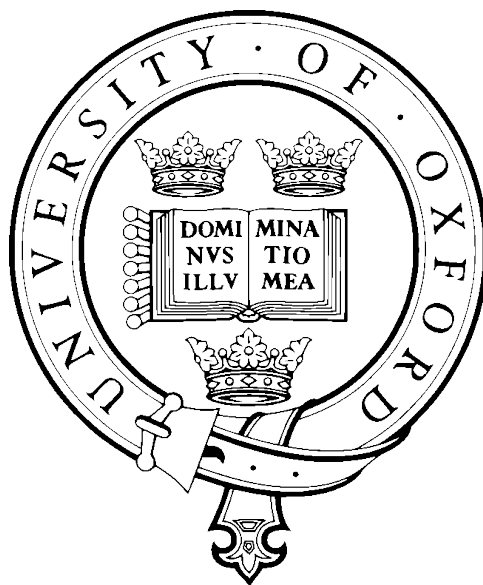


A survey of G protein-coupled receptor stoichiometry



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St. Cross College

Thesis submitted for the degree of Doctor of Philosophy
Trinity Term, 2013

Abstract

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G-protein coupled receptors (GPCRs) represent the largest family of transmembrane proteins in the human genome. Their biological and medical significance has driven extensive research into their structure and function, yet a number of important aspects of their behaviour remain unresolved. Arguably the most contentious debate in the field concerns whether or not the receptors form stable homo- and hetero-oligomeric interactions, and there is currently no consensus on the extent or purpose of GPCR oligomerisation. In this thesis, the 'typical' stoichiometry of *Rhodopsin* family GPCRs is investigated via the examination of more than 60 receptors natively expressed by human HEK 293T cells using bioluminescence resonance energy transfer (BRET). Assaying receptors in the cells in which they are natively expressed maximises the likelihood of authentic assembly and trafficking while simultaneously providing an unbiased cross-section of the whole GPCR family. In order to make such an investigation possible, the sensitivity of existing BRET approaches for partial homodimers had to be, and was, confirmed, and a complementary competition-based BRET method suitable for a semi high-throughput analysis was developed. Application of these assays to the HEK 293T GPCR repertoire revealed that the *Rhodopsin* family is very predominantly monomeric but contains a small fraction of independently evolved dimers comprising small phylogenetic clusters of receptors. The mechanism of *Rhodopsin* family dimerisation was in some cases found to be reliant on interactions between transmembrane helices, in contrast to other families of GPCRs, which were observed or are known to be exclusively dimeric due to interactions between their N-terminal domains. The mechanism of dimerisation in *Rhodopsin* family GPCRs may preclude constitutive dimerisation but allow heterodimerisation of closely related receptors, as observed for a subset of receptors using a third type of assay designed to detect heterodimers. Taken together, these observations suggest a model of GPCR evolution in which dimers either have a selective disadvantage compared to monomers, or for which dimerisation offers no apparent selective advantage. These findings suggest that receptor stoichiometry is at least partly responsible for several of the remarkable features of GPCR family structure, including the very large size of the family as a whole, the great diversity of *Rhodopsin* family GPCRs, and the origins of sensory receptors.

For Mum

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Abbreviations

5-HT	5-hydroxytryptamine (serotonin) receptor
7TM	Seven-transmembrane
α_2 AR	Alpha 2C adrenergic receptor
AR	Adenosine A receptor
ADP	Adenosine diphosphate
AT	Angiotensin receptor
ATP	Adenosine triphosphate
B ₂	Bradykinin receptor B2
β AR	Beta adrenergic receptor
BiFC	Bimolecular fluorescence complementation
BRET	Bioluminescence resonance energy transfer
BSA	Bovine serum albumin
C5R	Complement component 5a receptor
CASR	Calcium-sensing receptor
cBRET	Competition BRET
CCKBR	Cholecystokinin B receptor
CCL	Chemokine (CC-motif) ligand
CCR	Chemokine (CC-motif) receptor
CHO	Chinese hamster ovary
CMV-IE	Cytomegalovirus immediate-early
CNS	Central nervous system
Co-IP	Coimmunoprecipitation
CRD	Cysteine-rich domain
CRF ₁ R	Corticotropin-releasing factor receptor 1
CXCL	Chemokine (CXC-motif) ligand
CXCR	Chemokine (CXC-motif) receptor
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DP	Prostaglandin D2 receptor
EDNR	Endothelin receptor
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMR	Epidermal growth factor-like module containing mucin-like receptor
EP	Prostaglandin E receptor
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FKBP	FK507-binding protein
FP	Prostaglandin F receptor
FPKM	Fragments per kilobase of exon per million fragments mapped
FPLC	Fast protein liquid chromatography
FPR	N-formyl peptide receptor
FRAP	Fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer

FSHR	Follicle-stimulating hormone receptor
GABABR	γ -amino butyric acid receptor
GCGR	Glucagon receptor
GFP ²	Green fluorescent protein
GPCR	G protein-coupled receptor
GPOR	G protein-coupled oestrogen receptor 1
GPR	G protein-coupled receptor
GPS	GPCR proteolytic domain
GRK	GPCR kinase
HBS	HEPES-buffered saline
HEK	Human embryonic kidney
HR	Histamine receptor
KYNA	Kynurenic acid
LB	Luria-Bertani
LDLa	Low-density lipoprotein receptor class A domain
LGR	Leucine-rich repeat containing GPCR
LHCGR	Luteinizing hormone receptor
LPA	Lysophosphatidic acid receptor
LRR	Leucine-rich repeat region
LTB4R	Leukotriene B4 receptor
M ₁	Muscarinic acetylcholine receptor M ₁
M ₃	Muscarinic acetylcholine receptor M ₃
MAPK	Mitogen-activated protein kinase
MAS	MAS1 oncogene
MCH	Melanin-concentrating hormone
MCS	Multiple cloning site
mGluR	Metabotropic glutamate receptor
MRG	MAS-related GPCR
NA	Numerical aperture
NEB	New England Biolabs
NEMCH	Nematode chemosensory
NPY1R	Neuropeptide Y receptor Y1
NTA	Nitrilotriacetic acid
OGR	Ovarian cancer GPCR
OPN	Opsin
OPRL	Opiate receptor-like
OR	Opioid receptor
OR4D1	Olfactory receptor, family 4, subfamily D, member 1
ORF	Open reading frame
OXER	Oxoeicosanoid receptor
OXGR	Oxoglutarate (alpha-ketoglutarate) receptor
P2YR	Purinergic receptor P2Y
PAR	Protease-activate receptor
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PTAFR	Platelet-activating factor receptor
QRFR	Pyroglutamylated RFamide peptide receptor

RET	Resonance energy transfer
Rluc	<i>Renilla</i> luciferase
RNA	Ribonucleic acid
S1P	Sphingosine-1 phosphate receptor
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SREB	Super-conserved receptor expressed in brain
SSTR	Somatostatin receptor
SV	Simian vacuolating virus
TAS1R	Sweet and umami taste receptor
TBE	Tris-borate EDTA
TIRFM	Total internal reflection fluorescence microscopy
TP	Thromboxane A2 receptor
TSHR	Thyrotropin receptor
UTP	Uridine triphosphate
UTR	Untranslated region
UV	Ultraviolet
VFTM	Venus flytrap motif

CHAPTER 1

Introduction

1.1 G protein-coupled receptors

1.1.1 Overview

Very few families of proteins can be said to have prominent functional significance across much of biology and, of these, the G protein-coupled receptor (GPCR) family is undoubtedly the largest. Comprising over 800 members in humans, the GPCR family represents a vast group of transmembrane receptors that are involved in regulating a broad range of physiological processes, including muscle contraction, neurotransmission, olfaction, inflammation, chemotaxis, vision, vasodilation, and many others. They are expressed in all human tissues where their role is the transmission of information from extracellular stimuli to intracellular signalling events. GPCRs fulfil this role by recognising extracellular ligands and subsequently activating cytosolic binding partners, typically heterotrimeric G proteins, which in turn affect intracellular signalling networks. The diversity of GPCR ligands is perhaps the most striking feature of their biology as they range from large molecules, *e.g.* peptides or full proteins; to small molecules, *e.g.* nucleotides, lipids, and amines; and finally to subatomic particles, *e.g.* protons and photons. This broad variety of ligands combined with their physiological importance means that GPCRs are of remarkable pharmacological significance, with perhaps up to 30% of all licensed drugs targeting GPCRs (1). Our understanding of these receptors has improved markedly in recent years, with both structural breakthroughs and functional studies providing key insights into GPCR biology.

Discovery and early investigation of cell surface receptors

The notion of cells' ability to recognise and respond to external chemical stimulus has been in existence since the beginning of the twentieth century (2, 3). The properties of 'receptive elements' at the surface of cells were initially explored as the effects of agonistic and antagonistic substances on tissue preparations between the 1900s and 1960s, which eventually led to the development of classical receptor theory in terms of dose-dependence and mass action contributions (4). Despite the actual

identities of the cell-surface receptors and their ligands being unknown at the time, a number of components of the signaling machinery were discovered through investigations of cellular changes upon stimulation. The first insights into receptor signalling came with the observation that cyclic AMP (cAMP) acts as an adenylate cyclase-dependent secondary messenger (5), which was subsequently followed by the discovery of cAMP-dependent protein kinases (6), and then heterotrimeric G proteins (7, 8). A significant advance was the successful development of receptor-specific radiolabelled ligands, initially for melanocortin receptors (7, 8), and subsequently for adrenergic receptors (9, 10) and others. This allowed the precise investigation of ligand-dependence of individual receptors, ultimately leading to the establishment of the ternary complex model of receptor signalling (11), in which the active signalling unit is formed from a complex of receptor, ligand, and effector molecules.

It was at around this time that the receptors responsible for signalling were being first isolated from membrane preparations (12-14), yet it was not until 1986 that the genes encoding the receptors were first isolated, initially those for the β -adrenergic (15, 16) and muscarinic acetylcholine receptors (17, 18). Apparent in these early sequences was the fact that the receptors in question clearly contained seven transmembrane portions, similar to the already-cloned rhodopsin protein (19). Rhodopsin had not previously been considered to be related to either the adrenergic or muscarinic acetylcholine receptors – indeed it had not even been known if the adrenergic and muscarinic acetylcholine receptors were related to each other – yet the obvious conserved topology as well as a significant degree of sequence homology between the three forms of receptor was clear evidence that all were part of the same receptor family. In the following years it emerged that this family, now known as GPCRs, constituted an extremely large and diverse group of proteins (20-24), although it was not until the publication of the human genome (25) that the total number of human GPCRs, over 800, was finally known.

1.1.2 Outline of this chapter

Extensive work into GPCR biology has radically improved our understanding of these receptors in recent years. Phylogenetic analysis of GPCRs in both humans and other species has identified the relationships between a number of distinct receptor families, as well as their hierarchical organisation throughout evolution. Mammalian GPCRs comprise five discrete families, each with distinct structural characteristics (26). Nonetheless, it is now well known that all GPCRs possess topologically identical seven-transmembrane (7TM) cores flanked by N- and C-terminal domains of varying size. The seven TM α -helices (TM1-7) of the 7TM core are joined by six inter-helical loops of varying length. The three extracellular loops (EL1-3) combine with the N-terminal portion of the protein to form the

extracellular domain of the receptor, while the cytoplasmic domain consists of the C-terminal region and three intracellular loops (IL1-3). Many GPCRs also possess an eighth helix (H8) within their C-terminal domains. Signalling through this structure is achieved by recognition of cognate ligands by the extracellular half of the receptor, either through a central TM cavity or by the N-terminal domain, which leads to a conformational change within the 7TM core that exposes intracellular regions of the receptor to interaction with G proteins and other signalling partners (27). Activated G proteins are then released from the GPCR and are able to activate downstream effector proteins, such as adenylate cyclase (28).

In this introduction, current understanding of GPCR evolution will be discussed, followed by a more detailed overview of general receptor structure, conformational changes upon ligand binding, and the signalling consequences of GPCR activation. Finally, secondary aspects of GPCR behaviour are examined – specifically, subcellular receptor distribution and membrane organisation, and GPCR stoichiometry. There is currently no consensus within the field regarding GPCR stoichiometry, hence this section consists primarily of a technical discussion regarding the methods used in the investigation of receptor stoichiometry.

1.2 GPCR classifications and evolution

1.2.1 General protein evolution

The evolution of new proteins within a species is an outcome of two distinct steps: the mutation of DNA encoding the protein in question, and the fixation of the new protein variant in the population. The likelihood of fixation of new proteins within the species depends on the selective effects that the mutations exert on the fitness of the whole organism. The neutral and nearly neutral models of molecular evolution suggest that the majority of mutations do not have significant impacts on the fitness of the organism as a whole and so are governed almost entirely by simple genetic drift (29, 30). A minority of mutations are either advantageous or deleterious for the fitness of the organism and so are respectively supported and opposed by selection. There is, however, a large body of evidence to suggest that proteins do not evolve wholly independently of one another. This is in part due to genomic influences on the fixation rate of individual genes that arises because genes cannot be inherited individually between generations but rather only in chromosome-defined units. Thus, a deleterious mutation that is part of the same chromosome as an advantageous one is less likely to undergo negative selection, and *vice versa* the advantageous mutation is less likely to become fixed in the population (31, 32). This effect is partially relieved by meiotic recombination, which breaks up the

linkage between neighbouring loci and so allows subsequent independent evolution. This is consistent with the observation that regions of the *Drosophila melanogaster* genome that undergo low levels of recombination also have reduced rates of protein evolution (33). Nonetheless, a number of confounding observations have led to the suggestion that the major factors influencing the rate of protein evolution are protein-specific rather than genomic (34), *e.g.* that interference between genomic loci can be greatly alleviated by low levels of recombination (35), and that the link between rates of recombination and evolution can be overcome by factors such as transcriptional activity (36). Instead, the rate and direction of protein evolution appears to be largely determined by a number of protein-specific variables, including fitness density (*i.e.* the proportion of its sequence that has implications for evolutionary fitness; 37), protein dispensability (38), timing and location of expression (39, 40), and expression level (41).

One important factor influencing a protein's rate of evolution is its degree of connectedness to the functions of other proteins and networks. Rates of evolution are lower for genes whose products are highly connected to complex processes and undergo more protein-protein interactions (42-45). This is because connections (both physical and indirect) to other proteins effectively increase a protein's fitness density and so correspondingly reduce its evolutionary plasticity. This effect is analogous to the well established principle of adaptive evolution that pleiotropy limits the rate of genetic evolution (46). It seems that this effect is most profound within stable protein complexes rather than for proteins participating in transient protein-protein interaction networks (47, 48).

Evolution of multigene families

Multigene families (*i.e.* groups of genes descended from a common ancestral gene) are generated by gene duplication events of parent genes giving rise to identical duplicates that subsequently undergo mutation to become distinct but closely related genes (49). Originally, multigene families were believed to undergo 'concerted' evolution, in which all members of the family evolve as a single unit and mutations are spread over the whole family by repeated unequal crossover and/or gene conversion events (50). More recently, however, consensus has shifted to the view that most multigene families are instead subject to a 'birth-and-death' model of evolution, in which the products of gene duplication events are able to evolve in a genetically independent manner rather than as a single unit (51). Genes under this model can be thought of as having independent lifetimes in which they are 'born' through gene duplication and subsequently undergo independent mutation until they either fulfil their own physiological niche independent of the parent gene, or lose functionality and degrade into a pseudogene ('death'). The factors that determine the rate and direction of evolution within this

process differ from one protein to the next, as discussed above. This model has been used to explain the observed evolutionary lineages of numerous multigene families, including the major histocompatibility complex (52), immunoglobulin (52), histone (53), and ubiquitin (54) gene families.

1.2.2 Overview of GPCR evolution

GPCRs in humans are categorised into six principal families based on their phylogenetic relationships: *Glutamate*, *Rhodopsin*, *Adhesion*, *Frizzled*, *Taste2*, and *Secretin*, known collectively as the GRAFS nomenclature (26). Under a separate system that groups GPCRs based on their cognate ligands, group A corresponded to *Rhodopsin*, group B to *Adhesion* and *Secretin*, and group C to *Glutamate*. These six families comprise the GPCRs in all mammals, and also those present in large numbers in a remarkably diverse range of bilateral species, including *Caenorhabditis elegans* (nematode), *Drosophila melanogaster* (fruit fly), *Strongylocentrotus purpuratus* (sea urchin), *Ciona intestinalis* (sea squirt), and all vertebrate species. However there also exist a number of non-vertebrate receptor families found in more evolutionarily distant species. The most prominent of these are the *cAMP* family of amoebae (55), the large nematode chemoreceptor family (56), and the fungal pheromone receptors (57).

Evolution of the GPCR families

The precise origin of mammalian GPCRs is uncertain. Vertebrate-type GPCRs are common to virtually all eukaryotic species and are present across all of the eukaryota, but are not found in bacteria (58). The archaeal 7TM protein bacteriorhodopsin, which acts as light-driven proton-pump, does not signal via G proteins and shows no significant sequence identity with classical GPCRs (59). It is therefore unclear how bacterial and archaeal 7TM proteins relate to GPCRs. The most evolutionarily ancient vertebrate-type GPCRs currently identified are found in the Dictyostelid *Dictyostelium discoideum*, which possesses 25 members of the *Frizzled* family, 17 from the *Glutamate* family, one *Adhesion*-family receptor, and 12 members of the *cAMP* family (60). This is the first known occurrence of the *Frizzled* and *Adhesion* families but *Glutamate* and *cAMP* receptors have been also found in species even more evolutionarily distant from humans than *D. discoideum*. It is therefore now believed that most, if not all, mammalian GPCRs are descended from an ancient common ancestor of the *cAMP* and *Glutamate* families (61). Sequence analysis of the GPCR repertoires of 12 evolutionarily diverse species has indicated that the early *cAMP* family gave rise to the *Frizzled* and *Adhesion* families at similar times during the evolution of the earliest eukaryotes, approximately 1400 million years ago (61). *Trichoplax adhaerens*, which was among the earliest species to split from the vertebrate lineage, possesses

members of the *Rhodopsin* family indicating that this family of GPCRs appeared soon after the emergence of the *Frizzled* and *Adhesion* families from *cAMP*. Mutually exclusive motifs characteristic of both *Adhesion* and *cAMP* family members are found in *T. adhaerens* *Rhodopsin*-family receptors, meaning that the direct relationship is unclear, however Nordstrom and colleagues have proposed that the most likely scenario is the divergence of *Rhodopsin* from *cAMP* as motif conservation is more significant between these families than between *Rhodopsin* and *Adhesion*. The *Rhodopsin*-family receptors most similar to the *cAMP* and *Adhesion* families are those found within the melatonin receptor subgroup, indicating that these were among the earliest GPCRs to emerge in this family.

The remaining human GPCR families emerged much later. The *Secretin* receptors diverged from the *Adhesion* family approximately 1000 million years ago – at roughly the time of the common ancestor between humans and sea anemones – and the two families share numerous sequence motifs and splice-sites that are not conserved in the other GPCR families (58). The *Taste2* receptors appear to have diverged from the *Rhodopsin* family at a time near to the split between the mammalian and avian lineages, roughly 300 million years ago. Their inclusion, under the GRAFS nomenclature system, in a family with the *Frizzled* GPCRs is due to a number of similarities between the consensus sequences of the two groups, *e.g.* the conserved motifs of *IFL* in TM2, *SFLL* in TM5, and *SxKTL* in TM7, none of which is found in the other GPCR families (26).

Humans express a number of GPCRs that do not group into the GRAFS families, namely the vomeronasal type 1, ocular albinism-like, GPR108-like, and ITR-like receptors; however these groups comprise only 8 receptors and so they are not considered informative examples of general GPCR biology. An overview of the topology, size, and evolutionary lineage of the GPCR families in human is given in Figure 1.1.

1.2.3 The human *Rhodopsin* family

By far the largest GPCR family in humans, and indeed all bilateral species with the exception of nematodes, is the *Rhodopsin* family. Of the approximately 800 GPCRs encoded by the human genome, 687 belong to the *Rhodopsin* family, which leads to a strikingly unbalanced phylogenetic distribution of receptors across the whole GPCR family. This is in part due to the large number of olfactory receptors contained within the family (~460 genes), yet the non-olfactory *Rhodopsin* family receptors still outnumber the combined membership of all other GPCR families by a factor of more than two. These receptors are the most diverse family in terms of both physiological function and ligand selectivity, and as such include the most studied GPCRs and the vast majority of licensed drug targets. They are

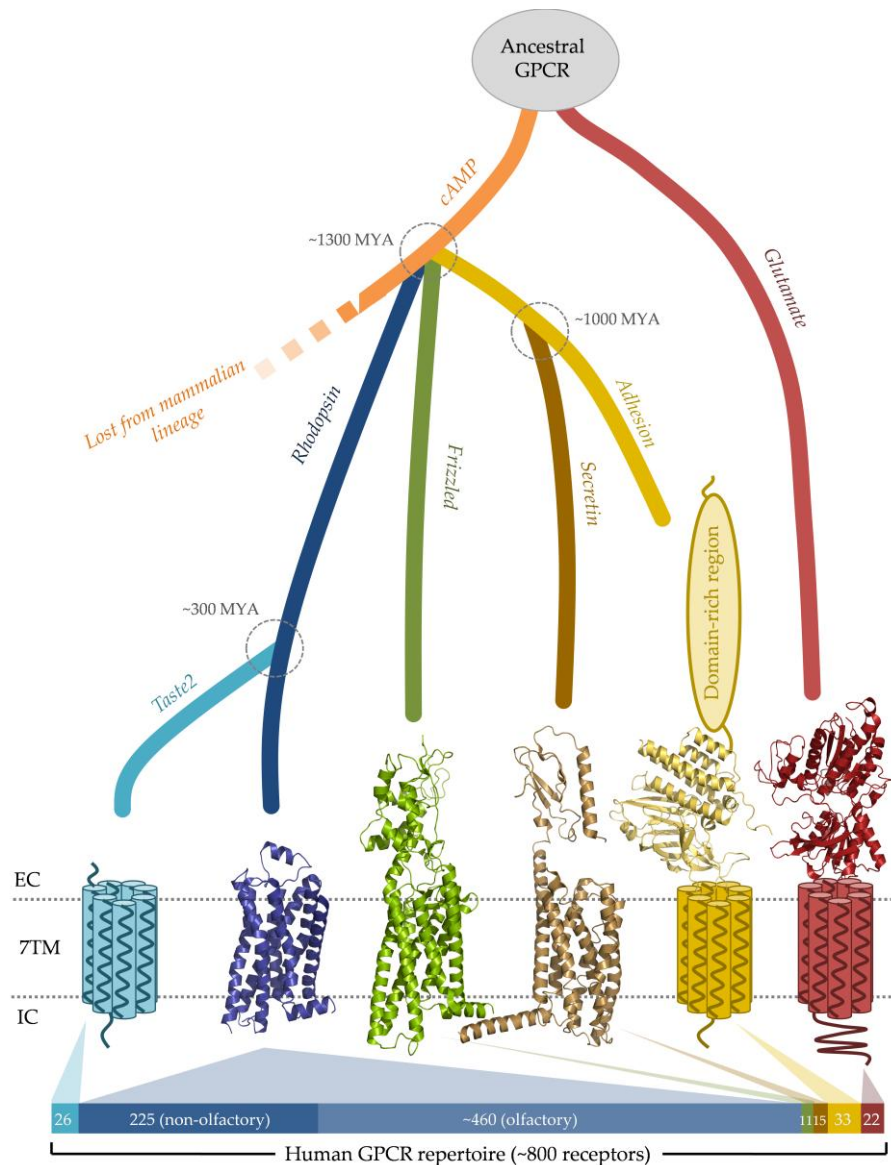


Figure 1.1: Overview of topology, size, and evolutionary lineage of the six main GPCR families in humans. The *cAMP* family, no longer present in humans, was the parent family for the *Rhodopsin*, *Frizzled* and *Adhesion* GPCRs, all of which emerged from *cAMP* ~1300 million years ago (MYA). The *Taste2* and *Secretin* families diverged subsequently from the *Rhodopsin* and *Adhesion* families, respectively. The *Glutamate* family appears to have diverged from the other families at an earlier point of evolution. The *Rhodopsin* and *Taste2* receptors are topologically simple, with small extra- and intracellular domains, whereas the other families are more complex as they possess large N- and/or C-terminal domains. Structures shown are representative of the families in question (PDB codes: *Rhodopsin* = 3NYA; *Frizzled* = 4JKV & 1IJY; *Secretin* = 4L6R & 4ERS; *Adhesion* = 4DLQ; *Glutamate* = 1EWV). The *Rhodopsin* family is by far the most numerous group of GPCRs in the human genome, with nearly 700 members.

further divided into four subfamilies, termed α , β , γ , and δ , based on their phylogenetic relationships (26).

Despite their extremely diverse biology, there are several conserved structural elements that identify *Rhodopsin* family receptors from the other GPCR families, including the *NSxxNPxxY* motif in TM7, and the *DRY* motif at the border between TM3 and IL2, which are found in almost all *Rhodopsin* GPCRs (26). The broad range of ligands that recognise these receptors are almost universally bound by residues lining a cavity formed between the TM helices, and the similarities between receptors for which structures have been solved suggest that most if not all *Rhodopsin* GPCRs mediate signal transduction via similar conformational changes (62). The need for a ligand-binding cavity is evident in most receptors because they lack significant extracellular domains that could otherwise mediate binding. Indeed, the vast majority of *Rhodopsin* receptors have only short extracellular loops and N-terminal domains of fewer than 70 amino acids (63). Nonetheless, there are exceptions to this. The leucine-rich repeat-containing GPCRs (LGRs) constitute a subfamily of five receptors that contain a distinct leucine-rich repeat region (LRR) within their N-terminal regions, which are over 500 residues in length and partially glycosylated (64). In addition, the relaxin-binding LGRs, LGR7 and LGR8, also possess a low-density lipoprotein receptor class A domain (LDLa) adjacent to their LRRs (64). The possession of LRR domains is shared with the follicle-stimulating hormone (FSHR), luteinizing hormone (LHCGR), and thyrotropin receptors (TSHR) (63), all of which cluster together with the LGRs in the δ subfamily (26). The only other *Rhodopsin* GPCRs that possess large N termini with conserved domains are the protease-activated receptors (PAR1-4), which have an intrinsic cleavage site within their N-terminal regions (65). The cleavage of this site by thrombin (PARs 1, 3, and 4) or trypsin (PAR2) exposes a tethered ligand, which is then able to activate the receptor. The unique coupling of receptor and ligand in this case explains the necessity for an N terminus larger than that found in most *Rhodopsin* GPCRs.

The human *Rhodopsin* α subfamily

The α subfamily in humans consists of 89 receptors subdivided into five main clusters: the prostaglandin, amine, opsin, melatonin, and MECA clusters (26). Several similarities are immediately observable across these groups. Firstly, all of the known ligands for receptors in this subfamily are small molecules rather than the peptide or protein ligands bound by other subfamilies. These include small amine molecules, *e.g.* serotonin or adrenaline; nucleotides; sphingolipids, *e.g.* sphingosine-1-phosphate (S1P); phospholipids, *e.g.* lysophosphatidic acid (LPA); or protanoids, *e.g.* prostaglandin and thromboxane. Functional similarity is also observed across α subfamily GPCRs, as most

contribute in some way to neuronal or central nervous system (CNS) function. In some cases receptors directly mediate neurotransmitter signalling, *e.g.* the serotonin or adrenaline receptors (66, 67), whereas others are involved more in correct neuronal and CNS development, *e.g.* the LPA or prostaglandin receptors (68, 69).

Of the five main clusters of the α subfamily, the amine cluster is the largest, containing 40 receptors for which the known ligands are all small amines with single aromatic rings. These include serotonin, dopamine, adrenaline, histamine, and acetylcholine, and so this cluster contains some of the most highly studied GPCRs, *e.g.* the α - and β -adrenergic receptors, the muscarinic acetylcholine receptors, and the histamine receptors. Interestingly, receptors in this cluster do not always share maximum identity with receptors for the same or similar ligands, and there are numerous cases in which receptors of one ligand are represented across the cluster. For example, the β -adrenergic receptors are more closely related to dopamine receptors 1 and 5 than to α -adrenergic receptors, while histamine receptors 2 and 4 are quite distantly related and instead share greater homology with the trace-amine and acetylcholine receptors, respectively (26).

The MECA cluster is so-called because it is comprised of the melanocortin receptors, endothelial differentiation GPCRs, cannabinoid receptors, and adenosine-binding receptors. It is the second largest α cluster with 22 members, of which three are orphans (GPR3, GPR6, and GPR12). Interestingly, the known ligands of MECA GPCRs are structurally very diverse, unlike the broadly similar ligands of the amine cluster. Moreover, the MECA cluster segregates into phylogenetically distinct subfamilies that share ligands: the cannabinoid receptors, for example, are 39% identical and are more closely related to the endothelial differentiation GPCRs than the adenosine-binding receptors, whereas the melanocortin receptors are between 39 and 56% identical and share several short motifs with the three orphan receptors.

The prostaglandin cluster contains eight prostaglandin receptors and seven orphan GPCRs. The prostaglandin receptors share 19-41% sequence identity and a number of unique conserved motifs in TM1 (*LxxTDxxxG*) and TM7 (*IxDPW*), whilst the orphan receptors are less closely related and are split into two groups: the SREB (super-conserved receptor expressed in brain) and non-SREB receptors. The smallest cluster within the α subfamily is the melatonin receptor cluster, which consists of only three receptors: the melatonin receptors MTNR1A and MTNR1B; and the orphan receptor GPR50. These receptors are closely related in their TM regions, especially in TM3, which is almost identical across the group. GPR50 is distinct from the other receptors because it possesses an extended C-terminal domain that is missing in the melatonin receptors; however there is still evidence that it contributes to melatonin signalling despite this divergence from the other receptors (70).

The most functionally distinct cluster within the α subfamily is the opsin cluster, which contains nine receptors that all mediate light-dependent signalling via coordinated retinal molecules (71). As already noted, despite their functional similarities, mammalian opsins and archaeal bacteriorhodopsin do not share any significant sequence identity (59), and instead the opsins are most closely related to the melatonin receptors (26). Interestingly, given the neuronal function of many α subfamily GPCRs, some opsin receptors are known to contribute to non-visual signalling in brain and neuronal tissues (72), suggesting a preserved functional relationship with the other α clusters.

The human *Rhodopsin* β subfamily

The β subfamily (35 members) is the only *Rhodopsin* subfamily in humans that does not display any significant clustering of GPCRs into distinct branches, although the receptors in this subfamily with shared ligands are usually the most closely related (26). The known ligands for β subfamily GPCRs are exclusively peptides, including hypocretin, neuropeptide Y, tachykinin, neuromedin B, and oxytocin. This consistency in ligand selection suggests a conserved or partially conserved mechanism of ligand recognition throughout the β GPCRs. It is only recently, with the determination of the structure of the rat neurotensin receptor 1 (NTR1) in complex with a neurotensin fragment (73), that details of ligand recognition have begun to emerge. In this structure, the majority of receptor-peptide interactions are formed by residues in EL2, EL3, TM6, and TM7. These take the form of extensive van der Waals interactions, a small number of hydrogen bonds, and ionic interactions between positively charged arginines on neurotensin and negatively charged residues towards the extracellular side of NTR1. The most striking difference between this mechanism of ligand-binding compared to other *Rhodopsin* receptors is that the peptide ligand enters far less deeply into the core of the 7TM region than small molecule ligands. Indeed, the C terminus of neurotensin is positioned more than 5 Å nearer to the extracellular side of NTR1 compared to the agonists of rhodopsin, and the α - and β -adrenergic receptors (74-76). It is unknown whether a similar mechanism of binding is conserved throughout the β subfamily GPCRs, but comparable interactions occur for the γ subfamily receptors CXCR4 and OPRL1 and peptide mimetic antagonists (77, 78), suggesting conservation of peptide-binding across the *Rhodopsin* family.

The human *Rhodopsin* γ subfamily

Humans possess 59 receptors in the *Rhodopsin* γ subfamily, which is more closely related to the β subfamily than either α or δ (26). This is reflected in the fact that most, but not all, γ subfamily GPCRs

also recognise peptide ligands. The receptors in this subfamily branch into three major clusters, by far the largest of which is the chemokine receptor cluster with 42 members. This group is so-named because it contains all of the classic CC- and CXC-motif chemokine receptors (CCRs and CXCRs), which are more closely related to one another than to any other GPCRs. This, combined with the observation that all but three chemokine receptor genes belong to the same HOX paralogon (79), suggests that all human chemokine receptors derive from a common ancestor. Interestingly, however, the CC- and CXC-motif chemokine receptors do not form two separate families and instead comprise subgroups that are independent of CC- or CXC-motif selectivity. For example, CCRs 7 and 9 are more closely related to CXCR4 than to CCR10, and all four of these receptors are closer relatives of CXCRs 1, 2, and 6 than of CCRs 1-5. This implies that the chemokine-binding mechanism is likely to be broadly conserved with small alterations generating CC- or CXC-selectivity, rather than there being large differences in binding-site structure that would have necessitated significant sequence divergence. As mentioned previously, there is evidence to suggest that this mechanism may be closely related to those used by other peptide-binding GPCRs.

The chemokine receptor cluster also contains a number of GPCRs that do not recognise chemokines. These also primarily recognise peptide ligands, *e.g.* the angiotensin, bradykinin, and N-formyl peptide receptors, although there are several exceptions. The complement component 5a receptors C5R1 and C5R2, and the chemerin receptors CMKLR1 and GPR1 recognise larger proteins or protein fragments, perhaps in a similar manner to the peptide receptors. More unexpectedly, three chemokine cluster receptors recognise eicosanoid molecules, *i.e.* the leukotriene B₄ receptors LTB₄R1 and LTB₄R2, and the prostaglandin D₂ receptor 2 (DP₂), and so might have been expected to share a closer relationship to the *Rhodopsin* α subfamily GPCRs. In actual fact, DP₂ shares only 8% identity with the α subfamily prostaglandin D₂ receptor 1, and only ~13% identity with the other prostaglandin receptors, whereas it is ~30% and ~25% identical to the N-formyl peptide and angiotensin receptors, respectively. The presence of these non-peptide receptors in the chemokine cluster is extremely interesting as it suggests a potential overlap between the mechanisms for recognition of peptide and small-molecule ligands by γ subfamily GPCRs, although as yet there is no structural clarification of this matter.

The remaining members of the γ subfamily group into two branches: the SOG and melanin-concentrating hormone (MCH) clusters. The MCH cluster consists of only two GPCRs, MCHR1 and MCHR2, which exhibit general γ subfamily ligand-selectivity as both recognise the cyclic neuropeptide MCH. The SOG cluster, containing five somatostatin receptors, 3 opioid receptors, and 3 galanin receptors, also includes the neuropeptide W receptors GPR7 and GPR8, the RF-amide receptor

GPR54, and the nociceptin/orphanin FQ peptide receptor OPRL1. Thus, the receptors in this cluster are all known to recognise peptide ligands, although the ligands themselves do not share structural similarities with each other or with other γ subfamily ligands.

The human *Rhodopsin* δ subfamily

The δ subfamily of human *Rhodopsin* GPCRs is by far the largest mainly because it contains ~460 olfactory receptors. There are 58 non-olfactory δ subfamily receptors, a number comparable with the other *Rhodopsin* subfamilies, and these are grouped into three main clusters. The largest of these is the purin cluster containing 42 GPCRs (26). The ligand-specificity of the purin cluster is diverse and restricted to small molecules with a limited number of exceptions. The ten P2Y purinergic receptors, for example, recognise a range of nucleotide molecules (80); whereas the two cysteinyl leukotriene receptors, the platelet-activating factor receptor, and a number of other purin cluster GPCRs bind to lipid-derived ligands. The PARs are also members of the purin cluster but do not bind small-molecule ligands and are instead activated by proteolysis.

The remaining non-olfactory δ subfamily GPCRs are grouped into the MAS and glycoprotein clusters, both of which contain eight members. The MAS cluster is named after its archetypal member, the MAS1 oncogene (MAS), which is closely related to the seven MAS-related GPCRs (MRGs) that make up the cluster: MRG, MRGD, MRGF, MRGX1, MRGX2, MRGX3, and MRGX4 (26). The ligand of MAS is the peptide hormone angiotensin-(1-7) (81), which has also been shown to activate MRG and MRGD (82), although the mechanism of binding is unknown.

The glycoprotein cluster is made up of the five LGRs, FSHR, TSHR, and LHCGR, which, as discussed previously, possess the largest N-terminal regions of the *Rhodopsin* family in the form of LRR and LDLa domains. These GPCRs all recognise glycopeptides ligands via their N-terminal domains. The crystal structure of the LRR domain of LGR4 in complex with an N-terminal fragment of its ligand R-spondin (83) reveals that the receptor forms a horseshoe-like structure consisting of two β -sheets of 10 and 5 β -strands separated by two additional β -strands. This curves around the glycoprotein ligand and forms several polar and non-polar sidechain interactions. A highly similar structure has been observed for FSHR in complex with follicle stimulating hormone (84), revealing a common ligand-binding mechanism across the cluster.

Also grouped into the *Rhodopsin* δ cluster are the olfactory receptors, which outnumber all the non-olfactory GPCRs combined across all families. In fact, the ~460 functional olfactory receptor genes identified in the human genome are matched by a similar number of pseudogenes, therein generating a total of over 900 olfactory receptor genes and pseudogenes (85). The primary function of

olfactory receptors is the detection of odorant molecules at the surface of olfactory receptor neurons (86), however they are also known to be expressed in a number of tissues outside the canonical olfactory system, *e.g.* airway epithelia (87), gut epithelium (88), and sperm cells (89), where they fulfil chemosensory functions. Given the extreme size of the group, it is noteworthy that the average sequence identity between randomly selected olfactory receptors is 35-40% (90), much higher than that observed within other, far smaller, GPCR clusters. This relatively low degree of divergence is reflected in their length, which varies only from 291 to 355 amino acids, with an average length of 315 residues (90). This is shorter than the vast majority of non-olfactory human *Rhodopsin* GPCRs, as are the N- and C- terminal domains of olfactory receptors, which are only ~25 and ~20 residues, respectively. It seems likely, therefore, that the olfactory receptors exist as highly compact structures *in vivo*, with very little extension from the 7TM region into the extracellular or cytoplasmic environments. This may partially explain why ligand-selectivity among olfactory receptors is relatively relaxed, with one receptor able to recognise numerous odorant molecules, and one odorant molecule able to be recognised by numerous receptors (91, 92). A loose system of ligand-discrimination is compatible with olfactory receptors being simple receptors that do not possess extensive ligand-recognition domains and bind a large variety of small molecules. The sheer number of human olfactory receptors suggests a strong positive selection for diverse olfactory capabilities during vertebrate evolution. The distribution of olfactory receptors within the genome as well as the large proportion of pseudogenes is consistent with the model of birth-and-death evolution (51, 93). The application of this model to the olfactory receptors is further supported by the observation that numerous mammalian species possess far more functional olfactory GPCRs than humans – mice, for example, have over 1000 (51) – and a smaller proportion of pseudogenes. It is believed that this is due to the relaxation of selective pressure for broad olfactory ability in humans due to improvements in the other senses. Indeed, the degeneration of the primate olfactory receptor family coincides with the evolution of full trichromatic vision (94). This mechanism of birth-and-death evolution may explain the minimal sizes of the olfactory receptors compared to other human GPCRs, as a functionally simple receptor might more readily diversify its specificity with a small number of mutations from the parent gene, thereby generating a large, functionally diverse family with relatively high sequence conservation.

1.2.4 Other human GPCR families

Compared to the *Rhodopsin* receptors, the other GPCR families in humans are extremely small in number, with a combined total of ~107 receptors. These are divided into 33 *Adhesion*, 15 *Secretin*, 22

Glutamate, 11 *Frizzled*, and ~26 *Taste2* GPCRs. All of these families share the conserved 7TM topology of the *Rhodopsin* receptors, and crystal structures of three non-*Rhodopsin* receptors indicate similar organisation of the TM helices to the known structures of the *Rhodopsin* family. Two of these structures were solved for *Secretin* family receptors: corticotropin-releasing factor receptor 1 (CRF₁R; 95), and the glucagon receptor (GCGR; 96); while the third was for the *Frizzled* GPCR smoothened (SMO; 97). The overall 7TM architecture in the *Secretin* receptors is broadly similar to that observed previously in *Rhodopsin* family structures, although TM7 of CRF₁R is rotated relative to the equivalent helix in most GPCRs. This gives the receptor a distinctive 'V' shape that exposes a large ligand-binding cavity towards its extracellular side. A similar cavity is observed for GCGR, suggesting that such clefts may be a feature of several *Secretin* family receptors. Several other features not seen in *Rhodopsin* family GPCRs are the extremely long H8 of GCGR, and the absence of a conserved TM3-TM6 salt-bridge in either receptor. The structure of SMO shows that the overall 7TM architecture is highly similar to that of *Rhodopsin* family receptors, more so than the *Secretin* receptors, which is likely to be the case across the *Frizzled* family as a whole given their relatively high levels of sequence conservation. Despite these similarities, there are a number of striking differences in aspects of the structure and function of the non-*Rhodopsin* receptors compared to the *Rhodopsin* GPCRs.

Extracellular domain size

The most conspicuous structural difference between the *Rhodopsin* receptors and most of the other families is the size of their extracellular domains. Unlike the *Rhodopsin* receptors, GPCRs from the *Adhesion*, *Secretin*, *Glutamate*, and *Frizzled* families almost all possess large N-terminal domains with highly conserved structures. Only the *Taste2* receptors are topologically similar to the *Rhodopsin* GPCRs, as they in fact contain the shortest N-terminal domains of all the GPCR families, ranging from 6 to 19 residues in length (98). The largest N-terminal domains are found in the *Adhesion* receptors, which have extremely long and diverse N termini containing numerous functional domains *e.g.* lectin, laminin, cadherin, immunoglobulin, olfactomedin, and thrombospondin domains (63, 99). These regions vary in length from 200 to 2800 residues and appear to be highly glycosylated (26). This, coupled with their high proline content, has led to the suggestion that these domains form rigid, mucin-like structures that protrude away from the cell surface to form contacts with other cells or extracellular matrix components (100), hence the '*Adhesion*' label. The ligands of the relatively small number of de-orphaned *Adhesion* receptors support the model of their acting as cell-cell or cell-matrix linkers. Epidermal growth factor-like module containing mucin-like receptor protein (EMR) 2 interacts with the glycosaminoglycan chondroitin sulphate, a major component of the extracellular matrix (101),

whilst EMR3 and 4 are known to form cell-cell contacts with as yet unidentified ligands on the surface of macrophages and lymphoma cells, respectively (102, 103). Similarly, GPR56 interacts with tissue transglutaminase TG2 in the extracellular matrix and thereby aids in the suppression of metastasis in melanoma (104). The N-terminal domain of *Adhesion* receptors is made larger still by the presence of a GPCR proteolytic domain (GPS) between the domain-rich region and the start of TM1. This is an autocatalytic cleavage site that allows proteolysis of the receptor into two non-covalently attached subunits whilst still in the endoplasmic reticulum or early Golgi compartments and appears to have a role in correct folding and trafficking to the plasma membrane (105).

Both the *Glutamate* and *Frizzled* family receptors also contain large N-terminal domains relative to the *Rhodopsin* GPCRs. The human *Glutamate* family is formed of 22 receptors forming two subgroups based on sequence similarities of their extracellular domains (106). Eight metabotropic glutamate receptors (mGluRs), the sweet and umami taste receptors (TAS1R1-3), the calcium sensing receptor (CASR), and GPRC6A form one subgroup, while the other is formed of two γ -amino butyric acid receptors (GABABR2 and three GABABR1 splice variants – a, b, and c). There are also seven orphan receptors that share some features with the other *Glutamate* receptors but differ significantly in key structural elements. Both major subgroups contain so-called Venus flytrap motifs (VFTMs) in their N-terminal domains, which are 280-580 residues in length (26) and are formed of two distinct lobes surrounding a central cleft (107). This domain in the mGluR-like subgroup shares structural similarities with bacterial amino-acid binding proteins, *e.g.* LIVBP (108), but is distinct from that in the GABABRs despite their similar overall shape (109). Moreover, mGluR-like *Glutamate* receptors also share a cysteine-rich domain (CRD) between the N-terminal VFTM and TM1, which contains nine conserved cysteine residues forming three predicted disulphide bonds (110) and is thought to act as a relay motif for the transmission of ligand-binding events into the 7TM region (111). The N-terminal domain of the *Frizzled* receptors also contains a large CRD of 200-320 amino acids containing nine conserved cysteine residues, one of which is missing in SMO (26). Alongside SMO, humans possess ten frizzled receptors (FZD1-10), which were initially identified as 7TM proteins in *D. melanogaster* (26, 112, 113) and all of which possess the large CRD.

The final non-*Rhodopsin* family, *Secretin*, also possesses a notably larger N-terminal domain than the typical *Rhodopsin* GPCRs. All 15 known *Secretin* family members have N-terminal domains of 60-80 residues length with a highly conserved disulphide bridge network (114) that is important in stabilising the N-terminal structure (115).

Ligand recognition

Whilst the *Rhodopsin* family predominantly bind ligands within a cavity formed between TM helices at the extracellular side of the receptor, the same is not true for GPCRs from most other families. Again, the most similar receptors to the *Rhodopsin* family in this regard are the *Taste2* GPCRs, which mediate ligand binding primarily via residues in the interhelical loops and TM helices (116). This similarity is perhaps unsurprising given the evolutionary relatedness of the two families. In all other non-*Rhodopsin* families, ligand binding is at least in part performed by the aforementioned large N-terminal domains. As mentioned above, ligand recognition in the *Adhesion* GPCRs appears to be mediated by the numerous discrete domains within their N-terminal regions. Such domains are believed to directly contact the respective ligands of the receptor, as has been observed in the EMR2-chondroitin sulphate interaction, which is mediated by an epidermal growth factor domain (101). The need for such a mechanism of ligand recognition is perhaps due to the nature of the *Adhesion* family ligands, which are often components of the extracellular matrix or adhesion proteins on other cells, and so would not be able to diffuse into a ligand-binding cavity within the 7TM region.

The ligands of the *Frizzled*, *Glutamate*, and *Secretin* receptors do not experience such constraints on their diffusion, and yet in each case they undergo recognition at least partly by the receptors' large N-terminal domains. Within the *Frizzled* family, the FZD receptors bind to secreted glycoproteins termed Wnt (117) as well as other glycoprotein ligands, *e.g.* R-spondin (118), whereas SMO apparently functions in a ligand-independent manner as the signalling component of the SMO-patched-sonic hedgehog complex (119). Ligand recognition by the FZD receptors is performed almost entirely by their N-terminal CRD (118), although there is some evidence that Wnt may also contact additional sites formed by extracellular interhelical loops (120). The structure of the FZD CRD is predominantly α -helical and stabilised by intra-domain disulphide bridges, although the residues that contact Wnt are generally located in non-helical regions (121). Interestingly, despite the apparent ligand-independence of the receptor, the N-terminal domain of SMO also contains eight of the conserved FZD cysteines as well as having strong conservation of the chemical properties of the residue side chains involved in FZD-Wnt interactions (63). Mutations of a number of these conserved residues disrupt SMO function (122, 123), suggesting an important functional, albeit non ligand-binding role for this region.

In a similar manner, it is the VFTM regions of the *Glutamate* GPCRs that mediate ligand recognition in these receptors. Glutamate and other endogenous ligands bind within this cleft in a manner reminiscent of a Venus flytrap (107), hence their name. The VFTMs are well conserved between the mGluR-like receptors and so it is unsurprising that ligand-selection between members of

this subgroup is broadly similar, consisting of glutamate and other amino acids or metabolic products. Indeed, even though the primary ligand for CASR is Ca^{2+} ions, the receptor also binds aromatic amino acids (124). Similarly, Ca^{2+} enhances glutamate-binding in some mGluRs (125), suggesting a degree of functional cross-over between CASR and the mGluRs. Despite the apparent necessity for VFTMs in ligand recognition by the *Glutamate* GPCRs, there are seven members of the family that lack the domain entirely: GPRC5A, GPRC5B, GPRC5C, GPRC5D, GABAbRL, GPR158, and GPR158L (106, 126-130). No ligand has yet been identified for any of these receptors and so it is possible that they function in a ligand-independent manner having lost their VFTMs.

Ligand recognition by *Secretin* receptors is initiated by N-terminal domains forming contacts between their proximal regions and ligand molecules, which then in turn form additional interactions with juxtamembrane portions of the N terminus as well as TM6 of the 7TM region (115, 131, 132). This bridges the N-terminal and 7TM regions of the receptor, stabilising the active conformation of the receptor. This conserved mechanism of activation is only possible because all known *Secretin* GPCR ligands are peptide hormones; including calcitonin, glucagon, secretin, gastric inhibitory polypeptide, and vasoactive intestinal peptide (63). Peptide ligands are better-suited to serving as linkers between distant receptor domains due to their large size and length compared to the small-molecule ligands of many *Rhodopsin* family receptors. So, again, ligand-recognition is markedly different to that of the *Rhodopsin* GPCRs.

