Using Biosensors to Study Free Fatty Acid Receptor Pharmacology and Function

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Abstract

The free fatty acid (FFA) family of G protein coupled receptors (GPCRs) has generated significant interest for exploiting its members as potential drug targets. However, unravelling the complex pharmacology of this family of receptors has proven challenging. In recent years the use of biosensor technologies capable of assessing biological functions in living cells, and in real time, has greatly enhanced our ability to study GPCR pharmacology and function. These include genetically encoded sensors that change the intensity or wavelength of light emitted from a bioluminescent or fluorescent protein in response to a stimulus, as well as non-genetically encoded sensors able to measure more global cellular changes, such as mass redistribution within a cell. This chapter will examine

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how these sensors can be used to study GPCRs, and in particular how they are helping uncover the pharmacology of the FFA family of receptors.

Keywords
Bioluminescence resonance energy transfer (BRET) • Biosensor • Fluorescence resonance energy transfer (FRET) • Free fatty acid • G protein coupled receptor (GPCR) • Label-free biosensor

1 Introduction

Fatty acids are biomolecules that mediate many important biological processes. Fatty acids are defined chemically as a carboxylic acid head group attached to an aliphatic tail, and thus are structurally diverse, based on the composition of their aliphatic tail. The fatty acid aliphatic tail varies by carbon chain length, where examples shorter than 6 carbon are deemed short chain fatty acids (SCFAs), those between 7 and 12 carbon are medium chain fatty acids (MCFAs) and those longer than 12 are long chain fatty acids (LCFAs). In addition to chain length, the MCFAs and LCFAs are also defined by the number, position and conformation of unsaturations in their aliphatic tail. Together, the fatty acids constitute a complex group of biomolecules associated with diverse biological effects.

Historically fatty acids were believed to produce their biological effects primarily as building blocks for the phospholipids, or as a source of energy. However, it has become clear that fatty acids are also signalling molecules. Of particular interest, a family of four G protein coupled receptors (GPCRs) is now recognized as the free fatty acid (FFA) receptor family. This currently includes four members: FFA1 (previously GPR40), FFA2 (previously GPR43), FFA3 (previously GPR41) and FFA4 (previously GPR120) (Davenport et al. 2013; Stoddart et al. 2008). Of these FFA1 and FFA4 respond to MCFAs and LCFAs, while FFA2 and FFA3 respond to the SCFAs. Fatty acid signalling through each receptor regulates key aspects of metabolism and/or inflammation and, therefore, it is not surprising that each FFA receptor has received interest as a possible target for metabolic and/or inflammatory disease. However, before the full therapeutic potential of these receptors can be realized there is a need to better understand the complex pharmacology of this family. In this chapter I will examine how biosensor technologies are being used to study GPCRs and, specifically, uncover the detailed pharmacology and function of the FFA family.

2 GPCR Pharmacology and Function

The GPCR superfamily is the single largest family of membrane proteins, comprised of more than 800 members (Fredriksson et al. 2003). GPCRs are classically defined by their conserved seven transmembrane structures, as well as

by their ability to interact with and transduce signals through guanine nucleotide binding G proteins. These features allow GPCRs to activate intracellular signalling pathways in response to diverse extracellular stimuli, which has made these receptors the most historically successful drug targets. In recent times, however, it has become apparent that the signal transduction, pharmacology and function of GPCRs are far more complex than previously believed and, indeed, that there is a clear need to develop new approaches to study the complex actions of these receptors.

2.1 GPCR Signal Transduction

The classic GPCR signal transaction pathway involves in order: (1) agonist ligand binding to the GPCR; (2) the GPCR adopting an active conformation; (3) the active GPCR interacting with a heterotrimeric ($\alpha\beta\gamma$) G protein; (4) exchange of GTP for GDP on the G protein α subunit causing activation and dissociation of the α and $\beta\gamma$ subunits and (5) the Gα and G$\beta\gamma$ subunits regulating downstream effectors. The specific signalling pathways activated are largely defined by the particular Gα involved where: $G\alpha_s$ activates adenylyl cyclase to increase cAMP; $G\alpha_{i/o}$ inhibits adenylyl cyclase to decrease cAMP; $G\alpha_{q/11}$ activates phospholipase C leading ultimately to an increase in intracellular Ca^{2+} concentration and $G\alpha_{12/13}$, which typically activates a small GTPase RhoA-mediated signalling pathway. Traditionally, a given receptor was associated with one specific Gα, thus, for example, the β_2-adrenoceptor was commonly associated with $G\alpha_s$, therefore activation of this receptor would be primarily viewed to result in an increase in intracellular cAMP.

In addition to the classic GPCR activation pathway, there is also a well-established pathway for the inactivation of GPCR signalling. This involves: (1) phosphorylation of serine/threonine residues in the third intracellular loop or carboxy-terminal of the GPCR by a G protein receptor kinase (GRK); (2) recruitment of an arrestin to the phosphorylated receptor; (3) internalization of the arrestin-bound receptor via clathrin-mediated endocytosis and (4) dissociation of the arrestin and dephosphorylation of the receptor allowing for the receptor to either recycle back to the cell surface or be degraded.

2.2 Expanding Complexity in GPCR Signalling

Although these GPCR activation and deactivation pathways are well established, in recent times there has been an appreciation that GPCR signalling is far more complex. Indeed, it is now clear that individual GPCRs may couple to multiple G proteins and that the specific G protein activated by a receptor may depend on the cellular context, other GPCRs or adapter proteins present, or by changes that occur to the receptor during the kinetics of activation (Baillie et al. 2003; Rashid et al. 2007; Steen et al. 2014). Further, while arrestins were traditionally viewed as adaptor molecules responsible primarily for turning off GPCR signalling, it is

now recognized that arrestins are signalling molecule in their own right. Indeed, GPCRs are capable of arrestin-dependent, but G protein-independent, signalling (Shukla et al. 2011). Further, it is apparent that different ligands for the same receptor are able to generate unique responses, either through differences in their binding/activation kinetics, or by stabilizing distinct receptor active conformations, a concept often now referred to as 'biased agonism' (Steen et al. 2014). Many GPCRs are also recognized to possess allosteric binding sites, distinct from the orthosteric site of the receptor's endogenous ligand, and ligands binding to allosteric sites often generate distinct signalling responses (May et al. 2007). Adding a final level of complexity, there is substantial evidence that GPCRs form hetero-oligomers with other GPCRs, and that these interactions alter nearly every aspect of receptor function (Milligan 2013). To accommodate our increased understanding of GPCR complexity, there has been a need to develop new tools and technologies to study GPCR function. One area in particular that has evolved to meet this need is that of biosensor technologies capable of assessing nearly every aspect of GPCR function in living cells and in real time.

