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REVIEW ARTICLE

# Fluorescent ligands: Bringing light to emerging GPCR paradigms

Mark Soave[1,2] | Stephen J. Briddon[1,2] | Stephen J. Hill[1,2] | Leigh A. Stoddart[1,2]

[1]Cell Signalling and Pharmacology Research Group, Division of Physiology, Pharmacology and Neuroscience, School of Life Sciences, University of Nottingham, Nottingham, UK

[2]Centre of Membrane Proteins and Receptors (COMPARE), University of Birmingham and University of Nottingham, Midlands, UK

**Correspondence**
Leigh A. Stoddart, Cell Signalling and Pharmacology Research Group, Division of Physiology, Pharmacology and Neuroscience, School of Life Sciences, University of Nottingham, Nottingham, UK.
Email: leigh.stoddart@nottingham.ac.uk

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In recent years, several novel aspects of GPCR pharmacology have been described, which are thought to play a role in determining the in vivo efficacy of a compound. Fluorescent ligands have been used to study many of these, which have also required the development of new experimental approaches. Fluorescent ligands offer the potential to use the same fluorescent probe to perform a broad range of experiments, from single-molecule microscopy to in vivo BRET. This review provides an overview of the in vitro use of fluorescent ligands in further understanding emerging pharmacological paradigms within the GPCR field, including ligand-binding kinetics, allosterism and intracellular signalling, along with the use of fluorescent ligands to study physiologically relevant therapeutic agents.

## 1 | INTRODUCTION

GPCRs are membrane proteins characterised by seven transmembrane spanning domains, an extracellular N-terminus, and an intracellular C-terminus and are responsible for the transduction of an extracellular stimulus into an intracellular response. Accounting for approximately 4% of the protein-encoding human genome (Bjarnadottir et al., 2006), they represent the largest family of proteins targeted by drugs in clinical use, with approximately 30% of all drugs targeting a GPCR. However, these targeted GPCRs represent only 30% of the non-olfactory GPCR population, and approximately 16% of all GPCRs in the human genome (Sriram & Insel, 2018), providing ample further opportunity for drug development.

Over the past 20 years, a number of novel facets of GPCR pharmacology have been described, which considerably extend the ternary complex model of GPCR activation (De Lean, Stadel, & Lefkowitz, 1980) and which play a significant role in ultimately determining the in vivo efficacy of a given compound. These include biased agonism (Kenakin & Christopoulos, 2013), binding kinetics (Swinney, Haubrich, Van Liefde, & Vauquelin, 2015), allosterism (Wang, Martin, Brenneman, Luttrell, & Maudsley, 2009), receptor dimerisation (Guidolin, Agnati, Marcoli, Borroto-Escuela, & Fuxe, 2015), and signalling from intracellular compartments (Thomsen, Jensen, Hicks, & Bunnett, 2018; Figure 1). The study of each of these phenomena has required the development of new experimental approaches, which, in many cases, have utilised fluorescently tagged ligands.

Fluorescent ligands for GPCRs are composed of an agonist or antagonist that is chemically linked to a fluorescent molecule (Vernall, Hill, & Kellam, 2014). The attachment of a fluorescent molecule can drastically alter the pharmacology of a compound, such as a change in selectivity (Vernall et al., 2013) or a significant loss in affinity compared with the parent compound (Baker et al., 2010; Sexton et al., 2011). A variety of fluorophores can be attached to the parent molecule, depending on the final application of the ligand, and these can vary significantly in size (Kuder & Kieć-Kononowicz, 2014; Vernall et al., 2014). In many cases, fluorophores that emit in near-IR wavelengths are favoured, such as Cy5 and BODIPY630/650. This avoids interference with autofluorescence from endogenous proteins and spectral crossover with commonly used fluorescent proteins such as GFP. With the development of better molecular models, based on the high-resolution structural data for a range of GPCRs, there is a greater

**Abbreviations:** BRET, Bioluminescence resonance energy transfer; FCS, fluorescence correlation spectroscopy; HILO, highly inclined and laminated optical sheet; RET, resonance energy transfer; TR-FRET, time-resolved FRET.

  

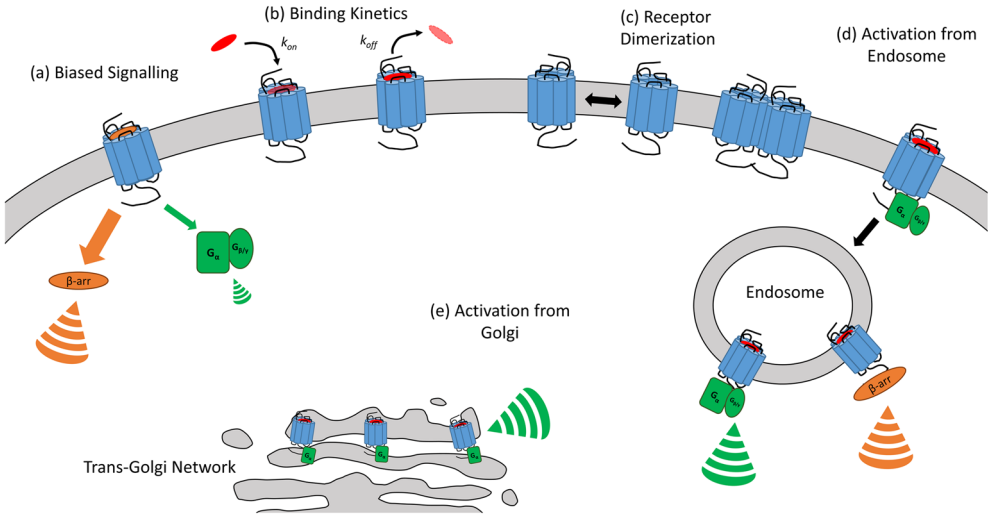


**FIGURE 1** Schematic of emerging paradigms in GPCR pharmacology. (a) Biased agonism, whereby a ligand binding to the receptor stabilises a receptor conformation that selectively couples one effector protein over another, in this case β-arrestin over heterotrimeric G proteins. (b) Ligand binding to the same receptor can have different binding rate constants, and there is a growing understanding that many clinically used drugs have non-equilibrium binding kinetics. (c) Receptor dimerisation (either homodimers or heterodimers) can alter the localisation, ligand affinity, and signalling of the respective protomers. (d) Finally, there is increasing evidence of GPCRs signalling from intracellular compartments. This has been shown at the early endosome following receptor internalisation. (e) Interestingly, there is also evidence of active GPCRs signalling from the Golgi apparatus prior to trafficking to cell membrane