Stoichiometry

A notable feature of at least some non-*Rhodopsin* GPCRs is their ability, and indeed sometimes functional necessity, to form stable dimers. The issue of *Rhodopsin* family stoichiometry is discussed in detail in Section 1.4. Briefly, the notion of *Rhodopsin* GPCR dimerisation is still contentious and, even in cases where it is thought to exist, is not generally believed to be a stable arrangement but rather a transient state in equilibrium between monomers and dimers. This is in stark contrast to the *Glutamate* GPCRs, which form stable homo- and heterodimers *in vivo* (133). The structure of the entire N-terminal domain (*i.e.* VFTM and CRD) of mGluR1 has been solved crystallographically and found to consist of two protomers linked by a disulphide bond between Cys140 of each monomer (107). It is believed that ligand binding within VFTMs effects a reorganisation of the two monomer units with the dimer complex, which is translated to the 7TM regions to affect signalling potential (107). So, in striking contrast to *Rhodopsin* GPCRs, the mechanism of signal transduction in *Glutamate* family GPCRs appears to be wholly dependent on the existence of receptor dimers.

The importance of dimerisation to *Glutamate* receptor function is confirmed by VFTM-independent mechanisms of dimerisation in these receptors. In the mGluR-like receptors, the CRD also appears to be capable of stabilising dimeric receptor complexes via the formation of disulphide bonds between neighbouring CRDs. This has been observed for the mGluR1 (134), mGluR4 (135), and CASR (136) CRDs in the absence of their 7TM regions. Similarly, the GABABRs (including GABABRL) also possess VFTM-independent dimerisation potential in the form of an intracellular coiled-coil domain adjacent to their C termini that is capable of forming contacts with the neighbouring coiled-coil in the dimer complex (137). These domains are of considerable functional significance as the GABABR receptors exist primarily as heterodimers in which the GABABR1 subunit contributes the ligand-binding capability and the GABABR2 mediates intracellular signalling (138, 139).

Considering the obvious functional similarities (*i.e.* ligand-binding) between the N-terminal domains of the *Glutamate* and *Frizzled* receptors, it is interesting to note that the *Xenopus* FZD3 homologue exists as a homodimer *in vivo* in a CRD-dependent manner (140). Moreover, mutant forms of rat FZD1 and FZD4 homologues interact with and disrupt correct trafficking of wild-type receptors, generating the phenotype of familial exudative vitreoretinopathy in an autosomal dominant manner (141). It is, however, still unclear how strong this CRD-CRD interaction is *in vivo*, as both mouse FZD 8 and the secreted FZD-related protein 3 are monomeric in solution at 100 μ M but dimerise during crystallisation (121). The dimer interface is equivalent in both homodimers, with two parallel intermolecular contacts at regions near short β strand elements at opposite ends of the CRD (121). Thus it is possible that the FZD receptors interact stably *in vivo* in the manner observed in the crystal structure, but not strongly enough to allow dimerisation of the CRDs in solution. Nonetheless, the structural and functional parallels of the *Glutamate* and *Frizzled* CRDs offer the intriguing possibility of analogous mechanisms of receptor dimerisation based on modular domains involving CRD-dependent mechanisms distinct from those of other GPCR families.

Diversification

Another notable difference between the *Rhodopsin* and non-*Rhodopsin* families is the rate of diversification of their constituent receptors. While the *Rhodopsin* GPCRs are extremely diverse in their sequence identity, receptors in the *Adhesion*, *Secretin*, *Frizzled*, and *Glutamate* families are relatively closely related by comparison (26), which indicates the existence of a constraining factor on the rate of receptor diversification in these families. This slower rate of diversification is evident in the extreme difference in size between the *Rhodopsin* and non-*Rhodopsin* families despite the fact that the *Rhodopsin*, *Frizzled*, *Adhesion*, and *Glutamate* families are all of a similar age (61). This size difference is not limited

to humans, as similar distributions of GPCRs across the various families are observed in many diverse species. For example, the chordate *Branchiostoma floridae* possesses 10 *Frizzled*, 17 *Secretin*, 11 *Adhesion*, 17 *Glutamate*, and 673 *Rhodopsin* receptors, and the sea anemone *Nematostella vectensis* has 6 *Frizzled*, 25 *Adhesion*, 34 *Glutamate*, and 826 *Rhodopsin* GPCRs (61). The constraint upon non-*Rhodopsin* GPCR diversification therefore appears to be maintained across much of eukaryotic biology.

A prominent exception to this is the *Taste2* family. Although there are only ~26 known *Taste2* proteins in humans, there are 28 full-length genes as well as 16 pseudogenes (98, 142). Sequence conservation between *Taste2* receptors varies from 21-90% and no significant identity is found within the N- and C-terminal regions (98). Given their relatively recent divergence from the *Rhodopsin* family, this indicates a rapid rate of *Taste2* receptor diversification. This is supported by the fact that some mammals possess far more *Taste2* receptors than are found in humans, as well as the large number of human *Taste2* pseudogenes that presumably once encoded functional receptors (142). This may be related to their function, as *Taste2* receptors are expressed primarily in the tongue and palate epithelium where they mediate sensation of bitter-tasting compounds (143) and there is a clear evolutionary advantage to sensing a wide range of bitter compounds, many of which are poisonous (144). Such positive selective pressure, as well as the rapid rate of diversification and large proportion of pseudogenes, is reminiscent of the olfactory receptors. As discussed above, such a scenario may favour functionally simple receptors that are able to diversify more easily than their more complex equivalents. It is perhaps unsurprising, therefore, that the *Taste2* receptors are similarly minimal in structure as the olfactory receptors. This may explain why the other non-*Rhodopsin* families have undergone much slower rates of receptor diversification and family expansion, as their greater levels of structural complexity may have precluded rapid evolution in some way.

1.3 GPCR structure, signalling, and organisation

1.3.1 GPCR structure

GPCRs possess one of the most distinctive topologies of any family of membrane proteins. It was clear from sequence analysis of early cloned GPCR genes that the receptors contain a core domain of seven transmembrane α -helices (TM1-7), which is characteristic of all members of the family and contributes the main ligand-binding and signal-transduction capabilities. These helices are joined by six inter-helical loops of varying lengths. The three extracellular loops (EL1-3) combine with the N-terminal portion of the protein to form the extracellular domain of the receptor, which also contributes to

ligand-specificity, while the cytoplasmic domain consists of the C-terminal region and three intracellular loops (IL1-3) and interacts with secondary signalling components *e.g.* G proteins.

The first high resolution GPCR structure was achieved in 2000, of bovine rhodopsin in its inactive conformation (145), and was the first to show the overall organisation of the transmembrane (7TM) region and the position of the individual TM helices and inter-helical loops. The second GPCR, and first human GPCR, to have its structure solved was the human β_2 -adrenergic receptor (β_2 AR) in 2007 (146), though the first structure of a GPCR in its active state was not determined until 2008 (147), a year in which several other GPCR structures were also solved, including squid rhodopsin (148), the turkey β_1 -adrenergic receptor (β_1 AR; 149), and the human adenosine A_{2A} receptor (A_{2A} R; 150). The relatively slow pace at which GPCR structures were forthcoming began to increase dramatically towards the end of the 2010s due to a number of technical innovations that overcame the high thermal lability and high TM-content of GPCRs (151), such as mutagenesis approaches for obtaining thermostabilised receptors. To date, 21 different GPCRs have been solved in 77 individual structures of various activity states. These receptors are diverse in their ligand-selectivity and represent examples of GPCRs from numerous functional groups, including the adrenergic (146, 149, 152), muscarinic acetylcholine (153, 154), chemokine (77), and opioid (78, 155-157) receptors. Thus, the archetypal structure of GPCRs is now well understood. Given the prevalence of the *Rhodopsin* family, these can be thought to represent 'standard' GPCR structure, and so all further discussion of receptor structure is specific to *Rhodopsin* family GPCRs.

7TM region organisation

The arrangement of TMs 1-7 within the 7TM body of GPCRs is strikingly similar across different receptors despite markedly diverse primary sequences. The seven TMs of GPCRs form a core bundle of a roughly cylindrical shape, with the helices arranged 1-7 in an anticlockwise direction if viewed from the extracellular side (Fig. 1.2). The receptors are almost entirely contained within the bilayer environment, although TM3, 5, and 6 each protrude 2-3 helical turns into the aqueous phase on the intracellular side. With the exception of TM3, the helices are oriented at roughly 90° relative to the plane of the membrane throughout most or all of their lengths. TM3 is unique in having a ~35° offset relative to the other helices, meaning that it is ~1 helical turn longer than the other helices and is able to contact more diverse areas of the 7TM domain. This angle means that it crosses over position with TM4 approximately half way into the membrane, such that it is flanked by TM2 and 4 on the extracellular side, but by TM4 and 5 on the cytoplasmic side. Proline- or glycine-induced kinks are

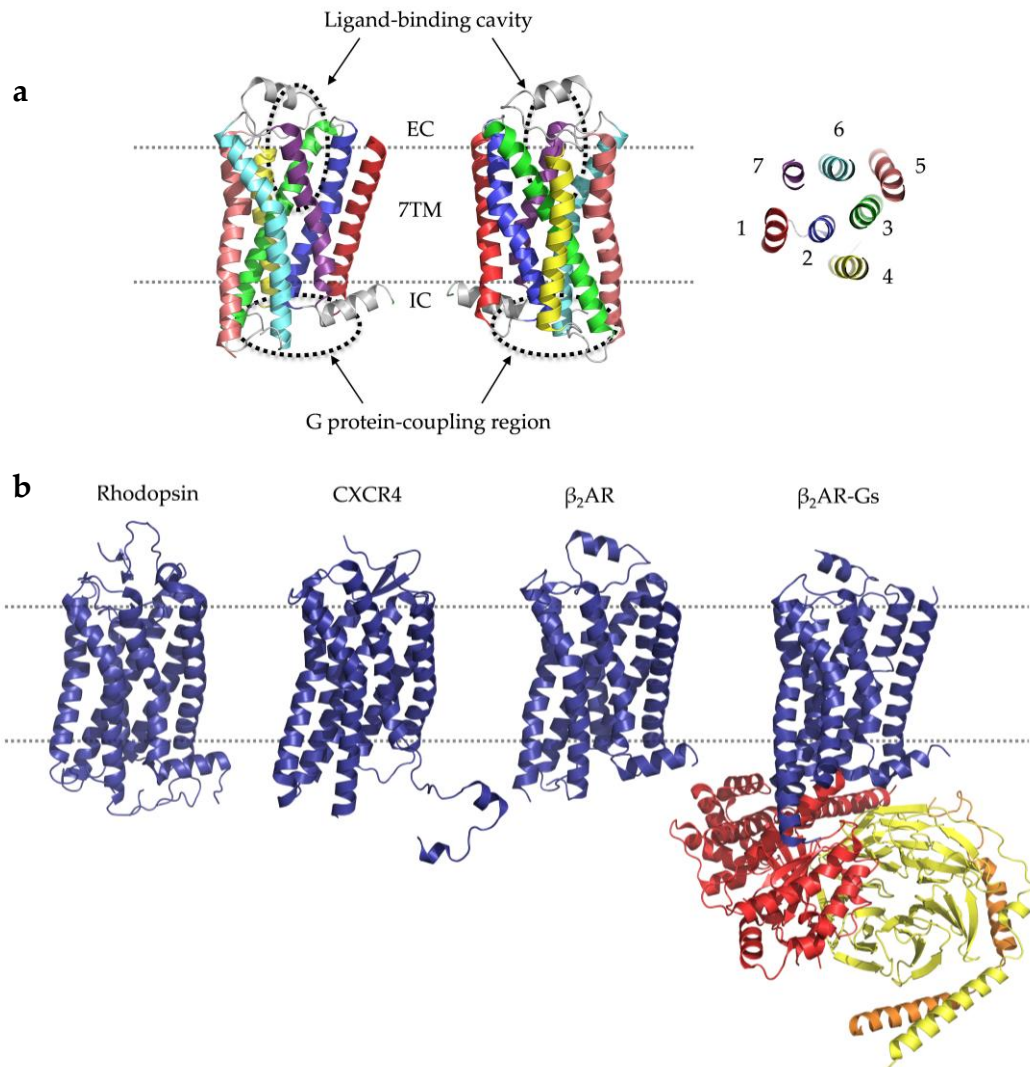


Figure 1.2: Overview of GPCR structure. (a) Typical organisation of GPCR domains. TM helices are shown as viewed from opposite sides of the receptor and as a cross-section viewed from the extracellular side. TM1 = red, TM2 = blue, TM3 = green, TM4 = yellow, TM5 = pink, TM6 = cyan, TM7 = purple. All non-TM regions are shown in grey. Ligand binding typically occurs in a cavity towards the extracellular side of the GPCR, whilst G proteins are bound by the intracellular region. Representative structure shown is β_2 AR (PDB: 3NYA). (b) GPCR structure is highly similar between diverse receptors. Organisation of the 7TM domain is similar in all GPCR structures currently solved. Examples of diverse *Rhodopsin* family receptors in their non-signalling conformation are shown for comparison. β_2 AR in complex with its cognate Gs protein is shown as an example of a typical GPCR in its signalling conformation. α , β , and γ subunits are coloured red, yellow, and orange, respectively. PDB codes: Rhodopsin = 3CAP; CXCR4 = 3OE0; β_2 AR = 3NYA; β_2 AR-Gs = 3SN6.

present in TM2, 4, 5, 6, and 7, causing them to splay out slightly from the central axis of the receptor on the extracellular side, thereby generating the ligand-binding cavity.

Within the 7TM region, there exist many conserved interhelical contacts that are found across the majority of receptors independent of sequence conservation. Analysis of the interhelical interactions in the currently available GPCR structures has revealed a network of 24 TM-TM contacts mediated by 36 topologically equivalent amino acids (158). Each of the interactions within this consensus network is maintained in either all or all but one of the known GPCR structures regardless of activation state. These contacts are mainly positioned towards the central and cytoplasmic areas of the 7TM region, and are most common at the interfaces between TM1-TM2 (4 contacts), TM3-TM4 (5 contacts), TM3-TM5 (4 contacts), and in the tripartite interface of TM3-TM6-TM7 (5 contacts). Although many of these interactions are mediated by highly conserved amino acids, *e.g.* Asn^{1.50}, Asp^{2.50}, Trp^{3.50}, and Pro^{4.50}, others demonstrate substantial amino acid variation¹. One case in point is the interaction between residues 2.42 and 3.46, which is conserved in all known structures yet shows no common identity across different GPCRs, constituting, for example, a Tyr-Met interaction in human kappa opioid receptor (κ -OR) but Ile-Leu in bovine rhodopsin (158). The universality of these interactions suggests that they are required to generate the core GPCR scaffold around which the key activation-dependent conformational changes can occur. Given the extent of its conserved contacts with the other helices, TM3 appears to be the most significant component of this central GPCR fold, implying that its uniquely tilted angle within the membrane might be functionally significant.

Ligand-binding within the 7TM region

Despite the remarkable diversity of their ligands, GPCRs exhibit significant levels of conservation in the location of residues that contribute to ligand-receptor interactions. The ligands of all *Rhodopsin* GPCRs for which structures are available are known to bind in a cavity formed in the extracellular side of the 7TM region. Within this cavity the ligand forms various polar and non-polar interactions with residues of the TM helices, primarily TM3, TM5, TM6, and TM7 (158). TM2 and TM4 contribute to ligand-binding in only a minority of cases, while TM1 does not interact with ligand in any of the currently available GPCR structures. Some ligands contact several residues in the extracellular domain of the receptor. Currently these are only the peptide ligands of the CXC-motif chemokine receptor

¹ Numbering denotes the Ballesteros-Weinstein nomenclature (159), in which residues are assigned a first number, 1-7, denoting their TM helix, and a second number corresponding to their position relative to the most highly conserved residue in the helix, which is assigned the number 50. One position towards the N terminus decreases the number by one and one position towards the C terminus increases the number by one.

CXCR4 (77) and the neurotensin receptor NTSR1 (73), both of which are too large to be fully accommodated in the conventional ligand-binding cavity and use additional means of stabilising the receptor-ligand interaction.

Although the ligand-binding mechanism appears to be broadly conserved across many GPCRs, the precise depth of ligand insertion into the 7TM region is markedly variable. Smaller ligands often infiltrate more deeply into the receptor than their larger equivalents, although this is not always the case. For example, doxepin binds approximately 14Å from the extracellular side of human histamine receptor H₁ (H₁R; (160); the larger peptide CVX15 ligand of CXCR4 inserts ~6Å (77); and the small molecule ligands caffeine and adenosine of the human A_{2A}R bind only to depths of ~5Å and ~7Å, respectively (150). It thus appears that ligand-binding varies significantly between GPCRs in terms of the exact extent and nature of the interactions, but that this variation exists within broadly conserved parameters, perhaps constrained by the tolerance of the signal-transduction mechanism.

GPCR extracellular domains

A striking feature of the extracellular domains of many GPCRs is the conformational stability that they often possess. In many cases, the structures of the ELs are observed for several available structures of the same receptor despite differences in crystallisation approach, crystal packing orientation, and agonist/antagonist selection, perhaps implying functional significance for these structures. This has been supported by hydrogen-deuterium exchange experiments on β₂AR (161, 162), which revealed exchange rates consistent with highly ordered regions. Nonetheless, potential areas of instability in the extracellular regions of some GPCRs have also been identified, including small segments of EL2 in A_{2A}R (150) and H₁R (160), which have failed to be resolved in crystal structures.

Although extracellular regions are stable in many GPCRs, they exhibit little sequence or structural conservation (63, 163). Indeed, the extracellular region is the area of greatest sequence divergence between GPCRs, presumably because it has a primarily receptor-specific role in ligand recognition that does not impinge heavily on the highly conserved signal-transduction mechanism of the 7TM domain. Nonetheless, the relative importance of individual loops does appear to be similar across receptors. This is most obvious in EL2, which is the longest of the three ELs in most GPCRs and usually contains most of the secondary structure elements in the extracellular domain. EL2 almost invariably forms a disulphide bridge with Cys^{3.25} of TM3, which stabilises the correct orientation of TM3 relative to the ligand-binding pocket and reduces potential conformational changes in this region (158), though it is notably absent in the sphingosine-1-phosphate receptor 1 (S1P₁) structure (164). This has the effect of essentially splitting EL2 into two elements, EL2a and EL2b, with the former

protruding away from the membrane and the latter packing more tightly against the 7TM region (62). In spite of these common features of EL2, its secondary structural elements vary significantly between GPCRs. In β_2 AR, for example, EL2a contains nearly 2.5 turns of an α -helix and EL2b is in an extended conformation (146), whereas in A_2A R EL2a adopts a short β -strand and EL2b contains a one-turn α -helix (150). The precise structure and chemistry of EL2 seems to be important in the early ligand-recognition events preceding full ligand-binding (165).

EL1 and EL3 are, by comparison, much less structurally distinct. These loops generally lack secondary structure and instead exist in extended conformations. Nonetheless, their conformations are usually conserved and may contribute to ligand recognition in many cases. For example, EL1 in A_2A R combines with EL2 to form the entrance to the ligand-binding pocket and EL3 forms salt-bridges with EL2 that influence ligand binding (150, 166). EL3 domains in many GPCRs are stabilised by disulphide bonds (150, 160, 167), also suggesting that these loops contribute to receptor function.

The N-terminal segments of GPCRs are similarly varied with apparent receptor-specific roles in ligand-recognition. These regions often lack secondary structure elements, but are sometimes stabilised by disulphide bridges with ELs, as in the case of CXCR4 (77). Interestingly, the N-terminal regions of rhodopsin and S1P₁ each have secondary structure elements – a β -hairpin loop and three-turn α -helix, respectively – that appear to block access to the ligand-binding pocket from the extracellular environment (145, 164). This implies that the hydrophobic ligands bound by these receptors enter the receptor via the lipid phase of the membrane rather than via the aqueous phase, as has been suggested for the cannabinoid CB2 receptor (168), and so the N termini of these GPCRs may act as a barrier to conventional ligand dissociation. Nevertheless, these receptors are exceptional and the majority of GPCR N-terminal segments appear to have less obvious structural roles.

GPCR intracellular domains

The intracellular regions of GPCRs are far more highly conserved than their extracellular counterparts, which is perhaps unsurprising considering that they are responsible for interaction with downstream signalling components common to all GPCRs, *e.g.* G proteins and arrestins (169, 170). This conservation is most apparent in IL1 and IL2, which are broadly similar across most receptors. IL1 consistently comprises six residues in either an extended or short α -helical conformation with a conserved leucine side-chain that forms close contacts with the 7TM region (62). Similarly, IL2 interacts with the DRY motif of TM3 in most receptors (158), disruption of which via mutation (149) or post-translational modification (171) appears to alter the conformational stability of different receptor activation states.

More diverse are the IL3 and C-terminal regions of GPCRs. IL3 varies greatly in size between receptors, ranging from just five to hundreds of residues in length. This region is often unstructured in crystal structures or is unobservable because it has been replaced by a stabilising fusion protein *e.g.* T4 lysozyme (*e.g.* 164), but is known to have partial α -helical character in at least some receptors (172, 173). The presence of alternative conformations of IL3 in the inactive form of β_1 AR (173) suggests that this region is highly flexible and may be intrinsically disordered in the absence of a binding-partner. This is supported by proteolytic and hydrogen-deuterium exchange analysis of β_2 AR (161, 162), which suggest low stability of any secondary structure elements that may form in IL3. This is similar to the situation in the C-terminal region, which is extremely variable in both sequence and length between receptors, and appears to be largely unstructured (163). This is with the notable exception of the so-called helix 8 (H8) region, which is a 3-4 turn amphipathic α -helix observed in all known GPCRs except CXCR4 (62) and NTSR1 (73). H8 runs parallel to the membrane and is often anchored within the bilayer by palmitoylation modifications, which seems to be functionally important for its roles in regulating receptor expression and stability (174). The remaining, unstructured regions of the C terminus are believed to act in conjunction with the unstructured areas of IL3 to allow recognition of binding partners prior to full interaction with the receptor. This is facilitated by their lack of secondary structure as it exposes linear peptide motifs, which are a common feature of protein-protein recognition events in many signalling processes (175, 176). Pre-coupling of G protein to GPCR has been linked to a polybasic motif adjacent to H8 in many receptors (177), thereby providing a link between these disordered regions and the recognition of signalling partners. Moreover, these regions are often subject to extensive post-translational modifications (178), *e.g.* phosphorylation by GPCR kinases or insulin receptor tyrosine kinase, which in turn allows for regulation of receptor activity and internalisation through recognition of these modifications by associated proteins.

Conformational changes upon activation

The advent of high-resolution GPCR structures in different states of activation has allowed many of the predominant conformational changes that occur upon ligand-binding to be elucidated (27, 179, 180). These are broadly conserved for the receptors whose structures are now available. Firstly, ligand-binding in the central 7TM cavity (or retinal isomerisation in the case of rhodopsin) stabilises the rearrangement of a cluster of conserved hydrophobic and aromatic residues on TM3, 5, and 6, altering the TM3-TM5 and TM5-TM6 interfaces (27). In A_2A AR and β_2 AR, this is achieved by partially collapsing the ligand-binding pocket through simultaneous interaction between the ligand and TM3, 5, and 7, pulling them closer together (181); whereas in rhodopsin, retinal isomerisation actually causes an

increase in the volume of the ligand-binding pocket (182), although the overall effect is the same. The helix movements associated with receptor activation are summarised in Figure 1.3.

These small-scale rearrangements at the extracellular side of the receptor are amplified through the core of the receptor in order to generate more significant structural changes in the intracellular region. Much of this is achieved by TM6, which rotates partially along its axis due to altered interactions with TM5 (183-185). Accordingly, due to the strong kink of TM6, this results in the large-scale repositioning of its cytoplasmic end, thereby exposing the previously occluded G protein-binding cleft (186). The scale of this movement varies between GPCRs (27) but can be as much as 14Å (187). A similar change is observed in TM5, where a reorganisation of the helix around a conserved proline residue amplifies the repositioning of the TM5 cytoplasmic end (188). Indeed, most TM helices undergo some form of rearrangement upon receptor activation, although TM5 and 6 are the most dramatic. The exceptions to this are TM1 and 2, which do not undergo any significant movements upon ligand-binding, and so it has been suggested that they have a greater role in correct membrane insertion and folding of receptors rather than in signal transduction (189).

The effect of these rearrangements is to prime the intracellular regions of the receptor for the binding of G proteins or other signalling components *e.g.* arrestins. The binding mechanism of G proteins has been clarified somewhat by crystal structures of rhodopsin in complex with a peptide mimic of the Gt C-terminal tail (76), and of β_2 AR bound to intact Gs (187). These structures demonstrate that G proteins dock with GPCRs through their G α subunit, with extensive contacts forming between the G protein α 5-helix and the receptor TM3, 5, and 6, as well as IL2. Interaction with the active receptor causes a number of structural changes in the G protein, including a 6Å movement of the α 5-helix and a repositioning of the β 6- α 5 loop (187). These changes alter coordination of the guanine ring in the G protein nucleotide-binding pocket, altering its relative affinities for GDP and GTP and allowing its activation (190).

While the snapshots of different GPCR states afforded by crystallisation studies are extremely useful in elucidating the general mechanism of receptor activation, they do not offer a full insight into the dynamic properties of the receptors *in vivo*. A great deal of biophysical and biochemical evidence suggests that GPCRs do not adopt binary 'on/off' states, but instead exist in a range of distinct conformations that are stabilised to varying extents by different ligands (191-194). This may explain the necessity for artificial stabilisation of many receptors prior to crystallisation. Moreover, the observation of 'inactive' state receptors in complex with agonists (74, 195, 196) suggests that in some cases additional binding partners, *e.g.* G proteins, are required for full stabilisation of the active complex. Indeed, anti-Brownian electrokinetic trapping analysis of individual β_2 AR molecules

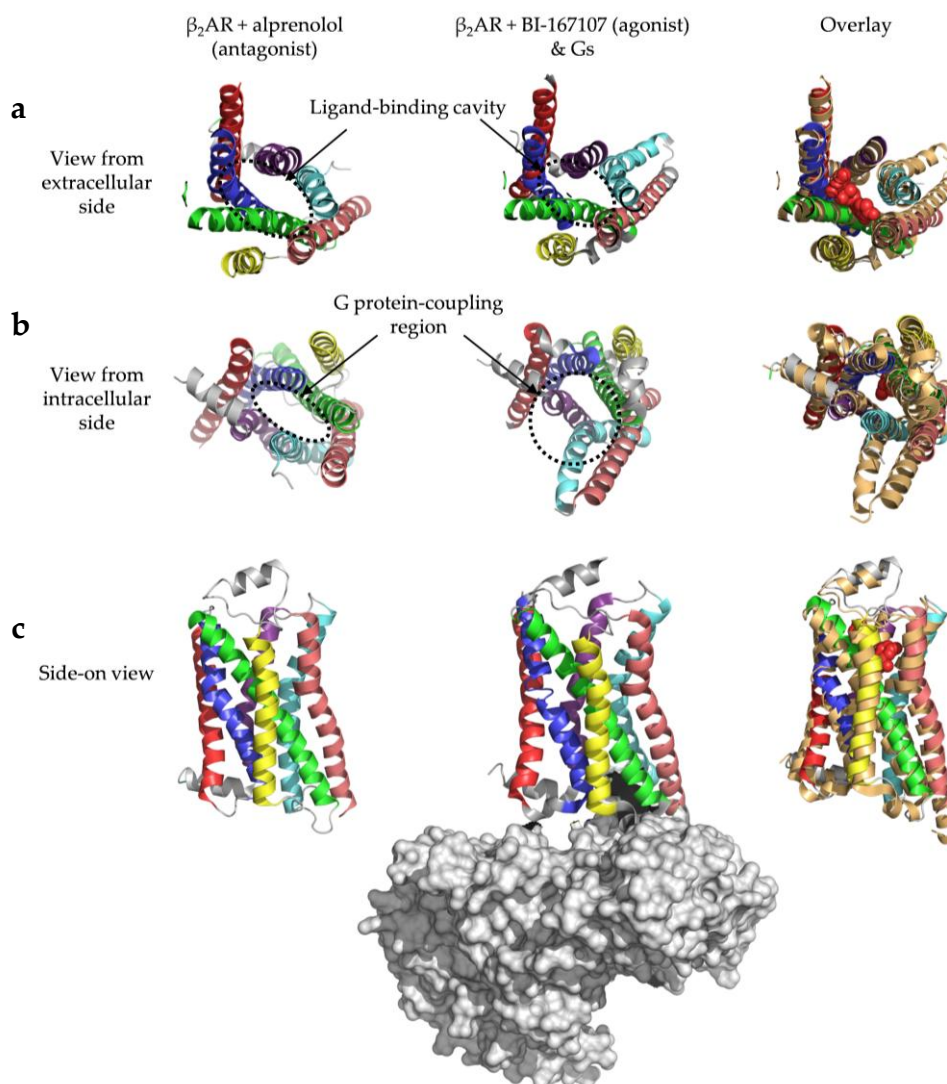


Figure 1.3: Conformational changes occurring upon GPCR activation. Binding of agonist within the ligand-binding cavity of the receptor stabilises an altered conformation that translates through the 7TM region to the intracellular side to allow interaction with G proteins. The example shown is β_2 AR in its antagonist-bound (PDB: 3NYA) and agonist-bound (PDB: 3SN6) states. (a) The presence of agonist in the ligand-binding region causes a partial collapse of the cavity due to simultaneous interactions with TM3, TM5, and TM7 (a space-filling representation of the agonist is shown in red in the overlay figure). This results in numerous conformation changes, including the partial rotation of TM5 and TM6, which causes their intracellular ends to splay outwards from the centre of the receptor by up to 14Å (b). This opens the previously occluded G protein interaction region, thereby allowing docking and activation of G protein (c). TM1 = red, TM2 = blue, TM3 = green, TM4 = yellow, TM5 = pink, TM6 = cyan, TM7 = purple. All non-TM regions are shown in grey. For overlay figures, colour scheme is maintained for the antagonist-bound structure while the agonist-bound conformation is shown in gold. The surface of the bound G protein in the presence of agonist is shown in grey.

suggests that agonist-binding alone is insufficient to lock the receptor into its active conformation, and that an equilibrium of states exists in both bound and unbound receptors (191). This allows for the intriguing possibility that intracellular binding events could alter receptor conformation in the absence of ligand and so allow for bidirectional allosteric communication between G protein and GPCR. This is supported by the observation that G protein-binding can increase the affinity of a receptor for its ligand by a factor of 100 (197) and that antagonistic antibodies can stabilise inactive forms of GPCRs by binding to intracellular regions (198). An important corollary of this is that different GPCRs could influence each other's behaviour by altering the availability of intracellular binding partners and in this way affect each other's equilibria of activation states. This would allow for functional communication between receptors without direct contact. Thus, our current structural understanding of GPCRs provides a detailed model for signalling from independent, monomeric receptor units.

1.3.2 G protein-coupled signalling

Alongside their conserved topology, the other feature revealed in early studies of 7TM receptors was their dependence on signaling via associated heterotrimeric G proteins, hence their being named GPCRs. G proteins form heterotrimers of α , β , and γ subunits (28), which exist in their non-activated state as a complex anchored to the inner leaflet of the plasma membrane via palmitoylation and myristoylation ($G\alpha$) and prenylation ($G\gamma$) sites (199). In this state, the G protein is also complexed with a bound GDP molecule within a nucleotide-binding pocket in the core of $G\alpha$. Recognition of a GDP-bound G protein by a ligand-activated GPCR causes the structural changes in $G\alpha$ that induce dissociation of GDP and its replacement with GTP (190). These conformational changes lead to the disaggregation of the G protein subunits: $G\alpha$ is released as an independent unit while $G\beta$ and $G\gamma$ remain associated as a heterodimer (200). Both halves of the G protein are then able to influence the activity of downstream effectors.

Whilst retaining a conserved mechanism of activation, G proteins differ in the signalling pathways that they target and so the selection of a G protein by a GPCR is intrinsically linked to its physiological function. Mammals possess up to 16 different $G\alpha$ proteins, and 5 β and 14 γ subunits (201), which can associate in a wide variety of combinations to form functionally distinct G protein complexes. $G\alpha$ subunits fall into four main families based on sequence identity and functional similarity: *Gai/o*, *Gas*, *Gaq*, and *G $\alpha_{12/13}$* (202). *Gai/o* and *Gas* proteins function primarily through the cAMP-dependent signalling network, in which they act as mutually opposed effectors of the enzyme adenylate cyclase that produces cAMP – *Gai/o* proteins inhibit adenylate cyclase whereas *Gas* proteins stimulate cAMP production (203). *Gaq* and *G $\alpha_{12/13}$* proteins do not influence cAMP levels but

instead activate phospholipase C to produce inositol triphosphate and diacylglycerol ($G_{\alpha q}$), or activate the small RhoA GTPase ($G_{\alpha 12/13}$) (203, 204). Although a primary role of the $G_{\beta\gamma}$ complex is to inhibit the GDP-bound G_{α} subunit, free $G_{\beta\gamma}$ directly influences signalling in its own right. Effects of active $G_{\beta\gamma}$ include the activation and inhibition of K^+ channels (205, 206), activation of phospholipase C (207), inhibition of Ca^{2+} channels (208), and activation of GPCR kinases (209).

The signalling potential of an individual GPCR therefore depends on which subsets of G proteins it preferentially activates. In many cases, GPCRs with closely related ligands activate G proteins of the same family – for example, the human opioid receptors couple exclusively to $G_{i/o}$ pathways, whereas the neurotensin receptors activate only G_q proteins (210). However, some ligands bind receptors with mutually opposed signalling effects, such that the outcome of binding on a particular cell depends on the relative abundance and activity of the two forms. This is the case with adrenaline, which can activate the G_s -coupled β -adrenergic receptors as well as the $G_{i/o}$ -coupled α_2 -adrenergic receptors and the G_q -coupled α_1 -adrenergic receptors (211, 212). Moreover, many GPCRs have promiscuous G protein preferences, recognising multiple G proteins to greater or lesser extents. For example, the orexin receptors activate both the G_s - and G_q -coupled pathways, the oxytocin receptor activates $G_{i/o}$ and G_q proteins, and the thyrotropin receptor couples to G_s , $G_{i/o}$, and G_q proteins (210). This allows single ligands to have complex signalling outcomes as the dominant effects are dependent on a wide range of variables, including the relative receptor affinities of the ligand, the G protein-selectivity of the receptors, the relative abundance of G protein types in the cell, the relative receptor expression levels, the abundance of downstream signalling components, and background signalling noise within the cell.

1.3.3 G protein-independent signalling

It has been known for many years that GPCR signalling is regulated by a number of negative feedback mechanisms that desensitise activated receptors. Typically, G protein signalling initiated by ligand-bound GPCRs activates GPCR kinases (GRKs), of which there are seven in humans (213). GRKs are activated in a variety of ways, including phosphorylation (214), allosteric regulation (215), and recruitment to the membrane (209). The GRKs phosphorylate serine and threonine residues in the C-terminal tails of activated GPCRs, which partially inhibits further signalling from the receptor, although it is not sufficient to fully quench signalling and varies in its efficacy for different receptors and GRKs (216). Instead, the major effect of GPCR phosphorylation is to allow binding of arrestin proteins, which allosterically block binding of new G proteins to the activated receptor as well as mediating internalisation of the GPCR by acting as a bridge between it and the clathrin-based

internalisation machinery (217). Humans possess four arrestin proteins (arrestins 1-4) with specificities for different receptors. For example, arrestin 1 (also known as visual arrestin) binds strongly to phosphorylated rhodopsin but not β_2 AR, whereas the converse is true for arrestins 2 and 3 (also known as β -arrestins 1 and 2, respectively; 218).

Alongside receptor desensitisation there is now good evidence that arrestins mediate additional signalling events by activated receptors, thereby providing a mechanism for GPCR signalling independent of G proteins. The principal route for this is the recruitment of c-Src family tyrosine kinases to the activated receptor, which in turn phosphorylate and activate mitogen-activated protein kinases (MAPKs), *e.g.* extracellular signal-regulated kinases (ERKs) 1 and 2 (219, 220). Arrestin-dependent signalling via MAPKs regulates a number of diverse cellular processes *in vivo*, *e.g.* neutrophil granule release (221), lymphocyte chemotaxis (222), and glucose transporter trafficking in adipocytes (223). Some GPCR agonists are biased in favour of arrestin-dependent over G protein-dependent signalling or *vice versa* (224), which may allow the development of more effective and specific drugs that induce or inhibit signalling through particular pathways. Early evidence suggests that arrestin-biased ligands have a preference for interaction with TM2 and 7 of the GPCR, and reduced interactions with TM5 (158), presumably inducing conformational states that are better recognised by GRKs than G proteins. This possibility has aroused considerable pharmacological interest, but is also an important reminder that the signalling and biology of GPCRs is far more complex than implied by the initial ternary complex model of receptor signalling.

1.3.4 GPCR organisation within the cell

Distribution within the plasma membrane

It is widely accepted that many membrane proteins are not subject to free diffusion within the plasma membrane and are instead highly constrained in their movement due to interactions with actin and/or other components of the membrane or cytoskeleton (225, 226). In many cases, membrane proteins are not homogeneously distributed across the entire cell surface due to compartmentalisation events that can range from small-scale 'clustering' to large-scale polarisation of the entire cell. It is only recently, with the advancement of a number of microscopy techniques, that such heterogeneous distribution can be investigated in detail. GPCR distribution within the plasma membrane is still poorly understood, but intriguing observations have nonetheless been reported. Initial studies of the photoreceptor GPCRs rhodopsin using atomic-force microscopy indicated that the receptor may exist in highly-ordered paracrystalline arrays within the membrane (227). This level of packing is not feasible for the majority of GPCRs, however, as rhodopsin is expressed at far higher concentrations

than other GPCRs. Nonetheless, evidence for the heterogeneous distribution of GPCRs with more typical expression levels has also been obtained. Single-particle tracking of the μ -opioid receptor (μ -OR) showed that it undergoes highly confined diffusion within the plasma membrane of normal rat kidney cells (228) and that its behaviour is consistent with hop-diffusion between distinct membrane compartments defined by the cytoskeleton (229). Similarly, near-field scanning optical microscopy of antibody-labelled β_2 AR has suggested that the majority of the receptors exist in clusters of ~ 150 nm average diameter in both human embryonic kidney (HEK) 293T cells and cardiac-derived H9C2 cells (230, 231). This is supported by super-resolution fluorescence microscopy of β_2 AR, which revealed the existence of protein clusters in H9C2 cells and suggested that the organisation is actin- rather than lipid raft-dependent (232). Interestingly, in the same study clustering was not observed in either Chinese hamster ovary (CHO) or HeLa cells, and a separate analysis of β_1 AR and β_2 AR in CHO cells also suggested homogeneous distribution (233). β_2 AR is not natively expressed in either of the CHO or HeLa lines whereas it is in both HEK 293T and H9C2 cells, perhaps indicating a relationship between expression of the receptor and the cellular machinery required for regulating its distribution. This is in line with a model in which individual receptor types are targeted by different regulatory proteins that influence their nanoscale organisation within the plasma membrane. Such a model suggests that some GPCRs will be highly organised within the membrane whereas others are largely freely diffusing. This is consistent with observations that the M_3 muscarinic acetylcholine receptor is not subject to the same organisation as β_2 AR in H9C2 cells (232). Thus, the diffusive behaviour and spatial organisation of GPCRs seems to vary across the family as a whole.

The functional relevance, if any, of heterogeneous GPCR distribution is still unclear. It has been suggested that differences in cell surface organisation of β_1 AR and β_2 AR in cardiac myocytes may explain subtype-specific differences in their signalling behaviour (234, 235). This may be due to the compartmentalisation of receptor subtypes into distinct domains containing different signalling components, possibly by proteins that form bridging interactions with the cytoskeleton, *e.g.* the ezrin-radixin-moesin family proteins, which bind to GPCRs (236). The C-terminal domains of β_1 AR and β_2 AR interact with PDZ domain-containing proteins in H9C2 cells, and this is proposed to be the driving force behind receptor confinement (237). The relative distributions of GPCRs and signalling elements may influence receptor signalling properties as certain G protein and adenylate cyclase components appear to be partially segregated from β_2 AR in HEK 293 cells, and alleviation of this segregation increases basal β_2 AR signalling (238). As well as this nanoscale segregation, there is also good evidence that some receptors are localised to distinct parts of the plasma membrane in cells with asymmetric morphologies. β_2 AR, for example, localises predominantly to the deep transverse tubule

regions of primary rat cardiomyocytes, whereas β_1 AR is distributed evenly over the whole cell (239). Localisation in this way could optimise the efficiency of ligand detection by concentrating receptors in the regions of greatest ligand concentration, or could allow polarised signaling for correct tissue development or cell movement. The combination of nano- and large-scale organisation of GPCRs and their signalling partners allows, in principle, greater complexity in the signalling outcomes following GPCR activation and could be important determinants of the cellular response to endogenous GPCR ligands in some cases.

Distribution within the cell

In addition to their potentially heterogeneous distribution at the plasma membrane, the distribution of GPCRs can vary within the cell as a whole. Many GPCRs are expressed predominantly at the plasma membrane until internalised upon activation, but this is not always the case. Several GPCRs exist predominantly within intracellular membranes and only traffic to the cell surface under certain circumstances. For example, the thrombin receptors are localised largely in the Golgi apparatus until activation of the small pool of cell surface receptors triggers their trafficking to the plasma membrane (240). Similar effects are observed for certain dopamine (241), opioid (242), and adrenergic (243) receptors, which are also recruited to the cell surface from intracellular stores in response to signals from the plasma membrane. This appears to be actively regulated by retention mechanisms rather than arising simply due to the absence of forward trafficking signals (244), suggesting that this is important in regulating receptor activity. Many other GPCRs undergo significant levels of intracellular retention (*e.g.* 245, 246, 247), and it is likely that this is a wide-spread phenomenon across the family as a whole. Thus, the distribution of many GPCRs appears to be regulated subcellularly and at the nanoscale level in the plasma membrane.

1.4 GPCR stoichiometry

1.4.1 Overview

Despite the tremendous progress made in understanding GPCR biology over the last half century, many aspects of their structure, function, and regulation are poorly understood. Perhaps the most significant and contentious such area is the question of receptor stoichiometry; whether GPCRs exist as monomers, dimers, or higher-order oligomers, and what functional importance this may have. As discussed above, several GPCR families dimerise through large N- and C-terminal domains, although

these are in the minority. The majority of human GPCRs and in particular the large *Rhodopsin* family, are structurally much simpler. It is the stoichiometry of these receptors, which lack large N and C terminal extensions that has been vigorously debated for over 20 years, with evidence both for and against oligomerisation emerging from a variety of experimental techniques.

Several studies have reported efficient G protein-coupling and activation by a number of monomeric GPCRs isolated in solution or in artificial bilayers, including rhodopsin (248, 249), β_2 AR (197, 250), and μ -OR (251). This strongly suggests that the basic functional unit of *Rhodopsin* family GPCRs is monomeric, and that oligomerisation, should it occur, fulfils a more subtle regulatory role. Much of the early evidence for oligomerisation came from studies of functional GPCR cooperativity, whereby the binding of one ligand to its receptor affects binding of a second ligand to another, distinct receptor. It has thus been suggested that oligomerisation mediates the mutual allosteric regulation of different receptors, allowing a relatively small set of receptors and ligands to produce complex signalling outcomes (*e.g.* 252, 253). Negative cooperativity has been reported for a variety of GPCRs, including opioid (254, 255), adrenergic (256, 257), muscarinic (258-260), and chemokine (261) receptors. Such cooperative effects have generally been interpreted as indications of GPCR oligomerisation (*e.g.* 262), however they do not provide actual physical evidence of this and other interpretations are possible. One possibility is based on the indirect effects that GPCRs can exert on one another via their influence on the pool of available G proteins and other binding partners (263). For example, if two GPCRs couple to the same G protein pool, signalling by one receptor will alter the size of the free G protein pool, by sequestering the G protein to the active receptor, and depleting the GDP-bound G protein population. Negative cooperativity would result from shifting the ligand-G protein interaction equilibrium of the ligand-free receptor towards the G protein-free species, leading to a reduced affinity for cognate ligand due to the absence of the stabilising effects of G protein interactions (264). That such effects might occur was first proposed over three decades ago (265), but it is only recently that clear downregulation of GPCR signalling by G protein-sequestration has been demonstrated (266). Tubio et al. showed that signalling from endogenous receptors in CHO cells is downregulated by overexpression of either the histamine H₂ receptor or β_2 AR, and that this effect is relieved by introduction of additional G proteins into the system.

Investigations of cooperativity and functional cross-talk have therefore not been sufficient to elucidate the genuine nature of GPCR stoichiometry and its possible role in signalling behaviour. For this reason, the direct examination of receptor stoichiometry has become one of the most pressing issues in GPCR biology, and has yielded a vast array of diverse, and sometimes contradictory, results. The early evidence relied on *ex situ* analyses of GPCRs, *e.g.* co-immunoprecipitation and Western

blotting, but these types of experiments have now largely been superseded by *in situ* techniques *e.g.* resonance energy transfer-based assays and single-molecule microscopy.

1.4.2 *Ex situ* studies of GPCR stoichiometry

Much of the evidence supporting GPCR oligomerisation has been obtained *ex situ* through investigations on either solubilised receptors or receptors studied in artificial or isolated membranes. A prominent early study used atomic force microscopy to examine rhodopsin distribution within isolated disk membranes obtained from mouse retina (227), revealing paracrystalline arrays of receptor dimers apparently associating via TMs 4 and 5. This represented the first direct visualisation of GPCR stoichiometry and distribution, and was pivotal in strengthening the emerging dimer hypothesis previously based on pharmacological studies. However, other studies had suggested that rhodopsin undergoes rapid diffusion within retinal disk membranes, both in terms of its rotation (267) and lateral diffusion (268). It also contradicted previous investigations of frog and toad rhodopsin showing that the receptor has no overall organisation at the membrane (269-272). It has been suggested that the structures observed using atomic force microscopy may have arisen artefactually due to partial lipid-protein phase separation in the disk membranes at sub-physiological temperatures (273), as observed for retinal samples obtained from cattle (271). This could lead to the formation of rows of evenly spaced receptors packed within partially ordered crystalline arrays in ways similar to those exploited by crystallographic approaches. It remains unclear to what extent the disk membrane preparations reflect physiological conditions.

Inferring stoichiometry from the size of solubilised receptors

There have been many sized-based stoichiometric analyses of GPCRs solubilised from their native membranes using detergents. Studies of solubilised rhodopsin suggest that it exists as monomers in detergent according to, *e.g.*, sedimentation velocity analysis (274, 275). However, other investigations of solubilised rhodopsin have reported dimer, trimer, and higher-order oligomer species alongside monomeric receptors (276, 277). These disparities may be explained by technical differences, since the choice of detergent as well as the methods of sample preparation and handling can have profound effects upon the aggregation of membrane proteins upon solubilisation. It has been known for some time that rhodopsin exhibits variable stoichiometry upon solubilisation depending on the detergent used; it is monomeric in dodecanoyl octaoxyethylene glycol but forms heterogeneous oligomers in the presence of sodium cholate (278). The same is likely true for many other membrane proteins. GPCRs

may be particularly sensitive to such effects given how much of their structure is normally embedded within the membrane, and how much this changes upon solubilisation. Many studies have concluded that the GPCR of interest is capable of forming dimers and higher-order oligomers based on the size of solubilised receptor complexes assessed by techniques such as SDS-PAGE, Western blotting, sucrose-density centrifugation, and analytical ultracentrifugation (*e.g.* 279, 280-285), yet these conclusions are complicated by the potential for artefactual aggregation upon solubilisation. That the majority of such investigations also report monomeric receptors as well as larger protein aggregates perhaps suggests that the oligomers are not physiologically relevant, as this heterogeneity is unlikely to be reproduced *in situ*. The interpretation of some studies is further complicated by the use of cross-linking agents prior to solubilisation (*e.g.* 276, 277), which introduces the possibility for non-specific cross-linking of receptors within the crowded membrane environment.

Thus, whilst size-based analysis of solubilised receptors has been extensively used in the investigation of GPCR stoichiometry, there are many potential complications in the interpretation of such data. Nonetheless, there is no consensus regarding the relative significance of these complications, and so the field is divided into those that attribute such observations to genuine oligomerisation (*e.g.* 286) and those that consider them artefacts of solubilisation (*e.g.* 287). This is not a trivial disagreement as it has the potential to seriously undermine our understanding of receptor function. Investigations of LTB4R1 solubilised using dodecyldimethylamine oxide or dodecyl maltoside were claimed by Baneres and Parello (280) to show that the receptor is predominantly a monomeric species that oligomerises upon ligand-binding, although the confounding effects of receptor clustering and internalisation were not addressed. In a subsequent study, a detergent that stabilises a dimeric fraction, hexadecyl β -D-maltoside, was used as a basis for claiming that the isolated receptor is essentially dimeric, in spite of the fact that five other tested detergents failed to stabilise LTB4R1 dimers (281). The ability of detergents to manipulate the solubility of membrane proteins, which is usually a valuable property, should in this case discourage their use in stoichiometric investigations.