3 Biosensor Technologies to Study GPCRs

A biosensor is an analytical approach that uses a biological element in order to measure a specific function, protein conformation or analyte concentration. Most commonly in the field of GPCR research, biosensors take the form of genetically encoded sensor proteins that incorporate intrinsically fluorescent or bioluminescent proteins to measure specific functions of these receptors. However, there are also examples of non-genetically encoded sensors, most notably the "label-free" technologies, including those that assess changes in dynamic mass redistribution (DMR) or cellular impedance as global unbiased measures of cellular function. Biosensor technologies have now been developed to assess nearly every aspect of GPCR function and, indeed, these have been critical in defining the pharmacology of the FFA receptor family.

3.1 Genetically Encoded Biosensor Technologies

Genetically encoded biosensors typically incorporate fluorescent or bioluminescent proteins designed to alter either their intensity or spectral properties in response to the function to be measured. There are three common approaches that can be utilized to achieve this (Fig. 1), including: (1) intrinsically context-sensitive biosensors; (2) resonance energy transfer (RET) approaches and (3) protein fragment complementation approaches (Hattori and Ozawa 2014; Wehr and Rossner 2016). While the first of these techniques requires only a single fluorescent/bioluminescent tag, the other two require two separate tags that may be incorporated into either an intramolecular or intermolecular biosensor (Fig. 2). In either case, the

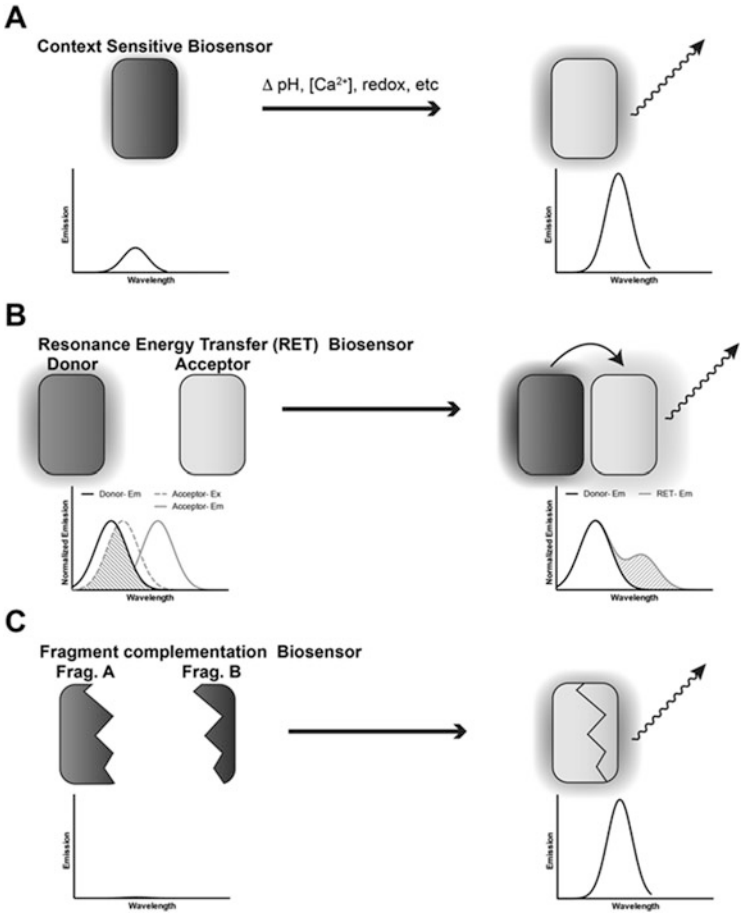

Fig. 1 Three common approaches utilizing genetically encoded bioluminescent and fluorescent biosensors. (**a**) In an intrinsically context-sensitive sensor the bioluminescent or fluorescent protein will alter its properties based on a change to the environment. This could result in either a change in intensity (shown) or the wavelength of emission. (**b**) Resonance energy transfer biosensors require a donor and an acceptor, where the emission spectrum of the donor overlaps the excitation spectrum of the acceptor (*left panel diagonal line fill*). When brought into close proximity, resonance energy transfer will occur, resulting in emission of the acceptor (*right panel diagonal line fill*). (**c**) Fragment complementation sensors split the bioluminescent or fluorescent protein into two non-functional halves, which become functional to emit fluorescence or bioluminescence when brought together

biosensor ultimately will measure changes in the distance between the two tags in order to assess various biological processes.

