yet, overall DMR profiles at later time points were comparable to those generated by C3 (**Figure 1D**). To consider these temporal differences, we quantified concentration-effect relationships for both ligands at early and late time points. This analysis revealed partial agonism of 4-CMTB for the first signaling impulse (**Figure 1E,F**), but full agonism at later time points (**Figure 1G,H**, and **Table S1**). All cell responses in the label-free readouts were specifically mediated via the hFFA2 receptor, since untransfected cells did not react upon compound addition (**Figure S1C,D**).

Structural integrity of the orthosteric site impacts on 4-CMTB signaling dynamics

A key residue within the orthosteric binding pocket of hFFA2 is R255$^{7.35}$ in helix 7 (Ballesteros-Weinstein indexing system in superscript), which - if mutated to alanine (hFFA2-R255A) - renders hFFA2 unresponsive to short chain fatty acids despite appropriate surface expression[17] ([30], see methods). Consistent with these findings, C3 was completely inactive on the hFFA2-R255A mutant receptor in both impedance- and optical-based label-free whole cell recordings (**Figure S1E,F**). As expected, 4-CMTB retained the capacity to trigger cell activation via the hFFA2-R255A receptor, corroborating its non-orthosteric mode of action. However, we noted that the kinetic profile of 4-CMTB differed significantly from that obtained at the wild-type receptor. Both label-free assays yielded temporal fingerprints for 4-CMTB indicative of impaired early but enhanced late cell responses (**Figure 1I,J;** for time-dependent quantification of label-free signatures at the hFFA2-R255A receptor see **Figure 1K,L**). Our mutagenic approach indicates that lack of orthosteric R255$^{7.35}$ impacts on signaling by 4-CMTB, either because signaling via the allosteric site requires the integrity of the orthosteric site and/or because 4-CMTB also interacts directly with the orthosteric receptor site.

CATPB serves as a selective orthosteric probe

To provide a complementary view on the biological role of the orthosteric binding site for 4-CMTB signaling, we also manipulated hFFA2 function using traditional pharmacological perturbation with CATPB, a small molecule previously reported to competitively antagonize hFFA2-wt receptor function[21] (**Figure 2A**). We initially verified competitive antagonism of CATPB with C3 using Schild analysis of DMR recordings at the hFFA2-wt receptor (pA_2: 7.61 ± 0.04, slope: 0.96 ± 0.01) (**Figure 2B,C**). Competitive inhibition of C3 function by CATPB was further substantiated in ERK1/2 phosphorylation assays (**Figure S2A,B**). In contrast, at no time did CATPB affect non-orthosteric 4-CMTB-mediated activation of the hFFA2-R255A mutant in label-free recordings (**Figure 2D,E;** for quantification of CATPB effects at early and late time points **see Figure 2F**). These data suggest occupancy of non-overlapping binding sites by CATPB and 4-CMTB at equilibrium and unambiguously

define CATPB as an orthosteric probe, competing with C3 for a common site within the orthosteric hFFA2 pocket.

Attenuation of orthosteric signaling by CATPB remodels the dynamics of 4-CMTB-mediated FFA2 activation

In accordance with an orthosteric mode of action, high concentrations of CATPB completely blocked C3-induced cell response in label-free assays (**Figure 3A, Figure S3A**). In contrast, saturating concentrations of CATPB exclusively blunted the first, but preserved or even enhanced the second signaling wave mediated by 4-CMTB (**Figure 3B, Figure S3B**, for quantification of early and late responses in label-free DMR and impedance assays, respectively, see **Figure S3C,D**). CATPB did not affect DMR or impedance responses triggered by endogenously expressed P2Y receptors, confirming the specific nature of hFFA2-wt receptor inhibition (**Figure S3E,F**).

To elucidate whether the characteristic temporal inhibition pattern of CATPB on 4-CMTB-induced cell responses is echoed in traditional GPCR signaling readouts, multiple parallel assays were employed that either capture rapid (mobilization of intracellular Ca^{2+}), delayed (accumulation of IP as well as inhibition of forskolin-mediated cAMP production) or both cell responses (ERK1/2 phosphorylation assays). Indeed, the transient rise of Ca^{2+}, which is detectable within seconds after addition of both C3 and 4-CMTB, is attenuated by CATPB (**Figure 3C,D)**. For both ligands, inhibition was complete and entirely consistent with competitive antagonism (**Figure 3E, Table S2**). Second messenger production in IP (**Figure 3F**) and cAMP accumulation assays (**Figure 3G**) after 4-CMTB stimulation was insensitive to inhibition with CATPB. This is in contrast to complete inhibition that was apparent when C3 was employed as the activating stimulus. Inhibition of early, partial inhibition of intermediate, but lack of CATPB sensitivity at late signaling time points is also recapitulated in ERK1/2 phosphorylation assays examining the time-dependence of interaction between 4-CMTB and CATPB (**Figure 3H**; see **Figure S3G** for unperturbed 4-CMTB pERK1/2 kinetics over time). Inhibition of pERK1/2 levels by CATPB was FFA2 receptor-dependent, since serum-induced controls were unaffected by the antagonist (**Figure**

S3H). In summary, as with real-time label-free data, second messenger assays provide strong support for the notion that 4-CMTB mediates early cellular responses via transient activation of the orthosteric site.