understanding of where a fluorescent molecule can be attached to extend out of the binding pocket and minimise its effect on ligand affinity (Stoddart et al., 2018). Although many fluorescent ligands are still custom-synthesised in academic and industrial settings, a number are now available commercially. This increase in availability, coupled with improvements in affinity and selectivity, has widened their suitability for a range of techniques from single-molecule microscopy to high-throughput binding assays, leading to a significant rise in their use. Although there have been several reviews on fluorescent ligands for GPCRs (Sridharan, Zuber, Connelly, Mathew, & Dumont, 2014; Stoddart, Kilpatrick, Briddon, & Hill, 2015), the rapid progress of research in both ligand design and GPCR pharmacology warranted an updated discussion, and therefore, this review will focus on publications in this area from the last eight years. We discuss the diverse ways that fluorescent ligands have been used experimentally to probe emerging paradigms in GPCR pharmacology with an emphasis on ligand-binding kinetics, allosteric modulation, and ligand bias.

## 2 | EQUILIBRIUM BINDING AND COMPOUND LIBRARY SCREENING

One of the initial steps in the identification of a new drug-like molecule for a GPCR is to determine its affinity for the receptor of interest. Affinities of unlabelled compounds are often determined through the use of equilibrium competition ligand-binding experiments, whereby the effect of increasing concentrations of a test compound on the binding of a labelled compound is measured (Hulme & Trevethick, 2010). The principles of competition binding assays are the same whether a radiolabelled or fluorescently labelled ligand is used, and the ultimate aim is to estimate the amount of ligand bound. For radioligands, the amount of ligand bound is typically measured using scintillation counting, which often requires separation of bound and free ligand, whereas there are many ways to measure the amount of fluorescent ligand bound (Figure 2). Methods using fluorescent ligands generally fall into two categories: direct measurement of fluorescence or indirect measurement using resonance energy transfer (RET; Stoddart, White, Nguyen, Hill, & Pfleger, 2016). Both of these techniques have been applied to measure the binding of known ligands for a variety of receptors and in general have found good agreement of affinity constants with radioligand-binding assays (Bosma et al., 2019; Leyris et al., 2011; Nederpelt et al., 2016; Soave, Stoddart, Brown, Woolard, & Hill, 2016). This has allowed fluorescence-based assays to be accepted as a robust way to measure affinity.

Drug discovery often relies on the ability to screen large compound libraries at a protein or receptor target. Although efforts in the literature have not been focused on miniaturisation, fluorescence-based binding assays can be easily used in both 96-well (Stoddart et al., 2012) and 384-well formats (Zwier et al., 2010). It has been noted that RET-based methods such as time-resolved FRET (TR-FRET) and BRET may be more suited to high-throughput screening due to their higher signal-to-noise ratios and lower non-specific signal compared with direct fluorescence measurement-based methods (Cottet et al., 2011). These high signal-to-noise ratios mean fluorescent ligands can be used at much higher concentrations than radioligands, and consequently, ligands with relatively low affinity ($K_D$ ~500 nM) have been successfully used in a BRET-based binding assay for the **$P2Y_2$ receptor** (Conroy et al. 2018). Fluorescent ligands have been used in a number of small-scale screens (~1,000 compounds) to successfully identify novel compounds that bind to the **formylpeptide**

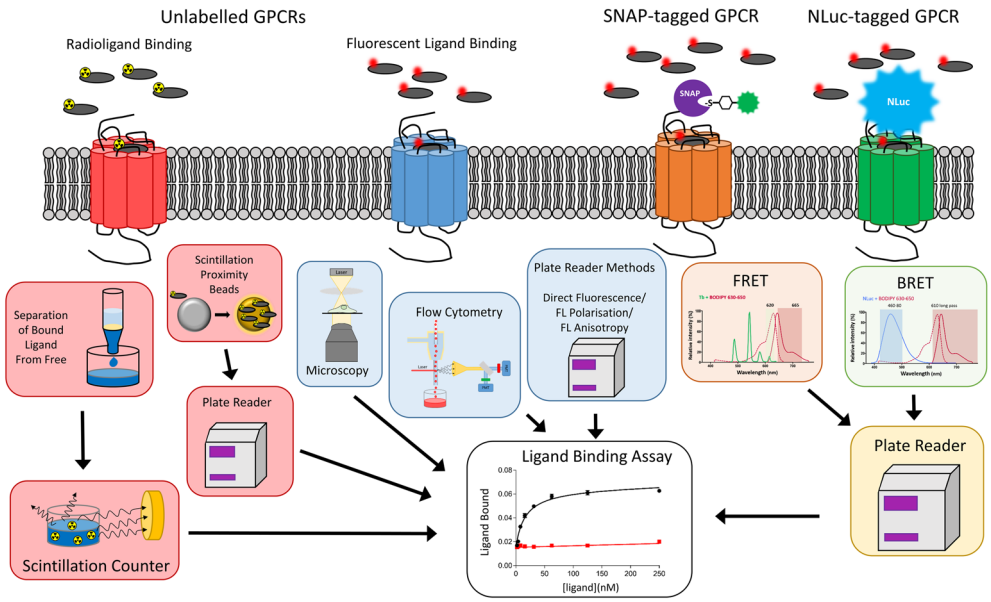


**FIGURE 2** Measuring ligand binding using fluorescence- or radioactive-based approaches. Using untagged receptors, radioligand-binding assays rely on the use of scintillation proximity technologies, or the separation of bound ligand from free ligand, followed by the detection of bound ligand using scintillation counters. Fluorescent ligands offer more versatility in the approaches that can be taken. Binding to unlabelled GPCRs can be monitored through microscopy, using plate readers to detect fluorescence, or through flow cytometry. Alternatively, the binding of fluorescent ligands to SNAP-tagged or NanoLuc-tagged GPCRs can be detected with RET approaches (FRET and BRET, respectively). These techniques often rely on plate readers to detect ligand binding, opening up the possibility of increasing the experimental throughput and miniaturisation

**receptor** (Young et al., 2005), **apelin receptor** (Iturrioz et al., 2010), and **adenosine $A_3$ receptor** (Arruda et al., 2017). These studies demonstrate the potential for fluorescent ligands to be used in screening large compound libraries at a GPCR, without the need for radioligands.