Co-immunoprecipitation

One way in which researchers have attempted to circumvent the problems arising from solubilisation is by using co-immunoprecipitation (Co-IP). Typically this is performed by dual expression of GPCRs tagged with different markers, *e.g.* influenza haemagglutinin or c-myc tags, that can be specifically recognised by monoclonal antibodies, and it has the advantage that it can be used to investigate both homo- and heteromeric protein complexes. Irrelevant controls are used to account for the effects of

non-specific aggregation caused by solubilisation, and so in principle the experiments should be more easily interpretable than simple size-based analyses. Co-IP has been used to investigate the stoichiometry of many GPCRs, including adrenergic (*e.g.* 288, 289-292), chemokine (*e.g.* 293, 294), muscarinic (*e.g.* 279, 295), and opioid (*e.g.* 290, 292, 296, 297) receptors. In all cases, homodimerisation was reported for the receptors of interest, and very often heterodimerisation. However, several potential issues complicate the interpretation of Co-IP analysis applied to GPCRs. Firstly, the technique is inherently biased towards observing oligomers, as only species containing both antibody markers will be detected. Monomeric species either do not undergo precipitation by the first antibody, or do not get recognised by the second. In one sense this is a strength of the technique as it allows the detection of oligomers within populations also containing monomers, but it is also a potential pitfall as it does not indicate what proportion of the protein is oligomeric and therefore whether the observations are functionally relevant. Secondly, attempting to account for non-specific aggregation by using irrelevant controls is risky because irrelevant controls only display equivalent levels of non-specific aggregation compared to the protein of interest if they also experience equivalent expression levels and surface distribution, which may not be the case². Thus, a GPCR may form more non-specific aggregates with itself or other GPCRs with similar distributions than with irrelevant controls, leading to over-estimation of the true extent of aggregation. For this reason, Co-IP experiments are not always unambiguous, and should always be interpreted with the understanding that artefacts can arise.

Crystallographic studies

Crystallography offers the appealing possibility of directly mapping potentially oligomeric interfaces within receptor complexes. However, the great majority of published crystal structures have failed to report physiologically feasible interactions between GPCR protomers, undermining the model of constitutive GPCR dimerisation. Moreover, many GPCRs exhibit different arrangements between crystal structures, making it difficult to determine which structure, if any, represents the physiological situation. For example, several studies have obtained crystal structures of rhodopsin in a monomeric form (*e.g.* 145, 298-300), however it also forms crystallographic dimers with an interface formed by TM1, TM2, and H8 (301). Similarly, although the turkey β_1 AR crystallises as oligomeric arrays (302), there are several solved structures of β_1 AR monomers (*e.g.* 149, 196). There is also little consistency between the observed stoichiometries of closely related receptors in their respective crystal structures. For example, the three opioid receptors share over 70% sequence identity, and whereas δ -OR

² Problems associated with irrelevant controls are discussed in more detail in 1.4.4.

crystallises as a monomer (157), κ -OR is dimeric (155), and μ -OR packs into higher-order oligomers (156).

Given this variation between studies, it is difficult to say with confidence whether the observed stoichiometries are genuine or an artefact of crystallisation. It is well known that crystallisation has the potential to stabilise interactions that do not occur under other conditions, in a similar manner to the artefactual aggregation associated with other solubilisation approaches. Crystallisation-induced interaction has been observed on many occasions for GPCRs in the form of non-physiological antiparallel dimers, in which the receptor protomers are inverted relative to one another (*e.g.* 145, 155, 157, 299, 303). Given that such arrangements are impossible *in vivo* they must be due to the stabilising effects of crystallisation, and so whether the topologically plausible oligomers that have been observed are genuine must be uncertain. Perhaps the best candidate for a genuine dimer solved by crystallography is CXCR4, as it forms nearly identical dimeric interfaces in five separate crystals (77). However, it is still possible that crystallisation is simply stabilising an energetically minimal arrangement of the receptors that does not exist physiologically, considering the extremely high concentrations of protein needed for crystallogenesis.

For the GPCR oligomers for which structures have been obtained, there appears to be little conservation of the interaction interface. The most common regions mediating parallel interactions within crystals are TM1, TM2, and H8, seen at the dimer interface for rhodopsin (301), κ -OR (155), μ -OR (156), and β_1 AR (302). Several other interfaces have been observed, however, including TM5-TM6 in CXCR4 (77) and μ -OR (156), TM3-TM4 in CXCR4 (77), and TM4-TM5 in β_1 AR (302). So, while GPCR oligomers are undoubtedly able to form in some cases under conditions of crystallisation, they are still subject to the same ambiguity associated with other *ex situ* approaches. Thus, their confirmation by *in situ* analysis is necessary to draw meaningful conclusions about the actual stoichiometry of a given receptor.

1.4.3 Resonance energy transfer

Resonance energy transfer (RET) is a physical phenomenon wherein excitation energy for one chromophore (the donor) is transferred to another chromophore (the acceptor) through non-radiative dipole-dipole coupling (304). This results in reduced emission by the donor chromophore and increased emission from the acceptor (Fig. 1.4). If the emission spectra of the acceptor and donor species are sufficiently different, it is possible to quantify the degree of RET occurring between them by direct measurement of the wavelengths being emitted. RET is separation distance-dependent, being inversely proportional to the sixth power of the mean distance (r) between the acceptor and donor

molecules (305). This, combined with a chromophore pair-specific constant, the Förster distance (R_0), determines the efficiency of RET between any two chromophores (Eqn. 1.1). R_0 is the separation distance at which 50% maximal RET is achieved between donor and acceptor (Fig. 1.4c).

$$RET_{max} = \frac{R_0^6}{r^6 + R_0^6} \quad (1.1)$$

R_0 values for frequently used chromophore pairs are typically in the range of 4-6nm, and the maximum separation distance at which RET can usually occur is approximately 10nm for most systems (306). RET techniques therefore have the potential to probe intermolecular interactions at a nanoscale level, a resolution that has yet to be matched by other *in situ* approaches *e.g.* fluorescence imaging. In practice, RET is used in the form of either Förster RET (FRET) or bioluminescence RET (BRET), which are physically analogous but employ different experimental tools. Nonetheless, in both cases RET is quantified as the efficiency of transfer (*i.e.* acceptor fluorescence per number of donors) rather than absolute acceptor fluorescence. FRET uses two fluorescent molecules with overlapping emission and excitation spectra as the donor and acceptor molecules. The most common donor and acceptor molecules used initially were fluorescent proteins such as green fluorescent protein (GFP) or mutants thereof, including cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), however small-molecule fluorophores conjugated to ligands or antibodies are becoming widely used (307). For such a system, the donor molecule is excited by an external light source that does not significantly excite the acceptor, and acceptor fluorescence is measured to determine the degree of FRET. BRET, however, requires no external light source as the donor is the bioluminescent enzyme luciferase, initially isolated from a number of marine animals *e.g.* *Renilla reniformis* or *Aequorea victoria* (308). This is usually coupled with a fluorescent GFP-derived acceptor, although recent advances in BRET technology are providing alternatives to this, *e.g.* the use of quantum dots as acceptors in ultrasensitive BRET assays (309). The use of a bioluminescent donor overcomes a number of technical problems associated with FRET, such as donor photobleaching or photoconversion, as well as providing a good signal-to-noise ratio, although it makes single-molecule analysis difficult due to the lower quantum yield of the luciferase donor compared to fluorescent molecules.

The BRET² assay is an optimised BRET approach that uses *Renilla* luciferase (Rluc) as the donor and a variant of GFP (GFP²) as the acceptor. The luciferase substrate used in this case is coelenterazine 400A, which is a derivative of the natural coelenterazine substrate that produces

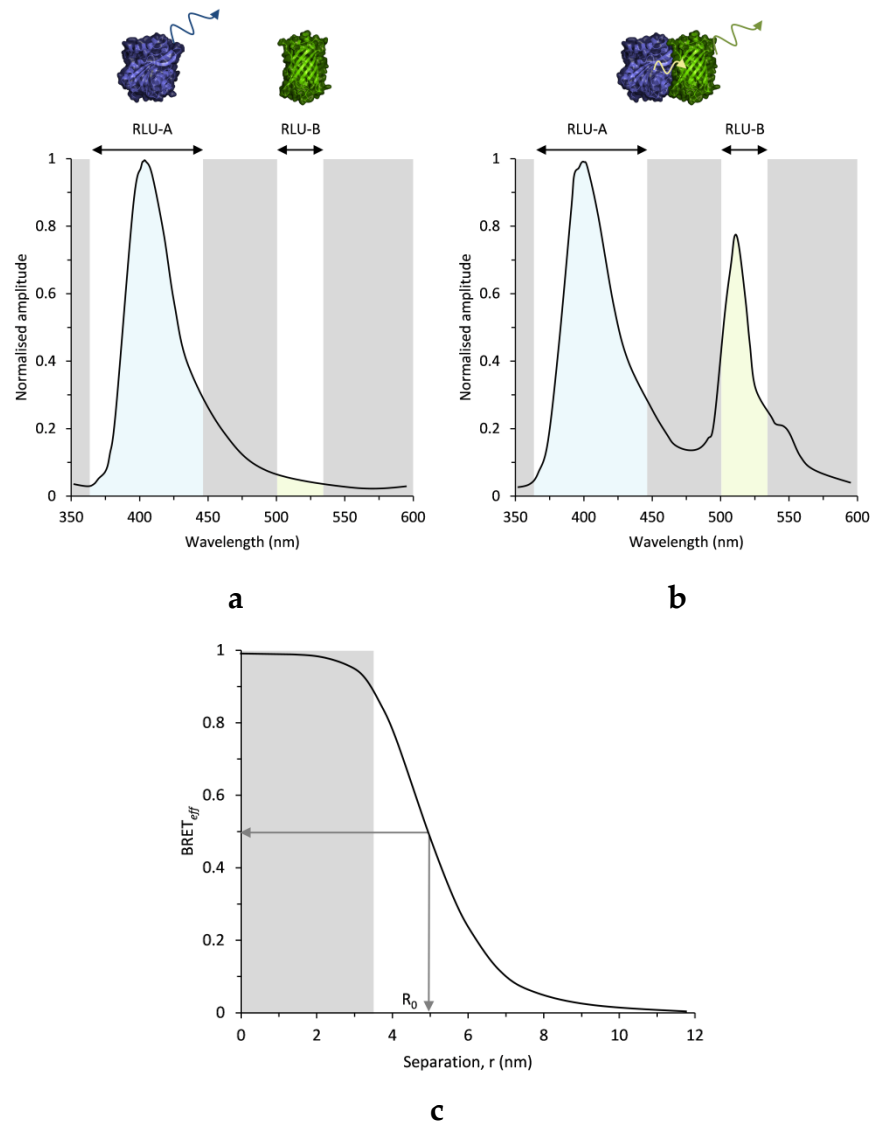


Figure 1.4: BRET leads to a distinct emission spectrum from the BRET pair in a separation distance-dependent manner. **(a)** In the BRET² assay, when the donor luciferase (blue) molecule is 100Å or more from the acceptor GFP² (green) emission is the characteristic spectrum of Rluc so the majority is within the RLU-A region of the spectrum (410 ± 40nm). Only ~7% is emitted within RLU-B (515 ± 15nm). **(b)** If GFP² is within 100Å, BRET occurs between the two molecules and above-background emission is observed in the RLU-B region. **(c)** Graphical representation of Equation 1.1. Almost no BRET occurs above 10nm separation and half-maximal BRET is achieved at R_0 , ~5nm. The hydrodynamic radii of GFP² and Rluc do not allow interactions closer than ~3.5nm separation.

maximal luciferase emission at 410nm. GFP² has an excitation maximum of 515nm, thereby giving a spectral separation of over 100nm between donor and acceptor. This allows for greater sensitivity due to reduced bleed-through from the donor into the acceptor wavelengths, but has the disadvantage of reduced signal intensity compared to bioluminescence using the conventional coelenterazine substrate. Recent advancements in luciferase technologies are, however, steadily improving the signal strength that can be obtained from BRET². For example, the Rluc mutant Rluc8 offers a 60-fold improvement in quantum yield in the presence of coelenterazine 400A compared to wild-type Rluc, as well as an 80-fold increase in light output from the GFP² acceptor (310). It also has a Förster distance of 8.15nm when used in conjunction with GFP², slightly larger than the R_0 of 7.5nm for the wild-type Rluc-GFP² pair (311), which may be useful in the investigation of proteins with relatively large hydrodynamic diameters, *e.g.* GPCRs.

1.4.4 Resonance energy transfer analysis applied to GPCRs

Both BRET and FRET have been used extensively to study GPCR stoichiometry. Their ability to probe stoichiometry *in situ* as well as their relative technical simplicity has made them very popular assays. The appearance of RET-based approaches applied to GPCRs only occurred in 2000, yet within just 5 years more than 60 studies of GPCRs using BRET or FRET had been published (312). The number now is likely double that, meaning that RET-based techniques have provided most of the stoichiometric GPCR data and are the principal foundation upon which many of the arguments, both for and against oligomerisation, rest.

Difficulties with early studies

Many of the first RET-based GPCR studies involved experiments in which acceptor- and donor-tagged receptors of interest were co-expressed at an equimolar ratio and the efficiency of RET (RET_{eff}) measured as a single value. A significant consideration in the interpretation of these original GPCR RET analyses now is the contribution of non-specific interactions to the RET_{eff} observed. Within the crowded membrane environment, donor and acceptor molecules will transiently pass within the RET-permissive ~10nm radius, allowing energy transfer in the absence of genuine interaction. This was illustrated for a pair of well-characterised type I membrane protein monomers (CD2 and CD86) that generated significant levels of energy transfer when expressed as BRET pairs – *i.e.* co-expressed as independent donor and acceptor fusion constructs (313). Indeed, the monomers transferred resonance energy at a level equivalent to 25% that of covalent homodimers such as CD28 and CTLA-4. It is

therefore not appropriate to interpret simply the presence of RET as indicative of *bona fide* interactions, as had been done previously (e.g. 292, 314).

Some studies attempted to account for non-specific interactions through the use of irrelevant controls. This involves the determination of background non-specific RET arising from an unrelated protein used as either donor or acceptor in a RET pair with the protein of interest (Fig 1.2c), and is an approach that has been used widely, e.g. in BRET, FRET microscopy, time-resolved FRET, and RET competition experiments. Examples include the use of CCR5 as an irrelevant control for both β_2 AR and CXCR4 (288, 315) and calcium receptor as a control in the study of angiotensin receptors (316), although, surprisingly, a single irrelevant control, GABABR2, is very widely used (e.g. 317, 318-321). In these studies reduced RET between the protein of interest and the irrelevant control is used as evidence that the higher levels of RET observed between the proteins of interest are due to genuine association. However, an important consideration in this approach is the level of expression of the two proteins, as unrelated proteins can be expressed at very different levels. If the irrelevant control were to be expressed either more or less strongly than the protein of interest, then the degree of non-specific transfer will increase or decrease, respectively (Fig. 1.5a,b). This is, unfortunately, not addressed by the majority of publications that employed this technique.

An additional limitation of the use of irrelevant controls stems from the aforementioned heterogeneity of GPCR distribution within the plasma membrane. Any control protein that is sufficiently 'irrelevant' to preclude specific interaction with the protein of interest is similarly unlikely to be subject to the same regulation of membrane localisation and so may not allow for appropriate comparisons (Fig. 1.5e). It is therefore very difficult to select an appropriate candidate for use as an irrelevant control in any RET assay between GPCRs without substantial understanding of their distribution within the cell and at the cell surface, which is not always available.

Finally, the stoichiometry of the control protein used will have a significant impact on the final RET_{eff} that is observed between it and the protein of interest. Dimerisation of an acceptor-fused irrelevant control effectively halves the number of acceptor molecules available to donors (Fig. 1.5d). This is analogous to the effect caused by heterogeneous distribution of the control proteins at the cell surface except in this case the acceptors are sequestered into receptor complexes rather than membrane sub-domains. Thus, an irrelevant control ought to undergo the same degree of dimerisation as the protein of interest in order to avoid such complicating factors, yet this is not possible when the stoichiometry of the protein of interest is inherently unknown. This seems to be

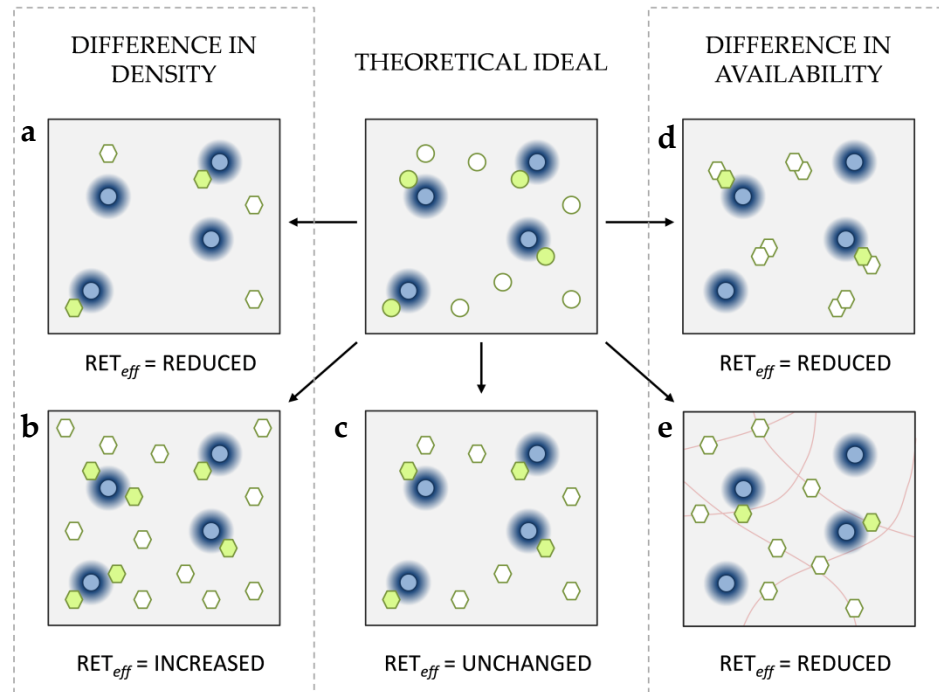


Figure 1.5: Problems in the interpretation of RET data obtained using irrelevant controls. The GPCR of interest is shown as a donor fusion protein and is represented by blue circles surrounded by a RET-permissible area. The acceptor is shown either as the GPCR of interest or as an irrelevant control, which is indicated by hexagons. Fluorescing and non-fluorescing acceptors are shown as green or white shapes, respectively. Cytoskeletal structures are represented as red lines. The theoretical ideal for use of irrelevant controls is that both proteins should exhibit equivalent density and availability within the plasma membrane (c). Any difference in density will cause either decreased (a) or increased (b) non-specific interactions and thereby alter RET_{eff} artefactually. Similarly, if the two proteins do not experience the same degree of mutual availability as the protein of interest does with itself then RET_{eff} will change. This can be due to differences in stoichiometry (d) or organisation within the membrane (e).

particularly relevant to the widespread use of GABABR2 as an irrelevant control, as it is widely accepted as forming stable dimers (138, 139, 322) and so is likely to result in artefactually reduced RET_{eff} if used as an irrelevant control for a protein that is actually monomeric.

Persistence of early problems into recent studies

The issues described above are still relevant even in studies that are far more technically advanced than the early studies using RET fusion proteins. The generation of fluorescently tagged GPCR ligands, for example, offers the possibility of highly targeted investigation of GPCRs within native tissues (307). Any such study ought to consider the potential artefacts that can arise due to non-specific interactions, yet this is not always done. For example, time-resolved FRET between fluorescent ligands for vasopressin, dopamine, and oxytocin receptors has been used to support a model of constitutive homodimerisation of these receptors in native tissues (323). However, FRET_{eff} in this case was measured as a single value with no assessment of non-specific interactions, and the conclusion was based solely on the existence of transfer. This is in spite of the authors' own observations that FRET_{eff} is highly dependent on receptor expression level in different tissues, which conflicts with a model of constitutive dimerisation. This is unfortunate as the technical achievement of developing ligand-based FRET suitable for use in native tissues is an impressive one. Direct imaging of FRET by Boyer et al. (324) at the surface of cells using new microscopy techniques also faces similar problems since dimerisation was once again judged by the extent of FRET alone and allowances were not made for non-specific interactions. Equally, a novel time-resolved fluorescence anisotropy approach was used to support a model of constitutive homodimerisation in serotonin 1A receptors (325, 326) without consideration of non-specific FRET effects and without comparisons to known monomers and dimers. These and other examples highlight the need for RET techniques that address the issue of non-specific interactions in a manner that does not depend on the use of irrelevant controls.

Saturation experiments

One technique developed with the aim of distinguishing between specific and non-specific RET is the BRET saturation assay. In this approach, cells are transfected with a constant amount of donor and systematically increasing amounts of acceptor (Fig. 1.8c). Theoretically, specific interaction between the acceptor- and donor-fused constructs should exhibit a hyperbolic relationship between BRET_{eff} and acceptor-donor ratio due to reduced donor-donor interactions (327). Conversely, BRET arising exclusively from non-specific interactions should result in a pseudolinear increase in BRET_{eff} with

acceptor-donor ratio, due to increasing protein density at the cell surface and a greater frequency of non-specific interactions. Thus, discrimination between specific and non-specific interactions is achieved by determining whether the data fit more closely to a hyperbolic or pseudolinear distribution. This has been used in many studies to investigate GPCR oligomerisation (*e.g.* 328, 329-331) and invariably used to support oligomerisation of the receptors studied.

Despite their widespread use, several pitfalls complicate the interpretation of BRET saturation assays. The first is a technical issue relating to the consistency of transfection efficiency in the presence of varying amounts of DNA. BRET saturation assays are generally performed on transiently transfected cells using one of several transfection protocols. The outcome of such transfections may change as the total amount of DNA is increased in order to allow greater acceptor construct expression. It is possible that at high acceptor:donor ratios, increasing the amount of acceptor-encoding DNA does not result in increased acceptor expression from transfected cells, and instead increases the proportion of successfully transfected cells. This would manifest as increased total acceptor levels when measured in bulk, but would not influence the $BRET_{eff}$, as the total protein concentration in individual cells would be largely unchanged. For monomers this would give an artificial plateau of $BRET_{eff}$ at high acceptor:donor ratios and an artefactual hyperbolic relationship despite BRET occurring entirely due to non-specific interactions. In order to avoid this possibility, care must be taken to ensure equivalent transfection conditions for all acceptor-donor ratios. This can be achieved by using 'buffer' DNA that does not encode protein but maintains constant total DNA amounts between transfections. This issue is not addressed in the majority of published BRET saturation studies, and it is often unclear which transfection protocols were used. The extent to which this might be a complicating factor across the current literature is therefore difficult to determine.

A second technical issue arises from the fact that both monomeric and oligomeric proteins produce increases in $BRET_{eff}$ as the amount of acceptor rises. This means that discrimination relies on interpreting which of two constantly increasing models fits better to the data obtained. This can be ambiguous in cases in which the data does not clearly fit to one model or the other, which may occur in cases of weak oligomerisation or low absolute $BRET_{eff}$, where experimental error becomes more significant. A possible example of this is the analysis of β_2AR in a BRET saturation assay (330) wherein the data appear to be neither fully hyperbolic nor completely pseudolinear, but is instead marginally non-linear making interpretation difficult. This and other examples of previously published cases where BRET saturation data may have been misinterpreted are given in Figure 1.6.

A more fundamental issue with interpreting BRET saturation assays is the assumption of pseudolinearity for all monomeric proteins. The distribution of data for monomeric proteins can

deviate from linearity for several reasons. Firstly, as discussed above, GPCRs can cross-talk with one another due to direct effects on the pool of signalling partners as well as indirect effects on downstream effectors, *e.g.* GRKs. Thus, as acceptor expression increases, donor expression may be reduced due to increased negative feedback. This effect might be negligible at high acceptor-donor ratios when increases in acceptor expression only minimally increase the total protein density. At low acceptor-donor ratios, however, this effect could be highly significant as the introduction of acceptor-tagged proteins will greatly increase total receptor numbers and could therefore cause the acceptor:donor ratio to increase more rapidly than total surface density due to donor expression down-regulation. This is significant because, at a constant surface density, $BRET_{eff}$ for a monomer only increases with acceptor:donor ratio until a ratio of ~ 2 , after which it is constant (332). Thus, if acceptor:donor ratio is increasing more quickly than total density, the net effect will be a more rapid rise in $BRET_{eff}$ at ratios below ~ 2 , giving a non-linear relationship between $BRET_{eff}$ and acceptor:donor ratio (Fig. 1.7)

Similarly, $BRET_{eff}$ at high acceptor:donor ratios may reach a maximum value after which it fails to increase further. This is because at very high levels of receptor expression its normal trafficking and distribution at both the plasma membrane and internal membranes may break down; expression may reach a point at which the receptors are no longer locally organised in their native fashion and begin to be distributed more evenly across the plasma membrane. Further increases in total expression would have a diminished influence on effective concentration and non-specific $BRET_{eff}$. Alternatively, additional basal signaling associated with higher expression may promote greater retention of the receptors in internal membranes, or increased rates of receptor internalisation, both of which would alter receptor distribution and change the dependence of $BRET_{eff}$ on total expression level. Overall, any effect that causes an expression-dependent redistribution of receptors will induce deviations from pseudolinear behaviour for monomers, which may lead to their incorrect assignment as oligomers.

The difficulties in interpreting BRET saturation assays have broad implications for much of the evidence used to propose GPCR oligomerisation, as the use of saturation approaches has yielded ambiguous results in many studies for the reasons discussed above. For example, in BRET saturation assays M_1 muscarinic receptor exhibits a hyperbolic relationship, as does M_1 when it is the donor and the unrelated SMO receptor is the acceptor (331). The authors in this case concluded that M_1 is a homodimer because the absolute $BRET_{eff}$ values are higher when it is expressed as a BRET pair than when expressed with SMO (Fig. 1.6c), but this conclusion is unsafe because absolute $BRET_{eff}$ values depend on many unpredictable variables including acceptor and donor orientation, effective and total protein concentrations, and protein geometry. Instead, BRET saturation data must be interpreted

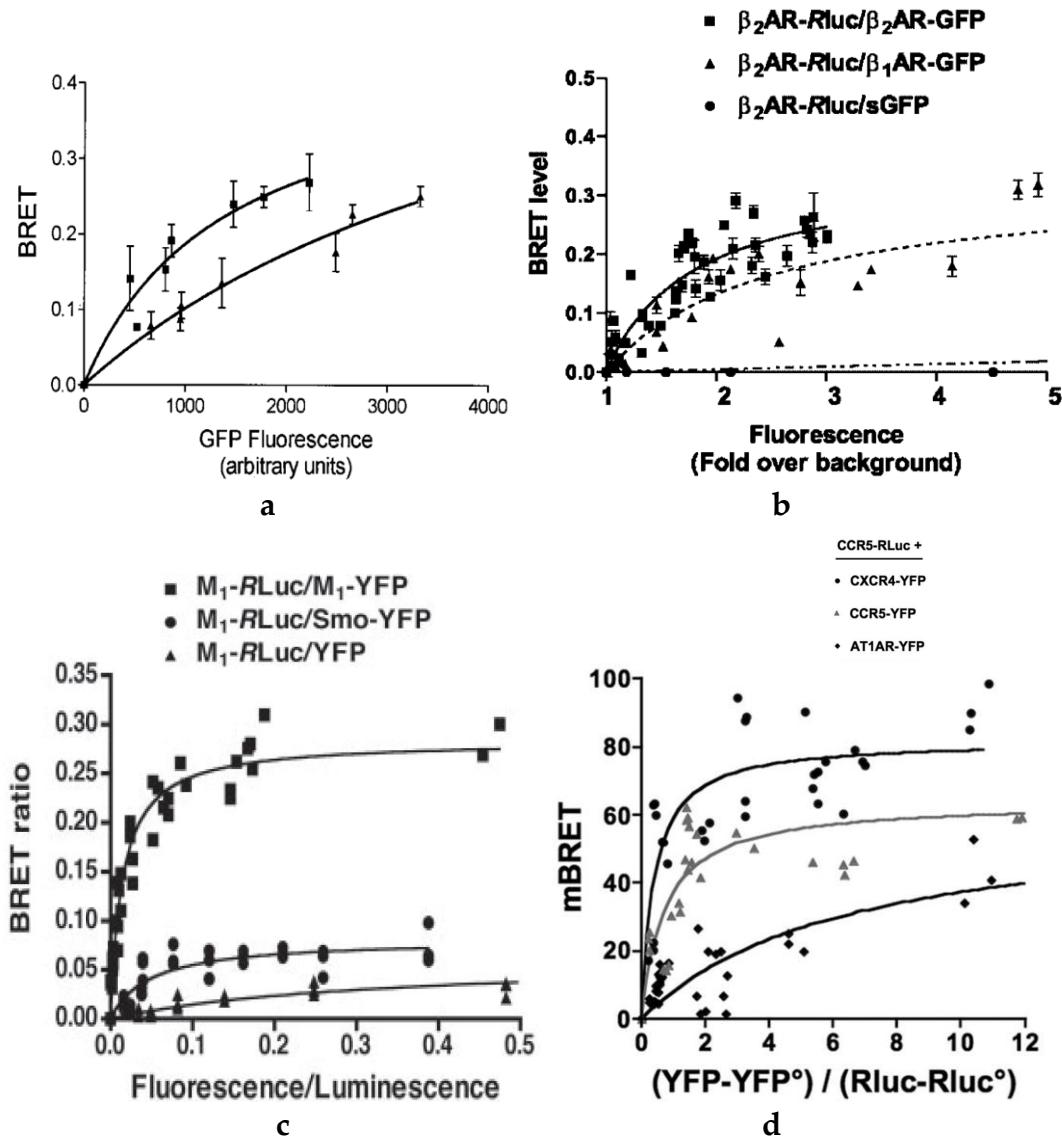


Fig. 1.6: Published examples of potential pitfalls of the BRET saturation assay. (a) As both monomers and dimers exhibit increasing BRET_{eff} in a BRET saturation assay, the difference between fits of the data can sometimes be minimal. Both data sets in this example (330) are only marginally non-pseudolinear, yet the top set is concluded to be a dimer. (b) The use of inappropriate controls may lead to misinterpretation of data. In this example (328) soluble GFP (sGFP) is used as a control for non-specific BRET, but this is inappropriate as sGFP is not membrane-associated and will undergo a much lower level of non-specific interaction than a protein diffusing only in the two dimensions of the membrane. (c & d) In some cases, irrelevant GPCR controls give equivalent hyperbolic relationships as those obtained for the GPCR of interest, yet this is disregarded as the absolute BRET_{max} is lower and so the GPCR of interest is concluded to be a dimer. This is the case in these examples where SMO is used as an irrelevant control for M_1 (c; 331), and AT1AR as an irrelevant control for CCR5 and CXCR4 (d; 329).

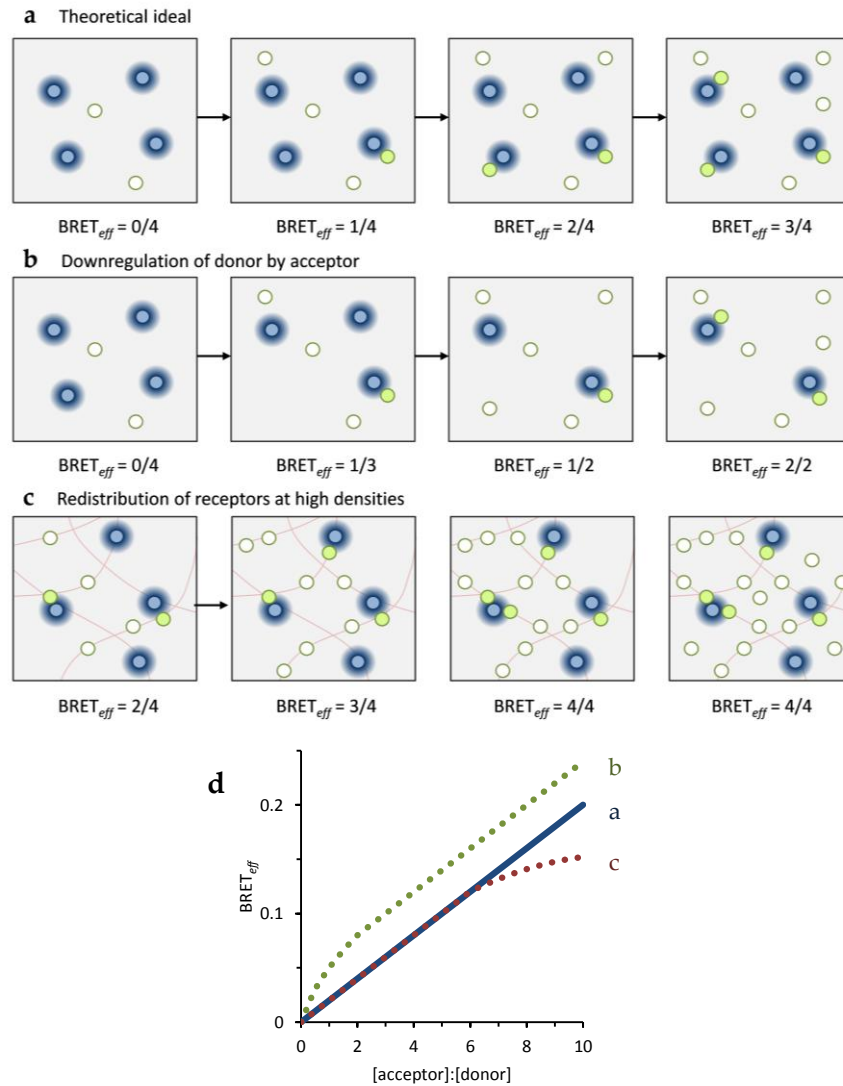


Figure 1.7: Potential problems with the BRET saturation assay. Fluorescing and non-fluorescing acceptor molecules are shown as green and white circles, respectively, and donors as blue circles. The BRET-permissible area surrounding donors is represented as a blue halo. Cytoskeletal structures are represented as red lines. (a) Ideally, for a monomer, addition of acceptor molecules into a system with a constant number of donors should result in an increase in BRET_{eff} that is linearly dependent on [acceptor]/[donor]. (b) In cases where additional acceptor-tagged receptors cause downregulation of their donor-tagged counterparts, BRET_{eff} will increase more quickly than expected for a monomer as the number of donor molecule will be reduced, thereby relieving competition between donors for the acceptor population. (c) If a receptor experiences heterogeneous distribution, *e.g.* though interaction with the cytoskeleton, this may begin to break down at high densities. Thus, addition of further acceptor molecules will have a diminished effect on BRET_{eff} as the effective concentration of donors and acceptors will increase more slowly. (d) Representation of how these effects would affect the apparent pseudolinearity of data obtained for a monomer. Colours denote the situation shown in the figure with the corresponding letter (a = blue, b = green, c = red).

according to the shape of the curves alone, which in this case imply that M_1 is both homodimeric and capable of forming heterodimeric contacts with SMO. Given the structural and functional unrelatedness of SMO and M_1 this seems unlikely, implying that the curve suggesting heterodimeric interactions is artefactual. But this in turn calls into question the reliability of the curve obtained for M_1 alone. The possibilities are either that M_1 forms homo- and heteromeric interactions with itself and SMO, or that it forms neither. It cannot be that it does the former without the latter.

Type 1 and 2 BRET approaches

Owing to the potentially complicating factors associated with BRET saturation experiments, two novel BRET approaches have been devised to allow more straightforward discrimination between specific and non-specific interactions (313). These approaches are based on theoretical considerations regarding the relationship between $BRET_{eff}$ and fluorophore concentrations for monomeric and oligomeric proteins, whose use was first explored in FRET experiments (summarised in 333). In the first ('type 1') experiment, total protein concentration is kept constant and the ratio of GFP²- to Rluc-tagged proteins is varied (Fig. 1.8a). For monomers, there is no dependency of $BRET_{eff}$ on acceptor:donor ratio above a certain threshold (332). This is because the acceptor:donor ratio reaches a point at which the acceptors are in large excess over donors and so donors no longer compete for acceptors. Thus, altering the ratio by removing donors and replacing them with acceptors has essentially no effect on the average distance between each remaining donor and its nearest acceptor, meaning that $BRET_{eff}$ remains constant. In marked contrast, $BRET_{eff}$ for oligomers shows a strong dependence on acceptor:donor ratio because, as the ratio increases, fewer unproductive donor-donor pairs stay formed and each donor is more likely to be paired with an acceptor, increasing $BRET_{eff}$. This dependence can be expressed as a hyperbolic relationship between $BRET_{eff}$ and acceptor:donor ratio (f) that is defined by the stoichiometry (n) of the oligomer (Eq. 1.2). The derivation of this relationship has been published previously (313).

$$\frac{BRET_{eff}}{BRET_{max}} = 1 - \frac{1}{(1+f)^{n-1}} \quad (1.2)$$

The theoretical framework for type 1 BRET is therefore similar to that of BRET saturation assays, as oligomers will exhibit a dependence on acceptor:donor ratio of a type that monomers do not. However, type 1 BRET is more easily interpreted than saturation approaches because it is reliant on a constant surface density. This overcomes the problems associated with unpredictable relationships between non-specific BRET and surface protein density. Moreover, it is easier to distinguish between

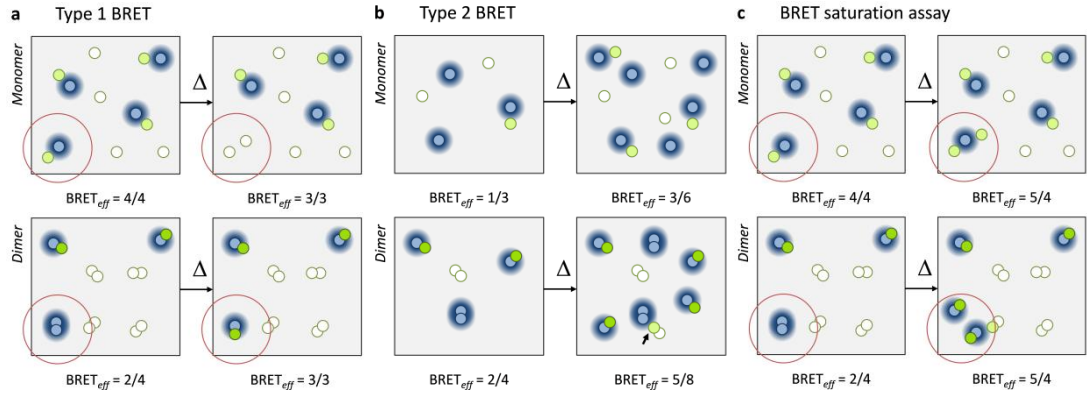


Figure 1.8: Principles of different BRET approaches. (a) In a type 1 BRET assay the acceptor/donor ratio is increased but surface density is kept constant. The increase in acceptor/donor ratio is obtained by exchanging a donor for an acceptor (the change is indicated within the red circle). For simplicity, $BRET_{eff}$ is defined here as the ratio of the numbers of fluorescent acceptors and luminescent donors. In the examples shown, for the monomer (top) $BRET_{eff}$ is unchanged (3/3 versus 4/4), whereas for the dimer (bottom) the ratio increases from 2/4 to 3/3 as the fraction of productive dimers increases. (b) In a type 2 BRET experiment, the acceptor/donor ratio is kept constant and surface density is varied, in this case by a factor of two. Due to the increase in monomer density (top) the likelihood of random collisions increases, and $BRET_{eff}$ increases from 1/3 to 3/6. For constitutive dimers (bottom), however, $BRET_{eff}$ is largely unchanged, increasing from 2/4 to 5/8, because the likelihood of dimerisation does not change. Only random interactions of dimers (arrow) contribute to increases in $BRET_{eff}$ (in reality these contributions will be significantly smaller than the effects of dimerisation). (c) In the BRET saturation assay, the acceptor/donor ratio is increased by keeping donor numbers constant and increasing the numbers of acceptors. In the examples shown, $BRET_{eff}$ for monomers (top) and dimers (bottom) both increase upon addition of one or two extra acceptors, respectively (from 4/4/ to 5/4 for the monomer, and from 2/4 to 5/4 for the dimer). This is due to the increased random interactions of monomers, and increased formation and random interactions of dimers. Assays in which $BRET_{eff}$ always increases may be more easily misinterpreted than assays in which changes in $BRET_{eff}$ vary systematically with receptor stoichiometry. Fluorescing and non-fluorescing acceptor molecules are shown as green and white circles, respectively, and donors as blue circles. The BRET-permissible area surrounding donors is represented as a blue halo.

the constant (monomer) and increasing (oligomer) $BRET_{eff}$ values in type 1 BRET than the corresponding stoichiometric signatures of saturation experiments, which both increase with acceptor:donor ratio but to different extents. This is particularly important in the case of partial dimers, as a weak dependence of $BRET_{eff}$ upon acceptor:donor ratio is more easily identified in type 1 BRET experiments.

The second ('type 2') experiment maintains a constant acceptor:donor ratio and instead varies the total density of protein within the membrane (Fig. 1.8b). Under such conditions, the relationship between $BRET_{eff}$ and surface expression should be pseudo-linear for non-specific interactions with transfer efficiency approaching zero at very low cell surface densities. Oligomeric interactions, however, should yield $BRET_{eff}$ that is partly independent of expression level as the separation distance between proteins within the oligomer is unaffected by their density at the cell surface. Non-specific interactions between oligomers will still increase with surface density and so $BRET_{eff}$ is not entirely independent of expression level even for constitutive oligomers.

Initial application of these BRET approaches to type 1 TM control proteins with known stoichiometries validated their ability to accurately distinguish between monomers and dimers (313). The same study also showed that both approaches detected dimerisation in the case of the well-established GPCR dimer, GABABR2, confirming that the increased hydrodynamic diameter of GPCRs compared to type 1 TM proteins is not an issue for the techniques. Intriguingly, analysis of human β_2AR and mouse cannabinoid receptor 2 using both approaches yielded unambiguous $BRET_{eff}$ data characteristic of monomers in both cases, contrary to the widespread belief in constitutive GPCR oligomerisation. Expression of β_2AR fused to the coiled-coil C-terminal domain of GABABR2 induced dimeric behaviour in both assays, showing that if wild-type β_2AR dimers were present they would have been detected.

The ensuing controversy

The results obtained using type 1 and 2 BRET were somewhat controversial given that they contradicted the consensus view that GPCRs generally dimerise, and the implication that many BRET studies may have been misinterpreted. The conclusions derived from the type 1 and 2 BRET assays were criticised in subsequent publications that claimed the data had been misinterpreted or that the assays had been incorrectly performed (334). The primary concern of Bouvier et al. was that the new approaches contradicted a large body of pre-existing evidence and so should not be taken at face value. Whilst this is a sensible precaution, Bouvier et al. otherwise failed to identify clear-cut technical problems with either technique that justified the use of the older methods. A specific criticism of the

type 1 assay levelled at James et al. was that $BRET_{max}$ is dependent on numerous factors and cannot be used as evidence for random or specific interactions alone. However, although James et al. do report increased $BRET_{max}$ values for known and predicted dimers versus monomers, conclusions regarding their stoichiometry were based entirely on the relationship between $BRET_{eff}$ and GFP:Rluc ratio, independently of $BRET_{max}$.

The criticism of the type 2 BRET data presented by James et al. was based on what Bouvier et al. claimed was a misinterpretation of the relationship between $BRET_{eff}$ and expression level. Bouvier et al. drew attention to the fact that the slope of the curve obtained in type 2 BRET assays of β_2AR by James et al. was more similar to that of the constitutive dimer CTLA-4 than that obtained for the monomers CD2 and CD86, supporting their claim of β_2AR dimerisation. However, this reflected a misunderstanding of the way in which stoichiometry is judged using the type 2 assay. The main factor that determines interpretation of type 2 BRET is the position of the y -intercept of the $BRET_{eff}$ -expression relationship, not its gradient. This is because the gradient is dependent on a variety of unpredictable variables including efficiency of transport to, and distribution within, the plasma membrane. Thus, only the y -intercept, which is independent of such variables, is a reliable indicator of stoichiometry. Both β_2AR and mCannR2 clearly exhibit y -intercepts that are not significantly non-zero, which is why James et al. concluded that they are both monomers according to this assay.

Finally, Bouvier et al. claimed that previous studies linking ligand binding to increased $BRET_{eff}$ cannot be reconciled with the type 1 and 2 BRET data, as non-specific interactions would not be affected by receptor activation. However, this does not allow for increased effective concentrations of the receptor upon activation due to, *e.g.* internalisation processes such as clustering within clathrin-coated pits. They also refer to the large body of non-BRET data suggestive of GPCR oligomerisation, without countenancing the complicating factors associated with these techniques as discussed above.

The experimental challenge to the conclusions of James et al. by Salahpour and Masri (335) was based on type 2 experiments in which non-zero y -intercepts were observed for β_2AR and two dopamine receptors, in contrast to the zero intercepts obtained for β_2AR by James and colleagues. Salahpour and Masri were critical of several aspects of the original BRET study, particularly that receptor numbers were not quantified and that positive controls were measured in a batch-wise manner rather than through the use of a batch-independent standard curve. Salahpour and Masri claim that a combination of these factors, as well as inadequate control of the constant GFP:Rluc ratio in type 2 assays, makes the work of James et al. difficult to interpret. In response, James and Davis (2007) argued that using batch-specific positive controls circumvented variation in the preparations of coelenterazine 400A used in the assay which can in turn cause variation in $BRET_{eff}$ measurements.

Secondly, they emphasised that absolute numbers of receptors are not required to distinguish between zero and non-zero y -intercepts. Finally, luminescence was argued by James et al. to be far more sensitive to low protein concentrations than radioligand-binding as used by Salahpour and Masri, allowing measurements to be performed at much lower surface densities. The lowest densities at which James et al. took measurements was ~1000 molecules/cell, compared to over 100,000 molecules/cell in the experiments of Salahpour and Masri. The difference in the expression ranges may explain the apparent differences in β_2 AR behaviour observed in the two studies, as the pseudolinearity of the BRET_{eff}-density relationship is observed to break down at higher receptor concentrations (313).

Thus, whilst the work of James et al. was controversial, it was not undermined by any significant theoretical or experimental challenge. Nevertheless, neither the type 1 or type 2 approaches have been used extensively before or since publication of the methods by James et al., presumably due in part to the critical reception of their study. However, when they have been used these types of approaches have supported a model of monomeric GPCRs. For example, FRET analysis of the neurokinin-1 receptor using a type 2 approach revealed a clear zero y -intercept and no detectable FRET at physiological expression levels (336), strongly suggesting that neurokinin-1 receptor is a monomer. Moreover, this study observed that the degree of non-specific FRET was strongly affected by cholesterol-dependent co-localisation of receptor monomers, emphasising the profound effects that constrained distribution can have upon the interpretation of RET studies.

Heterodimer assessment with BRET

Whether GPCRs form heterodimeric complexes, and which receptors they pair with, is presently highly contentious. A great deal of evidence is claimed to demonstrate receptor heteromerisation in varying combinations for many different receptors (337), although the vast majority of this data was obtained using Co-IP, single-point RET, or BRET saturation assays, each of which have their limitations. The nature of heteromeric interactions requires the use of irrelevant controls in many cases, which also greatly complicates interpretation. It would therefore be valuable to have a BRET-based technique that reliably reports GPCR heterodimers. Neither type 1 nor type 2 BRET is well suited to the investigation of heterodimers, however. This is because both approaches require constant total expression levels (type 1) or acceptor:donor ratios (type 2), which is difficult to achieve when different receptors are used as donors and acceptors since the number of proteins expressed from a single transfected gene often varies from one receptor to the next. In a type 1 experiment, therefore, as acceptor:donor ratio is varied, the total number of proteins changes. If the acceptor expresses more efficiently than the donor then surface density will increase with acceptor:donor ratio, whereas the

converse is true if the donor is expressed more efficiently – potentially leading to false-dimer and false-monomer signatures, respectively. Similarly, in a type 2 experiment acceptor:donor ratio will change with surface density if one receptor is expressed more rapidly than the other, leading to variations in $BRET_{eff}$ independent of expression level.