Mutational analysis unveils dual input control of 4-CMTB signaling at the wild-type receptor

A corollary of orthosteric receptor activation by 4-CMTB is selective abrogation of early, but not late, cell responses in mutant forms of hFFA2 lacking a functional orthosteric site. To test this prediction, we investigated the temporal signaling pattern of 4-CMTB at the hFFA2-R255A receptor in both Ca^{2+} and second messenger assays. Consistent with our prediction, rapid signaling of transient Ca^{2+} flux upon 4-CMTB stimulation was abolished (**Figure 3I**) but delayed cell responses in second messenger (**Figure 3J,K**) and ERK1/2 accumulation assays (**Figure 3L**) were preserved. Thus, we posit that 4-CMTB dually controls input at the wild-type receptor by sequentially activating the orthosteric followed by the allosteric site, respectively.

An ECL2 swap mutant exaggerates the transient orthosteric action of 4-CMTB

We next chose to investigate the mechanism of receptor activation by 4-CMTB using a chimeric hFFA2 receptor, in which the extracellular loop 2 (ECL2) of hFFA2 was exchanged for the counterpart of the cognate hFFA3 receptor (hereafter hFFA2-ECL). This mutant was designed previously in an effort to understand transmission of allosteric effects by 4-CMTB[19]. Both C3 and 4-CMTB displayed temporal activation patterns in hFFA2-ECL expressing HEK293 cells that were comparable with those observed at the wild-type receptor (**Figure S4A-D**, compare with **Figure 1C,D,F,H**)). Inhibition of C3 by CATPB remained competitive at the hFFA2-ECL receptor (pA_2: 7.00 ± 0.10; slope: 1.01 ± 0.04; **Figure S4E,F**). Consistent with this, saturating concentrations of CATPB completely inhibited C3-induced DMR responses at all times (**Figure 4A**). Intriguingly, CATPB inhibition of 4-CMTB signaling differed significantly from the pattern observed for the wild-type receptor because both initial and delayed activation of 4-CMTB was largely diminished (**Figure 4B**, compare with **Figure 3B**). When we compared CATPB modulation of C3 and 4-CMTB signaling at early time points at the hFFA2-ECL

receptor, we observed complete inhibition indistinguishable from competitive antagonism (**Figure 4C,D**) and reminiscent of the profile at the wild-type receptor (**Figure 4E,F**). Quantitative analysis of CATPB IC_{50}-shifts indicates competitive antagonism for both C3 and 4-CMTB and thus strengthens the conclusion of temporary orthosteric receptor activation by 4-CMTB (**Figure 4G,H, Table S3**). Moreover, CATPB remained able to partially inhibit delayed 4-CMTB-induced cell responses at the hFFA2-ECL mutant (**Figure S4G,H)**, whereas it gradually turned into enhancement over time at the wild-type receptor (**Figure S4I,J;** compare **G** with **I**, and **H** with **J**; for snap-shot quantification of time-dependent modulation by CATPB of 30 µM 4-CMTB see **Figure 4I**). These data argue for a gatekeeper role of ECL2 in determining the duration of orthosteric first phase agonism by 4-CMTB. To challenge this hypothesis, we introduced the Arg255Ala mutation into the hFFA2-ECL receptor resulting in the double mutant hFFA2-R255A-ECL. DMR recordings in hFFA2-R255A-ECL expressing cells mirror the traces obtained in hFFA2-ECL cells in the presence of CATPB: loss of the orthosteric contribution ("the fast component") but maintenance of the capacity to evoke activation via the allosteric site ("the slow component") indicate an initial adoption of an orthosteric pose that is temporally extended in the hFFA2-ECL chimera (**Figure 4J**). Consistent with this, Ca^{2+}-ionophore, A23187, but not C3 and 4-CMTB, elicited a calcium transient in hFFA2-R255A-ECL cells (**Figure 4K**). However, delayed activation via the allosteric site was still detectable for 4-CMTB as evidenced by robust accumulation of inositol phosphates (**Figure 4L**). These results led us to conclude that concomitant perturbation of the orthosteric binding pocket by Arg255Ala and of the gatekeeper function of ECL2 is well suited to illustrate the sequence of events during receptor activation by 4-CMTB, thereby associating the initial signaling impulse with an orthosteric and the prolonged signaling impulse with an allosteric mechanism.

A single amino acid replacement restricts 4-CMTB to an orthosteric signaling mechanism

The capacity to enhance duration of orthosteric 4-CMTB action in the ECL2 swap mutant prompted us to hypothesize that a similar effect might be achieved by replacement of key residues lining the allosteric FFA2 site by the corresponding FFA3 counterparts. Guided by homology modeling based on

the crystal structure of the related FFA1 receptor we replaced K65$^{2.60}$ by arginine (hFFA2-K65R), a residue which has not been probed previously as a determinant of 4-CMTB action (**Figure 5A,B** and [32]). Both C3 and 4-CMTB robustly activated hFFA2-K65R consistent with the mutated residue being outside the orthosteric area (**Figure S5A-E**). Most notably, however, and entirely consistent with our hypothesis, both C3 and 4-CMTB were now fully antagonized by CATPB (**Figure 5C-E**). Similar observations were made in IP accumulation assays: C3 and 4-CMTB-induced IP production was completely ablated by CATPB in a manner compatible with competitive antagonism for both ligands (**Figure 5F-H, Table S4**). Thus, at hFFA2-K65R the only mode of interaction available to 4-CMTB is occupancy of epitopes within the orthosteric pocket which manifests as complete sensitivity towards inhibition by CATPB.