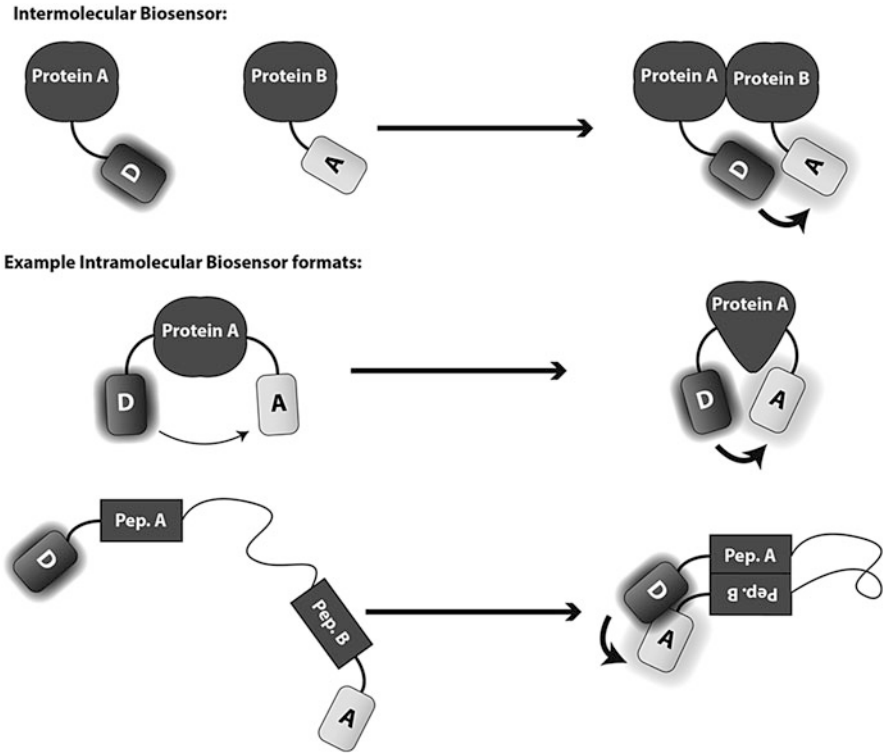

Fig. 2 Resonance energy transfer (RET) biosensor formats. General approaches to developing RET biosensors include intermolecular and intramolecular formats. In an intermolecular sensor, protein A is tagged with a donor (D), while protein B is tagged with the acceptor (A). If the two proteins come into close proximity RET will occur between donor and acceptor. There are several ways to generate intramolecular sensors, with two specific examples shown. In the first, protein A is tagged with both donor and acceptor in distinct locations, allowing for a basal RET. After the protein undergoes a conformational change, the relative distance of donor and acceptor decreases, resulting in increased RET. In the second example the sensor is constructed with the donor linked to peptide A, and the acceptor linked to peptide B. These are artificially separated with a linker, resulting in minimal basal RET. When a stimulus causes the two peptides to interact, it also brings the donor and acceptor into close proximity, increasing RET. Although this figure depicts RET biosensors, similar approaches are often suitable for fragment complementation sensors

3.1.1 Context-Sensitive Biosensors

It has long been known that both fluorescent proteins, such as green fluorescent protein (GFP) (Kneen et al. 1998), as well as bioluminescent luciferase proteins (Kitayama et al. 2003), are often sensitive to pH. Thus a number of context-sensitive biosensors have been developed utilizing GFP variants to assess changes in pH. For GPCRs, this may be of particular use since the luminal pH of secretory and endocytic compartments is acidic, and thus, pH sensors incorporated into the N-terminal of a GPCR may be used to assess cell surface delivery and internalization (Ashby et al. 2004). This has, for example, been useful in demonstrating the

dependence on receptor-activity-modifying proteins in delivering the calcium-sensing receptor to the cell surface (Bouschet et al. 2005). More recently, Robers et al. demonstrated that incorporating a pH sensitive NanoLuciferase (NLUC) (Hall et al. 2012), bioluminescent tag at the N-terminal of a GPCR allowed them to measure agonist induced internalization of several GPCRs (Robers et al. 2015). While these approaches are suitable to assess GPCR trafficking, they provide few advantages over tagging the receptor with a fluorescent protein at its intracellular C-terminal and using this to track subcellular location via either microscopy or high content imaging platforms (Zhang and Xie 2012). In addition to pH, context-sensitive biosensors have been described to measure the concentration of various other ions as well as redox state (Hochreiter et al. 2015). Most notably for GPCR studies, this includes sensors capable of assessing intracellular Ca^{2+} levels (Nakai et al. 2001), which may be useful as a readout of $G_{q/11}$ signalling.

3.1.2 RET-Based Biosensors

Fluorescent and bioluminescent RET biosensors have been by far the most widely employed technologies to study GPCR function. Fluorescent RET biosensors are based on the Förster resonance energy transfer (FRET) phenomenon dictating that when two fluorophores with overlapping emission and excitation spectra are in close proximity (<100 Å), excitation of the shorter wavelength donor will lead to a non-radiative transfer of energy to the acceptor, resulting in emission from the acceptor. The efficiency of energy transferred varies with the inverse of the distance between the donor and acceptor to the 6th power (Nakai et al. 2001), thus even very small changes in the distance between donor and acceptor, for example, those occurring within an intramolecular biosensor, will produce a measurable change in FRET. For bioluminescent RET sensors, bioluminescent resonance energy transfer (BRET) is employed, in which the fluorescent donor is replaced with a bioluminescent protein, but otherwise the same general principles apply (Pfleger and Eidne 2006). The scope for RET biosensors to study GPCRs expands to nearly every aspect of their function, with RET biosensors described to measure each of: ligand binding, receptor conformational changes, receptor/G protein interactions, G protein activation, intracellular levels of second messengers such as cAMP and Ca^{2+}, activation of downstream kinases, arrestin recruitment and receptor oligomerization, to name but a few (Lohse et al. 2012; Salahpour et al. 2012; Stoddart et al. 2015; van Unen et al. 2015). Considering this versatility, it is not surprising that RET-based biosensors have become an important tool in assessing the evolving complexities of GPCR pharmacology and function.

3.1.3 Fragment Complementation Biosensors

Protein fragment complementation-based sensors involve a fluorescent or bioluminescent protein split into two non-functional segments, able to recombine to form a functional protein only if brought into close proximity to each other. Therefore, complementation sensors function effectively as binary ‘on/off’ sensors and thus, are not well suited for measuring small changes in relative distance. This is

particularly true for the split fluorescent protein approach, bi-molecular fluorescent complementation (BiFC), where the process of combining the two non-functional halves is irreversible (Rose et al. 2010). While split luciferase sensors are reversible, and indeed have been developed to study many cellular processes and functions (Azad et al. 2014; Hattori and Ozawa 2014; Wehr and Rossner 2016); at least within the GPCR field, RET-based approaches remain far more widely used.