To address this issue, a novel BRET approach was developed by Mustafa and Pflieger (2011) for identifying GPCR heteromers. GPCR heteromer-identification technology (GPCR-HIT) works on the basis that signalling proteins recruited to heterodimers will be recruited to both receptors in a complex even if only one is signalling (338). In order to probe interactions between receptor A and receptor B, receptor A is expressed as a fusion with the donor luciferase while receptor B is expressed in an untagged form (Fig. 1.9a). Acceptor fluorophores are expressed as fusions with appropriate signalling proteins, *e.g.* G proteins or arrestins. The acceptor is recruited to receptor B by addition of an agonist specific to that GPCR. If receptors A and B interact, then the acceptor will be recruited to the donor attached to receptor A and $BRET_{eff}$ will increase, whereas no increase will be observed for non-interacting proteins. GPCR-HIT has in this way been claimed to identify heteromeric interactions between several GPCRs, including chemokine, angiotensin, bradykinin, and adrenergic receptors (339-341). A fundamental issue with the assay, however, is that GPCR-HIT assumes homogenous protein distribution, which may not be the case. As discussed above, a growing body of evidence suggests that GPCRs do not experience free diffusion within the membrane, and there have also been observations of heterogeneous subcellular distribution of G proteins and other signalling molecules (*e.g.* 342, 343, 344). Moreover, the distribution of G proteins and arrestins will be influenced by GPCR activity. This significantly complicates the interpretation of GPCR-HIT, as it cannot distinguish between recruitment of acceptor-tagged protein to a genuine donor-containing heterodimer or simply to a region of the cell surface that is enriched for donor-tagged receptors. Increases in $BRET_{eff}$ upon addition of agonist may arise due to changes in the degree of non-specific BRET resulting from signalling-dependent alterations in local acceptor concentration (Fig. 1.9b & c). Presently, there is no BRET approach that can unambiguously identify GPCR heteromers *in situ*.

1.4.5 Single-molecule microscopy

Recent advances in fluorescence microscopy now allow GPCR stoichiometry to be addressed by visualising individual fluorescently labelled receptors *in situ*. This has not been possible using standard epifluorescence microscopy because illuminating the entire sample volume results in background fluorescence that is too high to allow discrimination of single molecules. Instead, total

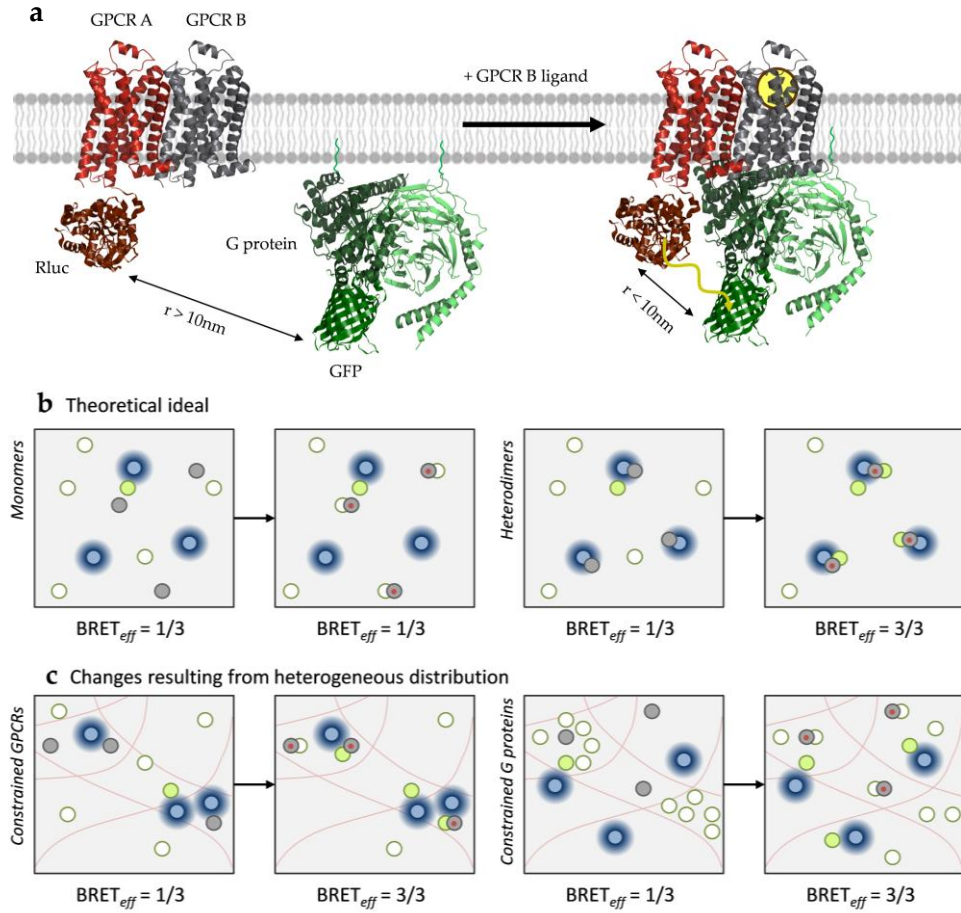


Figure 1.9: Experimental approach and potential problems of GPCR-HIT. (a) In GPCR-HIT, the two receptors of interest (GPCRs A and B) are co-transfected as an Rluc-fusion and untagged protein, respectively. GFP is attached to an appropriate signalling partner of GPCR B, such as a G protein or arrestin. (b) Addition of an agonist to GPCR B will recruit the GFP-tagged signalling partner to GPCR B and so result in an increase in BRET_{eff} if GPCRs A and B form heterodimers, but should have no effect upon BRET_{eff} in the case of monomers or non-interacting homodimers. Fluorescing and non-fluorescing acceptor molecules are shown as green and white circles, respectively, and donors as blue circles. The BRET-permissible area surrounding donors is represented as a blue halo. GPCR B is shown in grey, its agonist is represented by a red spot. (c) Increases in BRET_{eff} can occur upon addition of GPCR B agonist for reasons other than genuine heterodimerisation. Non-specific BRET_{eff} will be increased under circumstances where either GPCRs A and B are colocalised and so acceptor is recruited to an area of higher effective donor concentration (left), or where signalling by GPCR B causes a relaxation of constraints on acceptor diffusion (right).

internal reflection fluorescence microscopy (TIRFM) is used, wherein only those fluorophores within approximately 100-200nm of the glass coverslip on which the sample is mounted are illuminated. This is achieved by directing excitation laser(s) at an angle that gives total internal reflection of the beam due to differences in the refractive indices of the glass coverslip and mounting medium. An evanescent wave is generated with the wavelength of the incident laser, which propagates into the sample but undergoes rapid decay. The effect of this is that only the basal cell membrane is illuminated and interpretation is not complicated by the behaviour of intracellular fluorophores.

In principle, TIRFM is suitable for use with any fluorophore that can be successfully attached to the protein of interest. Several studies have used fluorescent proteins expressed as fusions with the protein of interest in order to allow direct visualisation of the target molecule (*e.g.* 345). There is now a range of alternative small-molecule fluorophores available that offer greater brightness and photostability than many fluorescent proteins. These fluorophores can be directly conjugated to monoclonal antibodies targeted to the protein of interest, or to ligands with high binding affinities (*e.g.* 346). Some fluorophores are also available as ligands for versatile enzyme-based tags *e.g.* SNAP (347), CLIP (348), or Halo (349). These employ an enzymatic tag that can be irreversibly labelled with a variety of fluorophores conjugated to their respective substrates. This, to some extent, overcomes potential effects that antibodies or ligands may have on protein behaviour.

The diffraction limit

A limitation of standard TIRFM is that resolution is constrained by the Abbe diffraction limit. Fluorophores separated by a distance smaller than this limit cannot be independently resolved due to diffraction, and as a result they appear as single objects. The size of the diffraction limit (d) is determined by the wavelength emitted (λ) as well as the numerical aperture (NA) of the instrument according to the relationship $d = \lambda/2NA$. Most current microscopes have NA values of roughly 1, and so the diffraction limit is approximately half the size of the fluorophore emission wavelength. Fluorophores used in TIRFM typically have emission wavelengths ranging from 400-700nm, resulting in diffraction limits of 200-350nm. This is one or two orders of magnitude larger than most molecules present at the plasma membranes of cells, and over 20 times larger than the effective resolution of most RET approaches, which is typically 5-10nm. The diffraction limit is therefore a significant obstacle to single-molecule microscopy, as it places a fundamental constraint on the minimum size of systems that can be visualised. Conventional single-molecule studies must be mindful of the potential effects of the resolution limit, as what may appear to be physical interactions of target molecules may instead be co-localisation within the diffraction limit.

1.4.6 Single-molecule microscopy applied to GPCRs

Initial disagreement with the constitutive dimer model

To date only a handful of single-molecule studies of GPCR stoichiometry have been undertaken, yet they have profoundly influenced general opinion regarding receptor oligomerisation. It now seems as if a paradigm shift is underway, from a one of constitutive dimerisation suggested by RET and Co-IP analyses, to another based on transient dimerisation (350). The status these studies have obtained is perhaps due to them being the first to offer stoichiometric information without the need to interpret bulk data. In the first investigation, TIRFM was used to image transfected M₁ muscarinic receptors at the surface of CHO cells (346). Measuring spot brightness for receptors labelled with Cy3B-conjugated antagonist allowed the number of points containing one, two, or more fluorescent antagonist molecules to be counted. Over 80% of spots had fluorescence intensities consistent with single antagonist molecules and underwent single-step photobleaching, strongly suggestive of monomeric receptor behaviour. The remaining spots were grouped primarily into those likely to contain two antagonists confined within the diffraction limit (~8%), or those that exhibited transitions between monomer and dimer states. Two-colour tracking of the receptors using Cy3B- and AlexaFluor488-conjugated antagonists employed in a 1:1 labelling strategy showed that ~80% of tracked receptors exhibited independent movement, where coincident movement was defined as spots staying within 300nm of one another for more than 10 consecutive frames (at 25-33 frames s⁻¹). The remaining 20% of receptors exhibited coincident movement for at least 10 frames, with a mean coincidence lifetime of ~0.5 s. The data obtained in this study lead the authors to conclude that M₁ receptors are predominantly monomeric, with ~30% of the receptors forming short-lived dimers at any one time.

These observations are highly significant as they argue strongly against a model of constitutive M₁ dimerisation proposed previously on the basis of RET and Co-IP experiments (*e.g.* 331). Unfortunately, the conclusion that ~30% of receptors form transient dimers is not as clear-cut. Whilst coincident movement is a pre-requisite of dimerisation, it does not necessarily indicate dimeric behaviour. Coincidence can be the product of co-localisation of receptors within the ~300nm diffraction limit rather than of direct interaction. Hern et al. used surface densities of up to ~2 receptors μm^{-2} , which equates to ~14% of the cell surface being covered by fluorescence assuming a 300nm spot size. At these high densities non-specific co-localisation within the diffraction limit is likely. Due to the nature of the labelling method used, it was not possible to make comparisons with known monomer controls and so instead the predicted level of non-specific coincidence was estimated assuming free diffusion and the observed diffusion rate of ~0.8 $\mu\text{m}^2\text{s}^{-1}$. The probability of non-specific

coincident movement within 300nm for >10 frames was calculated on this basis to be well below 1%, which suggests that coincident molecules are genuine dimers. However, if the assumption of free diffusion is incorrect then the probability of apparent dimers arising due to chance coincidence may be far higher than 1%. Hern and colleagues justify their assumption of free diffusion by observing that the mean square displacement of receptors is linearly proportional to time, consistent with a random walk. However, receptor clustering does not necessarily require anomalous diffusion, for instance if GPCRs transiently co-localise within structures that are themselves diffusing, *e.g.* in membrane domains or multi-protein assemblies. This would explain the observation that apparent dimers diffuse more slowly than monomers, although this may also be due to the increased hydrodynamic diameter of genuine dimers. Whilst this first single-molecule analysis clearly precludes constitutive dimerisation in the case of M₁, it remains unclear what fraction of the observed transient dimers result from genuine, specific interaction.

Subsequent single-molecule studies of GPCRs

The concept of constitutive dimerisation was also challenged by single-molecule analysis of the *N*-formyl peptide receptor (FPR) expressed in CHO cells (351). Spot intensity analysis revealed that ~60% of the observed objects possessed fluorescence intensities characteristic of monomeric receptors, consistent with the observations that FPR does not exhibit homodimerisation under Co-IP analysis, and that wild-type FPR does not cause an internalisation-deficient mutant to be internalised upon ligand-binding (352). The remaining ~40% were concluded to be dimers with an average lifetime of 91ms, far shorter than that observed for M₁ (346). However, these data suffer the same potential complications described above; principally that monomers co-localised within the diffraction limit cannot be distinguished from genuine dimers. Whilst this issue was addressed through the use of monomeric controls, it is unlikely that the proteins replicate the diffusive behaviour of FPR, particularly given that one was an unsaturated phospholipid likely to undergo much more rapid and unrestrained diffusion than a GPCR. Attempts to measure single-molecule FRET in this system failed to yield detectable levels of acceptor excitation, strongly suggesting that apparent rather than genuine dimers were being detected in the single molecule experiments. In an attempt to detect genuine FPR dimers, bimolecular fluorescence complementation (BiFC) was used, in which FPR or a monomeric control is expressed as fusion proteins with N- and C-terminal fragments of YFP, such that dimerisation allows reconstitution of a functional, fluorescent YFP molecule. Kasai et al. reported that BiFC was consistently higher for FPR than their monomeric control, but this may again simply reflect receptor organisation within the membrane. That the fluorescent BiFC spots had average lifetimes of

301ms (three-fold higher than the authors report for single-molecule tracking) also suggests that the interaction between YFP fragments may be stabilising indirect FPR dimers, as observed elsewhere (*e.g.* 353, 354, 355).

Similar considerations are relevant to the first single-molecule investigation of native M₂ muscarinic receptors in primary mouse cardiomyocytes (356), performed using the same fluorescently conjugated muscarinic receptor antagonist used previously to study M₁ (346). Photobleaching analysis revealed similar levels of monomeric M₂ species as reported for M₁: ~74% in perinatal cardiomyocytes and ~57% in myocytes from newborns. The remainder exhibit two-step photobleaching, suggestive of dimers. This again confirms that receptor dimerisation is not necessarily constitutive, but concerns about the authenticity of the dimers remain. Indeed, the variation in monomer fraction size between cell types (*i.e.* larger in myocytes) may reflect differences in receptor density, as the M₂ density in newborn myocytes is roughly twice that of perinatal cardiomyocytes.

Another single-molecule study (251) is much more easily interpreted because it was performed in high density lipoprotein particles and therefore not subject to issues arising from active receptor organisation within the membrane. μ -OR conjugated to Cy3 or Cy5 and visualised using TIRFM exhibited no specific co-localisation and were instead resolvable as individual receptors, strongly suggesting that, at a protein-intrinsic level μ -OR, is inherently monomeric, although this does not preclude the existence of oligomeric complexes arising from indirect interactions or active processes *in vivo*.

The only single-molecule study of GPCRs that has claimed extensive dimerisation of *Rhodopsin* family receptors was performed on both β_1 AR and β_2 AR in CHO cells using spot intensity analysis of SNAP-tagged receptors to estimate stoichiometry (233). This investigation reported between 30-85% dimers for β_1 AR, and 60-100% for β_2 AR, depending on receptor surface density. However, the strong dependence of the dimer fraction upon surface density for both receptors strongly indicates that at least some of this apparent dimerisation is a result of transient colocalisation within the diffraction limit. This is further supported by the fact that apparent trimers, tetramers, and higher-order oligomers of both receptors were observed at high densities. Again, the issue of non-specific colocalisation is addressed through the use of simulated non-specific interactions at the densities used, but once more the possibility of anomalous diffusion is not incorporated into the simulation and so it may not be an appropriate comparison. This is despite the observation in the same study of significant levels of cytoskeleton-dependent organisation for the GABA_BR1-2 heterodimer. The description of higher degrees of apparent dimerisation for β_2 AR compared to β_1 AR is consistent with previous observations that suggest in some cells β_1 AR is relatively freely diffusing

whereas β_2 AR is more tightly restricted in its localisation (239). An additional possible explanation for the high levels of apparent dimerisation seen by Calebiro et al. results from the use of intensity analysis rather than two-colour tracking. This is because any experimental factor that leads to more efficient detection of brighter spots will inherently bias towards the observation of dimers in an intensity analysis. This can be the result of inappropriate threshold levels below which fluorescence is discounted, or more subtle issues regarding the brightness and stability of the fluorophore used. Calebiro et al. report a 0% monomeric fraction for a two-fluorophore control, CD86 fused to two N-terminal SNAP-tags. The fact that no apparent monomers were reported for this construct indicates that a bias towards brighter spots may have been introduced into the experiment. This is because even a 100% dimer, such as a doubly labelled monomer like CD86, will always exhibit some degree of apparent monomericity as the folding and labelling efficiency of the tag used is never perfect. SNAP-tag is a derivative of the human DNA repair protein O⁶-alkylguanine-DNA alkyltransferase (347), and so is able to undergo low levels of conjugation to non-fluorescent benzylguanine moieties within the cell, which precludes 100% labelling. Moreover, the folding efficiency of the first generation of SNAP-tag, as used by Calebiro et al., is known to be significantly below 100% (Jonathan A. Javitch, Columbia University personal communication). These factors combined make a 0% apparent monomer fraction technically unfeasible, which indicates some degree of bright spot bias in the analysis of Calebiro et al. The extent to which this has affected their conclusions is impossible to say, but it seems likely that it will have increased the proportion of apparent dimers observed and so the observation of up to 100% dimerisation may not be reliable.

1.4.7 The current state of the GPCR oligomerisation debate as reflected by β_2 AR

Generally considered to be paradigmatic, β_2 AR is without doubt the most studied GPCR. This results from its prominence in early functional and genetic studies (*e.g.* 9, 12, 16) as well as its pharmacological importance (357). It was also the first soluble ligand-binding GPCR to have its structure solved (146). Nonetheless, despite numerous studies of its stoichiometry, there is no consensus over whether β_2 AR exists as a monomer, dimer, or higher-order oligomer *in situ*.

Several studies have shown that β_2 AR self-associates upon solubilisation *e.g.* in native gels (358, 359) and following Co-IP (*e.g.* 288, 289-292), yet homodimeric interactions are absent in all currently available crystal structures of the receptor (*e.g.* 146, 152, 187, 195, 303, 360). Moreover, isolated β_2 AR monomers are capable of activating G proteins under conditions that prevent

dimerisation (197, 250), and it crystallises in a stable 1:1 receptor-G protein complex (187). *In situ* analyses of β_2 AR ought to have helped reconcile these observations, but these investigations have mostly employed RET, which unfortunately have also yielded contradictory results. BRET assays of β_2 AR using single-point measurements and saturation assays were initially considered to support the notion of constitutive homodimerisation (288, 291, 292, 296, 320, 328, 330). Conversely, type 1 and 2 BRET assays strongly suggested that β_2 AR behaves as a prototypic monomer (313), despite the controversy (334, 335, 361, 362). An investigation of β_2 AR in artificial bilayers using time-resolved FRET indicated, on the other hand, that the receptor forms constitutive tetramers that are unaffected by ligand-binding (359). Conversely, a third-party BRET assay implied that β_2 AR forms constitutive homodimers *in situ* (363), whilst FRET experiments involving a novel coiled-coil labelling approach has concluded that β_2 AR is actually wholly monomeric (364).

In situ analysis of β_2 AR using non RET-based techniques is similarly inconclusive. Wild-type β_2 AR is retained within cells if co-expressed with retention-specific mutants (330), which is interpreted as being due to direct homodimerisation. Similarly, confocal microscopy suggests that agonist-induced internalisation of wild-type receptors causes co-internalisation of binding-deficient mutant receptors, again suggesting association between the two (365). However, a similar study using compartment-constrained BRET to report subcellular localisation of wild-type and mutant receptors did not observe any effect of the internalisation of one receptor type upon the other (366). Equally, whilst fluorescence recovery after photobleaching (FRAP) suggests that β_2 AR protomers mutually influence their diffusion through higher-order oligomerisation (367), histidine-tagged β_2 ARs are unable to recruit untagged receptors into artificially induced regions of receptor clustering (368), implying that the receptors do not influence each other's diffusion. Single-molecule, non-super-resolution fluorescence microscopy, however, suggests once again that β_2 ARs form transient homooligomers (233), though this is subject to the possible technical complications discussed above.

Despite many attempts to demonstrate β_2 AR oligomerisation there is also no consensus on its possible function or mechanism. Co-IP analysis initially suggested that TM6 is the principal mediator of receptor-receptor interactions (289), which seemed to be supported by BRET analyses indicating a role for L284 of TM6 (330). However, computational analysis of β_2 AR surface chemistry suggests that the lowest energy arrangement of two receptor protomers requires the interface to be formed by TM1 and H8 (369), as observed for the closely related β_1 AR in a recent crystal structure (302). Our understanding of how these interactions might influence receptor behaviour is further complicated by numerous studies implicating β_2 AR in a variety of heteromeric associations. For example, Co-IP and BRET approaches were used to argue that β_2 AR forms direct interactions with both β_1 AR (291, 328)

and β_3 AR (320). These complexes are proposed to have distinct signalling and trafficking behaviour versus their monomeric equivalents, although the possibility that the observed signalling effects are due to receptor crosstalk is yet to be ruled out. The observation that ligand-binding to both β_2 AR and β_3 AR is unaffected by heterodimerisation (320) argues against allosteric effects operating between the two receptors. Similar considerations apply to interactions of β_2 AR with δ -OR (290, 296) and κ -OR (292, 297), in studies that relied on Co-IP and single-point BRET measurements. In fact, type 2 BRET analysis of β_2 AR associations with δ -OR and κ -OR (292) yielded zero BRET_{eff} y -intercepts indicative of random interactions that were interpreted instead to indicate heterodimerisation. The argument for heteromerisation is further weakened by the observation that the ligand-binding ability of β_2 AR, δ -OR, and κ -OR is unaffected by their co-expression (297), and by the finding that β_2 AR- δ -OR association is undetectable in time-resolved FRET experiments (296). Thus, whilst it has been the focus of intense activity, the contradictory nature of much of the resulting data means that we are no closer to understanding the functional significance of homo- and heterodimerisation *in vivo*, or even whether it occurs at all.

1.5 Aims of this thesis

GPCRs form the largest family of receptors in the mammalian genome. The evolutionary success of these receptors is likely to be underwritten by a signaling mechanism shared by the great majority of receptors in the family. All existing studies have focussed on one or a small number of GPCRs, and so the general behaviour of the family presently has to be deduced by consolidation and extrapolation of these individual studies. Moreover, although the studies now number in the hundreds, there has been a disproportionate focus on a small number of prominent receptors, namely the β -adrenergic, opioid, adenosine, and muscarinic receptors, but also to a lesser extent the serotonin, chemokine, and histamine receptors. The stoichiometry of many GPCRs has never been assessed. Technical issues with many of the techniques used in these investigations makes their interpretation difficult, yet the only BRET approaches that appear to overcome these problems have thus far been applied to just two *Rhodopsin* family receptors, β_2 AR and mCannR2 (313), and remain controversial. Expansion of these approaches to a larger number of GPCRs offers the dual possibility of increasing our understanding of typical GPCR stoichiometry and at the same time increasing the likelihood of observing *Rhodopsin* family dimers, thereby confirming the efficacy of the technique.

The goal of this thesis is to undertake a semi-systematic of *Rhodopsin* family GPCRs, in order to (a) determine the extent to which stoichiometry varies across the family as a whole, (b) establish the

‘typical’ stoichiometry of these GPCRs, and (c) to offer possible explanations as to why stoichiometry may vary between receptors from an evolutionary perspective.

CHAPTER 2

General methods

2.1 Molecular cloning and vector assembly

2.1.1 General cloning overview

Standard molecular biology techniques were employed throughout the work detailed in this thesis (370). In order to generate expression vectors to allow expression of proteins of interest in target cell lines, genes were amplified from plasmid or cDNA templates using 5' and 3' oligonucleotide primers that introduced suitable restriction sites at either end. Amplified product and target vector were then digested with the appropriate enzymes and purified to obtain fragments of correct size, before being ligated together to form a continuous vector. This ligation product was used to transform bacterial cells and allow selection of whole vector-containing colonies based on antibiotic resistance, which would then be screened for correctly inserted and oriented vector and insert DNA sequences. Larger preparations of DNA for subsequent applications would be made after confirmation of DNA sequence and integrity.

Vectors used in this thesis

pRLuc N3 (PerkinElmer) This vector was used in the generation of all Rluc-tagged constructs for use in the BRET² assay. The vector contains a multiple cloning site (MCS) followed by the *N*-terminus of the *Renilla* luciferase (*Rluc*) gene. Insertion of a gene of interest into the MCS with in-frame restriction sites allowed the generation of fusion proteins where the *C*-terminus of the protein of interest is joined to the *N*-terminus of a proceeding *Rluc*. This fusion protein is expressed from the cytomegalovirus immediate-early (CMV-IE) promoter in order to provide constitutive high-level expression. Selection for this vector in bacterial cells was facilitated by a kanamycin-resistance cassette within the plasmid backbone.

pGFP² N3 (PerkinElmer) This was used in conjunction with pRluc N3 to complete the BRET pairs required for the BRET² assay. pGFP² is equivalent to pRluc but the MCS is followed by the *GFP²* gene rather than *Rluc*. The genes in both vectors are in the same reading frame relative to the MCS and so sub-cloning was possible between them. pGFP² also contains the CMV-IE promoter but confers zeocin[®]-resistance rather than kanamycin-resistance to transformed bacterial cells.

pU N3 Expression of genes from this vector results in a protein lacking any C-terminal tag such that it may be used in competition BRET assays. This vector was made from the pGFP² N3 plasmid by replacing the *GFP²* gene with an in frame *TAG* stop codon by excision of *GFP²* using the restriction enzymes and *Bam*HI and *Not*I. The MCS and resistance cassettes are otherwise identical to pGFP² N3.

pJET1.2 (Fermentas) This was used in the amplification of low-yield PCR products and as a shuttle vector during some cloning processes. pJET1.2 is supplied in a blunt-end linearised form in the CloneJET PCR cloning kit (Thermo Scientific). Blunt end PCR products can be ligated into the vector for subsequent amplification in bacterial cells, selected for by an ampicillin-resistance cassette within the plasmid backbone. Self-ligation of an empty vector results in the expression of the lethal restriction enzyme *Eco47I* that kills host *E. Coli* upon transformation.

2.1.2 Molecular biological methods

RNA extraction and cDNA preparation

In order to extract RNA from cell lines of interest, cells were diluted to a concentration of 1×10^7 /ml in TRIzol[®] (Invitrogen) before 0.2ml chloroform per 1ml TRIzol[®] was added. The cell suspension was shaken for 15 seconds to ensure homogeneity, incubated at room temperature for 3 minutes, and then centrifuged at 3500rpm for 30 minutes at 4°C. Following centrifugation, the aqueous phase was transferred into a clean tube containing an equivalent volume of isopropanol and shaken again to precipitate any RNA, which was then pelleted by centrifugation at 3500rpm for 30 minutes at 4°C. After centrifugation, the supernatant was removed from the pellet, which was then washed with 1ml 75% ethanol and air-dried to remove remaining ethanol. The dried RNA pellet was initially resuspended in 250µl RNase-free H₂O before the concentration was determined spectrophotometrically at 260nm. The integrity of the extracted RNA was assessed by agarose gel

electrophoresis whereby the presence of intact RNA could be confirmed by the visualisation of bands corresponding to the 60S and 18S ribosomal RNA molecules.

For cDNA preparation, extracted RNA was diluted in ddH₂O to a final concentration of 500ng/μl and aliquoted into 10μl reaction volumes. 1μl of 500ng/μl oligodeoxythymine (oligo dT; Invitrogen) was added to each 10μl sample of RNA, which was then heated to 70°C for 10 minutes and cooled to room temperature to allow the oligo dT to anneal to mRNA 3' polyadenosine tails. Reverse transcription of the primed mRNA was then performed at 42°C for 40 minutes in the following mixture before heat inactivating the enzymatic components at 90°C for 10 minutes:

	Company	Concentration	Volume
Reverse Transcriptase buffer	Invitrogen	5x	4μl
Dithiothreitol	Invitrogen	100mM	2μl
dNTP	Sigma-Aldrich	10mM	1μl
RNAasin™ (ribonuclease inhibitor)	Promega		1μl
Superscript™ II Reverse Transcriptase	Invitrogen	200U/μl	1μl
RNA-oligo dT mix	-	-	11μl

The resulting cDNA was diluted 1:5 in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) for subsequent use. All RNA and cDNA preparation steps were performed using RNase-free equipment.

Polymerase chain reaction

The polymerase chain reaction (PCR) was used to amplify genes with the desired 5' and 3' restriction sites. Oligonucleotide primers used were designed complementary to the 5' and 3' ends of the target gene and included convenient restriction sites that are specified for individual constructs in subsequent sections. The reaction enzyme used was Phusion® High-Fidelity DNA polymerase (New England Biolabs; NEB), which contains an additional processivity-enhancing domain and 3'-5' exonuclease proofreading activity to ensure efficient, accurate amplification. For most amplification reactions, the following mixture was used:

	Company	Concentration	Volume
Polymerase buffer	NEB	5x	8μl
dNTP	Sigma-Aldrich	10mM	2μl
5' Primer	Sigma-Aldrich	10μM	0.4μl
3' Primer	Sigma-Aldrich	10μM	0.4μl
Template DNA	-	100ng/μl	1μl
DNA Polymerase	NEB	2U/μl	0.2μl
ddH ₂ O	Sigma-Aldrich	-	28μl

In most reactions the HF (high-fidelity) buffer was used, but for some more difficult templates the GC (GC-rich) buffer was used instead. In some cases, dimethyl sulphoxide (DMSO) was added at concentrations from 1-5% total reaction volume and the volume of ddH₂O adjusted accordingly. Amplification sequences were carried out using a T100™ Thermal Cycler (BioRad) using the following format in most cases:

	Stage	Temp. (°C)	Time
1	Enzyme activation	98	2 min
2	DNA denaturation	98	15 s
3	Primer annealing	55	30 s
4	Polymerase extension	72	30 s/kb
	<i>Repeat stages 2-4</i>		34 x
5	Final extension	72	5 min
6	Cooling	10	∞

For some primers or templates, the temperature of annealing was varied to achieve maximum product yield, typically between 50 and 70°C. In some cases where amplification was difficult or from a heterogeneous template such as cDNA, a gradient touchdown sequencing reaction was used:

	Stage	Temp. (°C)	Time
1	Enzyme Activation	98	2 min
2	DNA denaturation	98	15 s
3	Primer annealing	65	30 s
4	Polymerase extension	72	30 s/kb
	<i>Repeat stages 2-4</i>		34x
	<i>Reduce annealing temp. by 0.3 °C/cycle</i>		
5	Final extension	72	5 min
6	Cooling	10	∞

The presence of inserted DNA in transformed bacterial cells was confirmed by a low-fidelity PCR screen using *Taq* DNA polymerase (Qiagen) in the following reaction mixture:

	Company	Concentration	Volume
Polymerase buffer	Qiagen	10x	2µl
dNTP	Sigma-Aldrich	10mM	0.5µl
5' Primer	Sigma-Aldrich	10µM	0.4µl
3' Primer	Sigma-Aldrich	10µM	0.4µl
Bacterial cells	-	-	-
DNA Polymerase	Qiagen	10U/µl	0.2µl
ddH ₂ O	Sigma-Aldrich	-	16.5µl

A modified reaction cycle was used in order to lyse the bacterial cells prior to extension:

	Stage	Temp. (°C)	Time
1	Bacterial lysis	95	5 min
2	DNA denaturation	95	30 s
3	Primer annealing	55	30 s
4	Polymerase extension	72	60 s/kb
	<i>Repeat stages 2-4</i>		34 x
5	Final extension	72	5 min
6	Cooling	10	∞

For the insertion of point mutations or the generation of chimeric genes, multi-step PCR protocols were used. Initial amplification would be achieved in two or more separate PCR reactions using oligonucleotide primers with 20-30 overlapping nucleotides in order to generate amplification products with complementary 5' or 3' termini. These overlapping products were then used as products in one or more subsequent rounds of amplification (5µl each), according to the original protocol, in order to generate the full-length product.

Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA by size and to assess the purity and integrity of DNA fragments and PCR products. 1% (w/v) gels were prepared by dissolving high-quality agarose (SeaKem LE agarose, BioWhittaker) in Tris-borate EDTA (TBE) buffer (Sigma-Aldrich). Gels were melted using a microwave for 80s/50ml and ethidium bromide was added to a final concentration of 0.5µg/ml before the gels were cast with combs of appropriate size for sample loading. DNA was loaded into the comb wells after mixing with 10% loading buffer (40% (w/v) sucrose, bromophenol blue, xylene cyanol) and electrophoresed using a PowerPac 300 (BioRad) typically at 70mA for 50-80 minutes. DNA was visualised post-electrophoresis using ultra-violet (UV) irradiation in a GelDoc (BioRad). In some circumstances it was necessary to extract DNA from the agarose gels. In these cases, DNA bands were excised using a clean scalpel blade under conditions of low UV-irradiation, and then purified using a Gel Extraction Kit (Qiagen) as per the manufacturer's instructions.

DNA restriction digestion

To cleave DNA at defined restriction sites, purified DNA was incubated with the relevant restriction enzyme(s) (NEB) typically at 10U/ μ l and under the following conditions:

	Company	Concentration	Volume
Reaction buffer	NEB	10x	2 μ l
BSA	NEB	100x	0.2 μ l
DNA	-	-	5 μ l
Restriction enzyme(s)	NEB	10U/ μ l	1 μ l
ddH ₂ O	Sigma-Aldrich	-	11.8 μ l

Where possible, high-fidelity versions of the enzymes were used in order to minimise star activity. Most reactions were performed at 37°C for 60 minutes. In cases where simultaneous digestion with two enzymes was not possible due to competing requirements for buffers, sequential digests were performed with the DNA being purified between stages using a PCR Purification Kit (Qiagen) as per the manufacturer's instructions.

DNA dephosphorylation with Antarctic phosphatase

In order to minimise vector self-ligation during insertion of digested DNA, the 5' phosphate groups of the digested vector ends were removed using Antarctic Phosphatase (NEB) at 37°C for 60 minutes in the following mixture:

	Company	Concentration	Volume
Reaction buffer	NEB	10x	3 μ l
Digested Vector	-	-	10 μ l
Antarctic Phosphatase	NEB	10U/ μ l	1 μ l
ddH ₂ O	Sigma-Aldrich	-	16 μ l

Digested and phosphatased DNA was always resolved on a 1% agarose gel prior to purification and ligation.

Insert ligation into vectors

Digested DNA or PCR product was inserted in digested, phosphatased vectors using a DNA ligase reaction to covalently link DNA fragments with complementary sticky ends. Control ligations excluding the insert DNA or DNA ligase were also set up in order to assess the degree of vector digestion. The following mixture was used at 16°C overnight:

	Company	Concentration	Volume
T4 DNA ligase buffer	NEB	10x	2µl
Insert DNA	–	various	10µl
Vector DNA	–	100ng/µl	5µl
T4 DNA ligase	NEB	400U/µl	1µl
ddH ₂ O	Sigma-Aldrich	–	2µl

Transformation and selection of competent bacterial cells

Ligation products were inserted into competent bacterial cells for amplification and screening. TOP10 *E. coli* were made competent by transferring one ml of an overnight culture into a 75cm² tissue culture flask containing 30ml Luria-Bertani (LB) broth and incubating at 37°C for 2-2.5 hours whilst undergoing orbital shaking at 200rpm. LB broth was prepared from tablet (Sigma-Aldrich) and autoclaved prior to use. Following incubation, the optical density of the cells was confirmed to be between 0.3 and 0.6 at 600nm before centrifugation at 4000rpm for 10 minutes. The bacterial pellet was resuspended in 2.5ml ice-cold TSS buffer (prepared as: 5ml DMSO, 10g Polyethylene Glycol 600, 5ml 1M MgCl₂, 90ml LB broth - all components from Sigma-Aldrich). Competent cells were kept on ice and 100µl aliquoted into 1.5ml eppendorf tubes. 10µl ligation product was also added and incubated on ice for 15 min before the cells were heat-shocked at 37°C for 4.5 minutes and then returned to ice. 400µl LB broth was added to each tube and the cells incubated at 37°C for 1 hour to allow antibiotic-resistance genes to be efficiently expressed.

Transformed bacteria were plated onto antibiotic-containing agar plates and spread using an ethanol-sterilised glass spreader. LB agar was prepared from tablets (Sigma-Aldrich) and autoclaved. Antibiotics were added to the appropriate concentration when the agar was molten, and then poured into 90mm petri dishes (Sterilin) to set and dry. Plated bacteria were incubated overnight at 37°C and resultant colonies were screened for correct vector-insert combination using PCR as previously described.

Vector extraction

In order to prepare workable quantities of DNA for downstream applications, vector DNA was extracted from overnight cultures of transformed bacteria selected using the relevant antibiotic. Extraction was performed using standardised kits that work on the principle of bacterial cell alkaline lysis prior to adsorption of DNA onto silica resin. Adsorbed DNA can be thoroughly washed at low salt concentrations to remove cell debris, before being eluted at higher salt concentrations in an anion-exchange mechanism. Eluted DNA is then precipitated using isopropanol and washed with 70% ethanol before being resuspended in TE buffer to an appropriate concentration. The PureLink™ HiPure Plasmid Miniprep Kit (Invitrogen) or QIAfilter™ Plasmid Midi Kit (Qiagen) were used according to the manufacturer's instructions to obtain small or larger-scale quantities of DNA, respectively. All DNA preparations were spectrophotometrically assessed for concentration at 260nm, and purity by the 260nm/280nm absorbance ratio, which was always ensured to be >1.8.

DNA sequencing and analysis

DNA sequencing was performed in-house using the reversible terminator base method with an ABI-3730 DNA Analyser (Applied Biosciences) and the BigDye® Terminator v3.1 Cycle Sequencing Kit and G5 dyeset (Applied Biosciences). Sequence integrity was analysed using the 4Peaks™ (Nucleobytes) software and alignment performed using the EMBOSS Needle global alignment system (European Bioinformatics Institute).

Preparation of bacterial glycerol stocks

Transformed bacteria were often stored for multiple rounds of culture and DNA extraction. Storage was at -80°C in a medium of LB broth containing 25% filter-sterilised glycerol (v/v) to prevent ice-crystal formation. 50µl of stored bacterial stocks would be used to inoculate 5ml cultures of LB broth containing an appropriate concentration of the relevant selective antibiotic.

2.2 Cell culture

2.2.1 General techniques

All work using mammalian cells was conducted under sterile conditions using disposable plastic pipettes for cell and medium handling, and in HEPA-filtered microbiological safety cabinets. All cell lines were cultured in dedicated tissue culture incubators under conditions of 37°C and 5% CO₂.

Cell lines used in this thesis

HEK 293T The transformed human embryonic kidney (HEK) 293T cell line is adherent and easily transfected, demonstrating good expression of most mammalian genes from the CMV promoter, and so was used for all transient expression assays in this study. This line was derived by transformation of primary cultures of HEK cells using sheared adenovirus (Ad)5 DNA (371), and then further transformed to contain the SV40 large T antigen.

SH-SY5Y This line is derived from one of three serially isolated neuroblast clones of the human neuroblastoma line SK-N-SH, extracted from a metastatic bone tumour (372). They grow largely as adherent cells, and have been demonstrated to have native expression of a number of receptors in the muscarinic acetylcholine family (373).

Cell subculturing

As all used cells were adherent, growth medium was removed and the cells washed with sterile phosphate-buffered saline (PBS). The cells were then incubated with an appropriate volume of trypsin for 5 minutes at room temperature to induce dissociation from the flask, which was aided by gentle shaking. A volume of growth medium twice that of added trypsin was then added to the cells to inhibit trypsin protease activity. The cell density was then determined and the cell suspension centrifuged at 1500rpm for 5 minutes before being resuspended in an appropriate volume of fresh medium.

Cells were typically grown in 25cm², 75cm², or 175cm² tissue culture flasks containing 10, 25 or 40ml growth medium, respectively. Cell lines were subcultured regularly to maintain optimum growth density, and were typically discarded after 8-12 passages.

Counting of cells

Cell density was determined by mixing 50µl cell suspension with an equivalent volume of trypan blue solution and then microscopically scoring cell numbers using a haemocytometer. Trypan blue staining was used as a marker for cells with compromised cell membranes.

Cell plating

Cells were plated out into 6- or 96-well plates prior to transfection. Cells were detached from culture and resuspended as described above, and then transferred to the plate at the desired density and in an appropriate volume. Incubation of plates overnight allowed cells to settle and re-adhere prior to transfection, typically at a density of ~80% confluence for optimum effects.

Storage and retrieval of cells

In order to freeze cells for storage, 6x10⁵ cells were harvested from culture and transferred to 1ml freezing medium (foetal calf serum with 10% (v/v) DMSO) in a Cryovial® (Simport). Cryovials were cooled to -80°C in a Nalgene® freezing container (Thermo Scientific) that ensures gradual cooling to preserve cell viability. Once frozen, cells were transferred to liquid nitrogen for long-term storage.

Cells were retrieved from storage by rapid warming to 37°C and transfer into 50ml pre-warmed growth medium. Cells were then centrifuged at 1500rpm for 5 min to remove residual DMSO and resuspended in an appropriate volume of fresh growth medium for culture. Cell viability was assessed microscopically 24-48h after thawing.

2.2.2 Growth media

The following media were used to culture mammalian cells. All components were either supplied sterile from the manufacturers, or sterilised before use by passing through a 0.2µm syringe filter.

TMEM – Medium for HEK 293T cells

	Company	Volume
Dulbecco's Modified Eagle's Medium (DMEM)	PAA	500 ml
Foetal Calf Serum	PAA	50 ml
L-Gluamine (200 mM)	Sigma-Aldrich	5 ml
Antibiotics (Pen/Strep/Neo)	Sigma-Aldrich	5 ml

SMEM – Medium for SH-5Y cells

	Company	Volume
DMEM	Gibco	250 ml
Ham's F12 Medium	Gibco	250 ml
Foetal Calf Serum	PAA	50 ml
L-Gluamine (200 mM)	Sigma-Aldrich	5 ml
Antibiotics (Pen/Strep/Neo)	Sigma-Aldrich	5 ml

2.3 Phenotypic analysis

2.3.1 Flow cytometry

Flow cytometry was used to assess the expression of proteins of interest in target cell lines. Cells were harvested from culture and resuspended in PBS containing 0.05% sodium azide (w/v) to a typical final concentration of 5×10^6 /ml. Cells expressing fluorescent reporters required no further labelling. In cases where cell surface proteins needed to be labelled with fluorescently-conjugated antibodies, cells were incubated with the relevant primary and secondary (if not a directly conjugated primary) antibodies for 45 min on ice before washing 5x in 4ml cold PBS-azide. All samples were resuspended to a final volume of 400µl cold PBS-azide for analysis and were maintained on ice throughout. Labelled cells were analysed using a CyAn ADP™ (Beckman Coulter) flow cytometer using the appropriate lasers and emission filters. Viable cells were gated using forward scatter and side scatter, and single cell events were gated for using pulse width. In cases where overlapping fluorophores were used, compensation was performed in each channel using singly-labelled samples.

2.3.2 Confocal microscopy

The expression and cellular localisation of fluorescently-tagged proteins was confirmed using confocal microscopy. Transfected adherent cells were grown on microscope coverslips and fixed using PBS + 4% para-formaldehyde for 10 minutes. Coverslips were then mounted onto glass slides in the presence of VectaShield mounting medium (Vectorlabs) and imaged using a Zeiss LSM-510 inverted confocal microscope under an oil-immersed 60x objective lens with excitation laser light typically at 405nm. Images were collected at the midpoint of the cell using a 515±15nm emission filter, and minimally manipulated to improve signal-to-noise.

CHAPTER 3

Expanding the use of BRET assays: I. the challenge of transient dimerisation

3.1 Introduction

3.1.1 Establishing the basis for a comprehensive investigation of *Rhodopsin* family GPCR stoichiometry

The first use of type 1 and 2 BRET approaches to investigate GPCR stoichiometry was highly controversial as it suggested monomeric behaviour of β_2 AR and mCannR2, contradicting a large number of previous studies (313). Whilst this has informed the discussion of the stoichiometry of these specific receptors, the initial investigation did not offer insights into the “typical” stoichiometry of the *Rhodopsin* family GPCRs. Nonetheless, these assays are theoretically sound and their validity was confirmed using well characterised control proteins, so their application to a larger range of GPCRs would allow the more general behaviour of the *Rhodopsin* family to be elucidated. One way to achieve this would be to investigate the entire GPCR repertoire of a single cell line, assayed in that same line. This should maximise the effectiveness of data collection by ensuring the greatest degree of correct protein folding and signalling, as all receptors will be native to the cells in question. Moreover, determining the subset of GPCRs to investigate by this means is essentially ‘blind’ as no researcher bias can be introduced by the selection of particular receptors. The best candidate cell line for such an approach is the HEK 293T line, as it is in these cells that the majority of previous BRET-based analyses of GPCR stoichiometry have been performed, owing to the ease with which they can be transfected. In order to allow such a study, two matters need to be clarified: (1) the capacity of the current BRET assay for detecting partial homodimers; and (2) the development of a complementary approach to type 1 BRET that is suitable for the investigation of GPCRs that are also expressed natively by the cellular host of the assay. The first issue is the subject of this chapter, and the second is considered in Chapter 4.

3.1.2 Detecting partial GPCR dimerisation

The recent emergence of transient GPCR dimerisation as a potential model of receptor behaviour (*e.g.* 350) has been driven largely by observations obtained using single-molecule microscopy of both transfected and native receptors (*e.g.* 346, 356). Given the widespread recognition of this new model, all studies of GPCR stoichiometry must now be mindful of the possibility of low levels of dimerisation, and so critically assess the techniques used accordingly. Although the ability of type 1 BRET analysis to distinguish between monomers and dimers has already been established (313), its sensitivity for the detection of partial dimers has not been thoroughly examined. Detection of transient CD80 dimers is unambiguous under type 1 BRET analysis (313), but the reduced hydrodynamic diameter of this type 1 TM protein relative to GPCRs is likely to enhance the sensitivity of the approach by increasing BRET within dimers. Thus, whether detection of transient dimerisation between GPCRs is similarly efficient has not yet been determined.

In this chapter, the sensitivity of the type 1 BRET approach to low levels of receptor dimerisation is assessed through the use of an inducible GPCR dimer system. This allows varying extents of dimerisation to be induced in the chemokine receptor CCR5, which is observed to otherwise yield data consistent with monomeric behaviour under both type 1 and type 2 analyses. The type 1 BRET approach is then applied to the muscarinic receptor M₁ in order to assess the prominent claims of transient dimerisation recently made (346).

CCR5

Chemokine receptors are expressed in all leukocytes, where they control cell recruitment to sites of inflammation and infection and coordinate leukocyte migration around the body (374). They respond to peptide cytokines called chemokines that are released from a wide variety of cell types in order to generate a gradient of chemoattractant signalling allowing leukocyte recruitment. CCR5 is involved in responses to a number of chemokines, primarily CCL5 (chemokine (CC-motif) ligand 5; 375). Therapeutic interest in CCR5 is focussed predominantly on the fact that it is exploited by HIV-1 during the infection of CD4⁺ T cells (376, 377). CCR5 was selected for use in this study as it is the subject of ongoing parallel work using single-molecule microscopy, due to both the availability of suitable high-affinity antibodies against CCR5, and the fact that is of local interest because of its role in T cell function.

The nature of CCR5 stoichiometry is not well understood. Co-IP- (378) and BRET-based (379) analyses of CCR5 have concluded that the receptor is a constitutive oligomer, whereas Co-IP in other investigations has suggested only ligand-dependent dimerisation of otherwise monomeric receptors (380). Indeed, even immunoblot and FRET analysis in the same study has given contradictory results, with FRET data being interpreted as indicating dimers in the absence of ligand, and immunoblot analysis only showing dimers upon CCL5-binding (381). Expression of wild-type CCR5 at the surface of cells is reduced by the presence of a mutant truncated receptor (CCR5 Δ 32), a finding also interpreted as evidence of homodimerisation (378), but one that could also be explained by indirect effects of the mutant upon wild-type receptor expression. A study employing immunoblot and FRET analysis of wild-type and mutant CCR5 receptors concluded that residues in TM1 and TM4 are required for receptor dimerisation (381), but this was subsequently challenged by Co-IP and BRET analyses that indicated no effect of mutation of these residues upon CCR5 stoichiometry (382).

M₁

Muscarinic acetylcholine receptors are widely expressed in the central and peripheral nervous systems where they sensitise neuronal cells to the small-molecule neurotransmitter acetylcholine. They are involved in a wide variety of neuronal processes, including smooth muscle contraction, glandular secretion, motor control, cardiovascular regulation, and memory (383). M₁ is the predominant post-synaptic acetylcholine receptor subtype in the CNS, and is a significant drug target due to its role in the development of a number of neurological disorders, such as Alzheimer's disease and schizophrenia (384).