Structural analysis defines receptor engagement by 4-CMTB

To further corroborate transient orthosteric first phase agonism of 4-CMTB, we examined its signaling pattern in mutant forms of hFFA2 designed to mimic the orthosteric site of FFA3: hFFA2-S86$^{3.29}$G-Y90$^{3.33}$F-I145$^{4.61}$Y-E166^{ECL2}L. Because 4-CMTB is selective for FFA2 over FFA3, we predicted the quadruple mutant to specifically lose rapid orthosteric but preserve allosteric activation. C3, in contrast, should retain functionality in both assays because it is unable to discriminate between FFA2 and FFA3. Indeed, C3 but not 4-CMTB induced intracellular Ca^{2+} flux (**Figure 6C,** left panel), however, both ligands produced substantial responses in IP accumulation assays (**Figure 6C,** right panel and **Figure S6**). In agreement with compromised orthosteric but functional allosteric signaling of the quadruple mutant, DMR recordings revealed rapid cell activation exclusively for C3 but delayed signaling that is superimposable for both ligands (**Figure 6D**). Based on these results, we predicted that combined substitution of a key orthosteric residue together with the allosteric Lys65Arg mutation should be sufficient to severely impair, if not ablate, 4-CMTB function. Indeed, complete lack of activation by 4-CMTB of hFFA2-K65R-R255A in DMR assays, despite appropriate surface expression, indicates experimental validation of our prediction (**Figure 6E** and **Figure S6**). Thus, we demonstrate that a single ligand may activate a GPCR in tandem via two topographically distinct

receptor sites (**Figure S6**), thereby introducing the novel pharmacological concept of sequentially activating ligands (SEALs).

Discussion

Pharmacological targeting of more than one receptor site has to date only been achieved with dualsteric/bitopic ligands[11,12]. This ligand class consists of hybrid molecules, in which orthosteric and allosteric building blocks are bridged by a molecular linker. Thus, dualsteric/bitopic ligands span and simultaneously bind to orthosteric and allosteric receptor sites[33]. Dualsteric pharmacology is a captivating concept that underlies receptor subtype selectivity and may even confer signaling bias[13,14]. 4-CMTB also engages ortho- and allosteric sites, however, this small molecule is distinct from dualsteric/bitopic ligands in that it has minimal interaction with both sites simultaneously, and does not contain individual building blocks that are separated by a defined molecular linker. Rather it achieves stepwise activation of two spatially distinct FFA2 receptor sites by passing through one binding mode (site 1 interaction) before proceeding to another (site 2 interaction). These differences in orientation convey dynamics to agonism whose cellular consequences are seen only in real time. There is ample evidence that signal dynamics are a common means/principle of nature to encode cellular information and regulate cell physiology (Behar, Cell 2013; Yosef, Cell 2011; Purvis, Cell 2013) but knowledge about the mechanisms that GPCRs use to transceive temporal signaling codes is incomplete (and relatively few experimental tools are available to achieve temporal control over signaling waves) (Calebiro, PlosOne 2009; Ferrandon, NatChemBio 2009; Irannejad, Nature 2013). The here presented data describe the basic concept for a stepwise activation mode of a GPCR by a ligand that sequentially adopts multiple binding poses and thereby encodes receptor-mediated cell activation in a temporal dimension.

A stepwise binding mode is not unprecedented in the literature, as several ligands are known to temporarily engage a disparate epitope before being routed to their terminal binding site. This has been shown for the orthosteric mAChR antagonists N-Methyl-scopolamine and Oxotremorine-M, which also bind to allosteric epitopes[34]. Molecular dynamics studies further proposed a temporary occupancy of an allosteric binding site by the orthosteric M2/3 mAChR antagonist Tiotropium[35]. Likewise, simulations with agonists and antagonists at β-adrenergic receptors propose an early

association with an unpredicted receptor site during the process of binding[36]. While all of these elegant studies underscore the importance of temporal aspects in ligand binding, none has linked receptor site occupancy with functional outcomes for each site. In fact, we speculate that GPCR ligands may frequently have a short-lived pause at an alternative site being guided to their final orientation. It is therefore conceivable that ligands classified as purely orthosteric or purely allosteric in fact halt *en route* of their binding pathway with potential functionality at distinct receptor sites. However, such a pharmacological phenotype may go unnoticed if compounds are exclusively analyzed in equilibrium assays. Thus it is likely that the concept discovered here for 4-CMTB does not merely reflect a new form of dynamic agonism of a single ligand but rather a more common phenomenon for both synthetic and even endogenous GPCR ligands. Indeed, two-step binding mechanisms are generally thought to occur when chemokines or large peptides bind their target receptors (Allen SJ et al., 2007 Annu rev Immunol; Castro M PNAS 2015). But even for small endogenous ligands of family A GPCRs such as acetylcholine multistep binding pathways have recently been suggested (Kappel 2015). Therefore, we speculate that our approach - if adapted to pharmacological analysis of other ligands - may affirm that sequential agonism, i.e. targeting more than one receptor site in a time-dependent manner, is more frequent than anticipated.

An intriguing feature of orthosteric-allosteric targeting of FFA2 by 4-CMTB is the chronological order of these events. The structure of class A GPCRs would suggest that ligands initiate contact within the allosteric vestibule followed by passage into the transmembrane binding pocket to elicit an orthosteric response[35,38]. 4-CMTB, however, appears to navigate via a different route. We speculate that 4-CMTB achieves sequential orthosteric-allosteric targeting by entering via the lipid bilayer. Such an entry mode has previously been shown for several class A GPCR ligands[39–44], particularly for those at lipid mediator GPCRs, such as the ago-allosteric agonist TAK875 (fasiglifam) at the related hFFA1 receptor[32]. Clearly, further studies are needed to investigate the entry mode of 4-CMTB at FFA2 in more detail. Nevertheless and to the best of our knowledge, we here detect 4-CMTB to be the first

GPCR modulator for which sequential binding is associated with sequential activation via occupancy of two distinct receptor sites in tandem.