3.1.4 Fluorescent vs Bioluminescent-Based Technologies

The general principles are similar for fluorescent and bioluminescent-based biosensors, thus it is important to consider the advantages and disadvantages of each technology. Most notably, fluorescence yields a greater intensity of light emitted, and thus has historically been the preferred option for imaging-based applications (Boute et al. 2002; Lohse et al. 2012). Although BRET-based microscopy was first described some time ago (De and Gambhir 2005; Xu et al. 2007), the low amount of light emitted from *Renilla* luciferase (RLUC), the most commonly used luciferase in BRET biosensors, has greatly limited its utility. However, the development of brighter luciferases, for example, NLUC (Hall et al. 2012), is beginning to change this and NLUC/BRET imaging yielding subcellular resolution is now being described (Machleidt et al. 2015).

Although BRET has lagged behind FRET for imaging, it does have advantages when employed in plate-reader-based applications. The use of FRET biosensors is complicated by photobleaching, phototoxicity, autofluorescence and direct excitation of the FRET acceptor, none of which will affect BRET-based sensors (Boute et al. 2002). Many of the limitations of FRET can also be avoided if a long fluorescent lifetime donor is employed, allowing for time resolved (TR)-FRET. Although there are no known fluorescent proteins with suitable lifetimes for TR-FRET, the ability to incorporate long fluorescent lifetime lanthanide fluorophores into a protein of interest using reagents such as the SNAP, CLIP and HALO tags is now well established, and indeed has been used extensively in sensors assessing GPCR oligomerization and ligand binding (Emami-Nemini et al. 2013; Marsango et al. 2015; Maurel et al. 2008). Despite the possibilities for TR-FRET, and the recent advances in BRET imaging, FRET continues to be the primary imaging approach, while BRET remains more widely used in high throughput plate-based assays.

3.2 Non-genetically Encoded Biosensors

The advances in genetically encoded biosensor technologies have in many ways revolutionized the way we are able to assess the function of GPCRs in real time in living cells. However, these biosensors are not without limitation. Most generally, the need to express an exogenous biosensor in the cells under study. Non-genetically encoded biosensor technologies avoid this limitation, allowing for the assessment of GPCR function in unmodified cells. This is most commonly

achieved through the so-called label-free detection platforms and sensors utilizing antibodies.

3.2.1 Label-Free Biosensors

Many of the underlying concepts and technologies used in label-free biosensors originated in the 1990s and at the time were primarily focused on developing methods to characterize ligand-receptor interactions without the need for an exogenous label on either the ligand or the receptor. These technologies were either optically based: measuring, for example, DMR (Fang 2006; Fang et al. 2006); or electrically based: commonly measuring changes in cellular impedance (Cooper 2006). It quickly became clear that the signals these sensors generated were far more data rich than initially anticipated, and in a sense, that label-free biosensors provide an unbiased measure of the global changes occurring within a cell (Fang et al. 2006; Grundmann and Kostenis 2015; Scott and Peters 2010). This makes label-free techniques particularly powerful to identify ligand bias (Fang 2013). However, as the signal measured represents a composite of all pathways being activated within the cell, it is often difficult to deconvolute meaning. Despite this challenge, it has now been shown that pharmacological inhibitors can be used to deconvolute label-free signals arising from GPCR activation, at least to the level of identifying signals arising from specific G protein families (Schroder et al. 2010).

3.2.2 Antibodies as Biosensors

With the inherent ability to bind nearly any target molecule, antibodies provide numerous opportunities for use as biosensors. In particular, as antibodies specific for post-translationally modified proteins can be generated, this creates the opportunity to use antibodies recognizing phosphorylated GPCRs as biosensors of receptor activation. Recently, this approach elegantly yielded a biosensor antibody that demonstrated the location of muscarinic M_1 receptor activation in mouse brain during learning and memory (Butcher et al. 2016). Beyond post-translational modifications, antibodies against a specific active conformation of a target protein can also be developed. This has, for example, been used to generate antibodies capable of selectively identifying active G proteins (Lane et al. 2008), which could also be viewed as biosensors to assess GPCR activation of these G proteins.

While antibodies are incredibly useful tools, they are limited by their inability to cross the cell membrane, and thus cannot generally be used as biosensors to study function in living cells or in real time. To overcome this limitation, technologies such as nanobodies, derived from camelid single domain antibodies, are now making it possible to develop genetically encodable antibody-like biosensors. Of particular note, Irannejad et al. employed a nanobody, originally developed to mimic $G\alpha_s$ interaction and facilitate crystallization of the active state β_2 adrenoceptor (β_2AR) (Rasmussen et al. 2011), to build a biosensor able to show the subcellular localization of activated β_2ARs (Irannejad et al. 2013). This work demonstrated that the β_2AR remained in an active state even after internalization, providing critical evidence for endosomal signalling of GPCRs. Currently the full potential of antibodies, nanobodies and similar technologies as biosensors

to study GPCRs is only beginning to be realized, and it will be exciting to see how these may be employed in the future.

4 Biosensors to Study the FFA Receptors

Since being identified as receptors for FFAs between 2003 and 2005, uncovering the pharmacology of members of the FFA family of GPCRs has proven challenging (Hudson et al. 2011). This has been influenced by several factors, including a lack of pharmacological tools with selectivity for individual family members, low potency of the endogenous fatty acid ligands for the receptors, complex allosteric modes of binding for several receptors and the potential to signal through multiple G protein-dependent and independent pathways (Milligan et al. 2016). Biosensor-based approaches have proven to be valuable tools to address some of these complex aspects of FFA receptor pharmacology and the following sections will discuss how both genetically encoded and non-genetically encoded biosensors have improved our understanding of this important family of GPCR.