The stoichiometry of the muscarinic acetylcholine receptors has been assessed in several studies using a variety of techniques, although there is no current consensus on the extent or nature of any interactions. Early investigations concluded that the receptors exist as covalently linked dimers (279), whilst more recently it has been proposed that they are constitutively oligomeric (385) or dimeric (331). It was an investigation of M₁ by single-molecule microscopy that initially proposed the now widely acknowledged notion of transient GPCR dimers (346). Although the structure of M₁ has yet to be solved, those of the closely related M₂ and M₃ receptors have (153, 154). Neither receptor dimerises upon crystallisation, arguing against a model of constitutive association for these receptors and probably for M₁ as well, and so is in closer agreement with the single-molecule analysis than the earlier bulk studies.

Materials and methods

3.2.1 Vector construction

BRET vectors for CCR5, β_2 AR, GABABR1b, CD86, and CD28 had been made previously by J. James (313) and were re-prepped and test digested (§2.1.2) to confirm integrity before use in BRET² assays and further cloning steps. M₁ BRET vectors were prepared by PCR amplification of the *CHRM1* gene from cDNA of SH-5Y neuroblastoma cells (§2.2.1) using oligonucleotide primers 2905 and 2906 (Table 3.1). The resultant product was inserted into the pGFP² and pRluc N3 vectors (PerkinElmer) using flanking 5' *MluI* and 3' *BamHI* restriction sites, and its sequence confirmed by reversible terminator base sequencing (§2.1.2).

CCR5-FKBP constructs were prepared by the amplification of *FKBP* from the pC₄-Fv1E vector (Ariad) using primers 2779 and 3016 and ligated between *CCR5* and *GFP²* or *Rluc* in the CCR5 N3 BRET vectors using the intermediate *BamHI* restriction site.

All novel constructs made for the work detailed in this chapter had their full sequences confirmed using reversible terminator base sequencing (§2.1.2).

No.	Target	Dir.	Primer sequence	Restriction
2905	<i>CHRM1</i>	5'	TAGTAGACGCGTGCCACCATGAACACTTCAGCCCCACCTGC	<i>MluI</i>
2906	<i>CHRM1</i>	3'	ATGAGGGATCCCGCATTGGCGGGAGGGAGTGCGGTG	<i>BamHI</i>
2779	<i>FKBP</i>	5'	TAGTAGGGATCCAGGAGTGCAGGTGGAAACCATC	<i>BamHI</i>
3016	<i>FKBP</i>	3'	CTACTAGGCTCCCCCTCCAGCTTCAGCAGCTCCACGTCG	<i>BamHI</i>

Table 3.1: Oligonucleotide primers used in this chapter.

3.2.2 Confocal microscopy

Surface expression of membrane proteins was assessed via confocal microscopy of their GFP fusion constructs. HEK-293T cells were transfected, 24h after plating, with 1 μ g pGFP² DNA per 6 $\times$ 10⁵ cells using GeneJuice® (Novagen) as per the manufacturer's protocol. Cells were then fixed and imaged as described in 2.4.2 using GFP-suitable excitation and emission filters.

3.2.3 Transfection

6-well plates were seeded with 6×10^5 HEK 293T cells in 2ml TMEM (§2.2.2) to ensure ~80% confluence after 24h. BRET constructs were co-transfected as BRET pairs consisting of both GFP- and Rluc-tagged proteins using GeneJuice® (Novagen) as per the manufacturer's instructions. DNA was always used at a final concentration of $0.05 \mu\text{g}/\mu\text{l}$ and total volume of $20 \mu\text{l}$ per well (*i.e.* $1 \mu\text{g}$). In the type 1 BRET assay pGFP²:pRluc ratios ranging from 1:2 to 66:1 were used as this gave the most useful spread of GFP:Rluc protein values. This was achieved by varying the volume of pGFP² and pRluc solutions in the final $20 \mu\text{l}$ volume; for example, a 3:1 pGFP²:pRluc ratio would constitute $15 \mu\text{l}$ pGFP² (*i.e.* $0.75 \mu\text{g}$) and $5 \mu\text{l}$ pRluc (*i.e.* $0.25 \mu\text{g}$). Transfection for type 2 BRET assays was done at a constant GFP:Rluc ratio of 12:1. All experiments included the treating of one well with $120 \mu\text{l}$ transfection medium with no added DNA as a negative control. $1 \mu\text{g}$ of expression vector for a soluble, fused form of Rluc-GFP (sGFP-Luc; PerkinElmer) was used to transfect one well as the positive BRET control.

3.2.4 BRET² assays

Type 1 BRET

Transfected 293T cells were collected from wells 24h after initial transfection and resuspended in PBS to a concentration of $\sim 1.5 \times 10^6$ cells/ml. BRET_{eff} ratios were obtained by adding DeepBlueC (PerkinElmer) to a final concentration of $10 \mu\text{M}$ in $100 \mu\text{l}$ cell suspension in a 96-well OptiPlate (PerkinElmer) and collecting light emission in the BRET-A ($410 \pm 40 \text{nm}$) and BRET-B ($515 \pm 15 \text{nm}$) wavelengths. Collection for each wavelength was performed 3 times integrated over 1 second on a Fusion® Microplate Analyzer (PerkinElmer), thus giving a BRET-A and BRET-B value for each transfection. BRET_{eff} values were calculated as BRET-B/BRET-A after background subtraction and correction for luciferase expression (Eq. 3.3). BRET_{eff} is normalised as a function of the BRET_{eff} measured for the sGFP-Luc positive control (Eq. 3.4), which was assigned a constant value of 1.

GFP expression was determined by exciting $100 \mu\text{l}$ cells (in a new well) at $425 \pm 25 \text{nm}$ and measuring emission at $515 \pm 15 \text{nm}$ 3 times over 1 second, thereby giving the total fluorescence units (FU). The same cells were then incubated for 2 min with $10 \mu\text{M}$ coelenterazine-*h* (final concentration) before measuring total emission 3 times over 1 second to give the total luminescence units (LU). Acceptor/donor ratio was calculated using the LU/FU value obtained from cell expressing the sGFP-Luc positive control (Eq. 3.5), since the acceptor/donor ratio for this construct is precisely one.

The voltage and gain settings used for all BRET_{eff} and GFP:Rluc measurements are shown in table 3.2. These settings were used for all BRET assays in this thesis unless otherwise specified.

$$\text{BRET}_{eff} = \frac{(RLU_B - RLU_B^{negative}) - ((RLU_A - RLU_A^{negative})Cf)}{RLU_A - RLU_A^{negative}} \quad (3.3)$$

$$Cf = \frac{RLU_A^{Rluc}}{RLU_B^{Rluc}}$$

$$\text{BRET}_{eff}^{normalised} = \frac{\text{BRET}_{eff}}{\text{BRET}_{eff}^{positive}} \quad (3.4)$$

$$\frac{[GFP]}{[Rluc]} = \frac{LU/FU}{LU^{positive}/FU^{positive}} \quad (3.5)$$

Type 2 BRET

This was performed essentially as described for the type 1 assay except that cells were harvested for analysis at various time points to allow systematic variation of total surface expression whilst maintaining a constant GFP:Rluc ratio. The earliest readings were performed approximately 10 hours post-transfection. Rluc emission was used as the readout for expression level as this is the most sensitive at low protein levels.

	BRET _{eff}	GFP fluorescence	Rluc luminescence
Lamp intensity	-	10	-
Excitation filter	-	425±10nm	-
Emission filter 1	410±40nm	515±15nm	-
Emission filter 2	515±15nm	-	-
PMT Voltage	1100V	500V	550V
PMT Gain	50	1	1
Read time	1 s	1 s	1 s
Number of reads	3	3	3

Table 3.2: Fusion® Microplate Analyzer settings used for all microplate assays in this thesis.

Statistical analyses

All data fitting was performed using the Prism5 (GraphPad) software. Type 1 data were fitted to models of both dimeric (Eq. 3.2) and monomeric (Eq. 3.6) behaviour using the non-linear regression least squares function. The lower and upper range limits of [GFP]/[Rluc] values included in the analysis were 2 and 15, respectively, as this describes the range over which differences in the two

models are most evident. The coefficient of determination (R^2) for the monomer fit is always zero as calculated $BRET_{eff}$ is a constant value. An R^2 value of less than zero for the dimer model indicates that it has a worse goodness-of-fit to the data than the monomer model, whereas an R^2 greater than zero indicates a better goodness-of-fit. Residual values (observed $BRET_{eff}$ – calculated $BRET_{eff}$) were also generated for each model and their moving average ($n=4$) plotted to further assess goodness-of-fit.

Type 2 BRET data were fitted to equation 3.7 using the linear regression least squares function. Y-intercept values and associated confidence limits were generated by regression of the fitted model to $x=0$, and goodness-of-fit was assessed using R^2 .

$$BRET_{eff} = BRET_{max} = c \quad (2 < [GFP]/[Rluc]) \quad (\text{Eq. 3.6})$$

$$BRET_{eff} = m(\text{expression}) + BRET_{eff}^0 \quad (\text{Eq. 3.7})$$

3.3 Results

3.3.1 Type 1 and 2 BRET analyses of CCR5 suggest monomeric behaviour

Confocal microscopy of CCR5 revealed that the receptor traffics well to the plasma membrane and does not appear to undergo aggregation (Fig. 3.1a). GABABR1b, which is used as a dimer control, also failed to exhibit aggregation, although it was largely retained within intracellular membranes, as reported previously (386). Protein aggregation was identified by the presence of non-uniform accumulations of GFP fusion protein with fluorescence intensities and z-plane distributions greater than that expected of protein retained in internal membranes, as has been demonstrated previously (387). An example of such aggregates is given in Figure 3.1b, in the case of CCR5 fused to the C-terminal domain of GABABR2 (CCR5_{coil2}). Accompanying type 1 BRET data for this construct is also shown to demonstrate the problems associated with attempting BRET on aggregated proteins. Typically, aggregated proteins will exhibit large $BRET_{eff}$ values as the GFP and Rluc proteins are closely apposed within aggregates. Lower than expected GFP:Rluc ratios are also observed for aggregated proteins, possibly because the protein is more rapidly degraded than properly expressed proteins and less GFP accumulates.

The single-cell images in Figure 3.1 and throughout this thesis are representative of the general fluorescence distribution observed across the wider cell population. The position of the plasma membrane was estimated using white light images of the cells in question. Examples of this selection process for CCR5 and M₁ are shown in Figure 3.2.

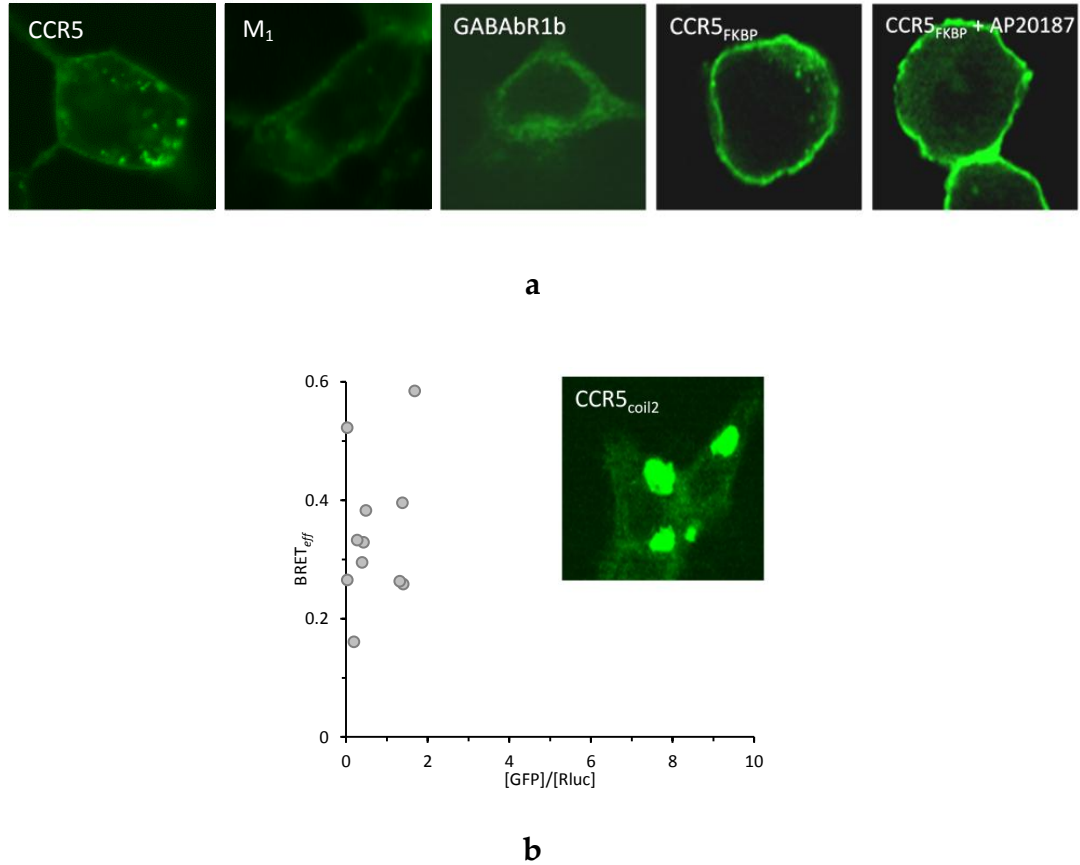


Figure 3.1: Representative confocal images of constructs used in this chapter. (a) CCR5 and M_1 both exhibit good levels of surface expression whilst GABAbR1b is largely retained within intracellular compartments but nonetheless does not appear to aggregate. CCR5_{FKBP} also reaches the surface well and this does not appear to alter upon addition of AP20187. (b) Example of protein aggregation in the case of CCR5_{coil2}. The accompanying type 1 BRET data shows a high level of BRET_{eff} due to the close proximity of GFP and Rluc in the protein aggregates, as well as low GFP:Rluc ratios.

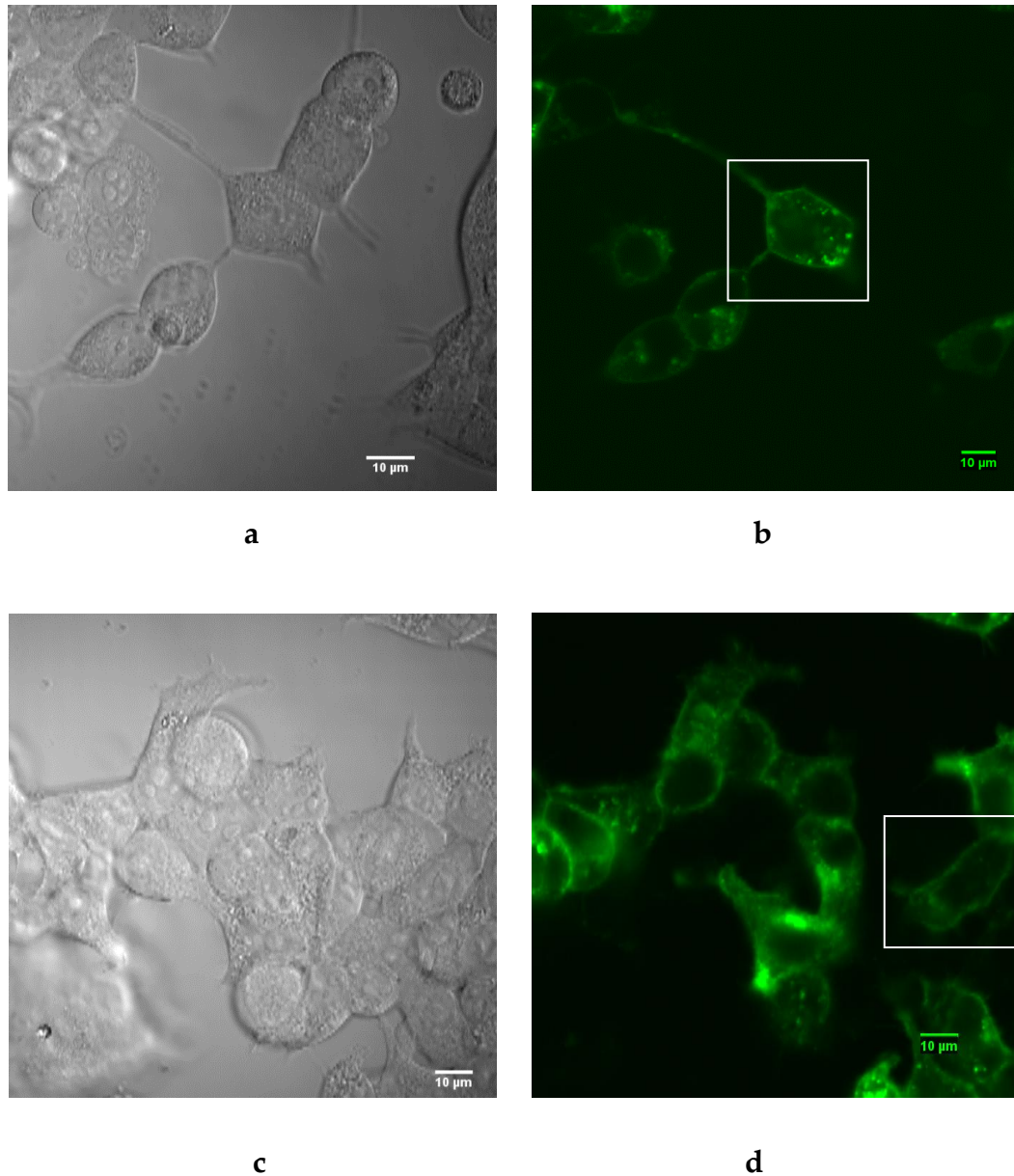


Figure 3.2: Examples of the image selection process undertaken in this thesis. The examples shown are images of CCR5_{GFP} (**a** & **b**) and M₁_{GFP} (**c** & **d**) transiently expressed in HEK 293T cells in both brightfield and GFP fluorescence channels. Individual cells representative of the general phenotype were selected from the wider field and cropped to an area of roughly 40μm × 40μm. White boxes indicate the area selected for representation in Figure 3.1. Cell boundaries were determined by reference to the brightfield image. Not all cells visible in the brightfield image exhibited detectable fluorescence in the GFP channel.

Type 1 BRET analysis of CCR5 yielded $BRET_{eff}$ values that are independent of $[GFP]/[Rluc]$ above a value of ~ 2 (Fig. 3.3a), consistent with monomeric behaviour. These data fit better to a constant (monomer) model than to the dimer model described in Equation 1.2. This was assessed using the absolute R^2 value of the dimeric fit; if the value is positive then this indicates a better fit to the dimer model, whereas a negative value indicated that a fit to a flat line would be better. The R^2 value of the dimer model applied to CCR5 is negative (Table 3.4). The better goodness-of-fit of the monomer model is also shown by plots of the moving average ($n=4$) of the residual values for each model (Fig. 3.3b) – the dimer fit deviates further from zero than the monomer fit above a $[GFP]/[Rluc]$ value of 2. Data collected for the *Glutamate* family dimer control GABAbR1b fit more closely to the dimer model. These models are only valid if total receptor expression is kept constant over the range of $[GFP]/[Rluc]$ values used in the type 1 experiments. In order to confirm that this was the case, relative total expression was determined for each measurement by converting GFP fluorescence units (FLU) into Rluc luminescence (RLU) units using the GFP:Rluc ratio, whereupon total relative expression was calculated as the sum of GFP and Rluc luminescence units (Eq. 3.1).

$$Expression_{total}^{RLU} = GFP^{RLU} + Rluc^{RLU} \quad (3.1)$$

$$GFP^{RLU} = f Rluc^{RLU} \quad (\text{where } f = \text{GFP:Rluc})$$

A plot of expression *vs.* $[GFP]/[Rluc]$ demonstrates that total expression level remained constant at different GFP:Rluc ratios for CCR5 (Fig. 3.4). This indicates that the models used to fit the data are being used appropriately and that the observed apparent monomer data are not the result of decreasing total expression at high GFP:Rluc ratios.

Type 2 BRET analysis of CCR5 yielded a y -intercept value for that was not significantly non-zero (Fig. 3.5), which is characteristic of monomeric behaviour. The dataset gave a good linear fit as indicated by its high R^2 values (Table 3.4), suggesting that stoichiometry is constant across the range of expression levels measured. Type 2 data can only be interpreted meaningfully if a constant acceptor:donor ratio is maintained for all expression levels. In order to assess this, $[GFP]/[Rluc]$ was

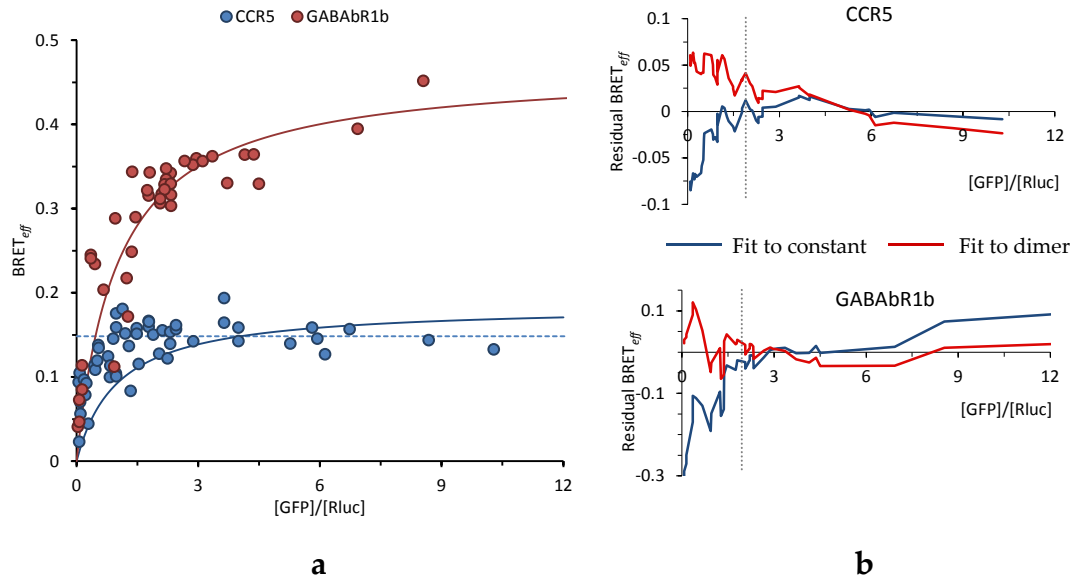


Figure 3.3: Type 1 BRET analysis of CCR5, and GABAbR1b. (a) CCR5 exhibits an independence of $BRET_{eff}$ upon $[GFP]/[Rluc]$ that fits more closely to a constant (monomer) model (broken line) than to a dimer described by Equation 1.2 (solid line). GABAbR1b is a dimeric control and yielded data that fit more closely to the fit expected of a dimer. Only points above a $[GFP]/[Rluc]$ value of 2 are used for data fitting (indicated by grey lines). (b) Plots of the moving average ($n=4$) of residual values between calculated and observed $BRET_{eff}$ show a better fit to the monomer model than the dimer for CCR5, but are better for the dimer for GABAbR1b. A good fit should reduce residual values to zero.

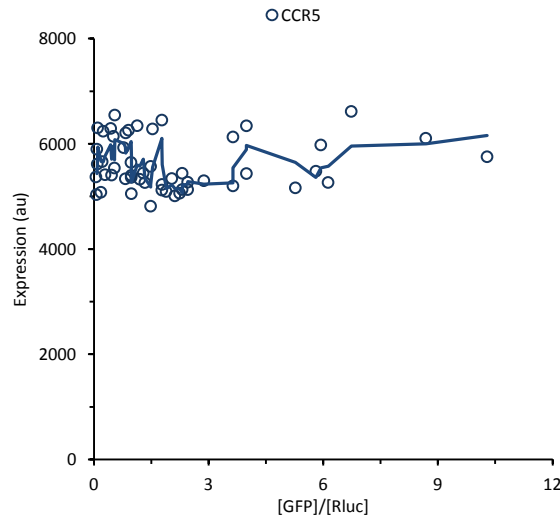


Figure 3.4: Total receptor expression level does not vary with $[GFP]/[Rluc]$ for CCR5 in a type 1 BRET experiment. Solid line is moving average ($n=4$) of individual data points.

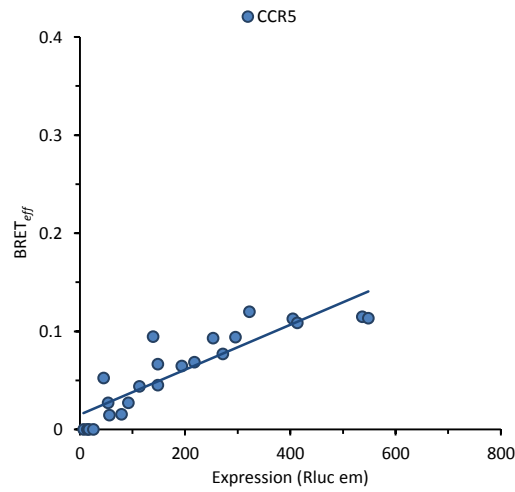


Figure 3.5: Type 2 BRET analysis of CCR5. Data fit well to a linear regression with a y -intercept that is not significantly non-zero (*i.e.* 95% y -axis confidence limits for $x=0$ include zero), indicative of monomeric behaviour.

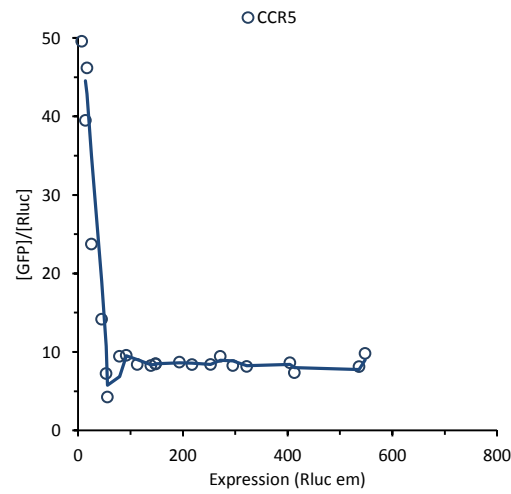


Figure 3.6: [GFP]/[Rluc] *vs.* expression level for CCR5 in the type 2 BRET assay. CCR5 demonstrates a constant GFP:Rluc ratio at all but the very lowest expression levels. Solid line represents the moving average ($n=2$) of the data.

determined for all expression levels in the same manner as type 1 experiments. This revealed that GFP:Rluc ratio is independent of expression level except at very low Rluc emission values (Fig. 3.6). At such low protein densities, GFP fluorescence is difficult to measure reliably due to increased contributions from background cellular autofluorescence. This is the cause of the significant experimental noise at these low Rluc values as small changes in GFP emission will translate to large changes in [GFP]/[Rluc]. Thus, it is highly likely that actual GFP:Rluc ratio is constant over all of the expression levels used. This highlights the need to use Rluc, rather than GFP, emission as the readout for expression at such low receptor densities, as it is a more precise indicator of relative protein concentration.

3.3.2 Type 1 BRET is capable of detecting weak GPCR dimers

In order to confirm the efficacy of type 1 BRET for detecting partial versus constitutive GPCR dimers, an inducible CCR5 dimer system was generated. This consisted of CCR5 expressed as a C-terminal fusion with FK507-binding protein (FKBP) as per the protocol of the Argent™ homodimerisation kit (Ariad). The FKBP was then followed by GFP or Rluc. Dimerisation could be induced by incubation with the small molecule AP20187 (ClonTech), which is a high-affinity bivalent ligand of FKBP. The observation of apparent monomericity for CCR5 under type 1 and 2 analyses suggests that the only dimerisation present in this system will be that induced by AP20187, which is important for more accurate determination of the sensitivity of the approach. The degree of relative dimerisation achieved at different AP20187 concentrations was assessed by incubating cells expressing CCR5_{FKBP}-GFP and CCR5_{FKBP}-Rluc at a 12:1 ratio at varying AP20187 concentrations for 45 minutes prior to BRET_{eff} measurement. The difference in BRET_{eff} between cells exposed to AP20187 or PBS was measured as the readout for change in the extent of dimerisation. These data fit well to a sigmoidal saturation curve with an EC₅₀ value of ~195nM (Fig. 3.7a), similar to previously reported EC₅₀ values of AP20187 in other systems (*e.g.* 388). When added to cells expressing CCR5_{FKBP}-GFP only, AP20187 does not affect total GFP fluorescence (Fig. 3.7b), which suggests that it does not influence receptor internalisation or degradation. This correlates with confocal microscopy imaging of CCR5_{FKBP} in the presence and absence of AP20187, which does not show any apparent internalisation upon induced dimerisation (Fig. 3.1a).

The determination of the dependence of relative dimerisation state upon AP20187 concentration allowed the calculation of the AP20187 concentrations required to cause varying

degrees of maximal dimerisation (Table 3.3). These may not correlate precisely with the actual degree of dimerisation induced in CCR5_{FKBP}, as the maximum level that can be achieved using this system is unlikely to be a 100% dimer population since some AP20187 molecules will be bound by only a single FKBP protein. Thus, 50% maximal dimerisation, for example, represents half the maximum level of association that can be reached using this system, and at equates at most to a 50% dimer population although probably less.

Application of the type 1 BRET assay to the CCR5_{FKBP} system at varying AP20187 concentrations revealed that even low levels of GPCR dimerisation can be detected in type 1 assays (Fig. 3.8). As expected, CCR5_{FKBP} in the absence of AP20187 (0%) yielded data that fit best to a monomer model when assessed using both dimer R^2 value (Table 3.4) and residual BRET_{eff} distribution (Fig. 3.9). Moreover, it demonstrated a similar BRET_{max} value to that observed for CCR5, indicating that the incorporation of FKBP did not significantly affect its expression or distribution. The addition of AP20187 at concentrations sufficient to induce 20% maximal dimerisation led to a better residual fit to the dimer model (Fig. 3.9) as well as a dimer fit R^2 value significantly greater than zero (Table 3.4). This was also observed for AP20187 concentrations that induce 30-100% dimerisation, with higher concentrations leading to better fits to the dimer model and increasingly poor fits to the monomer model. This is consistent with increasing levels of dimerisation from 20 to 100%. At 10% maximal dimerisation, the R^2 value obtained for the dimer model is only marginally above zero, and so would be difficult to assign unambiguously to dimerisation under circumstances where an induced system was not being used. This would therefore appear to represent the sensitivity limit of type 1 BRET assays, although the fact that a clear monomeric distribution – such as that observed for CCR5 – is not apparent would in itself be an indicator of low levels of dimerisation. Thus, it would appear that type 1 BRET is able to report dimerisation at levels at or above those corresponding to 10-20% of the maximum achievable using the CCR5_{FKBP} system, although less clear indications of dimerisation may be detectable at lower levels. This strengthens the conclusion that CCR5 is monomeric *in situ*.

3.3.3 BRET analyses do not observe transient M₁ dimerisation

Application of the type 1 BRET assay to M₁ yielded data that fit more closely to a monomer model than that of a dimer (Fig. 3.10). The R^2 value of the dimer model is -0.5646, which is substantially lower than that obtained for CCR5_{FKBP} at 10% maximal dimerisation (Table 3.4). This is consistent with M₁ being largely or exclusively monomeric, with any dimeric population representing a smaller fraction

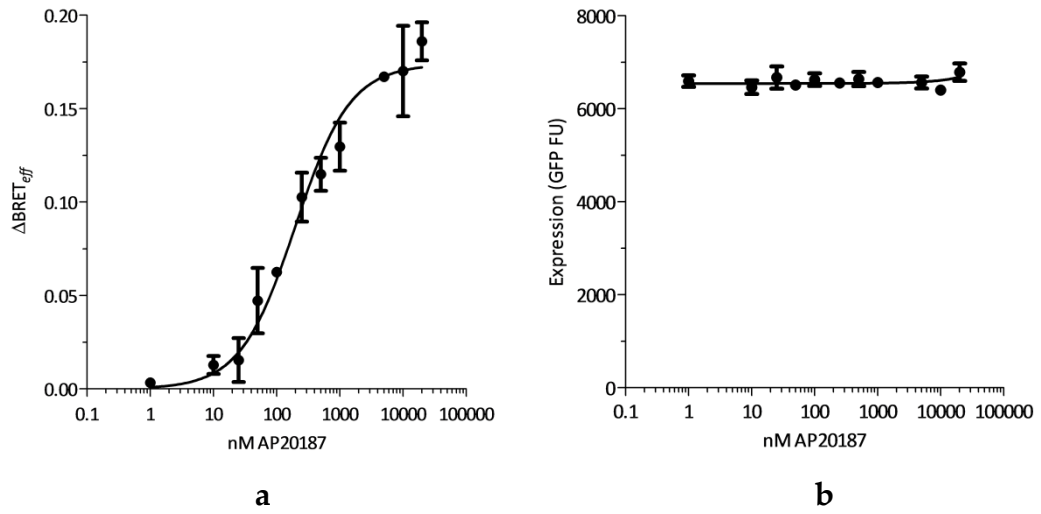


Figure 3.7: Effects of AP20187 on CCR5_{FKBP} dimerisation and expression. (a) BRET_{eff} increases with concentration of AP20187 following a sigmoidal saturation curve with an EC₅₀ of ~195nM. (b) AP20187 does not affect total expression of CCR5_{FKBP}GFP. All measurements were performed after 45 minutes incubation with the relevant AP20187 concentration. Data represent pooled data from three independent experiments. Error bars are SEM.

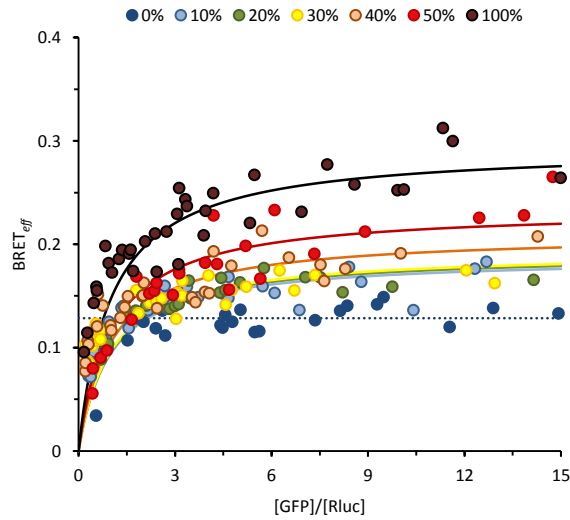


Figure 3.8: Type 1 BRET analysis of CCR5_{FKBP} in the presence of varying concentrations of AP20187. Percentages correspond to the AP20187 concentrations given in Table 3.3. Fits of the data to a dimer model are shown as solid lines. For 0%, the constant fit is shown as a broken line. CCR5_{FKBP} data fit progressively better to a dimer model as AP20187 concentration increases.

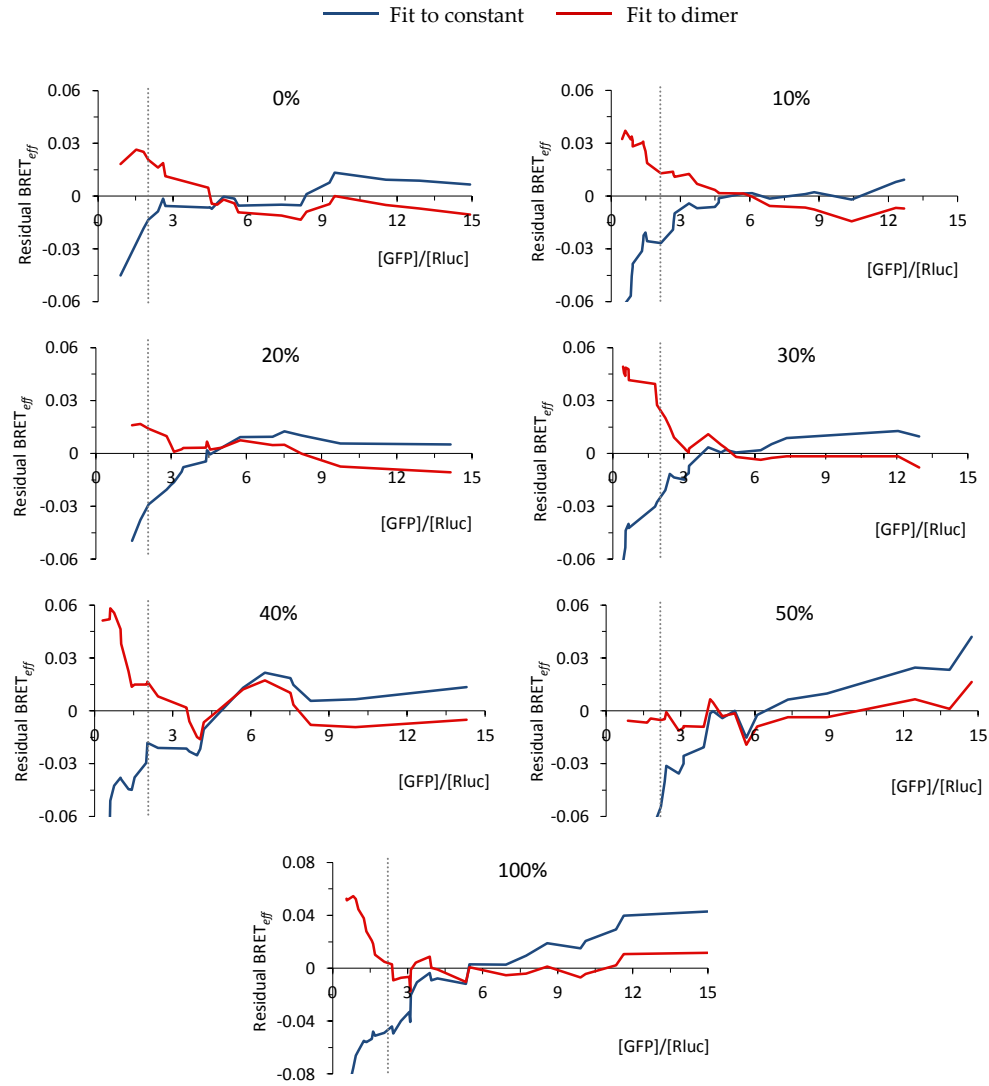


Figure 3.9: Residual $BRET_{eff}$ plots for monomer and dimer models of $CCR5_{FKBP}$ data at varying AP20187 concentrations. Percentages correspond to the AP20187 concentrations given in Table 3.3. The constant model results in the better fit at 0% maximal dimerisation. Neither model is significantly better than the other at 10% maximal dimerisation. At and above 20% maximal dimerisation, dimer fits agree increasingly well with the data and the constant fit agrees progressively worse.

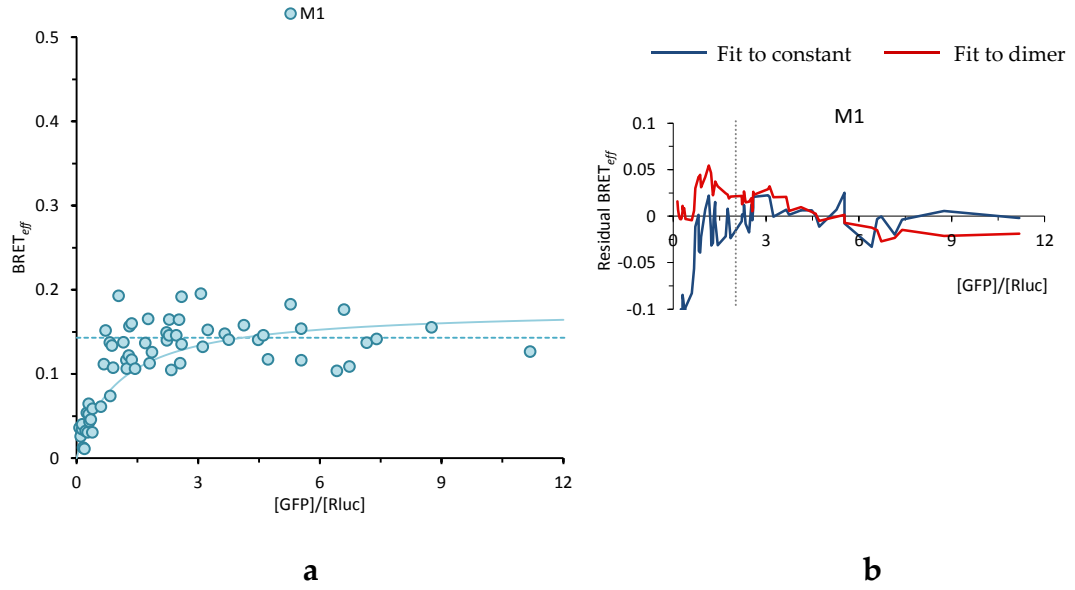


Figure 3.10: Type 1 BRET analysis of M1. (a) M1 exhibits an independence of $BRET_{eff}$ upon $[GFP]/[Rluc]$ that fits more closely to a constant (monomer) model (broken line) than to a dimer described by Equation 1.2 (solid line). (b) Plots of the moving average ($n=4$) of residual values between calculated and observed $BRET_{eff}$ show a better fit to the monomer model than the dimer.

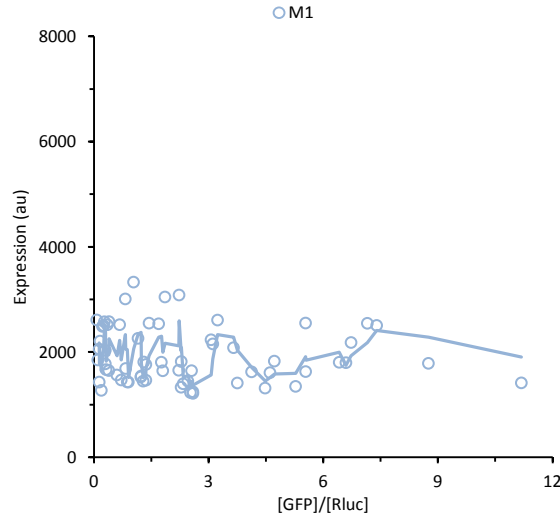


Figure 3.11: Total receptor expression level does not vary with $[GFP]/[Rluc]$ for M1 in a type 1 BRET experiment. Solid line is moving average ($n=4$) of individual data points.

of the total receptor population than the 10-20% apparent sensitivity of type 1 BRET. Again, the total receptor density of M₁ over varying GFP:Rluc ratios was assessed and observed to be constant (Fig. 3.11).

Type 2 BRET analysis of M₁ correlates with the data obtained by the type 1 assay; the calculated y -intercept is not significantly non-zero (Fig. 3.12), and is in fact lower than that observed for CCR5 (Table 3.4), further suggesting that M₁ is monomeric. As with CCR5, the GFP:Rluc ratio for M₁ was observed to remain consistent across all but the lowest expression densities (Fig. 3.13).

Interestingly, the slope of BRET_{eff} *vs.* expression is steeper for M₁ than for CCR5 (which is included in Figure 3.12 for comparison), suggesting that M₁ experiences more non-specific interactions at a given expression level than CCR5. This correlates with the observation that M₁ yields a similar BRET_{max} to CCR5 under type 1 analysis, despite having a lower total expression (Figs 3.3, 3.4, 3.10 & 3.11). For comparison, type 2 analysis of β_2 AR was also performed. Interestingly, both CCR5 and M₁ demonstrate steeper type 2 slopes than β_2 AR (Fig. 3.12), suggesting that both receptors undergo greater colocalisation within the cell than β_2 AR.

3.4 Discussion

The experiments described in this chapter set out to establish a framework for testing whether BRET could be used to detect partially dimeric receptors, following the proposal that M₁ and other GPCRs transiently dimerise. The application of both type 1 and 2 BRET to CCR5 yielded data that fit well to the predicted behaviour of a monomer. This is consistent with the previous analysis of *Rhodopsin* GPCRs, which also observed apparently monomeric receptors (313). Apparent CCR5 monomericity is not due to difficulties in establishing constant surface densities (type 1) or maintaining a constant GFP:Rluc ratio (type 2), as had been suggested (335), as these were observed to remain consistent throughout both types of experiment (Figs 3.3 & 3.10). No aggregation was observed for CCR5, although it is impossible to conclude with certainty that no misfolding occurs as this cannot be directly assessed using confocal microscopy. Nonetheless, this is unlikely as misfolded proteins are less likely to traffic to the plasma membrane, and are also more likely to aggregate and generate high BRET_{max} values, which were not observed. GABAbR1b, used as a dimeric control, does not efficiently traffic to the surface in the absence of equivalent levels of GABAbR2 (386), however this does not preclude its detection as a dimer by type 1 BRET, suggesting that surface expression is not a pre-requisite for investigation in this manner.

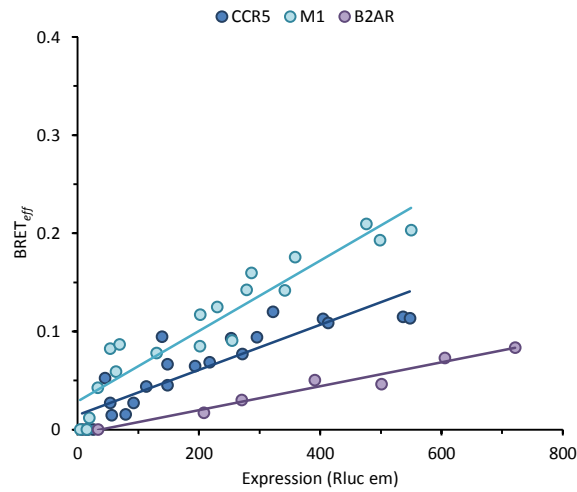


Figure 3.12: Type 2 BRET analysis of M1 and β_2 AR. CCR5 is also shown for comparison. Data obtained for all three receptors fit well to a linear regression with y -intercepts that are not significantly non-zero, indicative of monomeric behaviour.

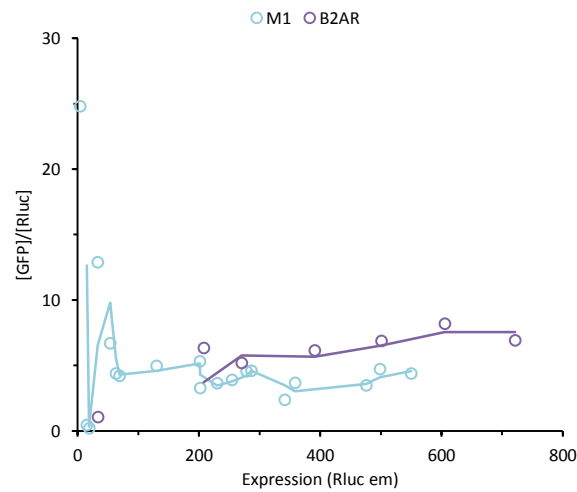


Figure 3.13: [GFP]/[Rluc] *vs.* expression level for M1 and β_2 AR in the type 2 BRET assay. Both receptors demonstrate a constant GFP:Rluc ratio at all but the very lowest expression levels. Solid lines represent the moving average ($n=2$) of the data.

% Maximal dimerisation	[AP20187]
0%	0nM
10%	25nM
20%	50nM
30%	90nM
40%	140nM
50%	195nM
100%	5 μ M

Table 3.3: AP20187 concentrations required for varying degrees of maximal CCR5_{FKBP} dimerisation.

Protein	Type 1 dimer fit R^2	Type 1 BRET _{max}	Type 2 R^2	Type 2 y -intercept
CCR5	-0.84	0.148	0.79	0.015 $\pm$ 0.016
M ₁	-0.56	0.143	0.88	0.028 $\pm$ 0.039
GABAbR1b	0.75	0.46	-	-
β_2 AR	-	-	0.96	-0.004 $\pm$ 0.012
CCR5 _{FKBP} – 0%	-0.30	0.153	-	-
CCR5 _{FKBP} – 10%	0.02	0.187	-	-
CCR5 _{FKBP} – 20%	0.33	0.191	-	-
CCR5 _{FKBP} – 30%	0.23	0.193	-	-
CCR5 _{FKBP} – 40%	0.45	0.210	-	-
CCR5 _{FKBP} – 50%	0.57	0.234	-	-
CCR5 _{FKBP} – 100%	0.58	0.293	-	-

Table 3.4: Goodness-of-fit, BRET_{max}, and y -intercept values for type 1 and 2 BRET experiments in this chapter. A negative type 1 dimer fit R^2 value indicates a better fit to a monomer model; a positive value indicates a better fit to a dimer model. Type 2 y -intercept values are given $\pm$ 95% confidence limits.