Our multifaceted approach identifies 4-CMTB as a small molecule altering its activation mechanism over time. Although compelling experimental evidence is provided in favor of this unique mode of action, we chose to verify this novel concept with a kinetic model to rationalize the sequence of events on a molecular level (see **Online Appendix**). We propose that 4-CMTB initially binds the orthosteric receptor site (site 1) to then traverse to the thermodynamically more favorable allosteric site (site 2), whereby occupancy of site 2 subsequently results in negative allosteric modulation of the 4-CMTB effect at site 1. The apparent transient orthosteric signaling phase is therefore rationalized if 4-CMTB functioned as a NAM on its own efficacy at a distinct binding site. The extracellular loop 2 (ECL2) likely contributes to cessation of orthosteric signaling, since the ECL2-swap from the FFA2 receptor to the cognate counterpart of FFA3 substantially enhanced orthosteric elements of activation. Within hFFA2-K65R, however, which lacks a key residue of the allosteric pocket, 4-CMTB was essentially restricted to an orthosteric mechanism, as evidenced by the enhanced sensitivity toward the orthosteric FFA2 antagonist CATPB. In line with these findings, partial agonism of 4-CMTB progressively increased with a rank order of hFFA2-wt < hFFA2-ECL < hFFA2-K65R implying a correlation between residence and efficacy at the orthosteric site. Thus, capacity to switch 4-CMTB pharmacology to orthosteric in mutants lacking key allosteric residues and *vice versa*, i.e. to achieve both orthosteric and allosteric trapping, can only be rationalized by occupancy of two distinct sites by the same molecule, yet in a sequential manner.

Significance: Drug recognition by GPCRs is a complex multistep process. Particularly for chemokines and large peptides two-site models are well established with ligands binding to site 1 before traversing to site 2 to induce a functional response. But also for marketed GPCR drugs time-dependent adoption of different binding poses is thought to occur. Our study is significant because it expands this concept to small molecule ligands, and because it assigns functionality to individual sites adopted by the molecule along its binding pathway. We realize that considerable efforts are put into

the discovery and characterization of orthosteric, allosteric and dualsteric/bitopic ligands that allow to fine-tune cell responses and even achieve signaling bias. We also note that of the methods frequently applied to study activation mechanisms, most provide only snapshots of ligand activity. Given the growing body of evidence on drug binding pathways for GPCR ligands and recently also for endogenous transmitters - often revealed by elegant computational techniques - it may be advisable to allow real time to become a variable in functional assays thereby unveiling kinetic effects of drugs. Our study took advantage of two label-free biosensor platforms to monitor GPCR-mediated cell activation in real time and resulted in the discovery of a novel pharmacological principle to govern cell function in response to extracellular signals. We are certainly aware that label-free whole cell sensing does not uncover the fine molecular details on the receptor level as opposed to elegant FRET- or BRET-based methods which may resolve binding events in the millisecond to second range. Nevertheless, our study highlights the potential of this methodology to support identification of previously undiscovered binding sites that may only temporarily host GPCR ligands and to discover novel activation mechanisms in real time, in living cells, and in the absence of radioactive or fluorescent labels.

Experimental Procedures

Chemical compounds

Propionic acid (C3) was purchased from Sigma-Aldrich. *(S)-3-(2-(3-Chlorophenyl)acetamido)-4-(4-(trifluoromethyl)phenyl)butanoic acid* (CATPB) and *2-(4-chlorophenyl)-3-methyl-N-(thiazol-2-yl)butanamide* (4-CMTB) were synthesized as described previously in [31] and [19], respectively. The identity and purity (>99%) of CATPB and 4-CMTB were confirmed by NMR and HPLC.

Plasmids and mutagenesis

Enhanced yellow fluorescent protein (eYFP) was C-terminally fused to FFA2 receptor cDNA and subcloned into pcDNA5/FRT/TO (Invitrogen) as previously reported[30]. Fluorescent microscopy images were obtained with cells either induced with 1 µg/mL doxycycline or transiently transfected with respective receptor mutant and subsequently visualized using a 20x objective (Leica DM IL LED Fluo, Leica Microsystems). Site-directed mutagenesis in receptor cDNA in pcDNA5/FRT/TO was carried out following the QuikChange® protocol (Agilent Technologies). DpnI was used to digest template DNA and the mutated constructs were sequenced to confirm correct mutations. ECL2-swap mutant hFFA2-receptor (hFFA2-ECL) was generated as previously described [19].

Cell culture and transfections

To generate stable cell lines inducibly expressing the receptors, Flp-In™ T-REx™ 293 cells (Invitrogen) were cotransfected with pcDNA5/FRT/TO containing the receptor of interest and pOG44 (Invitrogen) in a ratio of 1:9 using a calcium phosphate DNA precipitation method according to manufacturer's instructions. pOG44 drives expression of recombinase, which enables recombination of FRT sites in the receptor-cDNA carrying plasmid and the host genome of Flp-In™ T-REx™ 293 cells. In consequence, Hygromycin B resistance introduced by pcDNA5/FRT/TO can identify receptor-transfected cells. Stably transfected Flp-In™ T-REx™ 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL), Hygromycin B (100 µg/mL) and Blasticidin (15 µg/mL) at 37°C and 5% CO_2. Untransfected Flp-In™ T-REx™ 293 host cells were maintained in DMEM supplemented with 10% (v/v) FBS, penicillin (100 U/mL), streptomycin (100 µg/mL), 100 µg/mL Zeocin and 15 µg/mL Blasticidin at 37°C and 5% CO_2. All experiments were carried out after inducing receptor expression with 1 µg/mL doxycycline for approximately 18 hours.