4.1 Studying FFA Receptors with Genetically Encoded Biosensors

Genetically encoded biosensors have been used to characterize many aspects of FFA receptor pharmacology. This has largely focused on using BRET-based biosensors to study the pharmacology and function of heterologously expressed receptors. In particular, such approaches have proven useful in interrogating ligand binding, G protein activation and arrestin interactions of these receptors.

4.1.1 Ligand Binding Sensors

Radioligand binding assays have long been the standard approach to directly assess ligand/GPCR interactions. However, such assays require a suitable radio-labelled tracer ligand with both high affinity and low non-specific binding, which typically are not available for recently deorphanized receptors, such as the FFA family members. Thus, directly measuring ligand binding to the FFA receptors has been challenging (Hudson et al. 2011). Although in recent years radio tracers have been reported for both FFA1 (Lin et al. 2012) and FFA2 (Sergeev et al. 2016), this approach remains challenging for the FFA receptors, in large part due to high lipophilicity and resultantly high non-specific binding of most ligands. One alternative approach to assess ligand binding gaining significant traction within the GPCR field is the use of fluorescent tracers (Briddon et al. 2011). Indeed, several studies have now described fluorescent tracer agonists for the FFA1 receptor (Bertrand et al. 2016; Christiansen et al. 2016; Hara et al. 2009; Ren et al. 2016). Although these have proven useful, the high lipophilicity of FFA1 ligands has made it difficult to directly assess the binding of these ligands to the receptor. To

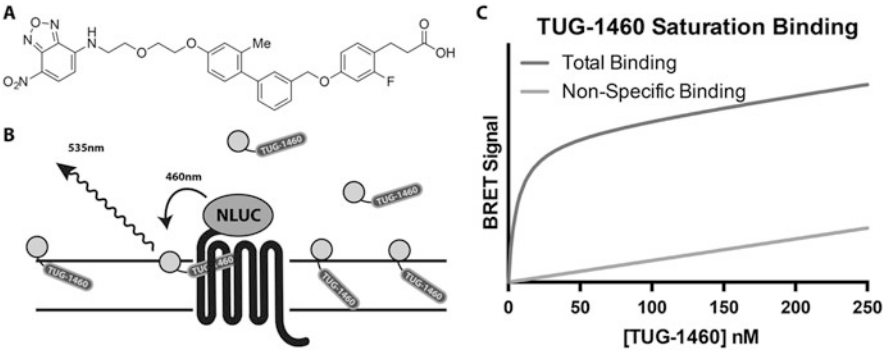

Fig. 3 A BRET-based ligand binding sensor for FFA1. The structure of TUG-1460, a fluorescent FFA1 agonist is shown in (**a**). Employing TUG-1460 in an intermolecular BRET biosensor utilizing NLUC-FFA1 allows for a BRET signal only from TUG-1460 bound to the receptor (**b**). This results in favourable specific to non-specific binding (simulated saturation binding data in **c**) despite the high lipophilicity of TUG-1460

overcome this, Christiansen et al. (2016) developed an intermolecular BRET biosensor approach to reduce the non-specific binding signal (Fig. 3).

By employing a system in which the FFA1 receptor was tagged at its N-terminus with the NLUC bioluminescent protein, BRET between the NLUC donor and the fluorescent tracer acceptor, TUG-1460, a high affinity FFA1 receptor agonist, should only be observed when the ligand is in close proximity to the receptor. This resulted in a very good specific to non-specific binding ratio for TUG-1460, despite its high lipophilicity. An additional benefit to this approach was that binding of the tracer to the receptor could be assessed in real time, which allowed Christenson et al. to measure the rapid binding kinetics of TUG-1460. In recent times there has been growing appreciation for the importance of binding kinetics in drug discovery (Swinney et al. 2015). Thus such BRET-sensor-based binding assays are likely to prove to be invaluable tools in the future. To date, FFA1 is the only FFA receptor for which such a BRET-binding assay has been reported; however, there is every likelihood that this approach can be extended to study the other members of the family if suitable fluorescent tracers can be developed.

4.1.2 G Protein Sensors

There are several RET-based biosensors designed to assess G protein interaction with GPCRs. These include: (1) intermolecular sensors where the receptor and G protein are tagged with either the RET donor or acceptor, and direct receptor/G protein interactions are assessed (Fig. 4a) (Ayoub et al. 2012); (2) intermolecular sensors where different subunits of the G protein αβγ heterotrimer are tagged with the RET donor and acceptor, allowing for dissociation/conformational rearrangement within the G protein to be measured (Fig. 4b) (Ayoub et al. 2012; Malik et al. 2013) or (3) intramolecular sensors where the receptor is tagged with both the RET donor and acceptor separated by a semi-flexible linker and by a peptide