Having established that CCR5 exhibits monomeric behaviour under both type 1 and 2 BRET, the extent to which weak dimers can in principle be detected by type 1 BRET was investigated using an inducible CCR5_{FKBP} system. It was found that type 1 BRET assays are capable of identifying ~10-20% of the maximum level of dimerisation that can be induced in this way. It is not possible from these data to speculate what level of dimerisation this equates to in real terms, but it is likely to be less than 10-20% genuine dimerisation as it is probable that not all CCR5_{FKBP} molecules were dimerised by AP20187. Therefore, although dimerisation at extremely low levels (*i.e.* 1-5%) will most likely be difficult to detect, type 1 BRET appears to be capable of detecting relatively weak dimers (*i.e.* dimers that represent only a small percentage of the total protein population at any given time). Nonetheless, the possibility cannot be ruled out that addition of AP20187 simply induces closer relative positioning of the GFP and Rluc proteins within a pre-existing CCR5 dimer, thus causing an associated increase in BRET_{off}. However, this seems very unlikely as it requires an extremely specific organisation of the two CCR5 protomers within the dimer complex, since only orientations in which the C termini are positioned opposite one another would preclude BRET within the native dimer. Moreover, if this were the case the C-terminal domains would have to undergo some degree of movement within the complex such that they are, at least occasionally, positioned in close proximity to one another, otherwise a single AP20187 molecule could never be bound by both simultaneously.

Taken together, these data appear to suggest that type 1 BRET is capable of faithfully reporting weak GPCR dimerisation, as well as providing additional insights into receptor behaviour. The application of this analysis to M₁, which has been prominently suggested as forming ~30% transient dimers (346), did not detect any evident receptor association. In light of the ability of the type 1 BRET assay to report weak dimerisation, and the fact that this observation was supported by type 2 BRET analysis, it appears that the transient dimers previously reported for M₁ are most likely the product of the poorer resolution of single-molecule microscopy relative to RET approaches. Nonetheless, despite the apparent technical strength of these approaches, dimerisation in *Rhodopsin* family receptors has yet to be observed in carefully performed type 1 and 2 BRET assays. Therefore the possibility exists that these approaches are not capable of detecting physiological (*i.e.* not induced) *Rhodopsin* family dimers for an unknown reason. This must in principle remain an open question until dimer-like behaviour of *Rhodopsin* family GPCRs is observed in type 1 or 2 BRET assays.

The data discussed in this chapter indicate that the use of type 1 BRET may now be expanded to a broader range of GPCRs with confidence that transient dimerisation can be detected. Nonetheless, if this is to be performed in HEK 293T cells using the native GPCR expression profile of this line, then

type 2 BRET is not a suitable complementary technique to the type 1 assay, for reasons discussed below (§4.1.1). Thus, such an analysis of a large number of GPCRs would benefit from the development of a new BRET-based approach that is suitable for use in the native cells of the receptor of interest, and complements the type 1 format in semi high-throughput assays. This is the subject of the next chapter.

CHAPTER 4

Expanding the use of BRET assays: II. A new competition approach

4.1 Introduction

4.1.1 Utility of the competition BRET assay compared to type 2 BRET

Assessing proteins by conventional type 2 BRET in cells in which they are also natively expressed may cause complications due to competition from native proteins for position within oligomeric complexes. This is naturally the case for all BRET assays performed in native cells, but is usually an insignificant effect due to the large excess of BRET proteins over their native counterparts. Type 2 BRET is particularly sensitive to this, however, because it operates in part at far lower BRET protein concentrations than other approaches. Thus, as expression reaches zero, competition from native proteins for position within dimers will become a more significant factor, thereby artefactually producing an apparently monomeric distribution of the data. Thus, the use of type 1 BRET in the native cells of the receptor of interest cannot be complemented reliably by type 2 analysis. Instead, one potential complementary approach is the competition BRET (cBRET) assay. This is based on the principle that the extent of RET between donor and acceptor molecules within oligomeric complexes is sensitive to the presence of untagged 'competitor' proteins. Such proteins compete with acceptor-tagged molecules for interaction with their donor-tagged counterparts, and thereby induce a reduction in the frequency of RET events for oligomers but not for monomers (Fig. 4.1 a,b). This approach is extremely attractive for the assessment of weakly interacting proteins because partially dimeric receptors that might be difficult to detect using a BRET saturation assay will nonetheless exhibit a decrease in energy transfer in a competition experiment.

The theoretical framework for such an assay was established over thirty years ago, revealing the distinct effects of competitor proteins on FRET signatures of monomers, dimers, and higher-order oligomers (389). It is more recently, however, that cBRET has been used in several studies of membrane proteins, most notably GPCRs, as a complementary but independent counterpart to other BRET approaches. The precise experimental approaches taken by various researchers in the

application of cBRET differ in a number of details, but all involve the introduction of untagged competitor proteins into a BRET system and assessing the changing $BRET_{eff}$ that results. In some cases, $BRET_{eff}$ is measured at a single GFP:Rluc ratio in both the presence or absence of competitor protein (317, 390), in a manner equivalent to the single-ratio BRET analyses described in the previous chapter. Others have transfected cells with a constant ratio and amount of donor and acceptor DNA, and then titrated in increasing amounts of competitor DNA (379, 391-393). A third approach is to perform BRET saturation experiments in both the presence and absence of competitor protein in order to assess its effects on $BRET_{max}$ and $BRET_{50}$ (394). In all of these studies, any decrease in $BRET_{eff}$ upon introduction of competitor proteins was interpreted as an indication of oligomerisation.

4.1.2 Potential difficulties in interpretation of conventional cBRET

A key consideration in the use of cBRET assays is the effect of competitor proteins upon the total expression of their acceptor- and donor-tagged counterparts. Most membrane proteins undergo stringent regulation of total expression through a combination of translational regulation and recycling/degradation at the cell surface (395). Many of these processes are in part regulated by negative feedback by the protein in question. Increasing total protein density within the plasma membrane may, in many cases, induce increased recycling of all relevant receptors, regardless of whether they are tagged or untagged. The outcome of this would be to reduce total density of tagged receptors, which would in turn cause a drop in the maximal BRET contribution by non-specific interactions and so result in a false-dimer signature for a monomer (Fig. 4.1 c,d). This may also apply to the addition of closely related competitors in the investigation of heterodimerisation, as such receptors could contribute to the same or connected regulatory networks. In the case of GPCRs this may manifest as increased activation of GRKs common to both receptors, or the indirect internalisation of one receptor due to its tight colocalisation with the other (396).

All of the currently published examples of cBRET applied to GPCR homodimerisation have reported decreases in $BRET_{eff}$ upon competitor addition, yet the effect of such competitors upon tagged protein expression is not addressed in any case. It is therefore not possible to assess the relative contributions of genuine competition and reduced expression to the observed reductions in $BRET_{eff}$. Several studies have attempted to control for non-specific effects of competitor addition through the use of irrelevant controls. In such cases, an unrelated GPCR is used as a competitor to demonstrate that no change in $BRET_{eff}$ is observed in the presence of untagged proteins other than the principal protein of interest. Examples include the use of rat gonadotropin-releasing hormone receptor as a control in cBRET of human thyrotropin-releasing hormone receptor (390); M₂ muscarinic acetylcholine

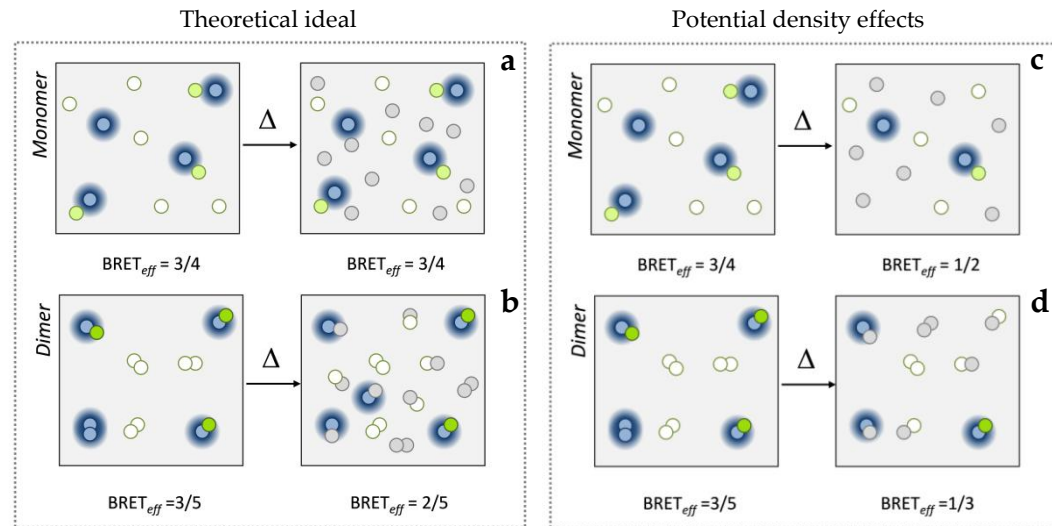


Figure 4.1: cBRET theory and potential density effects. Fluorescing and non-fluorescing acceptor molecules are depicted as green and white circles, respectively. Donor molecules are shown in blue, with the BRET-permissible area represented as a blue shadow. Untagged competitor molecules are depicted as grey circles. (a) In the absence of other effects, the introduction of competitor molecules into a monomeric system has no effect on BRET_{eff} as the likelihood of non-specific interactions between the monomeric donor and acceptor molecules is unchanged. (b) Conversely, the presence of competitor molecules in a dimeric system causes a reduction in BRET_{eff} as the untagged proteins compete for position within dimers, thereby resulting in a smaller proportion of BRET-productive dimers. (c and d) If the presence of untagged competitor leads to downregulation of tagged protein expression, non-specific interactions are reduced due to a lower concentration of acceptor and donor molecules within the membrane. For monomeric proteins the outcome is a reduction of BRET_{eff} attributable solely to density effects (c), whilst for dimers a reduction in BRET_{eff} is observed that is due to both reduced concentration and displacement of BRET-productive dimers (d).

receptor as an irrelevant control for the μ , δ , and κ -opioid receptors (393); and GABABR2 used in the study of vasopressin and oxytocin receptors (317). However such proteins are, by the very nature of their unrelatedness, unlikely to have the same impact on expression of the tagged proteins than untagged variants of the same GPCR or its close relatives. This gives rise to the potential for experimental bias in which any receptor that is of interest as a potential interaction partner with a certain GPCR is far more likely to produce false-dimer results than irrelevant controls due to its close functional relationship with that GPCR, which is usually what identified it as a potential interaction partner in the first instance. Thus, there is the constant possibility that any GPCR with sufficient functional connections to the main receptor of interest is inherently liable to generate false-dimer artefacts.

The work described in this chapter aims to assess the extent to which expression level changes pose potential difficulties in the interpretation of current cBRET studies, and also to develop a novel cBRET approach that overcomes these difficulties by incorporating theoretical considerations from type 2 BRET into the conventional cBRET framework.

4.2 Materials and methods

4.2.1 Cloning of competitor constructs

Vectors encoding untagged copies of CD2, CD28, CD80, CD86, β_2 AR, CCR5, CCR5_{FKBP}, GABABR1b and M₁ were generated by transferring the relevant gene from its pre-existing pGFP² vector into an 'untagged' vector (pU) using the following restriction endonuclease sites: CD2, CD80, β_2 AR, CCR5, CCR5_{FKBP}, and M₁: *MluI* and *BamHI*; CD28: *EcoRI* and *BamHI*; CD86: *MluI* and *AgeI*; GABABR1b: *MluI* and *KpnI*. Identity and integrity of the target gene was then confirmed using gene-specific PCR and test digestion of the product vector with the relevant restriction endonucleases.

The pU vector is identical to the pGFP² N3 vector (Perkin Elmer) but with the GFP²-encoding portion removed and replaced by three in-frame STOP codons. This was used to allow equivalent transfection efficiency and promoter activity as the pGFP² and pRLuc vectors. pU was initially generated by removal of the *GFP²* gene from pGFP² using restriction digestion with *BamHI* and *NotI*. Three STOP codons in the same frame as the removed *GFP²* were inserted into this space by ligation of a short dsDNA fragment formed from co-annealing oligonucleotides 5'-GATCCCTAGTAGTAGGC-3' and 5'-GGCCGCTACTACTAGG-3'. This fragment possessed complementary 5' *BamHI* and 3' *NotI*

overhangs to allow its ligation into the *GFP²*-deficient vector, as well as three consecutive TAG codons. Thus, proteins expressed from this vector will contain the sequence of their encoding gene followed by a short stretch of 2-6 amino acids encoded by the DNA that makes up the remainder of the MCS between their gene and the first STOP codon. The sequence integrity of pU was confirmed by reversible terminator base sequencing (§2.1.2).

4.2.2 Flow cytometry

Flow cytometry was used to assess expression of protein from the pU vector compared to the pGFP² and pRluc vectors. HEK 293T cells were transferred into 6-well plates at 3×10^5 cells/ml, 2 ml/well in DMEM and transfected 24 h post-plating with 1 µg of CD2-encoding pGFP², pRluc, or pU using GeneJuice® (Novagen), as per the manufacturer's instructions. Cells transfected with 1 µg of pU with no gene inserted (pU^{empty}) were used as negative controls. 24 h after transfection cells were sequentially labeled with 0.05 µg/µl of either mouse anti-human CD2 antibody (eBioscience: 12-0029-42) or mouse IgG isotype control (eBioscience: 12-4714-12), followed by labeling with 0.05 µg/µl of donkey anti-mouse IgG AlexaFluor 647-conjugated secondary antibody (Life Technologies: A31571). Cells were then assessed for AlexaFluor 647 fluorescence using flow cytometry. Cell preparation, labeling, washing, and measurement were performed as described in Section 2.3.1.

Additionally, flow cytometry was used to investigate the effect of pU-expressed protein on transfection efficiency and fusion protein expression. HEK 293T cells were plated as above and co-transfected with 0.5 µg pGFP² and 1 µg of either pU or pU^{empty}. Cells transfected with 1.5 µg pU^{empty} were used as negative fluorescence controls. Flow cytometry was performed 24 h post-transfection as described in 2.4.1. Transfection efficiency was calculated as the percentage of cells demonstrating above-background levels of GFP fluorescence, as gated by FL1 *vs.* FL2 fluorescence. Relative GFP expression was determined as the relative GFP fluorescence of individual cells, expressed as a histogram.

4.2.3 cBRET assays

HEK 293T cells were transferred into 6-well plates at 3×10^5 cells/ml, 2 ml/well in DMEM (§2.2.2). 24 h post-plating cells were transfected with 0.5 µg pGFP² and pRluc in a ratio of 12:1 pGFP²:pRluc, as well as 1 µg of either pU vector containing the gene of interest or pU^{empty}. Transfection was performed using

GeneJuice® (Novagen) as per the manufacturer's instructions. Cells were then incubated for varying periods of time before GFP emission, Rluc emission, and BRET_{eff} were measured as described for type 1 BRET (§3.2.4). For experiments replicating conventional cBRET, measurement was performed at a single time point 24 h after transfection. Data were combined from three independent experiments and treated as single values of BRET_{eff} and relative expression level. For 'type 2' cBRET multiple wells were transfected at the same time with the same transfection mixture and then measured at varying time points to ensure a spread of different protein expression levels. Typically, measurements were taken between 16 and 32 h post transfection. In some cases, cells transfected with pU were read at later time points than their pU^{empty}-transfected equivalents in order to achieve greater overlap between the expression levels of the two sets. Data from at least three independent experiments were pooled as a single dataset.

The relative expression level for all cBRET assays was calculated as the combined expression of the GFP- and Rluc-tagged proteins expressed as a function of Rluc emission. This was achieved by converting GFP fluorescence units (FLU) into Rluc luminescence (RLU) units using the GFP:Rluc ratio, whereupon total relative expression was calculated as the sum of GFP and Rluc luminescence units (Equation 3.1).

Cells expressing CCR5_{FKBP} were incubated with varying concentrations of AP20187 (ClonTech) for 45 min prior to reading in order to induce varying degrees of dimerisation. The appropriate final concentrations were determined previously (§3.3.2) as follows: 0nM (0% maximal dimerisation), 25nM (10%), 50nM (20%), 90nM (30%), 140nM (40%), 195nM (50%), 5µM (100%). AP20187 was not removed prior to measurement of BRET_{eff} and expression values.

4.3 Results

4.3.1 False dimers are observed using conventional cBRET

Flow cytometry analysis of the relative expression of CD2 from pGFP², pRluc, and pU (expressing untagged receptors) vectors revealed no difference in total CD2 expression between the different constructs (Fig. 4.2a). This means that the relative expression from the three vectors can be reliably controlled by simple variation of the DNA ratio of pGFP², pRluc, and pU in transfection reactions, as has been done previously for pGFP² and pRluc (§3.2.4).

In order to assess the potential contribution of expression level changes upon the analysis of conventional cBRET experiments, the expression levels of CD86, CD28, and β_2 AR were assessed using flow cytometry of HEK 293T cells transiently transfected with 0.5 μ g pGFP² and 1 μ g of either pU or pU^{empty}. In all cases, the efficiency of transfection was equivalent in both the pU and pU^{empty} cells, roughly 60% (Fig. 4.2c), yet a decrease in GFP expression was observed for all three proteins (Fig. 4.2b). This is most striking for β_2 AR yet less significant for CD28 and CD86. This is most likely due to the more complex signalling biology of β_2 AR compared to CD86 and CD28, and the fact that it is natively expressed in HEK 293Ts so is more likely to be subject to regulation within such cells. The fact that transfection efficiency was unchanged means that the decrease in GFP expression was not caused by a reduction in the number of copies of each plasmid entering the target cells, but was instead due to increased degradation and/or reduced production of the GFP-fused protein.

Conventional cBRET, in which BRET_{eff} is measured at a single time point in the presence or absence of competitor protein, was then applied to the monomers CD2, CD86, β_2 AR, CCR5 and M₁, as well as the dimers CD28 and CD80. The inducible dimer CCR5_{FKBP} system was also used in both the absence and presence of homodimer-inducing AP20187 (5 μ M final concentration), thereby acting as both monomeric and dimeric controls. Consistent with the flow cytometry data, a significant reduction in expression level was observed for the majority of proteins in the presence of competitor, although CD2, CD28, and CD80 showed small but non-significant decreases in overall expression level (Fig. 4.3). By far the greatest decreases in expression were demonstrated by β_2 AR, CCR5, and M₁, which suggests that GPCR expression levels may be particularly sensitive to the presence of competitor receptors, presumably due to the increased level of stochastic signalling introduced to the cell by the unlabelled GPCRs. With the exception of CD2, all investigated proteins demonstrated a significant decrease in BRET_{eff} in the presence of competitor protein (Fig. 4.4). For the dimers CD28 and CD80, this appears to be caused principally by genuine displacement of tagged proteins from BRET-productive homodimers by unlabelled competitors, as neither protein demonstrates a significant change in expression level between the two samples. However, given that CD86 is a known monomer, the reduction in BRET_{eff} it demonstrates must instead be the product of decreased non-specific interactions within the plasma membrane as a result of reduced BRET protein expression. CD2 shows no change in BRET_{eff} because its expression level is unaltered in the presence of competitor. β_2 AR, CCR5, and M₁ exhibit a similar dependence of BRET_{eff} change upon expression change as CD86 (Fig. 4.5), suggesting that they are also monomers undergoing reduced non-specific interactions in the presence of competitor. CCR5_{FKBP} shows a much greater reduction in BRET_{eff} when it is in its AP20187-bound

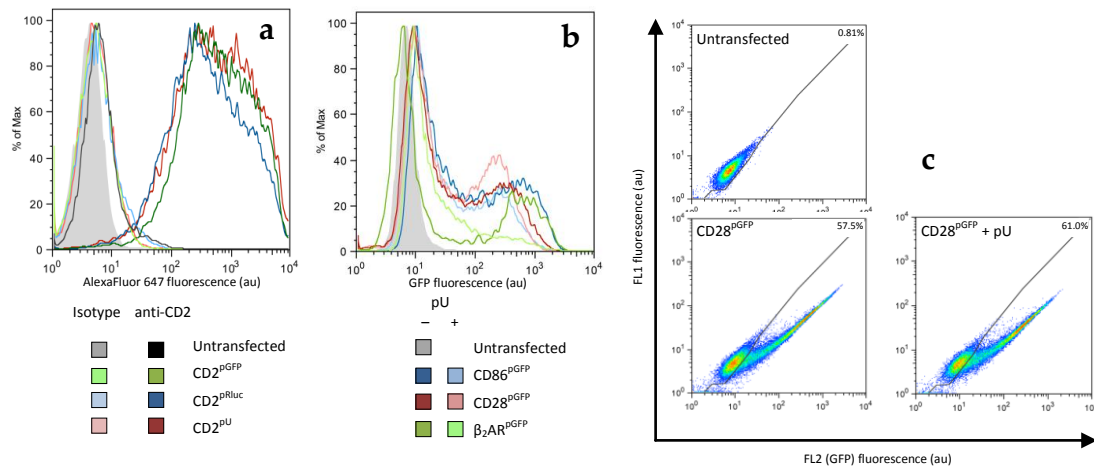


Figure 4.2: Flow cytometry analysis of untagged proteins demonstrates that they are expressed at the surface of HEK 293T cells with equivalent efficiency to GFP- and Rluc-tagged versions (a), and that their presence within the cell is capable of downregulating expression of their tagged counterparts (b). (c) Transfection efficiency is quantified as percentage of GFP⁺ cells when gated using FL1 *vs* FL2 fluorescence; CD28 data are shown and are representative of the behaviour of all samples.

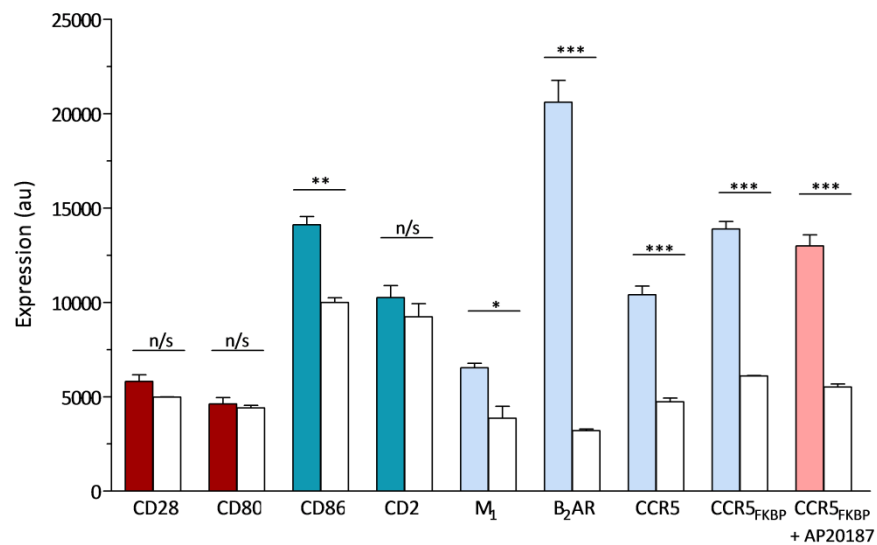


Figure 4.3: Introduction of competitor proteins results in reduction of protein expression for all GPCRs tested and CD86. CD2, CD28, and CD80 show no significant reduction in BRET protein expression in the presence of competitor. Expression values represent the combined GFP and Rluc values (as read in the cBRET assay) normalised to Rluc emission units using the GFP:Rluc ratio. In each case, expression measured in the absence of competitor is shown by a coloured bar, expression measured in the presence of competitor is shown by a white bar. Colours are as follows: dark red = type 1 dimer; dark blue = type 1 monomer; light red = GPCR dimer; light blue = GPCR monomer. Error bars represent SEM. *p < 0.05; **p < 0.01; ***p < 0.005.

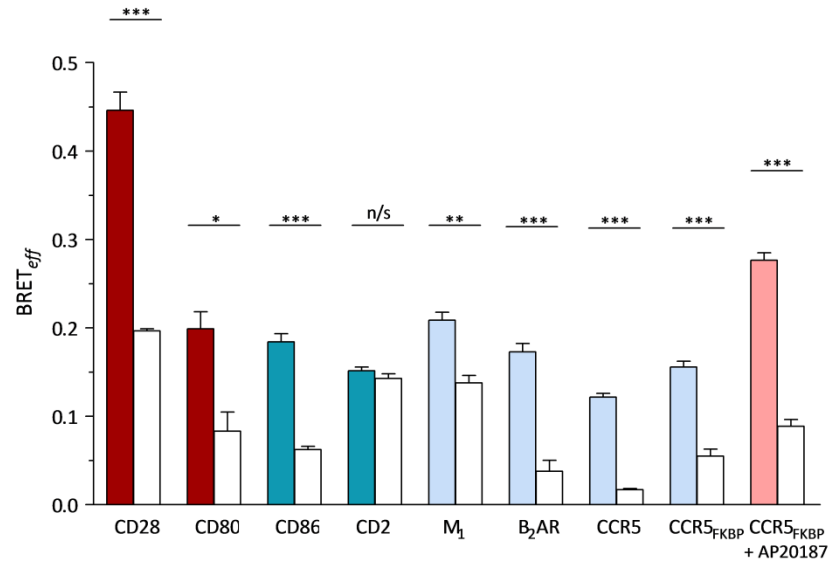


Figure 4.4: Conventional cBRET of type 1 transmembrane proteins and GPCRs demonstrates a significant decrease in BRET_{eff} in the presence of competitor protein for known monomers as well as dimers. CD2 is the only monomer to display no reduction of BRET_{eff}. In each case, BRET_{eff} measured in the absence of competitor is shown by a coloured bar, BRET_{eff} measured in the presence of competitor is shown by a white bar. Colours are as follows: dark red = type 1 dimer; dark blue = type 1 monomer; light red = GPCR dimer; light blue = GPCR monomer. Error bars represent SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

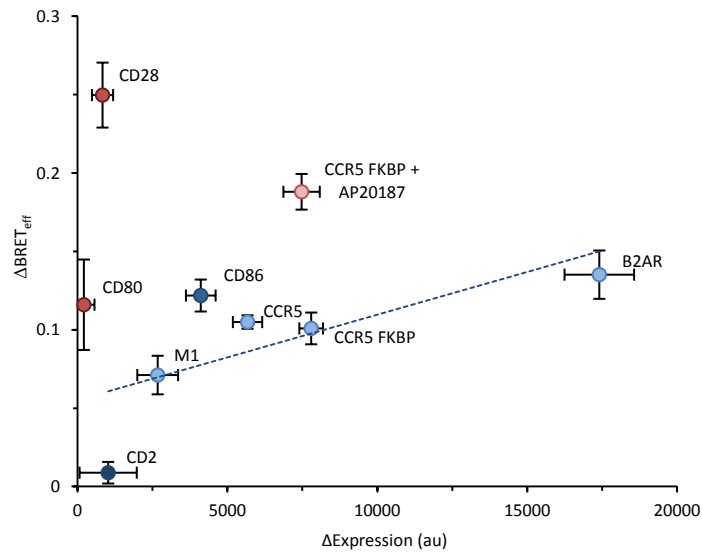


Figure 4.5: Relationship between change in BRET_{eff} and change in expression level under conventional cBRET analysis. ΔBRET_{eff} is dependent on Δexpression for monomers more than for dimers. Dimeric proteins show a much greater ΔBRET_{eff} than monomers for a given Δexpression. Line represents a linear least-squares fit of the monomeric data. Error bars represent SEM.

homodimer state than when it is in its monomeric form, despite an equivalent change in expression for the monomer and dimer states. This difference therefore represents the additional suppression of BRET that results from *bona fide* homodimer displacement.

From these data it is clear that any cBRET approach that does not take expression level into consideration is unlikely to correctly distinguish between monomers and dimers. The change in BRET_{eff} demonstrated by any given protein is a combination of genuine competition for position in homodimers, and changes in non-specific interactions that result from alterations in expression level. CD80 and CD86, for example, exhibit similar changes in BRET_{eff}, yet for CD80 this is the result of genuine competition, whereas for CD86 it is due to reduced effective concentration at the cell surface. Interpretation of these data is therefore made very difficult without careful consideration of the effects of competitor proteins on BRET protein expression.

4.3.2 Overcoming the problems of conventional cBRET

Due to the difficulties in interpreting results obtained by the conventional cBRET methodology, it was necessary to develop a new cBRET approach that is able to distinguish between changes in BRET_{eff} arising from reduced expression, and those arising from genuine competition. This was done by combining the theoretical considerations of type 2 BRET with the concept of competition from cBRET. In practice, data were collected in a manner equivalent to type 2 BRET, *i.e.* single transfections measured at varying time points in order to generate HEK 293T cells expressing varying levels of BRET proteins at a constant GFP:Rluc ratio. This was combined with the co-transfection of either untagged pU-encoded protein or an empty version of the untagged vector (pU^{empty}) that encodes no protein and is used only to ensure equivalent transfection conditions. In order to ensure efficient competition of any homo-oligomers, pU was used in excess over the pGFP² and pRluc plasmids to a final ratio of 1:12:26 pRluc : pGFP² : pU, with a total amount of 1.5µg DNA per 6x10⁵ cells. BRET_{eff} and expression were then measured at varying time points in order to allow determination of the dependence of BRET_{eff} upon expression level in the presence and absence of competitor proteins. Monomeric proteins should display no difference in this relationship under the two conditions even if the absolute expression levels and BRET_{eff} decrease. Oligomeric proteins should experience competition from the untagged proteins for position within protein complexes and so the proportion of BRET-productive associations will be reduced. This is then measurable as a changed BRET_{eff}.

expression relationship, in which $BRET_{eff}$ at any given expression level is lower than it would otherwise be in the absence of competitor. Unlike type 2 BRET, this cBRET approach does not require measurement at time points that give near-zero expression values, allowing a longer incubation time between transfection and initial measurements. This is more amenable to high-throughput applications as it eliminates the need for a 7 hour incubation followed by 8 hours of measurements. Because of these longer initial incubations, expression levels can be expressed as a function of both GFP and Rluc expression. In type 2 BRET, expression is described by Rluc emission alone because the low overall expression levels make accurate GFP measurements difficult. At the higher expression levels of this cBRET approach there is no such problem, and measuring total BRET protein concentration allows more appropriate comparison between different proteins, which may have differing final GFP:Rluc ratios and so could not be compared by Rluc emission alone.

Application of this new approach to the type 1 transmembrane controls confirmed its efficacy in distinguishing between genuine and apparent dimers. Both CD28 and CD80 showed significantly lower levels of $BRET_{eff}$ at a given expression level in the presence of competitor proteins (Fig. 4.6). Significance was determined using a two-tailed t test of least-squares linear regression for data collected with and without competitor to generate a probability value for difference between the two sets (P^{diff}). A P^{diff} smaller than 0.05 indicates a greater than 95% chance that there is a difference between the two conditions. The goodness-of-fit of all linear regressions were assessed using the R^2 coefficient of determination, which are given in Table 4.1. In addition to this change in $BRET_{eff}$ -expression relationship for CD28 and CD80, both proteins demonstrate a reduced projected $BRET_{eff}$ zero intercept and increased slope in the presence of competitor, which are consistent with increased monomeric behaviour when interpreted as independent type 2 analyses. Conversely, neither CD2 nor CD86 demonstrated any change in $BRET_{eff}$ -expression relationship in the presence of competitor (Fig. 4.7), which is consistent with the predicted behaviour for monomers. In these cases it was possible to model the values from both conditions as one dataset and so generate a single linear fit with one y -intercept. In both cases, this intercept was not significantly non-zero, which is also in line with monomeric behaviour.

The utility of this approach in identifying *bona fide* GPCR oligomers was assessed through its application to β_2AR , CCR5, and M_1 as well as the known dimer GABABR1b. None of β_2AR , CCR5, or M_1 demonstrates any change in $BRET_{eff}$ -expression relationship in the presence of competitor (Fig. 4.8), suggesting that they do not undergo homomeric interactions. This is in agreement with the type 1 and 2 analyses of these receptors detailed in the previous chapter. As expected for monomers –

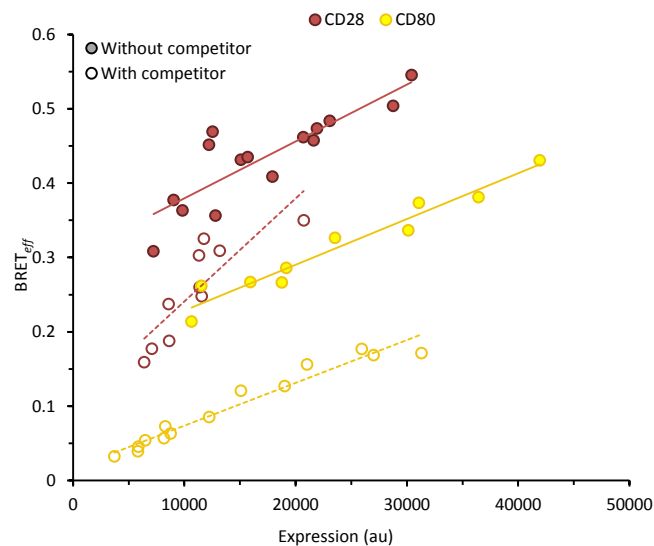


Figure 4.6: ‘Type 2’ style cBRET analysis of CD28 and CD80 demonstrates a significant reduction in the $BRET_{eff}$ observed at a given expression level in the presence of competitor protein. This is consistent with the formation of homodimers in both cases. Measurements taken in the absence of competitor are represented as solid circles; those taken in the presence of competitor are shown as empty circles of the same colour. Lines are linear fits to the data. Solid and broken lines indicate fits to data in the presence or absence of competitor, respectively.

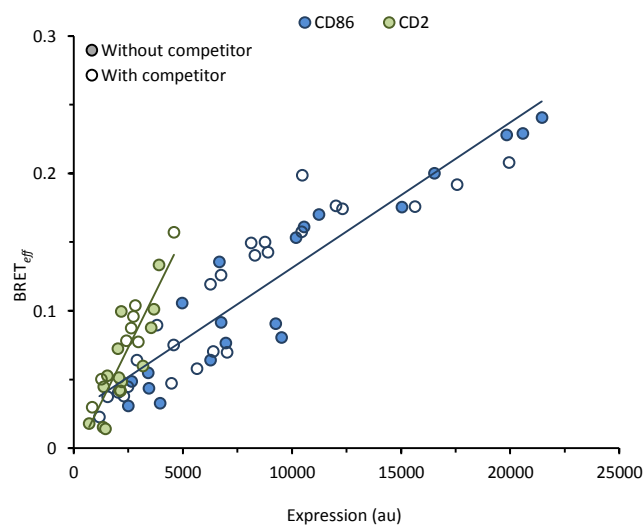


Figure 4.7: ‘Type 2’ style cBRET analysis of CD2 and CD86 demonstrates no significant change in the $BRET_{eff}$ -expression relationship in the presence of competitor in either case, consistent with monomeric behaviour. Measurements taken in the absence of competitor are represented as solid circles; those taken in the presence of competitor are shown as empty circles of the same colour. Lines are linear fits to the combined data both with and without competitor.

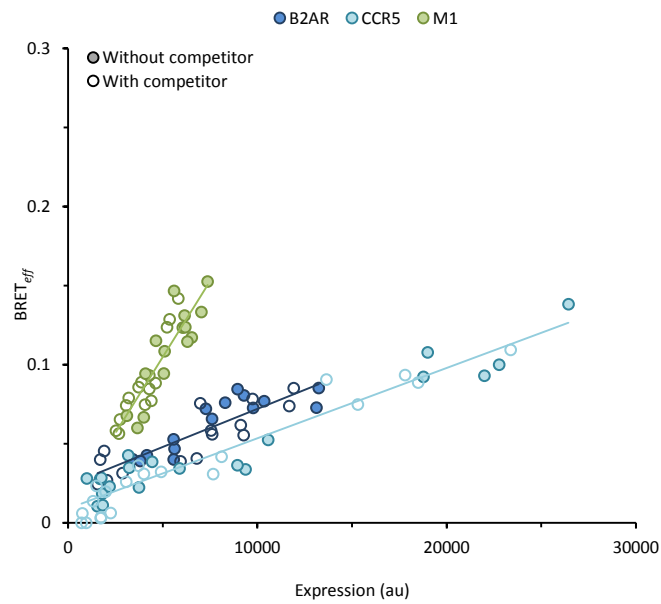


Figure 4.8: ‘Type 2’ style cBRET analysis of β_2 AR, CCR5, and M1 demonstrates no significant change in the $BRET_{eff}$ -expression relationship in the presence of competitor in any case, consistent with monomeric behaviour. Data is represented as detailed in Figure 4.7.

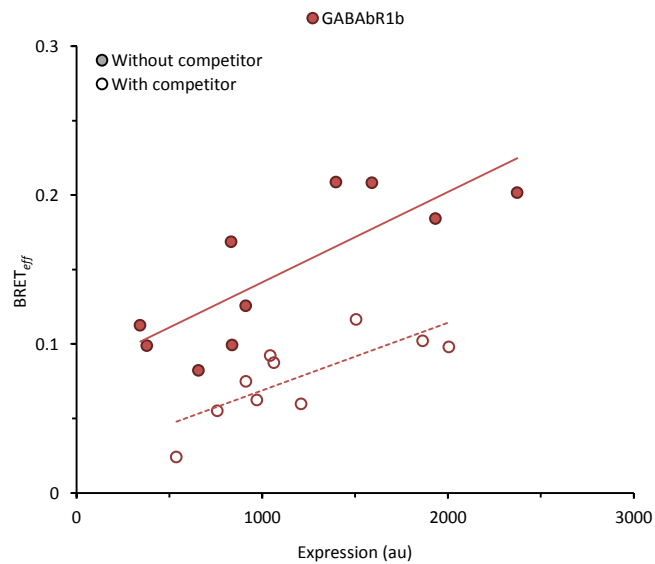


Figure 4.9: ‘Type 2’ style cBRET analysis of GABABR1b demonstrates a significant reduction in the $BRET_{eff}$ observed at a given expression level in the presence of competitor protein. This is consistent with the formation of homodimers. Data is represented as detailed in Figure 4.6.

and consistent with CD2 and CD86 – linear regression of the combined points for both conditions reveals y -intercepts that are not significantly non-zero. Interestingly, however, M_1 demonstrates a far steeper slope than CCR5 and β_2AR , consistent with the type 2 analysis (§3.3.3).

cBRET of GABAbR1b revealed a significant difference between BRET_{eff}-expression relationship in the presence and absence of competitor (Fig. 4.9). This clearly demonstrates that the new cBRET approach is capable of identifying GPCR dimers as well as type 1 transmembrane proteins.

4.3.3 Assessing cBRET sensitivity using an inducible dimer system

In order to investigate the ability of this new approach to identify partial dimers, the CCR5_{FKBP} inducible dimer system was used to give varying levels of dimerisation. This was done by using the different concentrations of AP20187 described in the last chapter to achieve 0, 10, 20, 30, 40, 50 and 100% of the maximal level of dimerisation that can be reached by this system.

As expected, CCR5_{FKBP} in the absence of AP20187 (0%) demonstrated cBRET behaviour equivalent to FKBP-free CCR5 (Fig. 4.10a), indicating that FKBP alone does not induce homodimerisation. At 10% maximal dimerisation, CCR5_{FKBP} displayed a very slight decrease in the BRET_{eff} arising at a given expression level that was just significant ($P=0.0457$), as well as a decrease in y -intercept value in the presence of competitor (Fig. 4.10b). This difference was markedly more significant at 20% ($P=0.01048$) and 30% ($P<0.0001$) maximal dimerisation (Fig. 4.10c), and was similarly accompanied by greater differences in y -intercept between the two conditions. Dimerisation levels above 30% gave highly significant differences in data distribution between the two conditions and increasingly large decreases in y -intercept in the presence of competitor (Fig. 4.10d). From these data, it therefore appears that the new cBRET approach is sensitive to dimerisation at levels above those equivalent to ~10-20% of the maximal dimerisation that can be achieved with the CCR5_{FKBP} system.

Taken alone, the measurements in the absence of competitor are equivalent to a type 2 BRET analysis. It is notable that the y -intercept of CCR5_{FKBP} does not become significantly non-zero until 20% levels of maximal dimerisation, and only rises above a lower confidence limit of 0.02 at 50% levels of dimerisation (Table 4.1). This can in part be explained by the fact that these data were taken at later time points than in a conventional type 2 experiment, which results in wider confidence limits as expression reaches zero. However, data obtained in the absence of competitor at levels of 0, 10, 20, and 30% maximal dimerisation are highly similar and would not be distinguishable if directly compared,

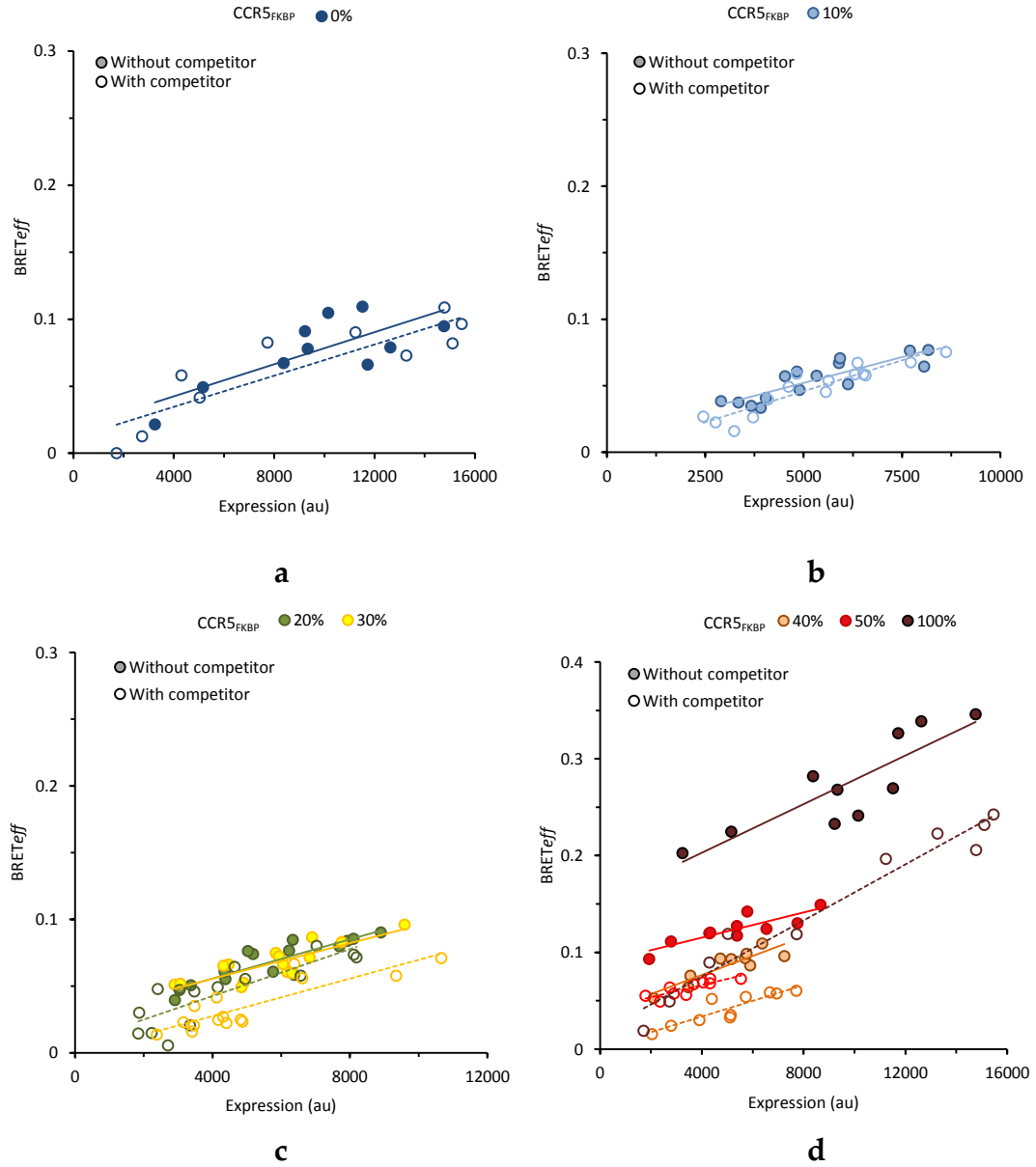


Figure 4.10: ‘Type 2’ style cBRET analysis of the inducible dimer CCR5_{FKBP} in the presence of increasing concentrations of AP20187 is consistent with increasingly dimeric behaviour. (a), No significant difference in BREteff-expression relationship is observed for CCR5_{FKBP} in the absence of AP20187, suggestive of monomeric behaviour. (b) Addition of AP20187 at a concentration that produces 10% maximal dimerisation causes a drop in BREteff in the presence of competitor that is narrowly significant ($p=0.0457$). (c & d) Concentrations of AP20187 that induce 20-100% maximal dimerisation demonstrate increasingly significant differences in BREteff-expression relationship in the absence and presence of competitor proteins, consistent with increasing levels of dimerisation.

Protein	R^2	y -intercept $\pm$ 95% confidence	P^{diff}
CD2			
– competitor	0.69	-0.007 ± 0.013	
+ competitor	0.87	-0.003 ± 0.012	0.1350
CD86			
– competitor	0.89	0.017 ± 0.021	
+ competitor	0.82	0.028 ± 0.019	0.1323
CD28			
– competitor	0.73	0.303 ± 0.051	
+ competitor	0.72	0.102 ± 0.081	<0.0001
CD80			
– competitor	0.95	0.167 ± 0.029	
+ competitor	0.80	0.102 ± 0.027	<0.0001
GABAbR1b			
– competitor	0.66	0.081 ± 0.019	
+ competitor	0.61	0.023 ± 0.016	<0.0001
β_2 AR			
– competitor	0.74	0.025 ± 0.014	
+ competitor	0.73	0.023 ± 0.012	0.2356
M ₁			
– competitor	0.75	0.006 ± 0.035	
+ competitor	0.83	-0.002 ± 0.024	0.2183
CCR5			
– competitor	0.91	0.013 ± 0.007	
+ competitor	0.93	0.004 ± 0.0060	0.2273
CCR5 _{FKBP} – 0% max dimer			
– competitor	0.59	0.018 ± 0.040	
+ competitor	0.76	0.011 ± 0.027	0.2817
CCR5 _{FKBP} – 10% max dimer			
– competitor	0.74	0.013 ± 0.014	
+ competitor	0.85	-0.001 ± 0.012	0.0457
CCR5 _{FKBP} – 20% max dimer			
– competitor	0.77	0.026 ± 0.014	
+ competitor	0.69	0.007 ± 0.016	0.01048
CCR5 _{FKBP} – 30% max dimer			
– competitor	0.70	0.028 ± 0.015	
+ competitor	0.74	-0.001 ± 0.013	<0.0001
CCR5 _{FKBP} – 40% max dimer			
– competitor	0.79	0.037 ± 0.021	
+ competitor	0.81	0.001 ± 0.017	<0.0001
CCR5 _{FKBP} – 50% max dimer			
– competitor	0.74	0.089 ± 0.017	
+ competitor	0.71	0.041 ± 0.011	<0.0001
CCR5 _{FKBP} – 100% max dimer			
– competitor	0.74	0.152 ± 0.061	
+ competitor	0.95	0.017 ± 0.037	<0.0001

Table 4.1: Goodness-of-fit, y -intercept, and P^{diff} values obtained under cBRET analysis in this chapter.

which suggests that this lower sensitivity is at least partially present in a conventional type 2 analysis. It would therefore appear that modification of type 2 BRET to the current cBRET approach increases its sensitivity to partial dimers 2-3 fold.

4.4 Discussion

4.4.1 Problems with interpretation of previous cBRET studies

It is clear from this analysis that conventional cBRET is not sufficient to distinguish between *bona fide* dimerisation and apparent dimerisation arising from non-specific interactions. This implies that the data obtained in previous cBRET analyses of GPCRs may have been misinterpreted, potentially attributing expression-dependent effects to competition within dimers. This is not to say that these results should be reinterpreted as indications of monomericity, simply that there is not sufficient information about the effect of competitor protein on the expression of tagged receptors to make an informed judgement in these cases. The need for a cBRET approach that takes expression level into consideration is therefore evident, and is fulfilled by the new combination of type 2 and cBRET.

4.4.2 Expanding the reach of BRET analysis with 'type 2' cBRET

Assessment of the sensitivity of the new cBRET approach has revealed that it is suitable for investigating the existence of partial dimerisation, which is particularly pertinent in the field of GPCR oligomerisation. Its capacity for unambiguously identifying partial dimers appears to be greater than that of either type 1 or type 2 BRET, which are otherwise the only BRET approaches that adequately address the issue of non-specific protein interactions. The precise sensitivity of cBRET appears to be a level of dimerisation equivalent to ~10% of the maximal dimerisation that can be achieved using the CCR5^{FKBP} system. Considering that this system is unlikely to achieve 100% dimerisation, this suggests that cBRET is capable of unambiguously identifying dimerisation even within a protein population of at least 90% monomers.