Fragment-based drug discovery is becoming a widely used tool in drug discovery and is based on screening a large number of low molecular weight (MW) compounds (typically less than 300 Da), with the aim of chemically expanding hit molecules into a lead compound with a high complexity with a relatively lower MW (Hopkins, Groom, & Alex, 2004). Fluorescence-based assays have been used to screen a small library of fragment-like molecules at the **adenosine $A_1$** and $A_3$ receptors and were successful in identifying a number of fragment molecules with moderate affinity for both receptors (Stoddart et al., 2012). In addition, this fluorescence-based study used intact, living cells for screening compounds in contrast to traditional fragment-based drug discovery methods that often used purified receptors. The use of purified receptors can expose sites in the receptor that are normally unavailable for ligand binding when the protein is within a membrane environment and lead to higher levels of false positive results. The use of a live-cell system can overcome some of these limitations and determine compound binding to a receptor in its native environment. The ability to screen low MW compounds using fluorescence assays has been further expanded to identify compounds that are selective for the $A_3$ receptor over the $A_1$ receptor (Ranganathan, Stoddart, Hill, & Carlsson, 2015). Using in silico models of the $A_3$ receptor and $A_1$ receptor, based on the then available crystal structure of the closely related **adenosine $A_{2A}$ receptor**, >0.5 million fragments were docked into these models. From this in silico modelling, a very small number of identified binders (21) were screened in the live-cell fluorescence ligand-binding assay, and eight were found to have medium affinity at the $A_3$ receptor ($K_i$ < 10 μM) with one compound displaying 29-fold selectivity over the $A_1$ receptor. Further elaboration of the hit compound using the in silico models resulted in a molecule with high affinity for the $A_3$ receptor and over 100-fold selectivity over the $A_1$ receptor (Ranganathan et al., 2015). A similar approach has recently used virtual screening that was used to identify compounds that bind to the **chemokine receptor CXCR4** (Adlere et al., 2019). In this study, the virtual hits were confirmed using a combination of radioligand and BRET ligand-binding assays, and there was a good correlation between the binding affinities obtained from both assays. Low MW compounds are thought to be a more attractive starting point in the development of a candidate molecule for clinical trials, and through the use of fluorescent ligand, it has been shown that they can be identified to bind to GPCRs in live cells, and when coupled with in silico modelling, this may be a powerful tool to develop new therapeutically active molecules.

## 3 | LIGAND-BINDING KINETICS

There is a growing understanding that measuring the affinity at equilibrium may not be the best predictor of the in vivo efficacy of a compound, as a number of clinically approved drugs show non-equilibrium modes of binding (Hothersall, Brown, Dale, & Rawlins, 2016; Kola & Landis, 2004; Schuetz et al., 2017; Waring et al., 2015). Equilibrium

affinity is a function of the association and dissociation rate constants ($K_D = k_{off}/k_{on}$) of a molecule binding to a receptor, and compounds with the same affinity can have different association and dissociation rates. Therefore, understanding a molecule's ligand-binding kinetics and incorporating this into the early stages of drug discovery may improve predictions of in vivo efficacy (Copeland, 2016; Schuetz et al., 2017). In some cases, such as for the use of **$\beta_2$-adrenoceptor** agonists in treatment of chronic obstructive pulmonary disease, compounds with slow $k_{off}$ have been shown to have better clinical outcomes (Beeh et al., 2015). Whereas in other clinical settings, such as in the targeting of the **dopamine $D_2$ receptor** in the treatment of schizophrenia, anti-psychotics with fast $k_{off}$ are thought to avoid long-term side effects (Kapur & Seeman, 2001). As with competition binding assays, ligand-binding kinetics has historically been quantified using radioligands. Multiple time points are essential to estimate kinetic rates, and in a radioligand-binding assay, a separate assay point is required for each time point. This can make them time consuming to perform and not well suited to high-throughput screening. Both direct measurement of fluorescence and RET-based fluorescent ligand-binding assays can be performed in real time using microplate readers or confocal microscopes with measurements as frequently as every second, if required, and have been widely adopted in the past 5 years to measure binding kinetics at GPCRs (Table 1 and references within table).

Fluorescent ligand-based techniques have made extensive use of the framework developed by Motulsky and Mahan (1984) to measure the binding kinetics of unlabelled ligands. In fact, one of the first applications of this method used a fluorescent peptide ligand to determine the binding kinetics of ligands to the formyl peptide receptor endogenously expressed on human neutrophils using flow cytometry (Sklar, Sayre, McNeil, & Finney, 1985). The Motulsky and Mahan method requires the kinetic ($k_{on}$ and $k_{off}$) parameters of a labelled ligand (radiolabelled or fluorescent-labelled) to be predetermined, and then by measuring the effect of an unlabelled ligand on these rates, the binding kinetics of the unlabelled ligand can be determined (Figure 3; see Bosma et al., 2019, for the Motulsky–Mahan equation used to simulate these data). Due to the ease of collecting larger datasets with fluorescence techniques, there have been several recent papers comparing the binding kinetics of unlabelled ligands measured using radioligand- and fluorescence-based methods (Bosma et al., 2019; Bouzo-Lorenzo et al., 2019; Nederpelt et al., 2016). Using the **gonadotropin-releasing hormone receptor**, Nederpelt et al. (2016)

**TABLE 1** Summary of GPCRs at which binding kinetics have been measured using fluorescent ligands

| Receptor | Fluorescent ligand | Assay format | Kinetics of unlabelled ligands determined? | Reference |
|---|---|---|---|---|
| Adenosine $A_1$ receptor | CA200645 | NanoBRET | No | Stoddart, Johnstone, et al., 2015 |
| Adenosine $A_{2B}$ receptor | XAC-X-BY630 | NanoBRET | No | White et al., 2019 |
| Adenosine $A_3$ receptor | XAC-ser-tyr-X-BY630, AV039 | NanoBRET | Yes | Bouzo-Lorenzo et al., 2019 |
| $\beta_2$-Adrenoceptor | Propranolol-ala-ala-BY630 | NanoBRET | No | Alcobia et al., 2018 |
| Calcitonin receptor | sCT8-32:AF568 | Fluorescence polarisation | Yes | Furness et al., 2016 |
| Dopamine $D_2$ receptor | PPHT-Red | TR-FRET | Yes | Klein Herenbrink et al., 2016; Sykes et al., 2017 |
| Formyl peptide receptors | Fluorescent fMLP derivative | Flow cytometry | Yes | Sklar et al., 1985 |
| Free fatty acid receptor 1 | Fluorescent derivative of TUG905 | NanoBRET | No | Christiansen, Hudson, Hansen, Milligan, & Ulven, 2016 |
| Free fatty acid receptor 2 | Fluorescent derivative of TUG-1609 | NanoBRET | Yes | Hansen et al., 2017 |
| Gonadotropin-releasing hormone receptor | Fluorescent buserelin | TR-FRET | Yes | Nederpelt et al., 2016 |
| Histamine $H_1$ receptor | mepyramine-ala-ala-BY630 (AV082), Gmep | NanoBRET, TR-FRET | Yes | Schiele et al., 2015; Stoddart et al., 2018; Bosma et al., 2019 |
| Histamine $H_3$ receptor | Bodilisant | Fluorescence polarisation | Yes | Reiner & Stark, 2019 |
| Histamine $H_4$ receptor | Clobenpropit-BY630 | NanoBRET | Yes | Mocking, Verweij, Vischer, & Leurs, 2018 |
| Melanocortin 4 receptor | UTBC101, UTBC102 | Fluorescence anisotropy | Yes | Link et al., 2017 |
| Relaxin family peptide receptor 1 | TAMRA-relaxin | NanoBRET | No | Hoare et al., 2019 |
| Oxytocin receptor | Fluorescein and DY647 derivatives of PF-3274167 | TR-FRET | No | Karpenko et al., 2015 |