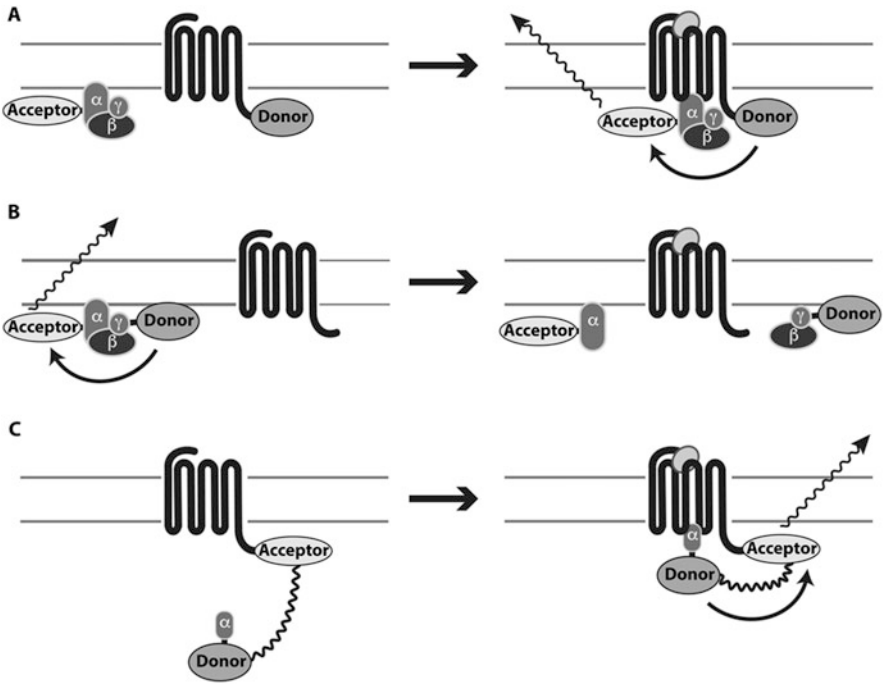

Fig. 4 Biosensor approaches to assess GPCR interactions with G proteins. (**a**) Intermolecular biosensors measure G protein recruitment through increased RET between tagged receptor and G protein. (**b**) Intermolecular biosensors where different components of the αβγ heterotrimer are labelled can measure dissociation of the G protein in response to activation as decreased RET. Although, the classic view of G protein signalling was that α subunit dissociates from the βγ upon activation, this may in reality only be a conformational rearrangement, which can equally be measured with this sensor as a change in RET. (**c**) An intramolecular RET sensor is shown where the receptor is tagged with the acceptor, which is then separated from the donor with a semi-flexible linker. The donor is then tagged with a peptide corresponding to the final 28 amino acids of a specific Gα subunit. Receptor activation leads to interaction between the Gα peptide and the receptor, resulting in increased RET. The specific locations of donor vs acceptor tags in this figure are used, for example, only, and in most cases these can be reversed

corresponding to the terminal residues of a specific Gα subunit; where if a ligand/receptor interaction leads to interaction between the G protein peptide and the receptor an increase in RET will be observed (Fig. 4c) (Malik et al. 2013).

To date, these approaches have not been widely employed in relation to the FFA receptors, with only one study using a BRET-based sensor to show Gq activation by the FFA1 receptor (Mancini et al. 2015). However, given that there is now good evidence that each of FFA1, FFA2 and FFA4 couple to multiple G proteins, and that this appears to vary depending on the specific cell type (Milligan et al. 2016), RET biosensors will be valuable tools to tease out factors that regulate G protein coupling preference (e.g., bias, cell type, oligomerization, etc.) of these receptors.

4.1.3 Arrestin Sensors

Arrestins were viewed historically as the 'off' switches of GPCR signalling, facilitating the removal of activated receptors from the cell surface. The fact that this process is conserved, and occurs with many different receptors has meant that arrestin interaction assays have become an important tool for GPCR drug discovery (Zhang and Xie 2012). In recent years, the importance of GPCR/arrestin interactions has only increased with the realization that arrestins not only turn off receptor signalling, but also generate their own G protein-independent signalling responses (Shukla et al. 2011). Although there are several assay formats able to assess GPCR/arrestin interactions, the most commonly employed biosensor capable of real time measurement of this interaction is an intermolecular BRET sensor, where the receptor and arrestin (either arrestin-2 or -3) are tagged with either of the BRET donor or acceptor (Fig. 5a). Over the years, this has become widely used as a key tool to characterize the pharmacology of several FFA family members, most notably for FFA4, which undergoes particularly robust interaction with arrestins-2 and -3.

One common application of the BRET arrestin-recruitment sensor has been to use this as a surrogate measure of agonist binding. As the sensor measures directly the interaction between receptor and arrestin, and this interaction should occur in a 1:1 stoichiometric ratio, the potency of an agonist to activate the BRET arrestin sensor is anticipated to be a good reflection of its affinity for the receptor (Hudson et al. 2011). This concept has been invoked as the basis for using a BRET arrestin-3-recruitment sensor to interrogate agonist binding at mutants intended to disrupt the orthosteric binding pockets of both FFA2 (Hudson et al. 2012, 2013a) and FFA4 (Hudson et al. 2013b, 2014). Likewise, this sensor has also been a useful tool in drug discovery efforts targeting FFA4, and indeed was the key assay used by Shimpukade et al. in identifying TUG-891 as the first potent and selective FFA4 agonist (Shimpukade et al. 2012).

The BRET arrestin-recruitment sensor has also proven useful in helping to define more complex aspects of FFA receptor pharmacology and function. This includes, for example, demonstrating arrestin bias agonism of certain synthetic agonists of FFA1 (Mancini et al. 2015) and FFA4 (Li et al. 2015), or confirming that arrestin recruitment to FFA4 occurs independently of $G_{q/11}$ activation (Schrage et al. 2015). The sensor has been particularly useful for defining the contribution of phosphorylated residues vs those possessing a fixed negative charge in the C-terminal tail of FFA4 for the recruitment of arrestin to the receptor (Butcher et al. 2014; Prihandoko et al. 2016).

Although one of the key advantages of using genetically encoded biosensors is the ability to assess receptor/arrestin interactions in real time, few studies have examined the kinetics of FFA receptor/arrestin interactions. While Watson et al. used a fluorescent protein fragment complementation sensor to study arrestin interaction with splice variants of the FFA4 receptor over time (Watson et al. 2012), it must be noted that the irreversible nature of this type of sensor makes interpretation of kinetic data challenging. More recently, Prihandoko et al. used a BRET arrestin-recruitment sensor to show the kinetics of arrestin interaction with mouse