Moreover, this new cBRET approach has the potential to expand the capacity for BRET analysis of proteins within their native cells. For reasons discussed above (§4.1.1), type 2 BRET is not a suitable companion to type 1 BRET analysis when investigating proteins within the context of their native cells. The extent to which type 2 BRET data would be difficult to interpret is unpredictable as it

depends on numerous variables that are generally unknown for most proteins, such as native protein expression levels and the nature of protein distribution within the plasma membrane. This is not a problem for cBRET as it operates at higher expression levels of the acceptor and donor proteins. What's more, even if native protein expression were extremely high, *i.e.* to the extent that type 1 BRET became ambiguous, cBRET would still be capable of providing meaningful information about protein stoichiometry. This is because the concentration of unlabelled protein will always be higher when competitor proteins are introduced through transfection rather than expressed solely at native levels. Considering that the vast majority of membrane proteins are more highly expressed than GPCRs, this is a potentially useful technique for the assessment of their stoichiometry *in situ*.

The new method also provides the potential for BRET analysis of GPCRs and other proteins in their native lines using lower levels of acceptor and donor expression. This may be informative as it more accurately replicates the conditions experienced by the protein *in vivo*. For example, some proteins may form part of hetero-oligomeric complexes at physiological expression levels but this is not observed upon over-expression as the protein of interest is now in large excess over its interaction partners. Investigation of such proteins using cBRET at low concentrations of acceptor and donor would allow the identification of genuine oligomers despite high degrees of competition from natively expressed proteins that would render other BRET approaches inadequate. Such an investigation is now technically feasible due to recent advancements in acceptor and donor technologies. Since cBRET is a new experimental approach rather than a new technology, it could easily be applied in conjunction with any of the recently developed improved acceptor or donor molecules, such as Rluc2 and Rluc8 (310), mOrange (397), Venus (398), or BRET-permissive quantum dots (309).

4.4.3 cBRET as a complementary analysis alongside type 1 BRET

cBRET has always been used as a secondary technique used to independently confirm conclusions derived by other BRET assays. The data reported in this chapter indicate that this new approach to cBRET is on its own sufficient to report membrane protein stoichiometry, however this does not mean it should not be used in conjunction with other methods. Of particular note is the type 1 BRET approach, which could be complemented by side-by-side use with cBRET. This is because type 1 BRET is very unlikely to generate false-dimer artefacts due to the highly diagnostic nature of data distribution for dimers, but is potentially at risk of providing false-monomer results in cases of higher-order oligomerisation or weak dimerisation. Conversely, cBRET is highly unlikely to give false-

monomer results as this would require a competitor-induced increase in tagged protein expression that precisely compensates for the drop in $BRET_{eff}$ caused by competition. It is possible, however, that cBRET could produce false dimers in certain cases where addition of competitor proteins causes a relaxation in the localisation of tagged proteins within the membrane, thereby reducing their effective concentration and, by extension, $BRET_{eff}$. Thus, complementary studies utilising both type 1 and cBRET may prove highly useful in assessing membrane protein stoichiometry, as any agreement between the two assays is very unlikely to be due to technical artefacts.

4.4.4 Further evidence for monomeric behaviour of representative GPCRs

The application of this new cBRET approach to the GPCRs β_2AR , CCR5, and M_1 strongly suggests that these proteins do not form homo-oligomeric complexes within the cell. This adds to the evidence presented in the last chapter that previous investigations of these receptors using RET may have been misinterpreted as suggesting dimerisation. Out of the three candidate GPCRs studied here, only CCR5 has been previously assessed using cBRET (379). In this study, the authors transfected HEK 293T cells with constant amounts of CCR5-Rluc and CCR5-YFP DNA as well as increasing amounts of DNA encoding either untagged CCR5 or CXCR4. They observed that $BRET_{eff}$ was reduced by untagged CCR5 in a concentration-dependent manner, but was not affected by CXCR4. However, they fail to address the potential of this reduction being caused by a CCR5-dependent decrease in BRET protein expression, despite the fact that they observed a strong dependency between $BRET_{eff}$ and expression level in a type 2 BRET analysis. In light of the data presented here it seems likely that the reduction in $BRET_{eff}$ observed in this previous study, which was attributed by the authors to constitutive dimerisation, was in fact due to decreased non-specific interactions arising from lower surface density of acceptor- and donor-tagged CCR5. Thus, the conclusion that these receptors exist as monomers is not in conflict with the data presented in this or other previous cBRET investigations.

An additional observation in this chapter is that M_1 demonstrates more BRET at a given expression level than do either CCR5 or β_2AR , consistent with the previous type 2 BRET analysis (§3.4). This indicates that M_1 experiences more non-specific interactions at a given surface concentration than do β_2AR or CCR5. This may be due to corralling of M_1 into defined regions of the plasma membrane, thereby increasing its effective concentration and hence the chance of non-specific interactions. Alternatively, constrained diffusion of β_2AR or CCR5, as has been described for β_2AR (237), may reduce the degree of non-specific interaction that these receptors experience at a given

surface concentration by anchoring a subset of the receptors at fixed positions that are separated by a distance greater than the BRET-permissive radius.

The successful application of both the type 1 and cBRET approaches to this small selection of GPCRs lays the foundation for a larger investigation of the stoichiometry of the native *Rhodopsin* family GPCR profile of HEK 293T cells, the subject of the next chapter.

CHAPTER 5

Application of reliable BRET assays to *Rhodopsin* family GPCR stoichiometry

5.1 Introduction

5.1.1 General *Rhodopsin* family stoichiometry

The issue of oligomerisation state is far from reconciled for even the most well studied *Rhodopsin* family GPCRs (§1.4.7). Although dimerisation and its functional importance have been confirmed for non-*Rhodopsin* receptors (*i.e.* the *Glutamate* family), the extent of *Rhodopsin* family GPCR dimerisation is still unknown as is the role, if any, that it plays in receptor function. As discussed previously (§1.4), the field remains divided on these issues, mainly due to disagreements over the interpretation of data obtained by various experimental techniques (*e.g.* 273, 286, 287, 313, 334). Concerns over the misinterpretation of various approaches have been further substantiated by the present investigation of β_2 AR, CCR5, and M_1 , which has come to conflicting conclusions compared to earlier studies (§3.4, §4.4). In addition to this, the fraction of *Rhodopsin* GPCRs that has been thoroughly studied is still comparatively small, and as a result there is no coherent understanding of how dimerisation varies within the family. There is therefore a need to more extensively investigate a wider range of *Rhodopsin* receptors using verifiably reliable techniques.

5.1.2 Systematic approach to investigate *Rhodopsin* GPCRs

A combinatorial approach using both type 1 and cBRET is, in principle, one way that GPCR stoichiometry can be investigated with high sensitivity (§4.4.3). Although the observation that β_2 AR, CCR5, and M_1 are monomeric is informative in itself, it does not offer substantial understanding of the

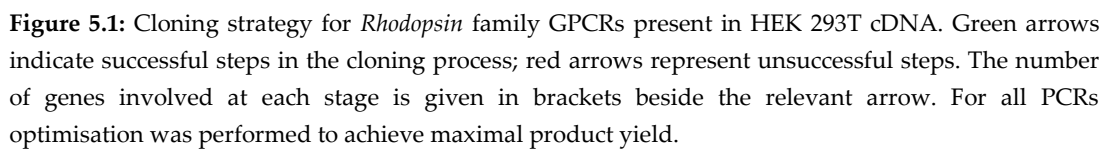
The work in this chapter was performed with the kind assistance of Rachel Knox, who aided in receptor cloning and cell preparation for data collection.

‘typical’ stoichiometry of *Rhodopsin* family GPCRs or why this group of receptors in particular has been so successful evolutionarily. In this chapter a semi-systematic approach is taken to address this issue via the investigation of all *Rhodopsin* receptors natively expressed in HEK 293T cells using the type 1 and cBRET assays. The native HEK 293T GPCR repertoire is used to maximise the likelihood of correct trafficking and organisation within the cell, as it is in these cells that BRET assays are generally performed. Moreover, determining the subset of GPCRs to investigate by this means is essentially ‘blind’ as no researcher bias can be introduced by the active selection of specific receptors. The direct analysis of a large fraction of the total *Rhodopsin* family thereby allows the typical behaviour of these receptors to be identified, as well as, potentially, offering insights into their evolution. The relative technical simplicity and efficiency of both the type 1 and cBRET approaches makes this an experimentally feasible goal.

5.2 Materials and methods

5.2.1 Vector construction

All genes were cloned into the pGFP², pRluc, and pU vectors for BRET using the strategy outlined in Figure 5.1. Briefly, using standard molecular cloning techniques described in 2.1.2, genes were amplified by PCR from cDNA generated from HEK 293T cells. This was initially attempted using oligonucleotide primers against the terminal 5′ and 3′ sequences of the gene open reading frame (ORF) with additional restriction endonuclease sites for subsequent digestion and ligation. In cases where no full length product could be amplified, nested PCR reactions were attempted using primers specific to the 5′ and 3′ untranslated regions (UTRs) of the target gene followed by a second round of amplification using the ORF-specific primers. If no product was obtained using a nested approach, PCR amplification was attempted in two stages whereby the 5′ and 3′ halves of the gene ORF were amplified independently and then used as template in the generation of a chimeric full-length product. Once amplified, genes were ligated into the shuttle vector pJET1.2 and their sequences confirmed using reversible terminator base sequencing. Correct sequences were then subcloned from pJET1.2 into pGFP² N3 by restriction digestion using the relevant endonucleases and subsequent ligation and clone selection (§2.1.2). After confirmation of surface expression by confocal microscopy (§5.2.2), genes were transferred into pRluc N3 and pU vectors in the same manner.



In some cases it was not possible to obtain workable quantities of the amplified target gene from cDNA. In such instances, the full length sequence of the gene of interest was synthesised directly by the GeneArt® gene synthesis service (Invitrogen) using the native codon sequence of the gene mRNA (*i.e.* codon optimisation was not performed). Such genes were synthesised with appropriate 5' and 3' restriction sites for subsequent cloning as above. The only exception to this was the *ADRB1* gene, which was obtained from Robert Lefkowitz, Duke University, in the form of a *FLAG-ADRB1* pcDNA3 vector (399) via the Addgene plasmid sharing service (plasmid #14698). This was used as template for full-length PCR amplification as above. The cloning route adopted for each GPCR gene is detailed in Tables 5.2 and 5.3, and the sequences of the oligonucleotide primers used in each case is shown in Tables 5.4 to 5.7. In cases of receptors existing in multiple isoforms, the isoform used for cloning and subsequent BRET investigation is given in Table 5.8.

5.2.2 Confocal microscopy

Surface expression of GPCRs was assessed via confocal microscopy of their GFP fusion constructs. HEK-293T cells were transfected, 24h after plating, with 1µg pGFP²-GPCR DNA per 6x10⁵ cells using GeneJuice® (Novagen) as per the manufacturer's protocol. Cells were then fixed and imaged as described in 2.4.2. Receptors were assessed qualitatively for cellular distribution and assigned an expression category (Table 5.1).

Category	Definition
A	Protein entirely in plasma membrane; almost no visible protein in internal membranes; no aggregation.
B	Protein almost entirely in plasma membrane; small amounts in internal membranes; no aggregation.
C	Majority of protein in plasma membrane; moderate amounts in internal membranes; no aggregation.
D	Some protein in plasma membrane; large amounts in internal membranes; no aggregation.
E	Some protein in plasma membrane; large amounts in internal membranes; small amounts of aggregation in some cells.
F	Some protein in plasma membrane; large amount in internal membranes; small amounts of aggregation in most cells.
G	Little or no protein in plasma membrane or internal membranes; large amounts of aggregation in most cells.

Table 5.1: Qualitative categories of GPCR expression based on cellular localisation and aggregation.

Expression (FPKM)	Gene name	Protein name	Alias	Common name	Subfamily	Cluster	Cloning route
16.4732	LGR4	LGR4	GPR48	Leucine-rich repeat containing GPCR 4	Delta	Glycoprotein	Chimera
15.3125	LTB4R1	LTB4R1	BLTR	Leukotriene B4 Receptor	Gamma	Chemokine	Full length
13.1097	F2R	PAR1	-	Protease-activated receptor 1	Delta	Purin	Full length
11.8567	F2RL1	PAR2	-	Protease-activated receptor 2	Delta	Purin	Full length
10.3164	LPAR3	LPA ₃	EDG7	Lysophosphatidic acid receptor 3	Alpha	MECA	Full length
9.82563	ADRA2C	α_2 CAR	-	Alpha 2C adrenergic receptor	Alpha	Amine	GeneArt®
9.61584	GPR27	SREB1	-	Super-conserved receptor expressed in brain 1	Alpha	Prostaglandin	GeneArt®
8.18117	GPR161	GPR161	RE2	GPCR 161	Delta	Purin	Full length
7.10933	CXCR4	CXCR4	-	Chemokine (CXC-motif) receptor 4	Gamma	Chemokine	Full length
6.67264	LTB4R2	LTB4R2	BLTR2	Leukotriene B4 Receptor 2	Gamma	Chemokine	Full length
6.65272	LPAR1	LPA ₁	EDG2	Lysophosphatidic acid receptor 1	Alpha	MECA	Full length
6.59387	GPR50	GPR50	-	GPCR 50	Alpha	Melatonin	Nested
6.48161	S1PR3	S1P ₃	EDG3	Sphingosine 1-phosphate receptor 3	Alpha	MECA	Full length
6.33240	GPR176	GPR176	-	GPCR 176	Beta	Beta	Full length
3.89113	P2RY1	P2Y ₁ R	P2Y ₁	Purinergic receptor P2Y 1	Delta	Purin	Nested
3.70002	ADORA2A	A _{2A} R	ADORA2	Adenosine A2A Receptor	Alpha	MECA	Full length
3.34284	P2RY11	P2Y ₁₁ R	P2Y ₁₁	Purinergic receptor P2Y 11	Delta	Purin	Nested
3.33991	OPN1SW	OPN1SW	-	Opsin 1, short-wave-sensitive	Alpha	Opsin	Nested
3.02577	OPN3	OPN3	ECPN	Opsin 3	Alpha	Opsin	Nested
3.02530	LPAR2	LPA ₂	EDG4	Lysophosphatidic acid receptor 2	Alpha	MECA	Full length
2.56134	S1PR2	S1P ₂	EDG5	Sphingosine 1-phosphate receptor 2	Alpha	MECA	Full length
2.03416	PTGER2	EP ₂	-	Prostaglandin E receptor 2	Alpha	Prostaglandin	Full length
1.96744	EDNRA	EDNRA	ET _A	Endothelin receptor type A	Beta	Beta	Full length
1.89578	LGR5	LGR5	GPR49	Leucine-rich repeat containing GPCR 5	Delta	Glycoprotein	Full length
1.84969	OPRL1	OPRL1	ORL1	Opiate receptor-like 1	Gamma	SOG	Full length
1.72930	QRFR1	QRFR1	GPR103	Pyroglutamylated RFamide peptide receptor	Delta	Purin	Full length
1.57948	ADORA1	A ₁ R	-	Adenosine A1 receptor	Alpha	MECA	Nested
1.54729	SSTR2	SSTR2	SS2R	Somatostatin receptor 2	Gamma	SOG	Full length
1.53152	S1PR1	S1P ₁	EDG1	Sphingosine 1-phosphate receptor 1	Alpha	MECA	Full length
1.50703	CCR10	CCR10	GPR2	Chemokine (C-C motif) receptor 10	Gamma	Chemokine	GeneArt®
1.44857	S1PR5	S1P ₅	EDG8	Sphingosine 1-phosphate receptor 5	Alpha	MECA	Full length
1.27402	OR4D1	OR4D1	OR4D3	Olfactory receptor, fam. 4, subfam. D, mem. 1	Delta	Olfactory	Full length
1.13756	PTGFR	FP	-	Prostaglandin F receptor	Alpha	Prostaglandin	Full length

Table 5.2: *Rhodopsin*-family GPCR genes cloned in this chapter and their protein products.

Expression (FPKM)	Gene name	Protein name	Alias	Common name	Subfamily	Cluster	Cloning route
1.12833	P2RY2	P2Y ₂ R	P2Y ₂	Purinergic receptor P2Y ₂	Delta	Purin	Full length
1.07180	OXR1	OXR1	TG1019	Oxoicosanoid receptor 1	Delta	Purin	Full length
1.05414	GPR35	GPR35	-	GPCR 35	Delta	Purin	Nested
0.94815	PTAFR	PTAFR	PAFR	Platelet-activating factor receptor	Delta	Purin	Full length
0.90909	HTR6	5-HT ₆	-	5-hydroxytryptamine (serotonin) receptor 6	Alpha	Amine	Nested
0.86951	AGTR1	AT ₁	AT1R	Angiotensin II receptor 1	Gamma	Chemokine	Full length
0.84491	TBXA2R	TP	TXA2-R	Thromboxane A2 receptor	Alpha	Prostaglandin	Chimera
0.80419	CCR11	CCR11	CCRL1	Chemokine (C-C motif) receptor 11	Gamma	Chemokine	Nested
0.80141	C5R1	C5R1	C5AR1	Complement component 5a receptor 1	Gamma	Chemokine	Full length
0.77396	EDNRBL	EDNRBL	GPR37	Endothelin receptor type B like	Beta	Beta	Nested
0.77190	NPY1R	NPY1R	NPYR	Neuropeptide Y receptor Y1	Beta	Beta	Chimera
0.74182	HTR7	5-HT ₇	-	5-hydroxytryptamine (serotonin) receptor 7	Alpha	Amine	Nested
0.71567	CCR6	CCR6	-	Chemokine (C-C motif) receptor 6	Gamma	Chemokine	Full length
0.64549	CHRM3	M ₃	-	Muscarinic acetylcholine receptor 3	Alpha	Amine	Full length
0.62961	GPR3	GPR3	-	GPCR 3	Alpha	MECA	Full length
0.52159	GPER	GPER	GPR30	G protein-coupled oestrogen receptor 1	Gamma	Chemokine	Full length
0.49975	BDKRB2	B ₂	BKR2	Bradykinin receptor B2	Gamma	Chemokine	Nested
0.48008	CCKBR	CCKBR	CCK2R	Cholecystokinin B receptor	Beta	Beta	Full length
0.47161	GPR83	GPR83	-	GPCR 83	Beta	Beta	Nested
0.47142	OGR1	OGR1	GPR68	Ovarian cancer GPCR 1	Delta	Purin	Full length
0.41269	HTR1D	5-HT _{1D}	-	5-hydroxytryptamine (serotonin) receptor 1D	Alpha	Amine	Full length
0.41085	OXGR1	OXGR1	P2RY15	Oxoglutarate (alpha-ketoglutarate) receptor 1	Delta	Purin	Full length
0.40592	HTR2B	5-HT _{2B}	-	5-hydroxytryptamine (serotonin) receptor 2B	Alpha	Amine	Nested
0.39794	PTGDR2	DP ₂	GPR44	Prostaglandin D2 receptor 2	Gamma	Chemokine	Nested
0.33038	GPR61	GPR61	BALGR	GPCR 61	Alpha	Prostaglandin	Nested
0.30210	HRH2	H ₂ R	-	Histamine receptor H2	Alpha	Amine	GeneArt®
0.27250	ADRB1	β ₁ AR	-	Beta-1 adrenergic receptor	Alpha	Amine	Addgene
0.26477	HTR2C	5-HT _{2C}	-	5-hydroxytryptamine (serotonin) receptor 2C	Alpha	Amine	GeneArt®
0.21784	PTGER3	EP ₃	-	Prostaglandin E receptor 3	Alpha	Prostaglandin	Nested
0.20203	PTGER4	EP ₄	-	Prostaglandin E receptor 4	Alpha	Prostaglandin	Full length
0.19944	GPR78	GPR78	-	GPCR 78	Alpha	Prostaglandin	GeneArt®
0.17996	HRH1	H ₁ R	-	Histamine receptor H1	Alpha	Amine	Full length

Table 5.3: *Rhodopsin*-family GPCR genes cloned in this chapter and their protein products

Gene	Target	Primer sequence	Restriction
<i>ADORA1</i>	Gene 5'	TAGTAGACGCGTGCCACCATGCCGCCCTCCATCTCAG	<i>MluI</i>
	Gene 3'	CTACTAGGTACCCCGTCATCAGGCCTCTCTTCTGGG	<i>KpnI</i>
	UTR 5'	TGCCCCTTGGTGCCCGTCTGC	-
	UTR 3'	TGGGCTGGTGGGAGCGGAAG	-
<i>ADORA2A</i>	Gene 5'	TAGTAGACGCGTGCCACCATGCCCATCATGGGCTCCTC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCGGACACTCCTGCTCCATCCTGGGC	<i>BamHI</i>
<i>ADRA2C</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGCGTCCCCGGCGCTGGC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCCTGCCTGAAGCCCCTTCTC	<i>BamHI</i>
<i>ADRB1</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGGCGCGGGGGTGCTCGTCC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCCACCCTGGATTCCGAGGCGAAGCCGGG	<i>BamHI</i>
<i>AGTR1</i>	Gene 5'	TAGTAGACGCGTGCCACCATGATTCTCAACTCTTCTACTG	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCCTCAACCTCAAAACATGGTGCAGGC	<i>BamHI</i>
<i>BDKRB2</i>	Gene 5'	TAGTAGACGCGTGCCACCATGTTCTCTCCCTGGAAGATATC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCCTGTCTGTCTCCCTGCCAGTCCTGC	<i>BamHI</i>
	UTR 5'	GGCCTCACTCACATCCCCACTTG	-
	UTR 3'	CTGAAAAGCAACTGTCCCTCAATC	-
<i>C5R1</i>	Gene 5'	TAGTAGACGCGTGCCACCATGAACTCCTTCAATTATACC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCCACTGCCTGGGTCTTCTGGGCCATAGTG	<i>BamHI</i>
<i>CCKBR</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGAGCTGCTAAAGCTGAACC	<i>MluI</i>
	Gene 3'	CTACTAGGTACCCCGCCAGGGCCCCAGTGTGCTGATGGTGG	<i>KpnI</i>
<i>CCR10</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGGGACGGAGGCCACAG	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCGTTGTCCAGGAGAGACTGTGGGTC	<i>BamHI</i>
<i>CCR11</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGCTTTGGAACAGAACCAG	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCAATGCTAAAAGTACTGGTTGGGTC	<i>BamHI</i>
	UTR 5'	AGACTGCTCTCTCTGCCGACTA	-
	UTR 3'	GTATCCAAGCAAAAGGCAGAGCA	-
<i>CCR6</i>	Gene 5'	TAGTAGACGCGTGCCACCATGAGCGGGGAATCAATGAATTC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCCATAGTGAAGGACGACGATTGTCTG	<i>BamHI</i>
<i>CHRM3</i>	Gene 5'	TAGTAGACGCGTGCCACCATGACCTTGCAACAATAACAGTACAAC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCCAAGGCCTGCTCGGGTGCGCGC	<i>BamHI</i>
<i>CXCR4</i>	Gene 5'	TAGTAGACGCGTGCCACCATGTCCATTCTTTGCCTCTTTTG	<i>MluI</i>
	Gene 3'	CTACTAGGTACCCCGTGGAGTGAAAACCTGAAG	<i>KpnI</i>
<i>EDNRA</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGAACCCCTTGCCTCAG	<i>MluI</i>
	Gene 3'	GAGCAGCCATAAGGACAGCATGAACAAGCTTTAGTAG	<i>HindIII</i>
<i>EDNRBL</i>	Gene 5'	TAGTAGACGCGTGCCACCATGCGAGCCCCGGCGCGCTTCTCG	<i>MluI</i>
	Gene 3'	CTACTAGGTACCCCGCAATGAGTTCCGACAGAAGCAAAAG	<i>KpnI</i>
	UTR 5'	GCAGGTGTCGATCCTAACTAAGCAG	-
	UTR 3'	AAATCTGACCCAACCAAGTACTG	-
<i>F2R</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGGGCCGCGGCGGCTG	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCAGTTAACAGCTTTTGTATATG	<i>BamHI</i>
<i>F2RL1</i>	Gene 5'	TAGTAGACGCGTGCCACCATGCGGAGCCCCAGCGCGCG	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCATAGGAGGTCTTAACAGTGGTTGAAC	<i>BamHI</i>
<i>GPER</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGATGTGACTTCCCAAGCCC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCACGGCACTGCTGAACCTCACATCCGAC	<i>BamHI</i>
<i>GPR161</i>	Gene 5'	TAGTAGACGCGTGCCACCATGAGCCTCAACTCCTCCCTCAGCTG	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCCTCTGTCTCGGCAGCTAAAACATC	<i>BamHI</i>
<i>GPR176</i>	Gene 5'	TAGTAGGATATCGCCACCATGGGACATAACGGGAGC	<i>EcoRV</i>
	Gene 3'	CTACTAGGATCCCCGGAATCCACCTTTGGAAAAATGCTCAC	<i>BamHI</i>
<i>GPR27</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGCGAACGCGAGCGAGC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCCTAAACCAATGCCTTCAGGTCG	<i>BamHI</i>
<i>GPR3</i>	Gene 5'	TAGTAGACGCGTGCCACCATGATGTGGGGTGACGGCAGC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCGACATCACTGGGGGAGCGGGATCGG	<i>BamHI</i>

Table 5.4: Oligonucleotide primers used in the generation of GPCR BRET vectors in this chapter.

Gene	Target	Primer sequence	Restriction
<i>GPR35</i>	Gene 5'	TAGTAGACGCGTGCCACCATGCTGAGTGGTTCCCGGGC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCGGCGAGGGTCACGCACAGAGAGTC	<i>BamHI</i>
	UTR 5'	CTTCATTCAACCTGGGCCCTTG	-
	UTR 3'	CTGGCCACAGCGCCACAGCAC	-
<i>GPR50</i>	Gene 3'	TAGTAGACGCGTGCCACCATGGGGCCACCCTAGCGGTTT	<i>MluI</i>
	Gene 5'	CTACTAGGATCCCCACAGCCATTTTCATCAGGATCATCTTC	<i>BamHI</i>
	UTR 5'	CTGAGCCTGCTGGGAGATCTTAA	-
	UTR 3'	ACTGCCTGGCCACCTACGAGAG	-
<i>GPR61</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGAGTCTCACCACATCCCCC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCCTGACTCCAGCCGGGGTGAGGCGGC	<i>BamHI</i>
	UTR 5'	GGTGCCCCCTACGAAACCAGG	-
	UTR 3'	CCCCATATCCCTCCGAGTGTC	-
<i>GPR78</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGGCCCCGGCGAGGCGCTGC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCGTGTGTCTGCTGCAGGCAGGAATCATTC	<i>BamHI</i>
<i>GPR83</i>	Gene 3'	TAGTAGACGCGTGCCACCATGGTCCCTCACCCTCTTGCTG	<i>MluI</i>
	Gene 5'	CTACTAGGATCCCCACTCATCGTCACAATGGGTTCCACAGATG	<i>BamHI</i>
	UTR 5'	GGCTCCTGCAAAATGGTCCCTC	-
	UTR 3'	TCCCACTCCCTCTTCCCAACC	-
<i>HRH1</i>	Gene 5'	TAGTAGACGCGTGCCACCATGAGCCTCCCCAATTCCTCC	<i>MluI</i>
	Gene 3'	CTACTAACCGGTCCGGAGCGAATATGCAGAATTCTCTTG	<i>HindIII</i>
<i>HRH2</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGCACCCAATGGCACAGCC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCCTAATTCCTGGCATGTGGTGGGAATTG	<i>BamHI</i>
<i>HTR1D</i>	Gene 5'	TAGTAGACGCGTGCCACCATGTCCCCACTGAACCAGTCAGCAG	<i>MluI</i>
	Gene 3'	CTACTAGGTACCCCGGAGGCCTTCCGGAAGGGACAATTTTC	<i>KpnI</i>
<i>HTR2B</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGCTCTCTCTTACAGAGTGTCTG	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCCTACATACTAAGTGTCTCTTCAGTTTTG	<i>BamHI</i>
	UTR 5'	GCTTAGCTACTAACCATGCTGAC	-
	UTR 3'	CATTTCATCATCTTACTCATCATTATGTTTG	-
<i>HTR2C</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGTGAACCTGAGGAATGCGG	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCACACTGCTAATCCTTTCGCTAACC	<i>BamHI</i>
<i>HTR6</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGTCCCAGAGCCGGGCCC	<i>MluI</i>
	Gene 3'	CTACTAGGTACCCCGTTCGTGGGGATGCCAAGTGGATG	<i>KpnI</i>
	UTR 5'	CCCTATCACTCCCTTGCCGTCCACC	-
	UTR 3'	CTCCCCATTGGCCAGCCCC	-
<i>HTR7</i>	Gene 5'	TAGTAGACGCGTGCCACCATGATGGACGTAAACAGCAGCGGCCG	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCCTGAATCATGACCTTTTTTCTACAGTAG	<i>BamHI</i>
	UTR 5'	GGCTCCATGGGCAGCGGCACAC	-
	UTR 3'	TTATTTTCATCTCCATTGTCTGCTTTC	-
<i>LGR4</i>	Gene 5'	TAGTAGACGCGTGCCACCATGCCGGGCCCCGCTAGGGC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCGTCTTTAACTCTTGGTAG	<i>BamHI</i>
	Chimera 5'	CAGTACCATATGCTTATCAGTGTGTGC	-
	Chimera 3'	GCACAGCACTGATAAGCATATGGTACTG	-
<i>LGR5</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGACACCTCCCGGCTCG	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCGAGACATGGGACAAATGCCACAGAG	<i>BamHI</i>
<i>LPAR1</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGCTGCCATCTCTACTTCCATC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCAACCACAGAGTGGTCATTGCTGTGAATC	<i>BamHI</i>
<i>LPAR2</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGTCATCATGGGCCAGTGC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCAAGGTGGAGTCCATCAGTGGGTGGC	<i>BamHI</i>
<i>LPAR3</i>	Gene 5'	TAGTAGACGCGTGCCACCATGAATGAGTGTCACTATGACAAG	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCGGAAGTGTCTTTATTGCAGAC	<i>BamHI</i>
<i>LTB4R1</i>	Gene 5'	TAGTAGGATATCGCCACCATGAACACTACATCTTCTGCAG	<i>EcoRV</i>
	Gene 3'	CTACTAGGATCCCCGTTCAGTTCGTTAACTTG	<i>BamHI</i>

Table 5.5: Oligonucleotide primers used in the generation of GPCR BRET vectors in this chapter.

Gene	Target	Primer sequence	Restriction
<i>LTB4R2</i>	Gene 5'	TAGTAGACGCGTGCCACCATGTCGGTCTGCTACCGTCCC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCAAGGTCCCATTCCGGACCGTCCTC	<i>BamHI</i>
<i>NPY1R</i>	Gene 5'	TAGTAGACGCGTGCCACCATGAATTCAACATTATTTCCAG	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCGATTTTTCATTATCATCATTGTTGTTG	<i>BamHI</i>
	Chimera 5'	GTATATATATCTTGAAGTAGCAAATAAATATA	-
	Chimera 3'	GCTACTTCAAGATATATATACGCCTAAAAAAG	-
<i>OGR1</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGGGAACATCACTGCAGAC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCGGCCAACCTGCCCCTGGGGAACCCGCC	<i>BamHI</i>
<i>OPN1SW</i>	Gene 5'	TAGTAGACGCGTGCCACCATGAGAAAAATGTCGGAGGAAGAG	<i>MluI</i>
	Gene 3'	CTACTAGGTACCCCGTTGGGGCCAACCTGGGTAGACG	<i>KpnI</i>
	UTR 5'	AATGTCGGAGGAAGAGTTTTATCTGTTCAAAAAT	-
	UTR 3'	TAAAAAGTAAAATTTAATTCTAGCTGTTGCAAAAC	-
<i>OPN3</i>	Gene 5'	TAGTAGACGCGTGCCACCATGTACTCGGGGAACCGCAG	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCAAGGACGAACCTGGATTACATCAAC	<i>BamHI</i>
	UTR 5'	CCGCCGAAGCTGAGCGCCTC	-
	UTR 3'	CCAATTTAAGGCCCCATCTTC	-
<i>OPRL1</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGAGCCCCTCTTCCCCG	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCTGCGGGCCGCGGTACCGTCTCAG	<i>BamHI</i>
<i>OR4D1</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGAACCACAGAACACCACAC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCCTCCCTGCAAATTACTAGGCAC	<i>BamHI</i>
<i>OXER1</i>	Gene 5'	TAGTAGACGCGTGCCACCATGTTGTGTACCCGTGGTGG	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCGCCCTGGGAGGAGCCTTCCTTTTC	<i>BamHI</i>
<i>OXGR1</i>	Gene 5'	TAGTAGACGCGTGCCACCATGAATGAGCCACTAGACTATTAG	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCAGGGTTGTTGAGTAACTAATTTTC	<i>BamHI</i>
<i>P2RY1</i>	Gene 5'	TAGTAGACGCGTGCCACCATGACCGAGGTGCTGTGG	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCAGGCTTGATCTCCATTCTGCTTGAAGTC	<i>BamHI</i>
	UTR 5'	CTCGGAGCCGCCGCTAAGTCG	-
	UTR 3'	CAACAGAGAGGTGTTGGAGATT	-
<i>P2RY11</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGCAGCCAACGTCTCGGG	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCCTGGCTCAGCTACGGGAC	<i>BamHI</i>
	UTR 5'	GACACAGGCTGAGGATCGGCAC	-
	UTR 3'	CCTAGGGTGAGGAGGCAGCTTC	-
<i>P2RY2</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGCAGCAGACCTGGGCC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCCAGCCGAATGTCCTTAGTGTTT	<i>BamHI</i>
<i>PTAFR</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGAGCCACATGACTCCTC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCATTTTGAGGGAATTGCCAGGGATC	<i>BamHI</i>
<i>PTGDR2</i>	Gene 5'	TAGTAGACGCGTGCCACCATGTCGGCCAACGCCACACTG	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCACTCGAGGTGCTGCTCAGCGCCC	<i>BamHI</i>
	UTR 5'	GAATCCTGTGTCCCTCTGTG	-
	UTR 3'	GTGAGTGCCGCCCTACGTGG	-
<i>PTGER2</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGGCAATGCCTCCAATGAC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCAAGGTCAGCTGTTTACTGGCATCTG	<i>BamHI</i>
<i>PTGER3</i>	Gene 5'	TAGTAGGATATCGCCACCATGAAGGAGACCCGGGGCTAC	<i>EcoRV</i>
	Gene 3'	CTACTAACCGGTCCATTTCCTCCAAAATTCCTCTTG	<i>KpnI</i>
	UTR 5'	GTCTGCCCCCTCCCGCTG	-
	UTR 3'	CTGTATCTGAGAGTTCTGCAAAAC	-
<i>PTGER4</i>	Gene 5'	TAGTAGACGCGTGCCACCATGTCCACTCCCGGGGTCAATTC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCATATACATTTTCTGATAAGTTCAG	<i>BamHI</i>
<i>PTGFR</i>	Gene 5'	TAGTAGACGCGTGCCACCATGTCCATGAACAATTCCAAACAGC	<i>MluI</i>
	Gene 3'	CTACTAGGTACCCCGGTGCTTGCTGATTCTCTGCAAC	<i>KpnI</i>
<i>QRFPR</i>	Gene 5'	TAGTAGACGCGTGCCACCATGCAGGCGCTTAACATTAC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCATGCCCACTGTCTAAAGGAGAATTC	<i>BamHI</i>

Table 5.6: Oligonucleotide primers used in the generation of GPCR BRET vectors in this chapter.

Gene	Target	Primer Sequence	Restriction
<i>S1PR1</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGGGCCCACCAGCGTCC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCGGAAGAAGAGTTGACGTTTCC	<i>BamHI</i>
<i>S1PR2</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGGCAGCTTGACTCGGAGTAC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCGACCACCGTGTGCCCTCCAGAAAC	<i>BamHI</i>
<i>S1PR3</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGCAACTGCCCTCCCCGC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCGTTGCAGAAGATCCCATTCTGAAGTGC	<i>BamHI</i>
<i>S1PR5</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGAGTCGGGGCTGCTGC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCGTCTGCAGCCGGTTCTGATACCAGAG	<i>BamHI</i>
<i>SSTR2</i>	Gene 5'	TAGTAGACGCGTGCCACCATGGACATGGCGGATGAGCC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCGATACTGGTTTGGAGGTCTCCATTG	<i>BamHI</i>
<i>TBXA2R</i>	Gene 5'	TAGTAGACGCGTGCCACCATGTGGCCCAACGGCAGTTCC	<i>MluI</i>
	Gene 3'	CTACTAGGATCCCCATCCTTTCTGGACAGAGCCTTC	<i>BamHI</i>
	Chimera 5'	CGAACC GCCGGGCGCGAGAAGGGCCGGTG	-
	Chimera 3'	GCTGGTGTGGGCGGCCGCGCTGGCGCTGGG	-

Table 5.7: Oligonucleotide primers used in the generation of GPCR BRET vectors in this chapter.

Protein	Isoform designation	NCBI reference
5-HT _{2c}	A	NP_000859.1
AT ₁	1	NP_000676.1
EDNRA	A	NP_001948.1
EP ₃	6	NP_942009.1
FP	A	NP_000950.1
GPR176	1	NP_009154.1
GPR35	B	NP_001182310.1
H ₂ R	1	NP_001124527.1
LGR5	1	NP_003658.1
TP	B	NP_963998.2

Table 5.8: *Rhodopsin* family GPCRs investigated in this chapter that have multiple expressed isoforms. In each case, the isoform investigated is given along with its corresponding National Centre for Biotechnology Information (NCBI) reference.

Protein aggregation was identified by the presence of non-uniform accumulations of GFP fusion protein with fluorescence intensities and z-plane distributions greater than that expected of protein retained in internal membranes, as has been demonstrated previously (387). Typically, such aggregates would be the brightest objects within the field of view. Genes that demonstrated an expression profile assigned to either category F or G were not progressed to pRluc and pU cloning, nor subsequent BRET analysis.

5.2.3 Type 1 and cBRET assays

BRET assays in this chapter were largely performed with the assistance of Rachel Knox, who aided in the preparation of cells for measurement.

Type 1 BRET and cBRET assays were performed on all GPCRs that demonstrated adequate surface expression under confocal microscopy. Type 1 BRET was performed as described in Section 3.2.4 on HEK 293T cells transiently transfected with BRET pairs of the target gene (§3.2.3). BRET_{eff} and GFP:Rluc ratio were measured 24h post transfection for the majority of receptors, however some GPCRs demonstrated insufficient protein expression to give reliable data after 24h and so were instead measured after 48h. GPCRs measured after 48h were AT₁, CCR11, GPER, NPY1R, OR4D1, and PAR1. Each receptor was assayed in a minimum of three independent experiments until a broad range of GFP:Rluc ratios had been achieved.

cBRET assays were performed as detailed in Section 4.2.3 in HEK 293T cells transiently transfected with BRET pairs of the target gene along with competitor or blank pU expression vector. In all instances, a 2:1 ratio of pU:(pGFP²+pRluc) was used to ensure an excess of competitor over labelled proteins, and a 12:1 pGFP²:pRluc ratio was used to ensure measurable levels of BRET. In the majority of cases this was achieved using a transfection strategy of 1µg pU, 0.462µg pGFP², and 0.038µg pRluc per well of 6x10⁵ cells, however in cases of low receptor expression these amounts were increased to 2µg pU, 0.924µg pGFP², and 0.076µg pRluc per well. Such an increase in DNA was required for 5-HT_{2B}, AT₁, B₂, CCR11, EDNRA, GPER, NPY1R, OR4D1, OXER1, and PAR1. Data collected are from a minimum of three independent experiments. For each experiment, five identical transfections were performed for each gene of interest and then assayed for BRET_{eff} and expression level at varying time points, typically between 16h and 32h post-transfection, in order to generate a range of protein densities whilst maintaining constant GFP:Rluc and untagged:tagged protein ratios.

Statistical analysis of all BRET data was performed using the Prism5 (Graphpad) software. Type 1 data were fitted to models of both dimeric (Eq. 3.2) and monomeric (Eq. 3.6) behaviour using the non-linear regression least squares function. The lower and upper range limits of [GFP]/[Rluc] values included in the analysis were 2 and 15, respectively, as this describes the range over which differences in the two models are most evident. The coefficient of determination (R^2) for the monomer fit is always zero as calculated BRET_{eff} is a constant value. An R^2 value of less than zero for the dimer model indicates that it has a worse goodness-of-fit to the data than the monomer model, whereas an R^2 greater than zero indicates a better goodness-of-fit. cBRET data were fitted to Equation 3.7 using the linear regression least squares function. y -intercept values and associated confidence limits were generated by regression of the fitted model to $x=0$, and goodness-of-fit was assessed using R^2 . P^{diff} values were determined as the probability that the two datasets were from populations with identical t distributions. The larger the P^{diff} value, the lower the probability of difference between datasets.

5.2.4 Quantitative flow cytometry analysis

Absolute receptor numbers were quantified using flow cytometry analysis of the GFP-tagged receptor variants. HEK 293T cells were transiently transfected with 1 μg pGFP²-GPCR vector per 6 $\times 10^5$ cells using GeneJuice[®] (Novagen) in the same manner as for type 1 BRET (§3.2.3), and incubated for an equivalent length of time as for the type 1 BRET assay (*i.e.* 24h for the majority of receptors, 48h for six exceptions). Transfected cells were analysed by flow cytometry (§2.4.1) for GFP expression. Data were collected for a total of 5 $\times 10^4$ cells for each gene in each experiment, and viable single cells were gated for using forward scatter, side scatter, and pulse width. GFP-positive cells were selected using a two-dimensional FL1 vs. FL2 gate in order to prevent artefacts arising from cellular autofluorescence. The geometric mean of GFP fluorescence was then determined for the GFP-positive population of each receptor.

GFP fluorescence was converted into absolute receptor numbers by reference to a standard curve of GFP fluorescence vs. surface protein expression. This standard curve was generated for each experiment using HEK 293T cells expressing a human CD2-GFP fusion protein labelled with phycoerythrin (PE)-conjugated mouse anti-human CD2 antibody (eBioscience 12-0029). Labelling was performed at an antibody concentration of 100 $\mu\text{g}/\text{ml}$ to ensure univalent binding to antigen. PE-GFP compensation was performed using unlabelled cells expressing CD2-GFP as a GFP-only control, and cells expressing CD2-Rluc labelled with PE-antiCD2 as a PE-only control. PE fluorescence on the

labelled CD2-GFP cells was then converted to antigen-binding events by reference to calibrated Quantibrite™ (BD Biosciences) PE beads as per the manufacturer's instructions. This allowed the generation of the GFP vs. surface protein expression standard curve by plotting relative GFP fluorescence against antigen-binding events, given that a univalent labelling strategy was used. Absolute protein numbers were determined in this manner for each receptor in three independent replicate experiments.

5.3 Results

5.3.1 HEK 293T cells display a partially neuronal GPCR expression profile

The GPCR expression profile of HEK 293T cells was determined by Yuan Lui. This was done by mining the Universal Protein Resource (www.uniprot.org) for genes associated with the keyword 'G protein-coupled receptor' (KW-0297), and then comparing the resulting list to a database of gene expression obtained by deep RNA sequencing (see 400) of cDNA derived from HEK 293T mRNA (Lui et al., unpublished data). Relative abundance of GPCR mRNA in HEK 293T cells was thereby obtained in fragments per kilobase of exon per million fragments mapped (FPKM).

In total, 65 *Rhodopsin*, 11 *Frizzled*, 8 *Taste2*, 12 *Adhesion*, 5 *Glutamate*, and 4 *Secretin* receptors are expressed in HEK 293T cells, as well as 20 GPCRs that were either not assigned a family in the analysis of Fredriksson et al. (26), or not included in that analysis at all. *Rhodopsin* family receptors expressed in HEK 293Ts are given in Tables 5.1 and 5.2, whilst non-*Rhodopsin* assigned GPCRs are given in Table 5.9. A number of genes were assigned as *Rhodopsin* family GPCRs despite not being assigned families by Fredriksson et al. because they returned close matches to numerous receptors in the same *Rhodopsin* subfamily when analysed using the Basic Local Alignment Search Tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). *GPER* was assigned to *Rhodopsin* γ as it is most closely related to *AGTR2*, *CCR1*, *CXCR1*, *CCR4*, and *CCR8*. *GPR176* was included in *Rhodopsin* β as its closest relatives are *HCRT2* and *HCRT1*. *OXER1* is most closely related to *HM74*, *FKSG80*, *GPR31*, and *P2Y4* and so was placed in the *Rhodopsin* δ subfamily. *HRH1* was included in the *Rhodopsin* α subfamily because it is closely related to *HRH2* but has a large insertion preventing it from clustering with the rest of the subfamily in Fredriksson et al. (2003).

The physiological ligands and functions of the HEK 293T *Rhodopsin* family receptors, where these are known, are given in Tables 5.10 and 5.11. A number of ligand families and types of

Family	Gene	FPKM
<i>Frizzled/Taste2</i>	<i>SMO</i>	17.683
	<i>FZD7</i>	13.889
	<i>FZD6</i>	13.6348
	<i>FZD8</i>	9.51027
	<i>FZD5</i>	8.25423
	<i>FZD3</i>	5.85188
	<i>FZD2</i>	5.10144
	<i>FZD4</i>	4.90713
	<i>FZD10</i>	4.40477
	<i>TAS2R14</i>	3.98896
	<i>FZD9</i>	3.27243
	<i>FZD1</i>	2.8725
	<i>TAS2R5</i>	2.556344
	<i>TAS2R20</i>	1.54932
	<i>TAS2R10</i>	1.21316
	<i>TAS2R4</i>	1.04428
	<i>TAS2R19</i>	1.03564
	<i>TAS2R3</i>	0.93946
	<i>TAS2R13</i>	0.67322
<i>Adhesion</i>	<i>LPHN2</i>	59.2576
	<i>LPHN1</i>	16.9396
	<i>CELSR2</i>	10.8531
	<i>CD97</i>	5.80254
	<i>BAI2</i>	4.30604
	<i>CELSR3</i>	3.1895
	<i>LPHN3</i>	2.06964
	<i>GPR64</i>	1.15453
	<i>EMR2</i>	0.49483
	<i>BAI3</i>	0.31717
	<i>GPR113</i>	0.19422
	<i>BAI1</i>	0.0704
<i>Glutamate</i>	<i>GABBR1</i>	17.482
	<i>GPR156</i>	1.43933
	<i>GABBR2</i>	1.0494
	<i>GRM8</i>	0.35944
	<i>GRM2</i>	0.24831
<i>Secretin</i>	<i>ADCYAP1R1</i>	13.6458
	<i>VIPR1</i>	3.92804
	<i>GIPR</i>	0.95769
	<i>PTH1R</i>	0.37527

Table 5.9: Non-*Rhodopsin* GPCRs natively expressed in HEK 293T cells and their relative mRNA abundance (FPKM), excluding genes that could not be assigned a family.

Protein	Known physiological ligand(s)	Known area(s) of physiological activity	Ref(s)
5-HT _{1D}	Serotonin	Neurotransmission; nociception	(68, 403)
5-HT _{2B}	Serotonin	Inflammatory cytokine production; vasodilation; neurotransmission	(68, 403)
5-HT _{2C}	Serotonin	Behaviour, memory, & CNS functions	(68, 403)
5-HT ₆	Serotonin	Neurotransmission; learning & memory	(68, 403)
5-HT ₇	Serotonin	Vasodilation; sleep, mood, & circadian rhythmic activity	(68, 403)
A ₁ R	Adenosine	CNS function; inflammation; regulation of heart rate; renal function	(404)
A _{2A} R	Adenosine	CNS function; anti-inflammation; vasodilation; platelet aggregation	(404)
α _{2C} AR	Adrenaline; noradrenaline	Sympathetic cardiovascular, pulmonary, CNS, & metabolic control	(69, 359)
AT ₁	Angiotensin II	Fluid homeostasis; regulation of blood pressure; neurogenesis	(405, 406)
β ₁ AR	Adrenaline; noradrenaline	Sympathetic cardiovascular, pulmonary, CNS, & metabolic control	(151, 213, 407)
B ₂	Bradykinins	Vasodilation; natriuresis; pain responses	(408, 409)
C5R1	Anaphylatoxin C5a	Inflammation; innate immune response	(410, 411)
CCKBR	Cholecystokinin; gastrin	Nociception; gastrointestinal secretion	(412, 413)
CCR6	CCL20	Leukocyte & epithelial migration; dendritic & B cell maturation	(414)
CCR10	CCL27; CCL28	Regulation of epithelial immune responses	(415, 416)
CCR11	CCLs 2,8,13,19, 21, & 25; CXCL3	Regulation of homeostatic leukocyte migration	(417-420)
CXCR4	CXCL12	Vascular, cardiac, & CNS development; haematopoiesis;	(421, 422)
DP ₂	Prostaglandin D ₂	Smooth muscle relaxation; leukocyte chemotaxis; CNS function;	(70, 423-425)
EDNRA	Endothelin	Vasoconstriction	(426, 427)
EP ₃	Prostaglandin E ₂	Smooth muscle contraction; neuronal activation; gastric secretion	(70, 423)
FP	Prostaglandin F _{2α}	Endothelial & myometrial cell proliferation; luteolysis	(70, 423, 428, 429)
GPER	Oestrogens	Diverse oestrogen signalling functions	(430, 431)
GPR3	None	Oocyte development; neuronal β-amyloid production	(432, 433)
GPR35	KYNA; Lysophosphatidic acid	Nociception; regulation of neuronal activity	(434-436)
GPR50	None	Melatonin signalling	(72)
GPR61	None	Likely involved in CNS function	(437, 438)
GPR78	None	Likely involved in CNS function	(439)
GPR83	None	Temperature homeostasis; immune tolerance	(440, 441)
GPR176	None	Vascular smooth muscle transformation	(442)

CCL = chemokine (cc-motif) ligand; CNS = Central nervous system; CXCL = chemokine (cxc-motif) ligand; KYNA = Kynurenic acid

Table 5.10: Known ligands and functions of *Rhodopsin* GPCRs natively expressed in HEK 293T cells.