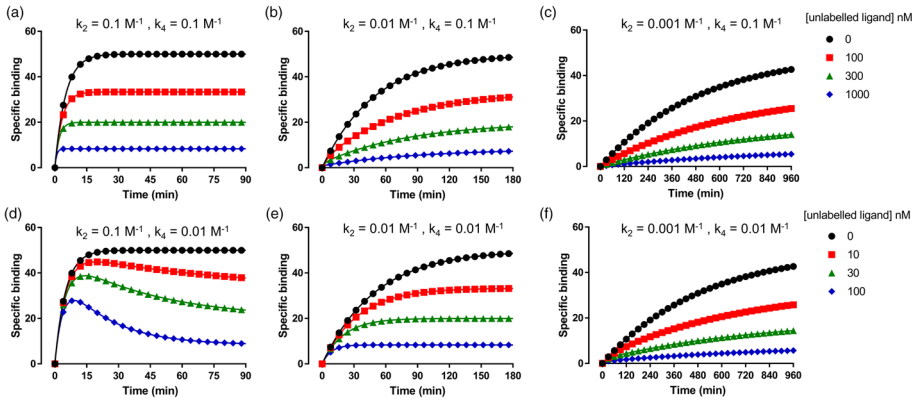


**FIGURE 3** Simulated competition association binding curves with increasing $k_{off}$ values of the labelled ligand simulations were generated in GraphPad Prism using the Motulsky and Mahan equation. $k_{on}$ (both $k_1$ and $k_3$) was set to $1 \times 10^6$ $M^{-1} \cdot min^{-1}$ for all simulated data (see Bosma et al., 2019, for full equation on the Motulsky–Mahan model). For (a, d), $k_{off}$ ($M^{-1}$) of the labelled ligand ($k_2$) was set to 0.1, (b, e) to 0.01, and (c, f) to 0.001, simulating a 10-fold increase in $k_{off}$ each time and a "fast," "slow," and "very slow" labelled ligand. The concentration of labelled ligand (L) was set to the $K_D$ of each ligand (100 nM for a and d, 10 nM for b and e, and 1 nM for c and f). For (a–c), $k_{off}$ of the unlabelled ligand ($k_4$) was set to 0.1, and for (d-f), it was set to 0.01, simulating an unlabelled ligand with a "fast" and "slow" $k_{off}$. The concentration of the unlabelled ligand was set to 1, 3, and 10 times its $K_D$ (100, 300, and 1,000 nM for a–c and 10, 30, and 100 nM for d-f). In (d), the classic "overshoot" of an unlabelled ligand with a slower $k_{off}$ than the labelled ligand can be observed, whereas when a labelled ligand with a slower $k_{off}$ value is used, it becomes more difficult to distinguish between "slow" and "fast" unlabelled ligand

compared the binding kinetics of a number of agonists measured in radioligand and TR-FRET assays. In this study, measured dissociation rates ($k_{off}$) at the gonadotropin-releasing hormone receptor were compared well across both assays, whereas the correlation in association rates ($k_{on}$) was poor. For the adenosine $A_3$ receptor, a comparison of radioligand and NanoBRET (BRET with the small bright luciferase Nanoluc as the donor) assays using labelled ligands with markedly different binding kinetics suggested that it is difficult to determine the kinetics of fast binding unlabelled ligands when using a probe with a very slow off rate (Bouzo-Lorenzo et al., 2019). Using the **histamine $H_1$ receptor** as a model system, Bosma et al. (2019) compared the binding kinetics of a number of unlabelled antagonists using two radioligands and both NanoBRET and TR-FRET fluorescence-based assays. This large dataset allowed multiple comparisons to be made and found that for the $H_1$ receptor, the association rates ($k_{on}$) compared well across all three assay set-ups, and there was good correlation between the dissociation rates measured in the radioligand and TR-FRET assays but not with the NanoBRET assays. By examining the accuracy of the binding rate constants (using the magnitude of error in the fit of the data to the non-linear equations), it was found that the error for both $k_{on}$ and $k_{off}$ of unlabelled ligands increased if they had considerably faster or slower binding rate constants than the labelled ligand (Bosma et al., 2019).

These data from both the $H_1$ receptor and the $A_3$ receptor indicate that the kinetics of the probe ligand can mask the binding kinetics of the unlabelled ligand if they are significantly different from those of the probe. This may help to explain the data with the gonadotropin-releasing hormone receptor, as there was a 50-fold difference in the association rate of the radioligand compared with the fluorescent ligand. As demonstrated by the simulated data in Figure 3 using the Motulsky and Mahan equations, binding rates of unlabelled ligands with significantly faster kinetics than the labelled ligand can be difficult to distinguish even with 10-fold difference in their $k_{off}$ values. As the Motulsky and Mahan method is the most commonly used method to measure binding kinetics of unlabelled ligands, the above examples emphasise the importance of selecting a labelled probe with the appropriate kinetics for accurate determination of test compounds. It is also becoming clear that a probe with fast binding kinetics may be more generally applicable for measuring the kinetics of an unlabelled ligand (Bosma et al., 2019; Sykes, Jain, & Charlton, 2019). As radioligands are only subtly different from the parent pharmacophore, differences between radioligand probe binding kinetics are often less pronounced. Fluorescent probes differ much more widely in their chemical structure as a result of using chemically diverse fluorophores and linkers. Consequently, marked differences have been observed in the binding kinetics of fluorescent ligands with the same pharmacophore but differing fluorescent labels and linker compositions (Bouzo-Lorenzo et al., 2019). With increasing knowledge of the structural and chemical factors influencing ligand-binding kinetics, rationale design of fluorescent ligands with the required binding kinetics for specific compound sets using the Motulsky and Mahan method may become possible.