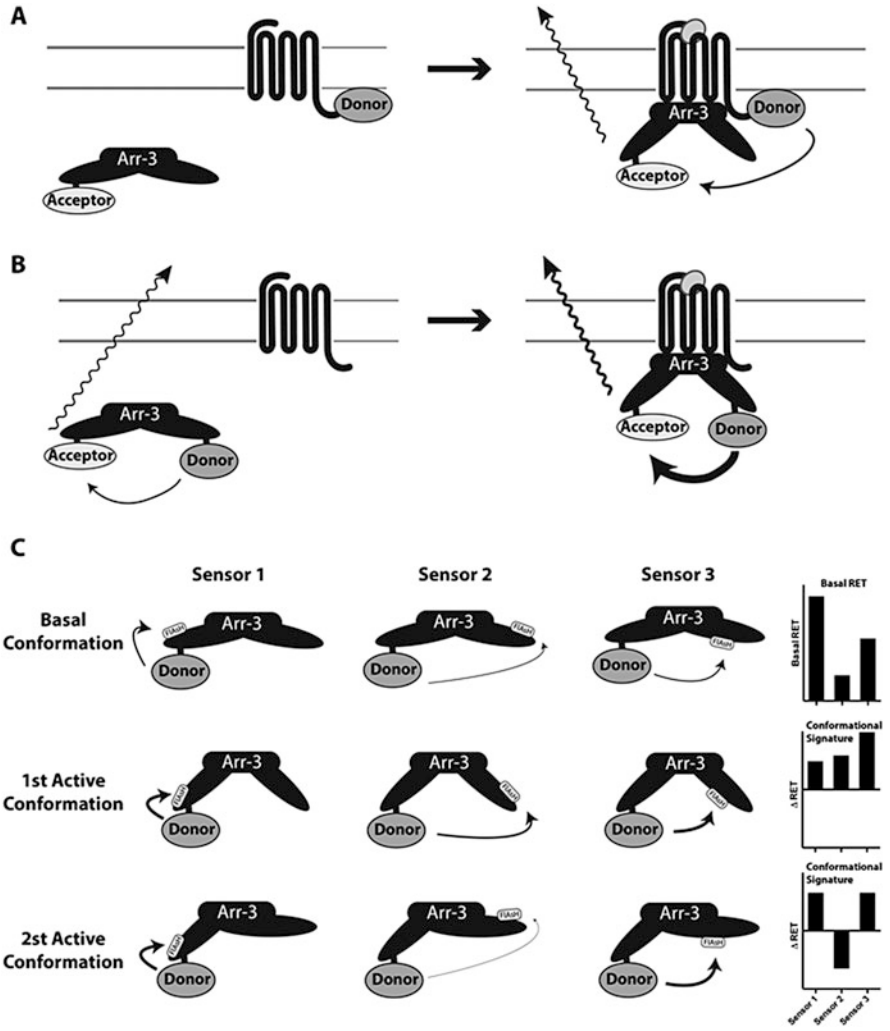

Fig. 5 Biosensors to assess GPCR interactions with an arrestin. (**a**) An intermolecular biosensor where the receptor and arrestin are tagged with RET donor and acceptor, respectively. Arrestin recruitment to the receptor increases RET. (**b**) An intramolecular arrestin sensor has the RET donor and acceptor at the N and C termini of arrestin. Interaction with an activated receptor results in a conformational change in arrestin, altering the RET. In (**c**), three examples of FlAsH-based arrestin conformational sensors are shown. In each case the six amino acid Cys-Cys-Pro-Gly-Cys-Cys sequence that binds the FlAsH fluorophore [4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein] is inserted into a different position of arrestin. Based on the location of FlAsH labelling, each sensor has a different basal RET between the donor and FlAsH acceptor. When arrestin adopts an active conformation (1st active conformation) the relative change in RET differs among the three sensors. The combined RET responses of all the sensors have been described as a conformational signature (Lee et al. 2016). If arrestin adopts a different active conformation (2nd active conformation), the conformational signature will be different, representative of differences in the relative distances between the RET donor and FlAsH acceptor between the two active conformations

FFA4 mutants lacking key phosphorylation sites (Prihandoko et al. 2016). Unravelling more details of the kinetics of FFA receptor/arrestin interactions is likely to be an important future application for these sensors.

In addition to sensors measuring receptor/arrestin interactions, there has also been some effort to develop intramolecular arrestin RET sensors able to assess the conformational changes that occur in arrestin itself in response to its interaction with an activated GPCR (Fig. 5b). This initially involved simple BRET-based sensors where arrestin-3 was tagged at its N-terminus with a luciferase and its C-terminus with a fluorescent protein (Charest et al. 2005). When the sensor interacted with an activated GPCR it resulted in a conformational change in arrestin, altering the relative distance between the two tags. Although simplistic, arrestin sensors based on this design were able to demonstrate that different ligands acting through the same receptor can generate distinct arrestin conformations (Shukla et al. 2008).

More recently, two groups independently developed more refined arrestin conformational sensors (one BRET- and one FRET-based) that employ the tetracysteine binding FlAsH fluorophore as the acceptor (Lee et al. 2016; Nuber et al. 2016). FlAsH binds to a six amino acid sequence, which provides more flexibility in terms of where within arrestin the acceptor can be inserted (Fig. 5c). This allowed both Nuber et al. and Lee at al. to generate multiple sensors reporting different aspects of the conformational changes that occurred after interaction with an activated GPCR (Lee et al. 2016; Nuber et al. 2016). Nuber et al. utilized their sensors to examine the conformational changes that occur in arrestin-3 after activation of the FFA4 receptor (Nuber et al. 2016). Of particular note, the conformational changes in arrestin-3 upon FFA4 activation were qualitatively different than those observed after β_2 adrenoceptor, or M_2 muscarinic receptor activation, providing evidence for receptor-specific arrestin active conformations. The full implications for this in terms of how it relates to arrestin-dependent signalling remain to be determined, but it is likely that these and similar arrestin conformational sensors will help define the finer details of FFA receptor/arrestin interactions and, potentially, signals generated.

4.2 Studying FFA Receptors with Non-genetically Encoded Biosensors

A key limitation to using genetically encoded biosensors is the need to heterologously express the sensor protein. Although not necessarily a requirement, in practice this means the majority of studies done using these sensors have been performed in common heterologous cell systems, such as HEK293 cells. Non-genetically encoded sensors have the potential to overcome this limitation, as at least conceptually, these should be suitable for use in any unmodified cell type. The most common of these approaches are the so-called label-free platforms, based on assessing either dynamic mass redistribution (DMR) or cellular impedance. These sensors have been important tools to study all four of the FFA family members. In addition to the label-free approaches, antibodies against residues

that alter phosphorylation status on addition of an agonist ligand may also be used as biosensors to assess GPCR activation and several studies have now developed these for the FFA4 receptor.