Dynamic mass redistribution (DMR) assay

DMR measurements were performed using either the Epic® System (Corning) or the EnSpire® System (PerkinElmer) as previously described in detail[28,29]. Briefly, 20,000 cells per well were seeded into a

384 well biosensor plate in culture medium and left to adhere for 4 – 6 hours at 37°C and 5% CO_2. Subsequently, medium was replaced by doxycycline (1 µg/mL) containing culture medium and the plate was incubated at 37°C and 5% CO_2 for approximately 18 hours to drive receptor expression. Cells were then washed at least twice with HBSS (supplemented with 20mM HEPES) and incubated for 1 hour at 37°C on the DMR reader. Compounds were diluted in HBSS (+20 mM HEPES) and added to the biosensor plate after 3 minutes of baseline read with a liquid handling system (CyBi®-SELMA, CyBio). Immediately after compound addition, DMR response was recorded for at least 1.5 hours. Where necessary, cells were preincubated with antagonist for 30 minutes.

Bioimpedance assay

Bioimpedance measurements were performed using the CellKey™ System (MolecularDevices). For this purpose, 15,000 cells were seeded into 384 well poly-*D*-lysine (PDL)-coated CellKey™ system microplates in culture medium and centrifuged at 150 x *g* for 1 minute. The plate was then incubated for 4 – 6 hours at 37°C and 5% CO_2 to allow cells to adhere to the biosensor plate. Thereafter, medium was replaced by doxycycline containing (1 µg/mL) culture medium and incubated for approximately 18 hours at 37°C and 5% CO_2. Cells were then washed with HBSS (supplemented with 20 mM HEPES and the appropriate amount of DMSO according to the compound dilutions) three times using a manifold, which leaves 5 µL left in each well. 15 µL of wash buffer was added and the plate was subsequently transferred to the impedance reader and incubated for 1 hour at 37°C. Compounds were diluted in HBSS (containing 20 mM HEPES) and DMSO amount was adjusted in all dilution steps. 25 µL of compound solution was dispensed into a 384 well compound plate and subsequently incubated in the CellKey™ system. A baseline read was then recorded for 5 minutes and compound solutions were added directly onto the biosensor plate. Changes in cellular impedance (ohms) were measured as a result of extracellular current (dZiec) for 1 hour. If needed, cells were preincubated with antagonist for 30 minutes.

Calcium flux assay

Intracellular calcium mobilization was measured using the FLIPR® Calcium 5 Assay Kit in conjunction with the FlexStation® 3 Multimode Benchtop Reader (Molecular Devices). Briefly, cells were seeded into poly-*D*-lysine coated 96 well microplates at a density of 60,000 cells per well. After 4 – 6 hours the medium was replaced by culture medium supplemented with 1 µg/mL doxycycline and incubated for 18 hours at 37°C and 5% CO_2. Thereafter, cells were loaded with the FLIPR® Calcium 5 dye for 30 minutes at 37°C and subsequently processed according to manufacturer's instructions. Where necessary, cells were preincubated with antagonist for 30 minutes.

IP and cAMP assay

Intracellular levels of the second messenger IP and cAMP were quantified with a Mithras LB 940 multimode reader (Berthold Technologies) using the HTRF®-IP-One kit and the HTRF®-cAMP dynamic kit (CisBio International), respectively, according to the manufacturer's instructions. Briefly, for the IP assay, 10,000 receptor-expressing cells were seeded into a 384 well microplate and incubated for 20 minutes at 37°C. Cells were then stimulated with agonist for 30 minutes and IP levels were quantified using the HTRF®-IP1 kit. For the cAMP assay, 3,000 cells were seeded into a 384 well microplate and incubated for 20 minutes at 37°C. Cells were stimulated with a mixture of agonist and forskolin for 30 minutes and intracellular levels of cAMP were subsequently analyzed using the HTRF®-cAMP dynamic kit. If needed, cells were preincubated with antagonist for 30 minutes.

pERK1/2 assay

Intracellular levels of phosphorylated ERK1/2 were quantified using the HTRF®-Cellul'erk kit (Cisbio International) and the Mithras LB 940 multimode reader (Berthold Technologies) following manufacturer's instructions. Briefly, 80,000 cells were seeded onto poly-*D*-lysine coated 96 well microplates and incubated for 4 – 6 hours at 37°C. Receptor expression was initiated by adding doxycycline (final concentration 1 µg/mL) and the plate was incubated for 18 hours at 37°C and 5% CO_2. Thereafter medium was replaced by starvation medium, lacking 10% FCS, and incubated for another 4 hours at 37°C and 5% CO_2. Compounds were added and pERK1/2 levels were determined

using the HTRF®-Cellul'erk kit at time points as indicated. Where necessary, cells were preincubated with antagonist for 30 minutes.

Molecular modeling and Molecular Dynamics Studies

The FFA1 crystal structure with PDB code 4PHU[32] was used as a template to generate the FFA2 homology model employing the Prime 3.0 program (Schrödinger, LLC, USA) with the default settings. The model was refined using a default energy minimization protocol implemented in Prime 3.0. Docking was conducted using Glide 6.5 (Schrödinger, LLC, USA) with the receptor grid defined by residues at positions 3.37, 4.57, 5.39, 6.51 and 7.53. The standard precision scoring function was used for docking. Modelling figures were generated with Maestro 9.9 (Schrödinger, LLC, USA). Molecular surface was built with probe radius of 0.9 Å.

Curve fitting and data analysis

All calculations were carried out using GraphPad Prism® 5.04 software (GraphPad Software). All label-free data from the DMR and bioimpedance assay were buffer-corrected and quantified as indicated. Calcium response was calculated using the maximal peak fluorescence within 80 seconds.