Investigating binding kinetics using fluorescence ligands can also reveal interesting aspects of GPCR pharmacology. The **relaxin family peptide receptor 1** (RXFP1) contains leucine-rich repeats in its N-terminus with which the endogenous ligand **relaxin** interacts (Bullesbach & Schwabe, 2005). Additional interaction points have been identified in an extracellular linker region (Sethi et al., 2016). To understand the interplay of these regions on binding of the ligand, Hoare et al. (2019) used a fluorescently labelled relaxin in a kinetic NanoBRET assay. Using kinetic assays, the authors uncovered the multi-step binding of relaxin to the RXFP1 receptor. By first assuming a single-step binding mechanism, they noticed a difference in the affinity of relaxin obtained under kinetic association experiments

compared with the equilibrium saturation assays. Upon further investigation, relaxin was shown to exhibit a two-phase dissociation profile at the RXFP1 receptor, indicative of a multi-step binding process. In addition, the authors demonstrated that the receptor had at least two relaxin-bound states and that, when the linker region was removed, a single-step binding mechanism was achieved confirming that this region is important for ligand binding (Hoare et al., 2019). The ability to gain detailed time-resolved NanoBRET data on fluorescent ligand binding to the receptor was crucial for the interpretation of these data, and this would have been much more challenging to obtain with a traditional radioligand-binding assay. An important recent study of dopamine $D_2$ receptor related binding kinetics to drug effects at this receptor. The $D_2$ receptor is the main target of antipsychotic drugs used in the treatment of schizophrenia, but extrapyramidal motor side effects caused by these compounds often limit their use (Miyamoto, Miyake, Jarskog, Fleischhacker, & Lieberman, 2012). Sykes et al. (2017) measured the binding kinetics of a wide range of antipsychotic drugs using a fluorescent $D_2$-antagonist in a high-throughput TR-FRET-based method and correlated these measured to side effects. They demonstrated that side effects correlated with association rates rather than dissociation rates (Sykes et al., 2017). This knowledge may help to direct future development of novel antipsychotics with optimal binding kinetics with the aim of reducing side effects. The increase in throughput associated with fluorescence-based kinetic assays allows much larger data sets to be generated and enables insights into both basic and therapeutic pharmacology of GPCRs.

## 4 | INFLUENCE OF BINDING KINETICS ON BIASED AGONISM

Many GPCRs have been suggested to exhibit biased agonism (Chen et al., 2012; Chen et al., 2013; Rankovic, Brust, & Bohn, 2016), namely, the ability of ligands acting at the same GPCR to stabilise different active conformations of the receptor. Typically, this has been characterised by the ability of a ligand to preferentially signal via G protein- or β-arrestin-mediated pathways, often measured with endpoint assays after a given stimulation time. The ability to use biased ligands to activate a given pathway has been potentially linked to improved therapeutic outcomes (Bohn et al., 1999; Rominger, Cowan, Gowen-MacDonald, & Violin, 2014).

A number of studies have described biased ligands at the $D_2$ receptor, such as **aripiprazole**, as having improved antipsychotic efficacy, but there have been contradictory studies as to the exact nature of this signalling bias (Allen et al., 2011; Masri et al., 2008; Szabo, Klein Herenbrink, Christopoulos, Lane, & Capuano, 2014; Urban, Vargas, von Zastrow, & Mailman, 2007). Many of these studies compare multiple endpoint signalling readouts from assays performed using different incubation times. To reconcile these discrepancies, Klein Herenbrink et al. (2016) considered the influence of ligand-binding kinetics on perceived biased agonism. Using a fluorescently labelled $D_2$ receptor agonist in a TR-FRET-based experimental system, it was possible to monitor the binding kinetics of a panel of dopamine ligands. The ligands with slower dissociation kinetics as determined with this approach (including aripiprazole) changed their apparent G protein versus β-arrestin bias profile over time (Klein Herenbrink et al., 2016). The data presented by Klein Herenbrink and colleagues suggested that the perceived biased agonism of compounds such as aripiprazole at the $D_2$ receptor was in fact due to differences in ligand-binding kinetics and differences in timings of the endpoint assays.

This is not a universal phenomenon, however, and not all observations of ligand bias can be explained by differences in binding kinetics. For the human **calcitonin receptor**, it has been shown that the human and salmon variants of the endogenous peptide **calcitonin** display differential potency and efficacy at the cAMP pathway (Andreassen et al., 2014). In contrast to the example of the $D_2$ receptor, kinetic assays using a fluorescent version of salmon calcitonin and chimeric peptides confirmed that the differential effects of human and salmon calcitonin were not due to differences in their binding kinetics (Furness et al., 2016). Signalling differences between these two variants of calcitonin arose instead from agonist-dependent differences in the conformational change induced in the receptor–G protein complex. These two examples highlight the importance of first determining ligand-binding kinetics before ascribing biased agonism. The relative ease of measuring binding kinetics using fluorescent ligands should allow binding kinetics to be evaluated routinely for ligands suggested to display biased agonism in endpoint signalling assays.

## 5 | GPCR ALLOSTERISM

Ligands that bind to a GPCR on a topographically distinct site from the orthosteric ligand are termed allosteric modulators. They can elicit a conformational change in the receptor that modulates the binding of orthosteric ligands (Kenakin, 2012; May, Leach, Sexton, & Christopoulos, 2007). This so-called cooperativity between the two ligand-binding sites is a central principle of allosterism (May et al., 2007), and the effect of the allosteric modulator on the affinity or efficacy of an orthosteric ligand is reciprocal. Furthermore, the reciprocity of the interaction gives rise to the phenomenon of probe dependence whereby the extent and nature of allosteric modulation can differ depending on the orthosteric ligand used (Kenakin, 2012).