4.2.1 Label-Free Biosensors

Since label-free biosensors measure global cellular changes, they are a useful way to assess the effect of a drug-GPCR interaction without concern for the specific pathway that is activated. Schmidt et al. took advantage of this and used a DMR assay to assess a library of small carboxylic acids (SCAs) at the FFA2 and FFA3 receptors (Schmidt et al. 2011). This identified *trans*-2-methylcrotonic acid and cyclopent-1-enecarboxylic acid as orthosteric agonists selective for FFA2 over FFA3. It is interesting to note that although most of the SCAs tested produced responses, clear differences in the nature of the responses were observed for individual SCAs, even when acting through the same receptor (Schmidt et al. 2011). This highlights another key advantage of label-free sensors, in that they are very good at showing functional differences, or perhaps bias, between ligands. A particularly interesting example of this came from Grundmann et al. when they compared both impedance and DMR label-free responses obtained with the endogenous SCFA, C3, to those obtained with a reported allosteric agonist, 4-CMTB [4-chloro-α-(1-methylethyl)-*N*-2-thiazolylbenzeneacetamide], at the FFA2 receptor (Grundmann et al. 2016). They noted that although C3 produced robust, rapid and sustained responses in both assays, 4-CMTB produced only a modest rapid response, followed by a robust sustained response. This demonstrated clear functional differences between the way these two ligands activate FFA2, which ultimately was attributed to an unusual ability of 4-CMTB to interact with the FFA2 orthosteric site to produce the rapid response, before producing its sustained response through interaction with a distinct allosteric site on the receptor (Grundmann et al. 2016).

In addition to demonstrating unique functional responses between two different ligands, label-free biosensors are also useful in demonstrating different responses to two different receptors, or receptor variants. Watson et al. used a DMR assay to show differences in the cellular response generated following activation of either short or long FFA4 splice variants (Watson et al. 2012). This difference was then attributed to an inability of the long variant to couple to $G_{q/11}$ pathways (Watson et al. 2012). The same DMR approach proved useful in demonstrating that a receptor activated solely by synthetic ligand (RASSL) variant of the FFA2 receptor displays signalling that is indistinguishable from the wild type receptor (Hudson et al. 2012), a critical property if this RASSL is to be used in future studies to help define FFA2 function (Bolognini et al. 2016).

Although the global nature of the label-free biosensor readout has advantages, it also can make it challenging to understand what exactly these sensors are measuring. To overcome this, a range of pharmacological inhibitors can be used to 'deconvolute' a label-free response into its individual components (Schroder et al. 2010). Such efforts, demonstrated that although FFA1 is traditionally viewed as a $G_{q/1}$-coupled receptor, treatment with a $G_{q/11}$ inhibitor, YM-254890, did not

fully eliminate the FFA1-DMR response (Schroder et al. 2010). Instead, it was only when cells were treated with both YM-254890 and the $G_{i/o}$ inhibitor, pertussis toxin, that the full FFA1-DMR response was eliminated (Schroder et al. 2010). This provided key support for the concept that FFA1 couples to multiple G protein families.

Despite a key advantages of the label-free biosensors being that they can be used in any cell type, to date the vast majority of work using these sensors on the FFA family has continued to utilize cells heterologously overexpressing the desired receptor. However, it has been shown that DMR can be used in MIN-6 and INS-1E insulinoma cell lines, as well as with primary rat islets, in order to assess endogenous FFA1 function (Grundmann and Kostenis 2015). Indeed, extending the use of these label-free biosensors to study endogenously expressed FFA receptors is likely to be a key use for these technologies.

4.2.2 Antibody Biosensors

There is an appreciation that antibodies able to recognize phosphorylated forms of a GPCR can, in effect, serve as biosensors for receptor activation (Butcher et al. 2016). Thus, it should be of note that antibodies specific to phosphorylated forms of both the mouse and human orthologues of the FFA4 receptor have now been described (Butcher et al. 2014; Prihandoko et al. 2016). To date, these antibodies have been employed only to show FFA4 activation in cells heterologously overexpressing the receptor. However, the antibody against mouse FFA4 was suitable to use in immunocytochemistry studies to visualize the location of activated receptor within cells (Prihandoko et al. 2016). Although still at an early stage, it will be exciting to see how these antibodies may be used in the future to assess FFA4 activation in cells and tissues endogenously expressing FFA4.

5 Conclusions and Future Perspectives

Biosensor technologies have proven to be important tools for uncovering the complex pharmacology of the FFA receptor family. This has included using sensors to identify new ligands, to define how these ligands interact with the receptors, to dissect signalling pathways and to understand the basis of receptor/arrestin interactions. There are, however, many questions that remain to be answered about the pharmacology and function of the FFA receptor family and biosensor technologies are likely to continue to be central to these efforts. Most notably, it remains unclear how the FFA receptors function under physiological conditions and how certain, rare fatty acids, for example, the omega-3 and fatty acid hydroxy fatty acids, that are believed to produce biological effects through the FFA receptors in vivo (Oh et al. 2010; Yore et al. 2014), are able to do so, even though these are at much lower levels in the body than the more common fatty acids that are also known to activate the receptors. Key to using biosensors to answer these questions will be to begin employing the sensors, both genetically encoded and otherwise, in more physiological situations. The recent revolution in CRISPR

genome editing technologies should facilitate the use of genetically encoded sensors in a wider range of cell types and at more physiologically relevant expression levels, allowing for studies on the FFA receptors in, for example, model cells relevant to the regulation of metabolism and inflammation. There has also been increased interest in using sensors to study GPCR function in synthetic organs and ultimately in intact animals (van Unen et al. 2015), which may provide key answers to where, when and how the FFA receptors are activated in vivo. Taken together, the use of biosensor technologies has, and will continue to, provide exciting opportunities to help unravel the complex pharmacology of the FFA family of receptors.

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