Author contributions

M.G. conceived the project, designed and performed the experiments, generated receptor mutants and cell lines, performed data analysis. I.G.T. conducted MD simulations, docking and homology modeling. B.D.H. generated receptor mutants. T.P.K. contributed to the hypothesis and developed the kinetic model for SEAL action. T.U. supervised chemical synthesis. G.M., B.D.H., N.J.S., T.U., I.G.T., T.P.K., K.M. contributed to discussions and edited the manuscript. M.G. and E.K. developed the hypothesis and wrote the manuscript. E.K. supervised the project.

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Figure legends

Figure 1. Label-free biosensors disclose kinetic differences between C3 and 4-CMTB

(A-D) Label-free real-time traces of HEK293 cells stably transfected with the hFFA2-wt receptor stimulated with C3 (A,C) and 4-CMTB (B,D) recorded with the impedance- and optical-based biosensor, respectively. (E-H) Concentration-response-curves (CRC) of FFA2 agonists calculated at early (E,F) and late (G,H) time points from bioimpedance (negative peak within 0 – 600 sec (E) and at 3,600 sec (G)) and DMR recordings (peak within 0 – 800 sec (F) and at 6,000 sec (H)). (I,J) Real-time signatures of 4-CMTB at the hFFA2-R255A construct in the impedance (I) and the DMR assay (J), respectively. (K,L) CRC of 4-CMTB at the hFFA2-R255A receptor calculated from early or late cell responses in the impedance (K) and DMR assay (L), respectively. Label-free signatures are shown as representative traces (mean + SEM), measured in triplicates. CRC are depicted as mean values ± SEM from three to six independent experiments. See also Figure S1 and Table S1.

Figure 2. CATPB is an orthosteric antagonist at the hFFA2-wt receptor

(A) Chemical structure of CATPB. (B,C) Effect of increasing concentration of CATPB on DMR-derived CRC of C3 at the hFFA2-wt receptor (B) with corresponding Schild plot (C). (D,E) Lack of effect of CATPB on 4-CMTB (30 µM) signaling at the hFFA2-R255A receptor in the DMR (D) and bioimpedance (E) assay. (F) Analysis of DMR data from panel (D) at different time points (500 sec vs. 6,000 sec.). Real-time recordings are shown as representative traces (mean + SEM), measured in triplicates. Quantified data are shown as mean values ± SEM of three to six independent experiments. Where not shown error bars lie within dimensions of the symbols. See also Figure S2.

Figure 3. 4-CMTB shows time-dependent sensitivity towards the orthosteric antagonist CATPB and a mutation of a key residue within the orthosteric site.

(A,B) Effect of CATPB on 30 µM C3 (A) and 4-CMTB (B)-mediated hFFA2-wt receptor activation in the optical-based label-free readout. (C,D) Effect of CATPB on hFFA2-wt Ca^{2+} flux evoked by varying

concentrations of C3 (C) and 4-CMTB (D). (E) Analysis of IC_{50}-shifts according to Cheng-Prusoff (slope: C3: 1.040 ± 0.024, r^2: 0.9946; 4-CMTB: 1.011 ± 0.045, r^2: 0.9805). (F) CATPB effect on C3 (pIC_{50}: 6.22 ± 0.39) or 4-CMTB-induced IP accumulation at the hFFA2-wt receptor. Data were baseline-corrected to remove the contribution of constitutive activity to IP signaling and CATPB responsiveness. (G) Impact of CATPB on C3 or 4-CMTB-mediated inhibition of 0.3 µM forskolin-induced cAMP production. (H) Time point-differentiated analysis of CATPB effect on 4-CMTB-mediated ERK1/2 phosphorylation. (I) Ca^{2+} flux in hFFA2-R255A expressing HEK293 cells upon stimulation with FFA2 agonists. Calcium-ionophore A23187 is shown as control. (J-L) Equilibrium CRC of C3 and 4-CMTB at the hFFA2-R255A receptor in the IP- (pEC_{50}: 4.70 ± 0.09) (J), the cAMP-(pEC_{50}: 5.96 ± 0.22) (K) and the pERK1/2-assay (pEC_{50}: 5.15 ± 0.04) (L). Label-free signatures are shown as representative traces + SEM, measured in triplicates. Bar diagrams and CRC represent mean values ± SEM of at least three independent experiments. Statistical significance was analyzed by two-tailed, unpaired Student's *t* test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. See also Figure S3 and Table S2.

Figure 4. Structural changes within the extracellular receptor region intensify susceptibility of 4-CMTB effect to interrogation at the orthosteric site level

(A,B) Effect of increasing concentrations of CATPB on DMR traces of 30 µM C3 (A) and 4-CMTB (B) activating the hFFA2-ECL receptor. (C,D) Comparison of CATPB inhibition at early time points (800 sec) on hFFA2-ECL activation by C3 (C) or 4-CMTB (D). (E,F) Comparison of CATPB inhibition at early time points (800 sec) on hFFA2-wt activation by C3 (E) or 4-CMTB (F). (G,H) Analysis of IC_{50}-shifts according to Cheng-Prusoff at the hFFA2-ECL (slope: C3: 0.992 ± 0.020, r^2: 0.996; 4-CMTB: 0.976 ± 0.046, r^2: 0.978) (G) and hFFA2-wt receptor (slope: C3: 1.049 ± 0.010, r^2: 0.999; 4-CMTB: 1.030 ± 0.029, r^2: 0.992) (H). (I) Temporal quantification of CATPB-sensitivity at the hFFA2-wt and the hFFA2-ECL receptor for 30 µM 4-CMTB. (J) 4-CMTB effect at the double mutant hFFA2-R255A-ECL in the DMR assay, Forskolin (fsk) is shown as control. (K) Ca^{2+} flux of FFA2 agonists at the hFFA2-R255A-ECL receptor, calcium-ionophore A23187 is shown as control. (L) Assessment of IP levels upon hFFA2-R255A-ECL receptor stimulation with C3 or 4-CMTB (pEC_{50}: 4.23 ± 0.03) under equilibrium conditions.