As with biased agonism, research into GPCR allosterism has been facilitated by specialised receptor-specific fluorescent ligand probes. For the adenosine $A_1$ receptor, negative allosteric modulators have a potential therapeutic application in conditions where high levels of adenosine are released, such as after an ischaemic incident. To understand how these molecules interact with the $A_1$ receptor bound to compounds with different intrinsic efficacies, Cooper et al. (2019) used NanoBRET saturation binding assays to directly measure the effect of the positive allosteric modulators VCP171 (Aurelio et al., 2009) and PD 81,723 (Bruns & Fergus, 1990) on fluorescent agonist and antagonist binding to human and rat $A_1$ receptors. The fluorescent agonists used in this study were derived from the endogenous $A_1$ receptor ligand, **adenosine** (ABA-X-BY630) or from the full agonist

NECA (ABEA-X-BY630, and AAG-ABEA-X-BY630), and the fluorescent antagonist was based on the non-selective adenosine receptor antagonist XAC (CA200645). While the allosteric modulators had little effect on the binding of the fluorescent antagonist, they enhanced the affinity of both fluorescent and non-fluorescent agonists. Interestingly, at the rat $A_1$ receptor, VCP171 increased the maximal binding capacity ($B_{max}$) with all three fluorescent agonists but only significantly increased the affinity of AAG-ABEA-X-BY630 (Cooper et al., 2019). This increase in agonist-specific affinity suggested that the allosteric modulators were allowing a larger proportion of the receptor population to adopt a high-affinity, R*, confirmation. The availability of fluorescent ligands with differing intrinsic efficacy (i.e., agonists and antagonists) was the cornerstone of the study and with the development of more fluorescent ligands will allow similar studies to be performed on different GPCR subtypes.

The five muscarinic acetylcholine receptors ($M_1$–$M_5$) have a principal role in the central and parasympathetic nervous system and as such have been the target of a number of drug development efforts over the past decades, including for the treatment of schizophrenia, Alzheimer's disease, and chronic obstructive pulmonary disease (Felder et al., 2018; Melancon, Tarr, Panarese, Wood, & Lindsley, 2013). These efforts have been hampered, as many of the developed ligands lack subtype selectivity due to the high conservation of the orthosteric binding site in these receptors (Kruse et al., 2013), and emphasis has been placed on the discovery of novel allosteric modulators that target less conserved allosteric sites (Felder et al., 2018). In one approach, the Ilien group developed a series of fluorescent bitopic ligands against the $M_1$ receptor. These ligands were based on the orthosteric antagonist pirenzepine (Daval et al., 2013) or the known $M_1$ allosteric modulator AC-42 (Daval et al., 2012). Pharmacological characterisation of this ligand series with FRET suggested a bitopic mode of ligand binding, with the fluorescent ligands able to occupy both the orthosteric and allosteric ligand-binding pockets on the same $M_1$ receptor. Altering the linker length between the pharmacophore and the fluorophore conferred differential abilities to occupy the allosteric binding site (Daval et al., 2013), demonstrating the application of allosteric fluorescent molecules for GPCR drug discovery.

However, it is not only low MW ligands that are allosteric modulators—proteins that interact with the receptor and change the pharmacology of a ligand are also considered to be allosteric modulators (Kenakin, 2012). This intermolecular allosterism can occur as a result of the receptor interacting with other membrane bound proteins, such as receptor activity-modifying proteins (Gingell et al., 2016; Hay & Pioszak, 2016), or can be applied to intracellular signalling proteins (such as G proteins or β-arrestin) as they also bind the receptor and result in changes in affinity or efficacy of particular agonists (Hill, May, Kellam, & Woolard, 2014; Kenakin, 2012). Of note for this review, the interactions between two or more receptors forming dimers or higher order oligomers can also be allosteric (Gherbi, May, Baker, Briddon, & Hill, 2015; Hill et al., 2014; Hiller, Kuhhorn, & Gmeiner, 2013; May, Bridge, Stoddart, Briddon, & Hill, 2011). Fluorescent ligands have been successfully used to detect the presence of GPCR heterodimers on the cell surface (Hounsou et al., 2014; Loison et al., 2012). This relies on the ability to use a fluorescent ligand to specifically label one protomer within a dimer whilst the other protomer is labelled with a fluorescent tag. However, a more nuanced approach is required for homodimers, as the fluorescent ligand in question has the potential to bind each protomer equally if there is no cooperativity. This obstacle has been tackled through the application of fluorescent ligands to continuously monitor the dissociation kinetics of the fluorescent ligand in real time using confocal microscopy coupled to continued perfusion of buffer in the presence or absence of other ligands (Gherbi et al., 2015; May et al., 2011). By using low concentrations of the fluorescent probe, it was possible to only occupy one of the protomers within a dimer. This particular methodology is highly sensitive to detecting cooperativity between two orthosteric binding sites that may exist within a GPCR dimer. May et al. (2011) used a fluorescently labelled adenosine receptor agonist (ABA-X-BY630) to monitor cooperativity across adenosine $A_1$ and $A_3$ receptor homodimers. Under these conditions, the dissociation rate constant of the fluorescent agonist was enhanced when an $A_3$ receptor homodimer was exposed to a second ligand. This was strong evidence for negative cooperativity across the dimer interface. Mutating a key residue in the orthosteric ligand-binding pocket of one of the dimer protomers abolished this cooperativity (May et al., 2011).

A similar approach was later taken at the $\beta_1$-adrenoceptor to determine if the observed secondary low-affinity conformation was a result of receptor oligomerisation. Non-conventional β-adrenoceptor ligands, such as CGP 12177 and pindolol, antagonised catecholamine-mediated responses with high affinity and were also able to stimulate a partial agonist response at much higher ligand concentrations. Gherbi et al. (2015) used BODIPY-TMR-CGP (a fluorescent analogue of CGP 12177; Heithier et al., 1994) to investigate whether this ability to exhibit high-affinity antagonism with low-affinity partial agonism was a result of cooperativity across $\beta_1$-adrenoceptor dimers. As with the adenosine $A_3$ receptor, the dissociation rate constant of the fluorescent ligand was increased when in the presence of second "orthosteric" ligand. This negative cooperativity was abolished by mutating residues in the transmembrane domain 4 of the $\beta_1$-adrenoceptor to that of the $\beta_2$-adrenoceptor (Gherbi et al., 2015). These data suggested that the secondary low-affinity pharmacology of the $\beta_1$-adrenoceptor was a result of negative cooperativity across a homodimer. These two studies were possible only with the ability to continuously monitor the dissociation kinetics of the tracer ligand in real time, an experimental facet that would have been technically challenging with radioligands.

## 6 | INTRACELLULAR SIGNALLING OF GPCRs

Another exciting recent development in the GPCR field has been the discovery that receptors can signal from intracellular compartments, including endosomes (Calebiro, Godbole, Lyga, & Lohse,

2015; Irannejad et al., 2013) or the trans-Golgi network (Godbole, Lyga, Lohse, & Calebiro, 2017). Fluorescent ligands have been used to monitor GPCR internalisation and have found a niche in labelling untagged or endogenously expressed GPCRs (Brand, Klutz, Jacobson, Fredholm, & Schulte, 2008; Lam et al., 2018; Margathe et al., 2016; Stoddart, Vernall, Briddon, Kellam, & Hill, 2015). It is important to note that the trafficking and organisation of GPCRs can change depending on the expression level (Ward, Pediani, Godin, & Milligan, 2015) and can be cell-type specific (Eichel & von Zastrow, 2018; Werthmann, Volpe, Lohse, & Calebiro, 2012). Calebiro et al. (2015) used a fluorescently labelled **thyroid stimulating hormone** (TSH) to monitor the internalisation of the **TSH receptor** in primary murine thyroid cells. The TSH receptor is a $G_s$-coupled GPCR, and thus, activation of these receptors results in an increase in cAMP production. They combined the fluorescent ligand with highly inclined and laminated optical sheet (HILO) microscopy to achieve the high degree of spatio-temporal resolution required to visualise internalisation of TSH receptors at endogenous levels of expression. Stimulation of the TSH receptor results in a sustained increase in intracellular cAMP levels, and by blocking internalisation through the use of dynamin inhibitors, this sustained response was lost (Calebiro et al., 2015). Further work demonstrated that this sustained response mediated by internalised receptors was occurring in the trans-Golgi network, and this localised perinuclear cAMP signalling was required for efficient CREB-mediated gene transcription (Godbole et al., 2017). Taken together, these data revealed the ability for the TSH receptor to signal from the endosomes and Golgi apparatus in primary cells, providing real insight into the mechanisms of TSH receptor action in an endogenous system. This study was possible due to the ability to use fluorescent TSH ligand to label TSH receptors with a high degree of specificity.

## 7 | FLUORESCENT LIGANDS FOR PHYSIOLOGICALLY RELEVANT SYSTEMS

The advent of CRISPR/Cas9-mediated genome engineering (Cong et al., 2013) has been a major breakthrough in cell biology. CRISPR/Cas9 has enabled the genetic modification of endogenously expressed proteins, including appending bioluminescent or fluorescent tags to the C-terminus of endogenous GPCRs (Figure 4a; White, Vanyai, See, Johnstone, & Pfleger, 2017). White, Johnstone, See, and Pfleger (2019) used CRISPR/Cas9 to generate an N-terminally NanoLuc™-tagged **adenosine $A_{2B}$ receptor** and performed NanoBRET ligand binding in HEK293 cells. The endogenous expression level of the $A_{2B}$ receptor in HEK293 cells has been shown to be sufficient for a robust functional responses upon ligand stimulation (Goulding, May, & Hill, 2018). White et al. (2019) found that the luminescence generated in the genome-edited cells was similar to those transiently transfected with a very low amount of NLuc-$A_{2B}$ cDNA. However, despite this low luminescence, robust fluorescent ligand binding could be measured to the genome-edited cells with NanoBRET. The resulting fluorescent ligand affinity was similar to that measured with HEK 293 cells transiently transfected with NLuc-$A_{2B}$ cDNA (White et al., 2019). Furthermore, competition and kinetic NanoBRET experiments yielded similar ligand affinities to those determined in cells exogenously expressing NLuc-$A_{2B}$. This study represents the first time NanoBRET ligand binding has been performed at receptors expressed under endogenous promotion. This opens up the possibilities of using fluorescent ligands to interrogate ligand binding and binding kinetics to receptors expressed at endogenous levels rather than in an overexpression system.

Fluorescent ligands have been used to elucidate new roles for endogenously express GPCRs in cellular responses. In neutrophils, the adenosine $A_3$ receptor has been shown to be a key regulator

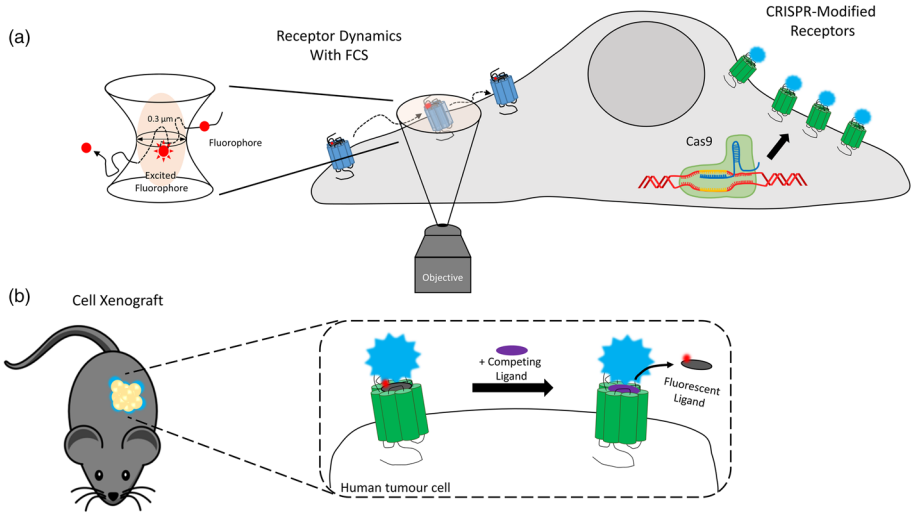


**FIGURE 4** Uses of fluorescent ligands to study GPCR pharmacology in vitro at endogenously levels of expression and in vivo. in vitro uses shown in (a) include using fluorescence correlation spectroscopy to measure receptor dynamics of an endogenously expressed GPCR labelled with a fluorescent ligand. This continues with using CRISPR/Cas9 to genetically modify the GPCR with NanoLuc to perform NanoBRET binding studies. In (b) human, tumour cells stably transfected with NanoLuc-tagged GPCRs were implanted into immune-compromised mice as a xenograft. NanoBRET binding studies were performed to monitor fluorescent ligand binding to these GPCRs in vivo

of function in response to chemoattractant stimulation and, in fixed cells, the receptor was localised in a highly polarised manner to the leading edge of the cell (Chen et al., 2006). Using a fluorescent antagonist for the $A_3$ receptor allowed the localisation of the receptor to be monitored in live cells and further investigate the role of the receptor (Corriden et al., 2013). It was shown that the $A_3$ receptor was localised predominantly in plaques at the base of cytonemes—filopodia-like projections that neutrophils use to tether bacteria ahead of phagocytosis. These experiments showed the $A_3$ receptor to have an integral role in the formation of these projections.