Representative real-time traces are shown as mean + SEM, measured in triplicates. Quantified data are depicted as mean values ± SEM of at least three independent experiments. See also Figure S4 and Table S3.

Figure 5. Identification of K65$^{2.60}$ as a key residue within the allosteric site that controls trapping of 4-CMTB in an orthosteric pose.

(A,B) Superimposition of FFA2 homology models. K65 is pointed towards the binding cavity in the FFA1-based model (white) due to the proline kink at position 2.58 that unwinds helix 2. This proline kink is conserved in the free fatty acid receptor family but absent in the previously used β2-adrenergic receptor-based model[45] (cyan). (C,D) Effect of increasing concentrations of CATPB on DMR signatures of 30 µM C3 (C) and 4-CMTB (D) activating the hFFA2-K65R receptor. (E) CATPB inhibition of hFFA2-K65R receptor-mediated cell activation by C3 (pIC_{50}: 6.82 ± 0.06) or 4-CMTB (pIC_{50}: 6.97 ± 0.10). (F,G) CATPB inhibition of C3 (F) or 4-CMTB (G)-elevated IP levels. (H) Analysis of IC_{50}-shifts according to Cheng-Prusoff (slope: C3: 1.007 ± 0.029, r^2: 0.990; 4-CMTB: 1.033 ± 0.050, r^2: 0.972). Representative real-time traces are shown as mean + SEM, measured in triplicates. Quantified data are depicted as mean values ± SEM of three independent experiments. See also Figure S5 and Table S4.

Figure 6.

(C) Ca^{2+}-flux and IP response after activation with C3 or 4-CMTB at the FFA2 quadruple mutant. (D) DMR traces to C3 and 4-CMTB stimulation of the FFA2 quadruple mutant transfected HEK293 cells. (E) DMR readout of HEK293 cells transiently expressing the hFFA2-K65R-R255A receptor mutant. Carbachol (Cch) is shown as control. Representative real-time traces are shown as mean values, measured in triplicates. Quantified data are depicted as mean values ± SEM of three independent experiments. See also Figure S6.

References

1. Lagerström, M. C. & Schiöth, H. B. Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nature reviews. Drug discovery* **7,** 339–357 (2008).
2. Overington, J. P. Al-Lazikani, B. & Hopkins, A. L. How many drug targets are there? *Nature reviews. Drug discovery* **5,** 993–996 (2006).
3. Conn, P. J. Christopoulos, A. & Lindsley, C. W. Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nature reviews. Drug discovery* **8,** 41–54 (2009).
4. May, L. T. Leach, K. Sexton, P. M. & Christopoulos, A. Allosteric modulation of G protein-coupled receptors. *Annual review of pharmacology and toxicology* **47,** 1–51 (2007).
5. Wootten, D. Christopoulos, A. & Sexton, P. M. Emerging paradigms in GPCR allostery: implications for drug discovery. *Nature reviews. Drug discovery* **12,** 630–644 (2013).
6. Kenakin, T. & Miller, L. J. Seven transmembrane receptors as shapeshifting proteins: the impact of allosteric modulation and functional selectivity on new drug discovery. *Pharmacological reviews* **62,** 265–304 (2010).
7. Kenakin, T. New concepts in drug discovery: collateral efficacy and permissive antagonism. *Nature reviews. Drug discovery* **4,** 919–927 (2005).
8. Kenakin, T. & Christopoulos, A. Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nature reviews. Drug discovery* **12,** 205–216 (2013).
9. Christopoulos, A. *et al.* International union of basic and clinical pharmacology. XC. multisite pharmacology: recommendations for the nomenclature of receptor allosterism and allosteric ligands. *Pharmacological reviews* **66,** 918–947 (2014).
10. Christopoulos, A. Advances in G protein-coupled receptor allostery: from function to structure. *Molecular pharmacology* **86,** 463–478 (2014).
11. Valant, C. *et al.* A novel mechanism of G protein-coupled receptor functional selectivity. Muscarinic partial agonist McN-A-343 as a bitopic orthosteric/allosteric ligand. *The Journal of biological chemistry* **283,** 29312–29321 (2008).
12. Antony, J. *et al.* Dualsteric GPCR targeting: a novel route to binding and signaling pathway selectivity. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **23,** 442–450 (2009).
13. Valant, C. Robert Lane, J. Sexton, P. M. & Christopoulos, A. The best of both worlds? Bitopic orthosteric/allosteric ligands of g protein-coupled receptors. *Annual review of pharmacology and toxicology* **52,** 153–178 (2012).
14. Lane, J. R. Sexton, P. M. & Christopoulos, A. Bridging the gap: bitopic ligands of G-protein-coupled receptors. *Trends in pharmacological sciences* **34,** 59–66 (2013).
15. Brown, A. J. *et al.* The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. *The Journal of biological chemistry* **278,** 11312–11319 (2003).
16. Le Poul, E. *et al.* Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. *The Journal of biological chemistry* **278,** 25481–25489 (2003).

17. Lee, T. *et al.* Identification and functional characterization of allosteric agonists for the G protein-coupled receptor FFA2. *Molecular pharmacology* **74,** 1599–1609 (2008).

18. Wang, Y. *et al.* The first synthetic agonists of FFA2: Discovery and SAR of phenylacetamides as allosteric modulators. *Bioorganic & medicinal chemistry letters* **20,** 493–498 (2010).

19. Smith, N. J. *et al.* Extracellular loop 2 of the free fatty acid receptor 2 mediates allosterism of a phenylacetamide ago-allosteric modulator. *Molecular pharmacology* **80,** 163–173 (2011).

20. Swaminath, G. *et al.* Mutational analysis of G-protein coupled receptor--FFA2. *Biochemical and biophysical research communications* **405,** 122–127 (2011).

21. Hudson, B. D. *et al.* Defining the molecular basis for the first potent and selective orthosteric agonists of the FFA2 free fatty acid receptor. *The Journal of biological chemistry* **288,** 17296–17312 (2013).

22. Swaminath, G. *et al.* Allosteric rescuing of loss-of-function FFAR2 mutations. *FEBS Lett.* **584,** 4208–4214 (2010).

23. Kenakin, T. P. Cellular assays as portals to seven-transmembrane receptor-based drug discovery. *Nature reviews. Drug discovery* **8,** 617–626 (2009).

24. Kenakin, T. A holistic view of GPCR signaling. *Nature biotechnology* **28,** 928–929 (2010).

25. Verrier, F. *et al.* GPCRs regulate the assembly of a multienzyme complex for purine biosynthesis. *Nature chemical biology* **7,** 909–915 (2011).

26. Scott, C. W. & Peters, M. F. Label-free whole-cell assays: expanding the scope of GPCR screening. *Drug discovery today* **15,** 704–716 (2010).

27. Schröder, R. *et al.* Deconvolution of complex G protein-coupled receptor signaling in live cells using dynamic mass redistribution measurements. *Nature biotechnology* **28,** 943–949 (2010).

28. Grundmann, M. & Kostenis, E. Label-free biosensor assays in GPCR screening. *Methods in molecular biology (Clifton, N.J.)* **1272,** 199–213 (2015).

29. Schröder, R. *et al.* Applying label-free dynamic mass redistribution technology to frame signaling of G protein-coupled receptors noninvasively in living cells. *Nat Protoc* **6,** 1748–1760 (2011).

30. Stoddart, L. A. Smith, N. J. Jenkins, L. Brown, A. J. & Milligan, G. Conserved polar residues in transmembrane domains V, VI, and VII of free fatty acid receptor 2 and free fatty acid receptor 3 are required for the binding and function of short chain fatty acids. *The Journal of biological chemistry* **283,** 32913–32924 (2008).

31. Hudson, B. D. Tikhonova, I. G. Pandey, S. K. Ulven, T. & Milligan, G. Extracellular ionic locks determine variation in constitutive activity and ligand potency between species orthologs of the free fatty acid receptors FFA2 and FFA3. *The Journal of biological chemistry* **287,** 41195–41209 (2012).

32. Srivastava, A. *et al.* High-resolution structure of the human GPR40 receptor bound to allosteric agonist TAK-875. *Nature* **513,** 124–127 (2014).

33. Mohr, K. *et al.* Rational design of dualsteric GPCR ligands: quests and promise. *British journal of pharmacology* **159,** 997–1008 (2010).

34. Redka, D. S. Pisterzi, L. F. & Wells, J. W. Binding of orthosteric ligands to the allosteric site of the M(2) muscarinic cholinergic receptor. *Molecular pharmacology* **74,** 834–843 (2008).

35. Kruse, A. C. *et al.* Structure and dynamics of the M3 muscarinic acetylcholine receptor. *Nature* **482,** 552–556 (2012).

36. Dror, R. O. *et al.* Pathway and mechanism of drug binding to G-protein-coupled receptors. *Proceedings of the National Academy of Sciences of the United States of America* **108,** 13118–13123 (2011).

37. Simpson, P. B. & Wafford, K. A. New directions in kinetic high information content assays. *Drug discovery today* **11,** 237–244 (2006).

38. Granier, S. & Kobilka, B. A new era of GPCR structural and chemical biology. *Nature chemical biology* **8,** 670–673 (2012).

39. Schadel, S. A. *et al.* Ligand channeling within a G-protein-coupled receptor. The entry and exit of retinals in native opsin. *The Journal of biological chemistry* **278,** 24896–24903 (2003).

40. Hanson, M. A. *et al.* Crystal structure of a lipid G protein-coupled receptor. *Science (New York, N.Y.)* **335,** 851–855 (2012).

41. Hurst, D. P. *et al.* A lipid pathway for ligand binding is necessary for a cannabinoid G protein-coupled receptor. *The Journal of biological chemistry* **285,** 17954–17964 (2010).

42. Pei, Y. *et al.* Ligand-binding architecture of human CB2 cannabinoid receptor: evidence for receptor subtype-specific binding motif and modeling GPCR activation. *Chemistry & biology* **15,** 1207–1219 (2008).

43. Picone, R. P. *et al.* (-)-7'-Isothiocyanato-11-hydroxy-1',1'-dimethylheptylhexahydrocannabinol (AM841), a high-affinity electrophilic ligand, interacts covalently with a cysteine in helix six and activates the CB1 cannabinoid receptor. *Molecular pharmacology* **68,** 1623–1635 (2005).

44. Kruijf, P. de *et al.* Identification of a novel allosteric binding site in the CXCR2 chemokine receptor. *Molecular pharmacology* **80,** 1108–1118 (2011).

45. Schmidt, J. *et al.* Selective orthosteric free fatty acid receptor 2 (FFA2) agonists: identification of the structural and chemical requirements for selective activation of FFA2 versus FFA3. *The Journal of biological chemistry* **286,** 10628–10640 (2011).