However, studying GPCRs at endogenous levels is technically challenging, often due to the low expression of these proteins found in many recombinant cell lines. Better understanding of GPCR dynamics requires the use of highly sensitive techniques to overcome these technical difficulties. The sensitive imaging technique of fluorescence correlation spectroscopy (FCS) represents a useful alternative experimental approach for monitoring the dynamics of GPCRs at low expression levels (Briddon & Hill, 2007; Briddon et al., 2018). Paired with selective fluorescent ligands, FCS can quantify the pharmacology of GPCRs at the subcellular level (Figure 4a). Rose, Briddon, and Hill (2012) used a fluorescent derivative of the histamine $H_1$ receptor antagonist **mepyramine** to characterise endogenous $H_1$ receptors in HeLa cells with FCS. This ligand showed high affinity for the $H_1$ receptor, but it also displayed high levels of non-specific binding and intracellular uptake of the ligand, which made the ligand unsuitable for the conventional confocal microscopy-based ligand-binding assays discussed previously (Arruda et al., 2017; Stoddart et al., 2012). However, the high sensitivity and diffraction-limited measuring volume of FCS allowed measurements to be taken in small areas of the cell membrane in the absence of significant cytosolic contamination and detect specific binding of ligand to the endogenous $H_1$ receptor (Rose et al., 2012). FCS measurements of the fluorescent ligand-bound $H_1$ receptor suggested a difference in the macromolecular organisation of the receptors in HeLa cells compared with that measured in CHO cells when the $H_1$ receptor was overexpressed. In an orthogonal approach to study endogenous expression of GPCRs, Soethoudt et al. (2018) utilised a modified antagonist for the **cannabinoid $CB_2$ receptor** that contained both a photoaffinity label and a ligation handle. The photoaffinity label covalently cross-linked the antagonist to the receptor upon UV irradiation, and the ligation handle allowed the conjugation of the antagonist, which is attached to the receptor, to a fluorophore. This permanent labelling approach allowed the expression of $CB_2$ receptors in peripheral blood mononuclear cells to be profiled using FACS analysis, and they demonstrated that the $CB_2$ receptor was highly expressed in $CD19^+$ B cells. These studies demonstrate that through the use of advanced imaging and labelling technologies, fluorescent ligands can be used to study GPCRs in endogenously expressing systems.

Although fluorescent ligands have been used to probe GPCR pharmacology in in vitro systems with great success, their ability to be used in vivo has been limited. Recently, however, several groups have used fluorescent ligands to monitor GPCR target engagement in vivo using emerging imaging technologies and new advances in fluorescent moieties. In one approach, Ma et al. (2016) developed an $\alpha_1$-**adrenoceptor** antagonist conjugated to a caged Cy5 moiety that would fluoresce brightly in the near-IR range (700–3,000 nm), which increases tissue penetrance of fluorescence in vivo. The quinazoline-conjugated Cy5 displayed a 10-fold increase in fluorescence upon binding $\alpha_1$-adrenoceptors in vitro. The fluorescent ligand (compound 3a in this study) bound $\alpha_1$-adrenoceptors located in ex vivo slices of murine prostate, demonstrating the ability to measure endogenous levels of receptor expression with compound 3a. Additionally, compound 3a was able to bind tumour xenografts that were implanted into mice containing cell lines that expressed the $\alpha_1$-adrenoceptor and found that compound was 3a was localised in organs with high $\alpha_1$-adrenoceptor expression when administered intravenously. Building upon the development of NanoBRET to monitor GPCR–ligand binding in living cells using the bright luciferase NanoLuc, Alcobia et al. (2018) sought to implement NanoBRET in vivo. They used a fluorescent analogue of the β-adrenoceptor antagonist **propranolol** (Prop-BY630) that had previously been characterised at β-adrenoceptors with NanoBRET in vitro (Soave et al., 2016; Stoddart et al., 2015). Alcobia et al. (2018) used the metastatic triple-negative breast cancer MDA-MB-231 cell line stably transfected with the NLuc-$\beta_2$-adrenoceptor as their model cell since propranolol has been shown to prevent breast cancer metastasis in murine models (Sloan et al., 2010). In a murine tumour xenograft model, the brightness of NanoLuc enabled the detection of luminescence from within a tumour cell mass upon the intravenous administration of the NanoLuc substrate, furimazine, and when Prop-BY630 was administered intratumourally, an increase in the BRET signal was observed (Figure 4b). The specificity of this signal was confirmed by the ability of the $\beta_2$-adrenoceptor selective antagonist **ICI 118551** to reduce the BRET ratio (Alcobia et al., 2018). This study demonstrated the ability to monitor target engagement in vivo using a fluorescent ligand and NanoBRET, and this approach could be implemented for other targets, including those involved with cancer progression, such as the **receptor tyrosine kinase VEGFR2** (Kilpatrick et al., 2017) or intracellular kinases (Robers et al., 2015).

## 8 | FUTURE DIRECTIONS

Advances in the field of GPCR pharmacology and drug discovery have been facilitated by the use of fluorescent ligands. With the increasing emphasis on improving efficacy rates in clinical trials, it is more important than ever to fully validate the pharmacology of lead candidates and to quantify this under non-equilibrium (kinetic) conditions. The kinetic approaches described above show the RET technologies can quantify ligand-binding kinetics in whole cells and membranes and how ligand-binding kinetics can potentially relate to side effects in the clinic. The combination of CRISPR/Cas9 and BRET techniques also offers an avenue to study ligand–receptor interactions at endogenous levels. Furthermore, improving subtype selectivity of fluorescent ligands will enable the labelling and monitoring of specific receptors in primary cells.

These represent more physiologically relevant models to study these emerging paradigms of GPCR pharmacology. With improved physiochemical properties of fluorescent ligands, such as improved hydrophilicity and fluorescence emission, these techniques can be applied in vivo as a powerful tool for monitoring target engagement and residence time.

## 8.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Christopoulos et al., 2019; Alexander, Fabbro et al., 2019).

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### CONFLICT OF INTEREST

The authors declare no conflicts of interest.

### ORCID

*Mark Soave* https://orcid.org/0000-0002-9199-1772
*Stephen J. Briddon* https://orcid.org/0000-0001-8514-0827
*Stephen J. Hill* https://orcid.org/0000-0002-4424-239X
*Leigh A. Stoddart* https://orcid.org/0000-0002-4469-0600

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