Protein	Known physiological ligand(s)	Known area(s) of physiological activity	Ref(s)
HLR	Histamine	Vasodilation; bronchoconstriction; CNS function	(443, 444)
H ₂ R	Histamine	Vasodilation; chronotropism & ionotropism; CNS function	(443, 444)
LGR4	R-spondin	Embryonic development; stem cell differentiation	(445, 446)
LGR5	R-spondin	Embryonic development; stem cell differentiation	(445, 446)
LPA ₁	Lysophosphatidic acid	CNS development & function; male reproductive function	(71, 447, 448)
LPA ₂	Lysophosphatidic acid	CNS development & function; male reproductive function	(71, 447, 448)
LPA ₃	Lysophosphatidic acid	CNS development & function; male reproductive function	(71, 447, 448)
LTB ₄ R1	Leukotriene B ₄	Inflammatory cell chemotaxis & survival	(449)
LTB ₄ R2	Leukotriene B ₄	Lymphocyte migration	(449)
M ₃	Acetylcholine	Bronchoconstriction; insulinotropism; regulation of mucosal activity	(450, 451)
NPY1R	Neuropeptide Y	Appetite regulation; anxiolysis	(452, 453)
OGR1	Protons	pH sensing & regulation	(454, 455)
OPN1SW	Light	Visual & non-visual phototransduction	(456, 457)
OPN3	Light	Regulation of apoptosis	(74, 458)
OPRL1	Nociceptin/orphanin FQ peptide	Restriction of neuronal excitability & neurotransmitter release	(80, 459)
OR4D1	Odorants	Olfaction	(460)
OXER1	5-oxoicosatetraenoic acid	Inflammation; leukocyte chemotaxis	(461, 462)
OXGR1	Alpha-ketoglutarate	Unknown – potentially mucin regulation	(463, 464)
P2Y ₁₁ R	ATP	Nociception leukocyte chemotaxis & viability; pancreatic function	(82, 465-467)
P2Y ₁ R	ADP	Platelet aggregation; nociception; pancreatic function	(82, 465)
P2Y ₂ R	UTP; ATP	Nociception; leukocyte chemotaxis; pancreatic function	(82, 465, 468, 469)
PAR1	Thrombin	Coagulation; platelet activation	(470, 471)
PAR2	Thrombin	Coagulation; platelet activation	(470, 471)
PTAFR	Platelet-activating factor	Inflammation; CNS function	(472, 473)
QRFR	QRFP peptides	Regulation of neuroendocrine function	(474, 475)
SIP ₂	Sphingosine 1-phosphate	Leukocyte migration & differentiation; anti-angiogenesis	(448, 476, 477)
SIP ₃	Sphingosine 1-phosphate	Leukocyte migration & differentiation; angiogenesis; vasculogenesis	(448, 476, 477)
SIP ₅	Sphingosine 1-phosphate	Natural killer cell function; CNS function	(448, 476, 477)
SREB1	None	CNS development	(478, 479)
SSTR2	Somatostatin	Regulation of neuroendocrine & gastrointestinal function	(480, 481)
TP	Thromboxane A ₂	Vasoconstriction; renal function; platelet activation	(482)

ADP = adenosine diphosphate; ATP = adenosine triphosphate; UTP = Uridine triphosphate

Table 5.11: Known ligands and functions of *Rhodopsin* GPCRs natively expressed in HEK 293T cells.

physiological activity are shared for many of the receptors. Principally these appear to be functions involved in vascular processes (e.g. vasodilation) or embryonic development (e.g. cell differentiation). The embryonic nature of HEK 293Ts is also apparent in their non-*Rhodopsin* GPCR expression profile, as they express all 11 *Frizzled* family members, which are highly active during early development. This is far more than other, adult-derived cell lines. For example, equivalent analysis of the monocyte-derived U937 line revealed only 6 natively expressed *Frizzled* receptors, whilst the natural killer cell-derived NK-92 line expressed only 2 (data not shown).

Almost half of *Rhodopsin* GPCRs in HEK 293Ts are to some extent involved in neuronal functions, either in the CNS or peripheral neurons. Some, such as the serotonin or acetylcholine receptors, are directly involved in neurotransmission; whereas others regulate CNS development or neurogenesis, e.g. *AT₁*, *LPA₁*, *SREB1* etc. Analysis of the HEK 293T *Rhodopsin* GPCR profile using the open-source Generic Gene Ontology (GO) term Finder software (481) returned a $\log_{10}$ p value of -13.3174 for the GO term 'neurological system process' (GO:0050877). This software searches for common semantic terms regarding gene function and assesses their significance relative to a wider database. Such a low $\log_{10}$ p value suggests that the presence of *Rhodopsin* receptors with neurological functions in HEK 293T cells is highly significant according to the automated annotation of human genes. After discarding GO terms common to all GPCRs (e.g. 'cell surface receptor signalling' etc.), only terms relating to anatomical tube size ($\log_{10}$ p = -27.8611), vascular processes ($\log_{10}$ p = -26.8642), and smooth muscle contraction ($\log_{10}$ p = -18.5101) had greater significance than those associated with neuronal function (Table 5.12). It therefore appears that the GPCR expression profile of HEK 293Ts combines that expected of embryonic cells, and that of cells with a more neuronal lineage. In any case, the investigation of these receptors is likely to provide a representative cross-section of the *Rhodopsin* family as a whole, as the receptors are apparently not restricted to a narrow range of ligands or functions.

5.3.2 GPCR overexpression is achieved using the pBRET vectors

Genes encoding all 65 *Rhodopsin* receptors native to HEK 293Ts were isolated and cloned into the pBRET vectors pGFP², pRLuc, and pU (§5.2.1). Absolute levels of GPCR expression from the pBRET vectors was assessed using flow cytometry of the GFP-tagged construct transfected into HEK 293T cells in the manner used during BRET assays. GFP fluorescence was converted into absolute protein numbers by reference to CD2-GFP-expressing cells labelled with a PE-conjugated anti-CD2 antibody,

GO term ID	Description	Log ₁₀ p
GO:0035150	Regulation of tube size (& anatomical structure size <i>etc.</i>)	-27.861
GO:0003018	Vascular process in circulatory system (& vasoconstriction, circulation <i>etc.</i>)	-26.864
GO:0006939	Smooth muscle contraction (& muscle system process <i>etc.</i>)	-18.510
GO:0050877	Neurological system process (& synaptic transmission <i>etc.</i>)	-13.317
GO:0006954	Inflammatory response (& defence response <i>etc.</i>)	-12.293
GO:0007610	Behavior (& response to stress, feeding <i>etc.</i>)	-9.776
GO:0040011	Locomotion (& cell chemotaxis, leukocyte migration <i>etc.</i>)	-8.822
GO:0046903	Secretion (& regulation of secretion <i>etc.</i>)	-7.419
GO:0007600	Sensory perception (& perception of pain <i>etc.</i>)	-7.352
GO:0003014	Renal system process (& glomerular filtration <i>etc.</i>)	-7.143
GO:0001659	Temperature homeostasis (& heat generation <i>etc.</i>)	-3.644

Table 5.12: Significant Generic Gene Ontology (GO) terms and their log₁₀ probability values for the *Rhodopsin* GPCR expression profile in HEK 293T cells. Descriptions include official GO term designations and examples of similar terms subsumed into that category.

and to Quantibrite™ (BD Biosciences) PE-conjugated beads (Fig. 5.2). The efficiency of transfection was similar for all receptors, roughly 60%, although the expression levels observed varied markedly between GPCRs. The lowest mean expression was observed for 5-HT_{2B} at ~15,000 receptors/cell, whilst the highest was observed for P2Y₂R at ~460,000 receptors/cell (Table 5.14). A typical expression level was ~100,000 receptors/cell, and almost half of receptors were expressed within the range 60,000-150,000 receptors/cell (Fig. 5.3). These receptor densities (equating to ~50-125 proteins/μm²) are 1-2 orders of magnitude above those reported previously for GPCRs in human cells (*e.g.* 356, 482) and so are sufficient that background interference from native receptors is unlikely to significantly impact on the apparent stoichiometry of the transfected proteins.

Despite the broad range of protein densities observed, no widely shared distribution of expression level was apparent across the *Rhodopsin* family when receptors were organised based on their phylogenetic relationships as determined by Fredriksson *et al.* (26); instead each subfamily exhibited broadly similar expression profiles, which varied considerably (Fig. 5.7).

5.3.3 *Rhodopsin* family GPCRs appear predominantly monomeric

As exemplified previously (Fig. 3.1), protein aggregation leads to artificially high levels of BRET that may cause the misidentification of proteins as oligomeric. In an effort to minimise potential aggregation effects, the expression quality of each receptor was assessed by confocal microscopy. GPCRs were placed into one of seven categories (A-G; Table 5.2) based on the apparent distribution of protein within the cell. Receptors exhibiting apparent aggregation (categories F and G) were not continued for BRET analysis. Protein aggregation was identified by the presence of irregular accumulations of protein with fluorescence intensities and z-plane distributions greater than that expected of protein retained in internal membranes, as has been described previously (387). Of the 65 *Rhodopsin* GPCRs natively expressed in HEK 293Ts, 60 had sufficiently good folding and trafficking to allow BRET analysis using both the type 1 and cBRET approaches (Figs. 5.4 & 5.5). The quality of expression was observed to be partially defined by phylogenetic position (Fig. 5.7). Some clusters, *e.g.* the α subfamily amine cluster, contained receptors assigned predominantly to categories A and B, whereas others were exclusively made up of receptors in categories C, D, and E, *e.g.* the α subfamily prostaglandin cluster. In the vast majority of cases, GPCR overexpression did not appear to have any obvious effects on cell morphology, growth, or behaviour, although this was only assessed qualitatively. The only exception to this was CXCR4, overexpression of which caused a

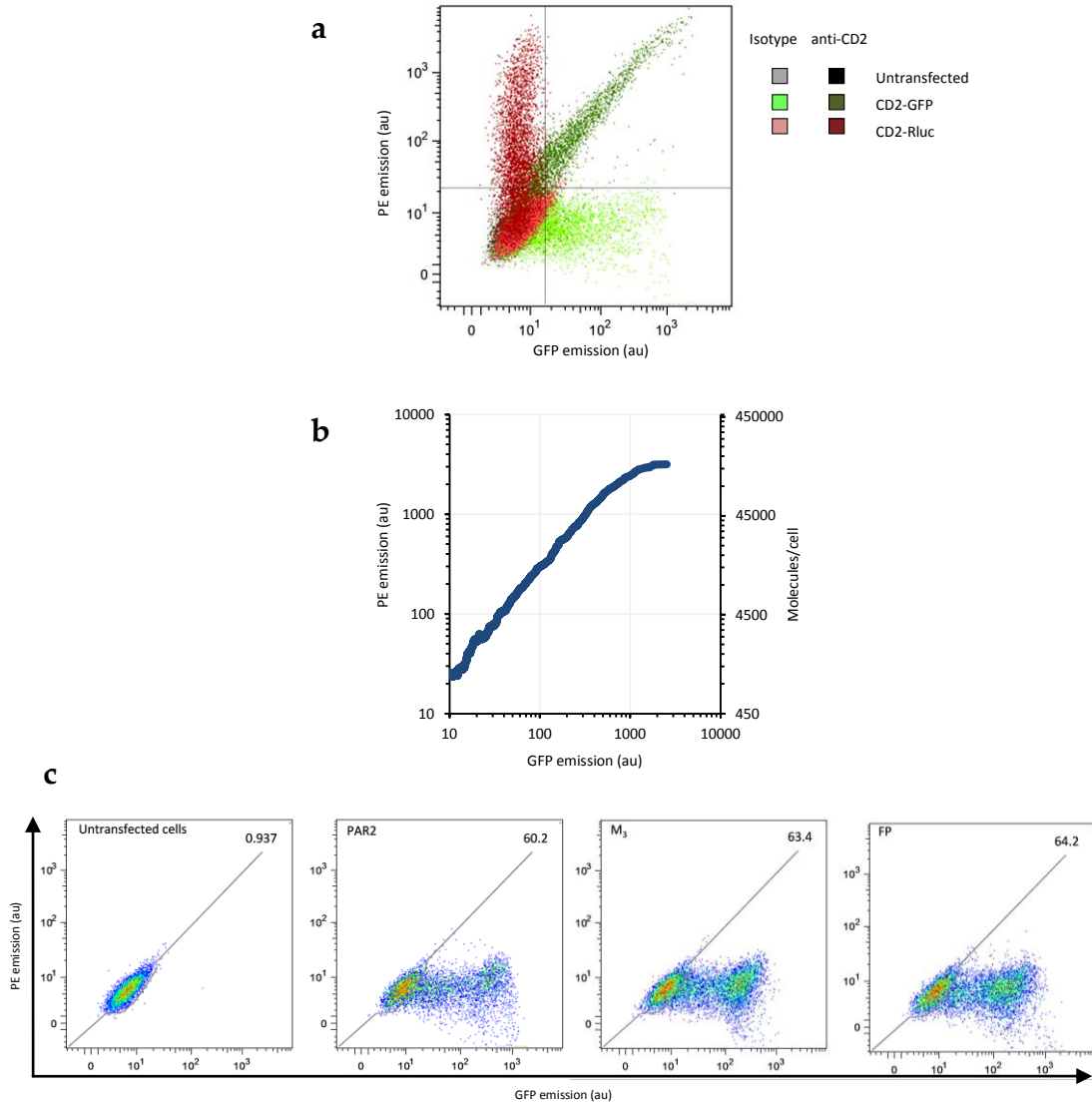


Figure 5.2: Transfected GPCR-GFP expression was quantified using flow cytometry. (a) CD2^{GFP}-expressing cells stained with anti-CD2 PE-conjugated antibodies exhibit a strong correlation between fluorescence in the GFP and PE channels. No such correlation was observed for unstained cells expressing CD2-GFP, or for stained cells expressing CD2^{Rluc} (b) PE and GFP emission values for labelled CD2-GFP cells was converted into absolute protein numbers by reference to QuantiBrite™ (BD Biosciences) PE-conjugated beads. Geometric mean GFP fluorescence values for individual GPCRs were then converted to absolute protein numbers by reference to the CD2-GFP standard, shown above as the moving average ($n=100$) of all points. (c) Representative examples of GPCR-GFP-expressing cells gated for GFP fluorescence. GPCR names are given. Values represent the percentage of cells gated as GFP positive and are a readout of transfection efficiency. Typically, transfection efficiency was ~60% of all cells after 24 h incubation, and was consistent between genes.

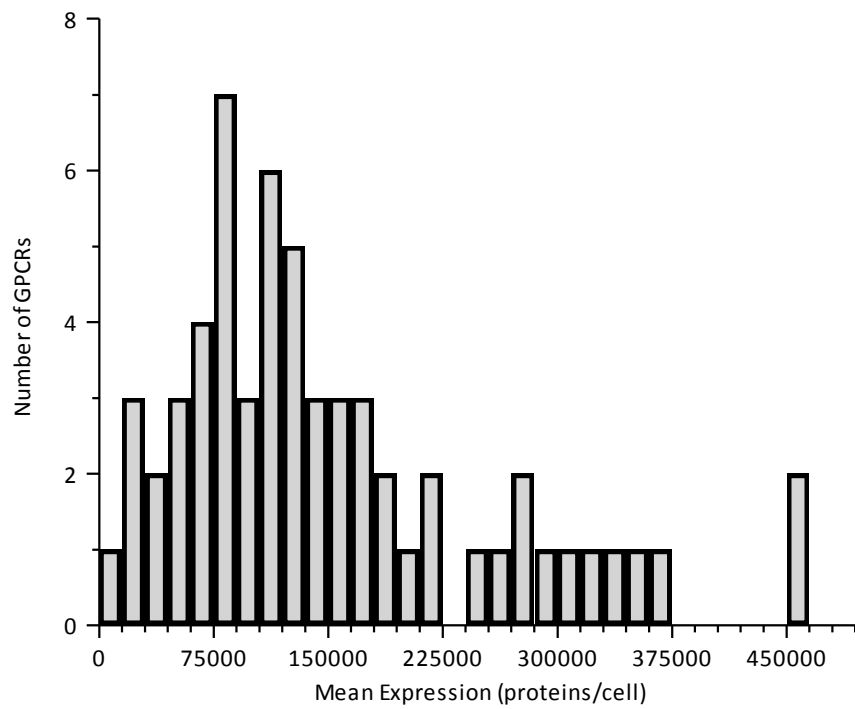


Figure 5.3: Histogram of mean expression of *Rhodopsin* family GPCRs expressed from the pGFP² vector in HEK 293Ts. Almost half of receptors are expressed within the range 60,000-150,000 proteins/cell. Absolute expression values are given in Table 5.14.

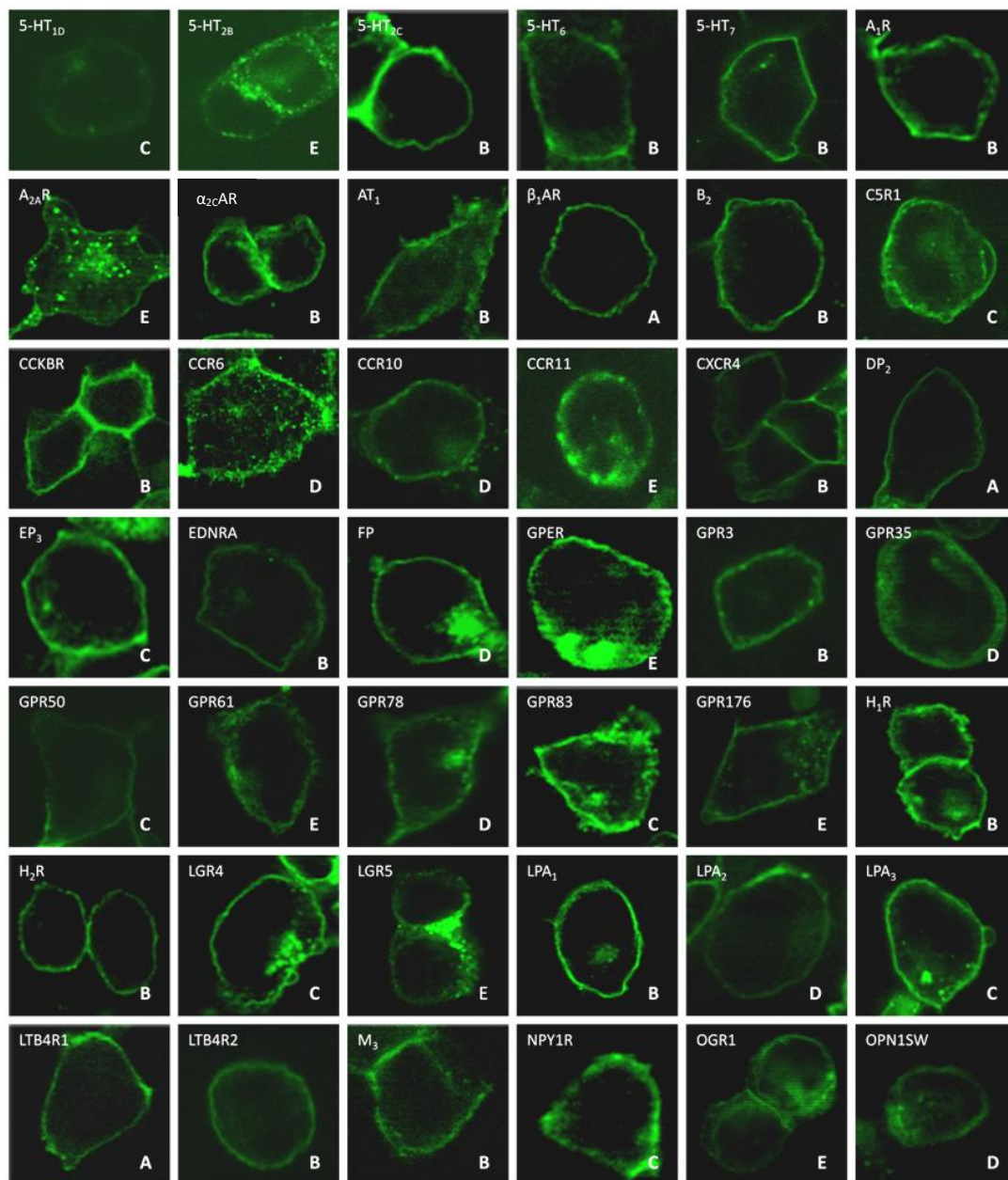


Figure 5.4: Representative confocal microscopy images of GPCR-GFP constructs expressed in HEK 293T cells. Receptors were placed into categories A-G (Table 5.1) based on their subcellular localisation and degree of observable GFP aggregation.

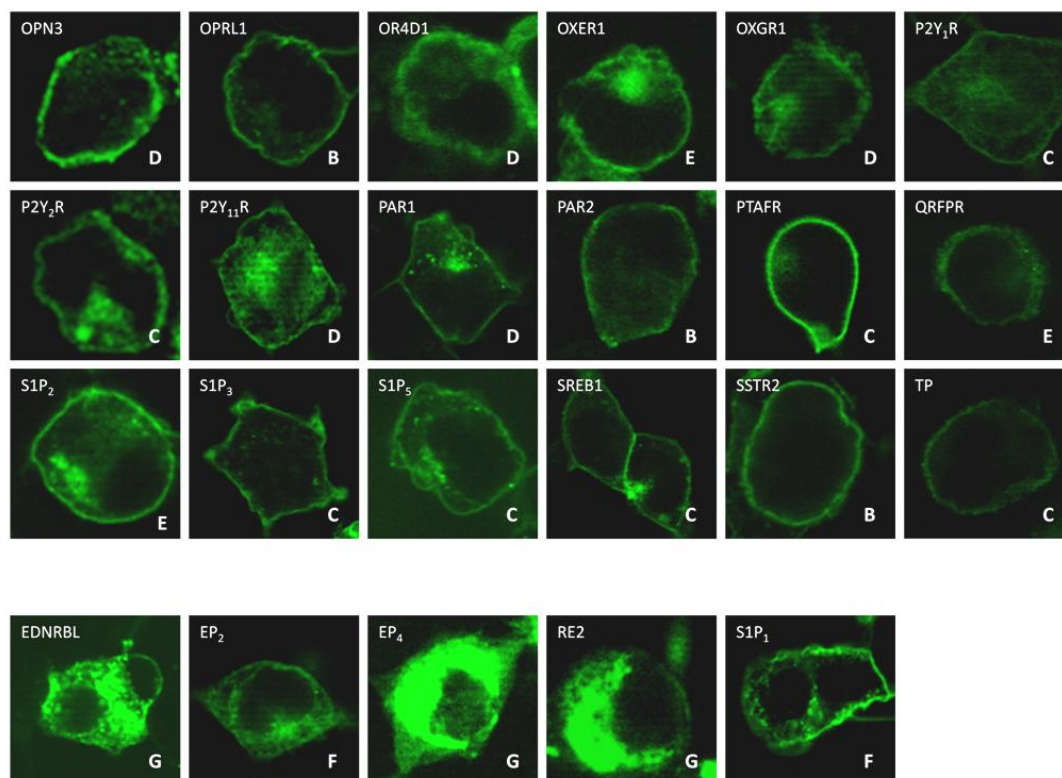


Figure 5.5: Representative confocal microscopy images of GPCR-GFP constructs expressed in HEK 293T cells. Receptors were placed into categories A-G (Table 5.1) based on their subcellular localisation and degree of observable GFP aggregation. Five receptors (bottom row) were identified as categories F and G, and so were not pursued for BRET.

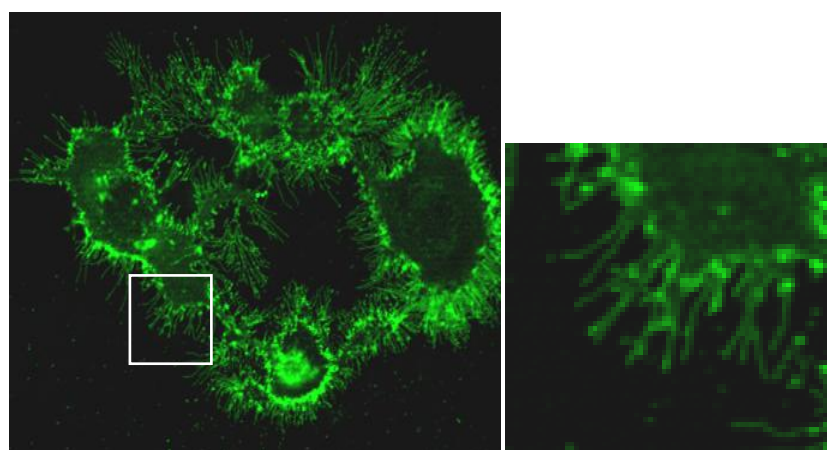


Figure 5.6: Altered HEK 293T cell morphology induced by CXCR4 overexpression. Approximately 50% of cells transfected with CXCR4 developed fibril-like protrusions extending from the main cell body, which were not observed for cells transfected with other GPCRs. White box defines the position of the enlarged selection (right) within the wider image.

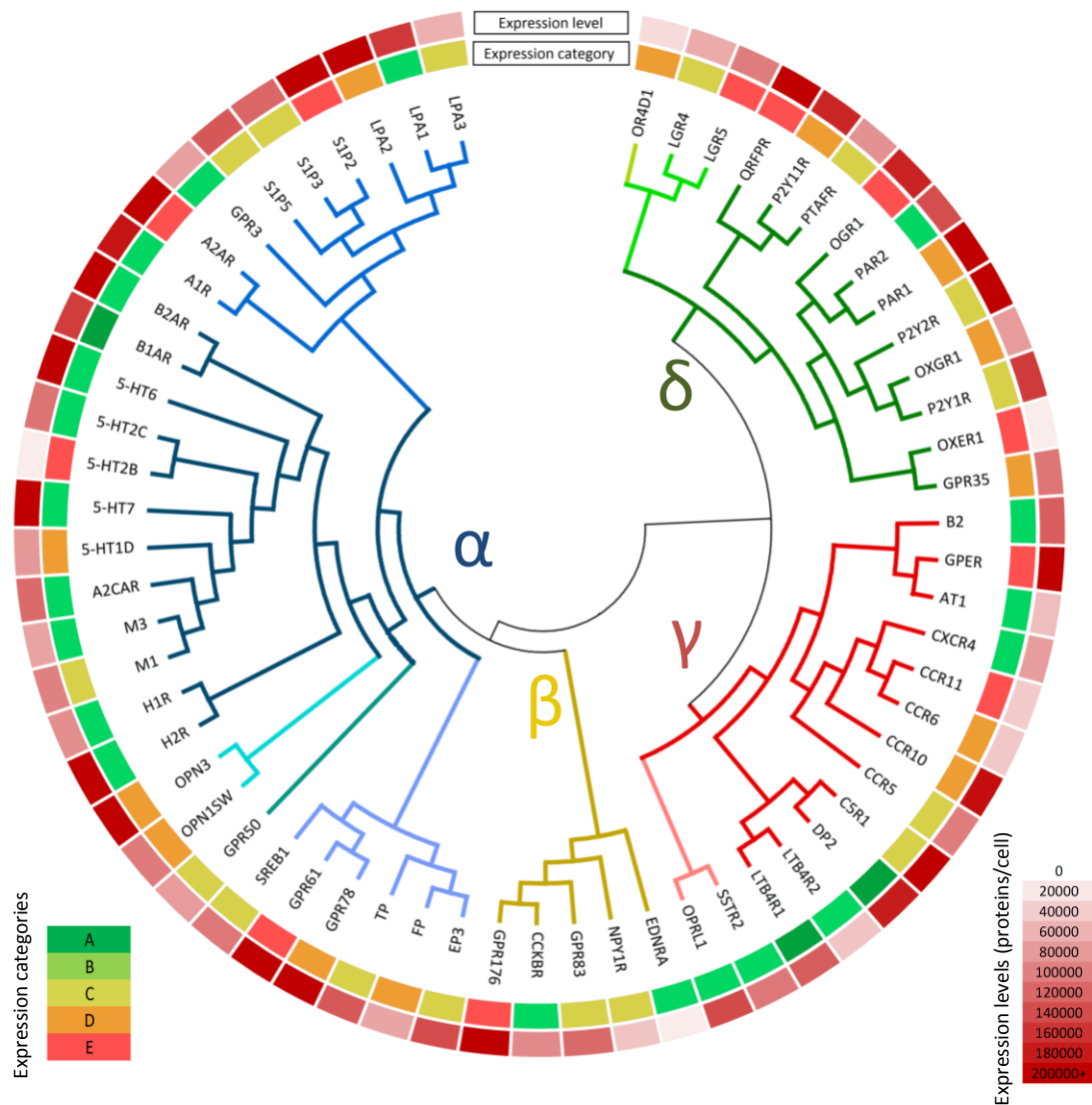


Figure 5.7 Visualisation of absolute expression levels and surface expression categories for all *Rhodopsin* GPCRs investigated in this thesis. Category assignments are represented in the inner ring of the circle corresponding to the adjacent receptor name, and are denoted by the colours shown in the key. Receptors in categories F and G are not shown. Expression levels are represented by the outer ring of the circle and are coloured as a gradient from zero (white) to 200,000 proteins/cell or above (red) as demonstrated in the key. Absolute expression values are given in table 5.14. Phylogenetic relationships are shown as a simple divergence tree based on the analysis of Fredriksson et al. (26). *Rhodopsin* subfamilies are represented by branch colour, α =blue; β =gold, γ =red, δ =green. Varying shades of each colour denote individual clusters within each subgroup. There is no clear correlation between expression category and expression level across the family. Expression category appears to show some correlation with phylogenetic position: e.g. within the α subfamily the amine receptors are predominantly categories A and B, whereas the prostaglandin receptor cluster fall exclusively into categories C, D, and E.

significant change in cellular morphology, inducing the formation of fibril-like structures protruding from the main cell body (Fig. 5.6).

Analysis of the 60 GPCRs by the type 1 and cBRET assays gave results that were in agreement for 57 receptors, allowing these receptors to be assigned as apparent monomers or dimers. Raw data for all investigated receptors are given in Appendix Figures A1–A15, whilst the type 1 assay R^2 and cBRET assay P^{diff} values are summarised in Figure 5.9 and given as absolute values in Table 5.13. Examples of the contrasting data obtained for apparent monomers and dimers are shown in Figure 5.8 in the form of data yielded by the LPA and S1P receptors. In total, of the 60 receptors investigated, 46 did not exhibit any evidence of dimerisation in either assay, suggesting that these receptors are wholly monomeric or form dimers at levels below the sensitivity of the techniques. Eleven receptors yielded data consistent with dimeric behaviour in both assays. Although the extent of dimerisation cannot be directly inferred from these data, it is probable that >10-20% of the GPCRs form dimers, as this is the apparent limit to the sensitivity of these assays. The correlation in outcome for both the type 1 and cBRET assays, as assessed by dimer model R^2 and cBRET P^{diff} values (Fig. 5.10), reinforces the conclusion that two distinct behavioural states have been observed within the *Rhodopsin* family.

The fraction of GPCRs assigned to each of the expression categories A-E was comparable for those suggested as monomeric and dimeric (Fig. 5.11), implying that the observed dimerisation is not an artefact of intracellular retention and/or aggregation. Moreover, there is no significant difference between the lengths of C-terminal domains in the apparently monomeric and dimeric populations (Fig. 5.12), suggesting that the observed monomers are genuine and not the product of large C-terminal domains that lead to donor-acceptor separation distances greater than the BRET-permissive 10nm radius. Analysis of three receptors (C5R1, DP₂, and LTB₄R1) gave conflicting results or non-assignable signatures, and are discussed below (§5.3.4).

Analysis of the signalling biology of the proposed monomeric and dimeric receptors yielded no obvious differences in either physiological ligand (Fig. 5.13) or G α protein-coupling (Fig. 5.14), suggesting that dimerisation might neither be a prerequisite for nor impediment to interaction with specific ligands or signalling partners. The only minor differences between the monomer and dimer populations is that no monomers, but three of the proposed dimers bind sphingolipid-derived ligands, *e.g.* sphingosine 1-phosphate, and no dimers but five proposed monomers to bind eicosanoid ligands, *e.g.* prostaglandins, thromboxanes. Overall, there are very few differences between the two groups based on general ligand chemistry – *e.g.* lipid-derived *vs.* peptide ligands.

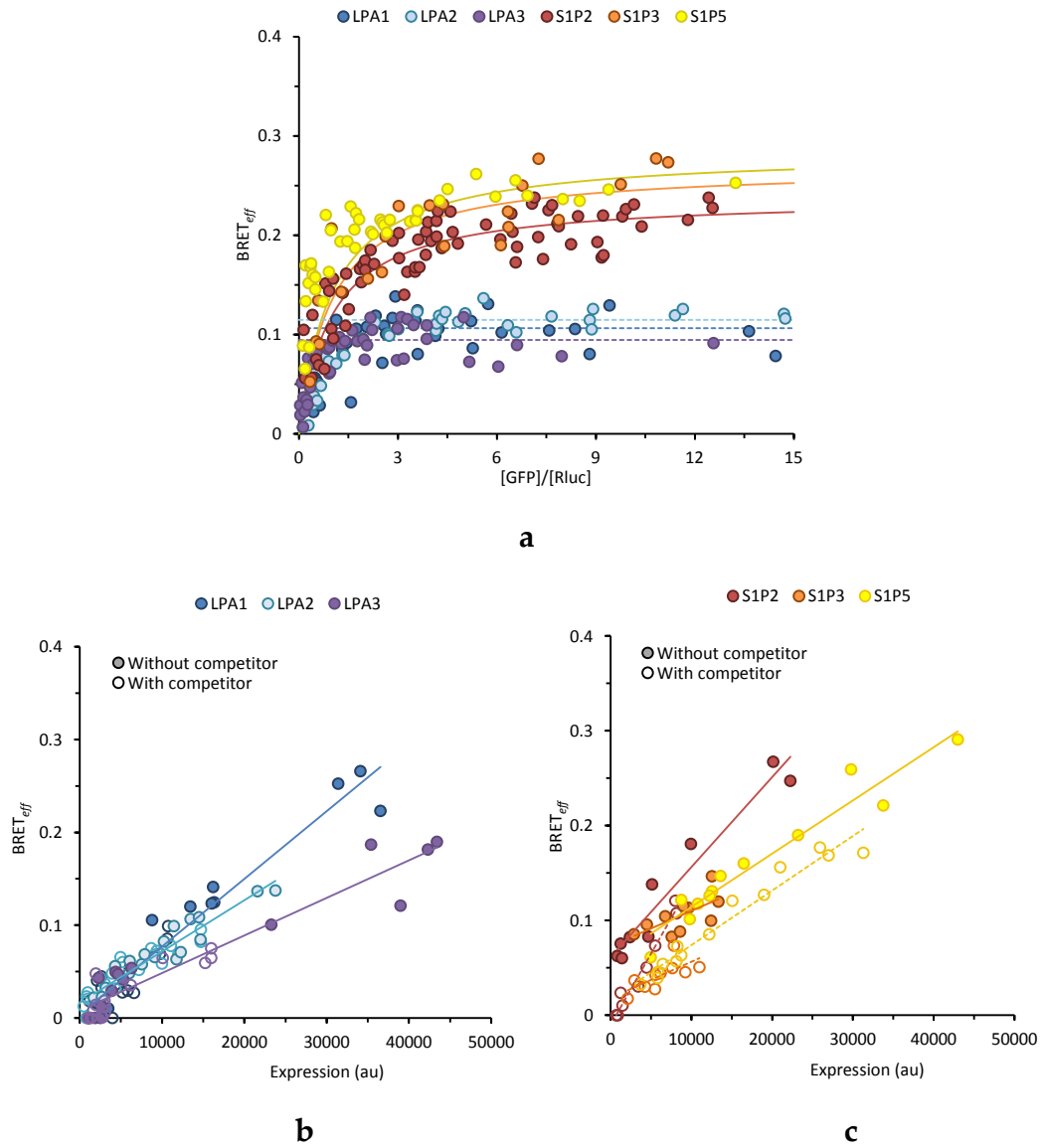


Figure 5.8: Example data obtained by type 1 and cBRET analysis in this chapter. Data shown were obtained for the S1P and LPA receptors, which make up the endothelial differentiation receptor family. (a) All three investigated LPA receptors yield type 1 $BRET_{eff}$ values consistent with those of a monomer, whereas the three S1P receptors give data that fit well to a dimer model. Data fits to a dimer model are shown with solid lines; fits to a monomer model are shown with broken lines. The optimal fit is shown in each case. (b) cBRET analysis of the LPA receptors displays no change in $BRET_{eff}$ -density relationship, consistent with monomeric behaviour. (c) S1P receptors demonstrate a significant drop in $BRET_{eff}$ relative to density in the presence of competitor receptors, indicating the presence of homodimers. For monomers, cBRET data are plotted as best fit of all points, indicated by a solid line. For dimers, 'with' and 'without' competitor data are fitted separately as solid and broken lines, respectively.

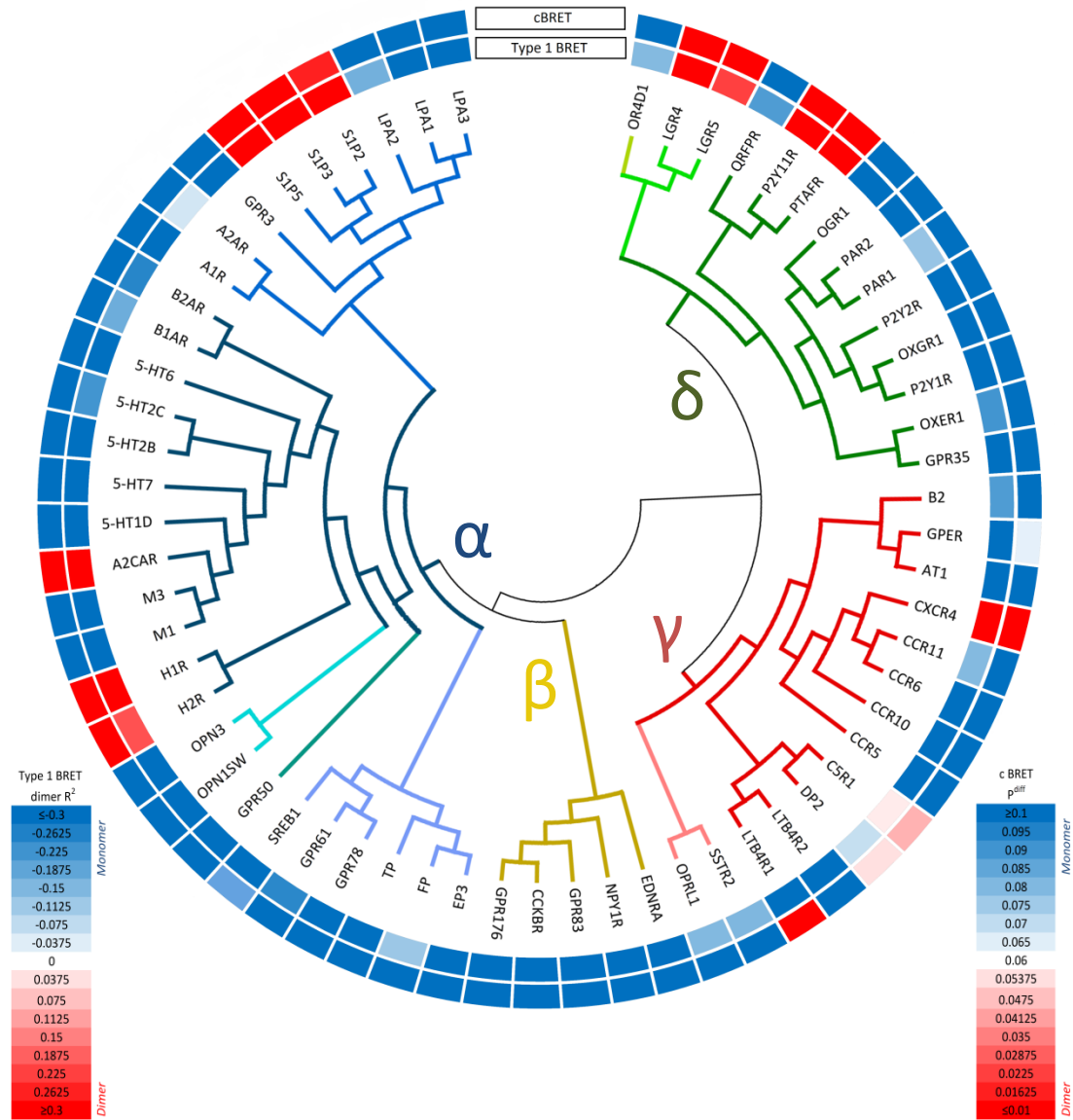


Figure 5.9: Summarised BRET-derived conclusions and phylogenetic relationships of all *Rhodopsin* GPCRs investigated in this thesis. Absolute values are given in Table 5.13. Type 1 BRET outcomes are represented by the R^2 value of the data in each case fitted to a dimer model. cBRET outcomes are represented by the P^{diff} value denoting significance of any difference between data taken in the presence and absence of competitor. In both cases, outcomes are shown as a gradient from blue (monomer) to red (dimer) corresponding to the values given in each key. The intensity of the relevant colour represents the degree of significance of the outcome: darker and paler colours denote more and less significant outcomes, respectively. The inner circle is coloured according to type 1 BRET, the outer is coloured according to cBRET. Phylogenetic relationships and branch colours are as in Figure 5.7. Both approaches agree in all cases except LTB4R1. C5R1 and DP₂ exhibit ambiguous outcomes in both experiments. The majority of receptors assessed exhibit apparently monomeric behaviour. Dimeric exceptions are often closely related, *e.g.* LGR4 and LGR5, H₁R and H₂R, and the S1P receptors.

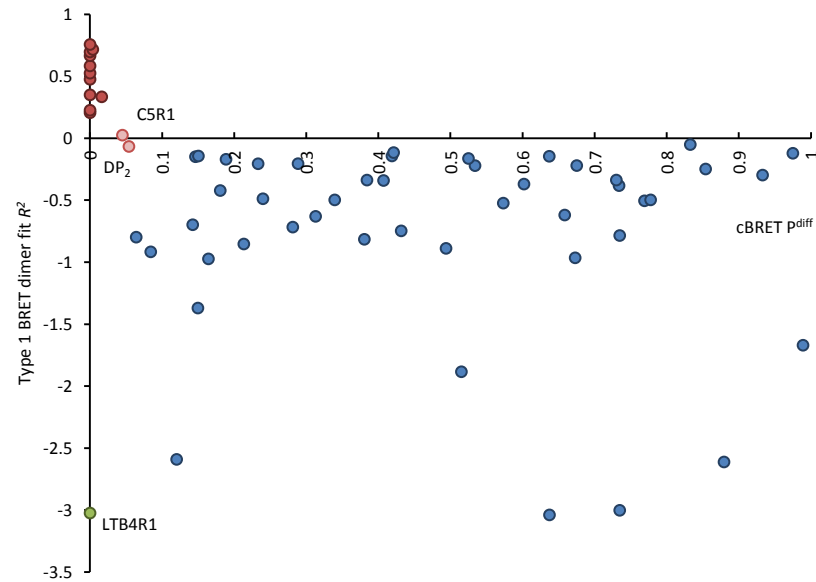


Figure 5.10: Concordance of outcomes obtained using the type 1 and cBRET assays. Data are summarised as the type 1 assay dimer fit R^2 value (a more positive value indicates a better fit to a dimer model) and the cBRET assay P^{diff} value (a lower value indicates a greater likelihood of specific interaction). Each point represents the data for a single receptor. Receptors concluded to be monomers are shown in blue, dimers in red. C5R1 and DP₂ are shown in pink, LTB4R1 in green. For the vast majority of receptors the data obtained under type 1 and cBRET analysis yield concurring outcomes, meaning that the apparently dimeric receptors cluster together in the region of high R^2 but low P^{diff} . C5R1 and DP₂ are ambiguous because they lie near the boundary of monomer *vs.* dimer outcome under both assays. LTB4R1 is the only receptor to yield conflicting results in the two assays, as it has a low dimer fit R^2 value but also a low P^{diff} value.

Protein	Type 1 dimer fit R^2	cBRET R^2 (- competitor)	cBRET R^2 (+ competitor)	cBRET P^{diff}	cBRET y -intercept	$\pm$	p=0.05 confidence
5-HT _{1D}	-0.33	0.80	0.44	0.7301	0.003	$\pm$	0.006
5-HT _{2B}	-0.50	0.63	0.51	0.7692	-0.006	$\pm$	0.019
5-HT _{2C}	-0.22	0.84	0.91	0.5341	-0.015	$\pm$	0.017
5-HT ₆	-0.71	0.80	0.68	0.2813	0.002	$\pm$	0.021
5-HT ₇	-0.85	0.96	0.57	0.2134	0.018	$\pm$	0.013
A ₁ R	-2.61	0.71	0.91	0.8795	-0.011	$\pm$	0.016
A _{2A} R	-0.05	0.82	0.77	0.8327	0.013	$\pm$	0.010
α_2 cAR	0.75	0.82	0.83	<0.0001	0.169	$\pm$	0.040
AT ₁	-0.81	0.93	0.92	0.3806	-0.016	$\pm$	0.039
β_1 AR	-0.17	0.70	0.43	0.1887	0.060	$\pm$	0.004
B ₂	-0.20	0.83	0.38	0.2886	0.015	$\pm$	0.007
C5R1	0.024	0.81	0.67	0.0450	0.071	$\pm$	0.010
CKBR	-0.52	0.97	0.63	0.5733	-0.003	$\pm$	0.007
CCR6	-0.63	0.87	0.72	0.3130	-0.010	$\pm$	0.012
CCR10	-1.37	0.77	0.75	0.1497	0.072	$\pm$	0.033
CCR11	-0.14	0.91	0.71	0.1504	-0.015	$\pm$	0.018
CXCR4	0.47	0.96	0.85	<0.0001	0.284	$\pm$	0.038
DP ₂	-0.06	0.83	0.68	0.0540	0.075	$\pm$	0.013
EDNRA	-0.29	0.80	0.73	0.9330	-0.008	$\pm$	0.008
EP ₃	-3.00	0.94	0.80	0.7348	0.029	$\pm$	0.014
FP	-0.11	0.84	0.85	0.4212	-0.010	$\pm$	0.014
GPBR	-0.79	0.76	0.55	0.0641	-0.006	$\pm$	0.017
GPR3	-0.62	0.96	0.97	0.6585	0.038	$\pm$	0.013
GPR35	-0.96	0.93	0.81	0.6730	0.015	$\pm$	0.021
GPR50	-0.37	0.91	0.76	0.6020	0.009	$\pm$	0.013
GPR61	-0.24	0.95	0.83	0.8540	0.000	$\pm$	0.003
GPR78	-0.78	0.63	0.74	0.7348	-0.025	$\pm$	0.034
GPR83	-0.42	0.96	0.79	0.1807	0.004	$\pm$	0.008
GPR176	-1.88	0.85	0.96	0.5152	0.000	$\pm$	0.025
H ₁ R	0.34	0.69	0.43	<0.0001	0.046	$\pm$	0.018
H ₂ R	0.20	0.69	0.61	<0.0001	0.058	$\pm$	0.020
LGR4	0.69	0.76	0.61	<0.0001	0.031	$\pm$	0.011
LGR5	0.22	0.80	0.83	<0.0001	0.072	$\pm$	0.013
LPA ₁	-0.49	0.94	0.61	0.7777	0.005	$\pm$	0.020
LPA ₂	-0.16	0.92	0.87	0.5250	0.023	$\pm$	0.008
LPA ₃	-0.48	0.90	0.77	0.2399	0.014	$\pm$	0.016
LTB4R1	-3.02	0.64	0.67	<0.0001	0.082	$\pm$	0.029
LTB4R2	-0.88	0.90	0.44	0.4939	-0.001	$\pm$	0.013
M ₃	-0.74	0.81	0.67	0.4317	-0.004	$\pm$	0.016
NPY1R	-0.49	0.92	0.91	0.3395	-0.005	$\pm$	0.007
O4RD1	-0.14	0.76	0.81	0.4189	-0.015	$\pm$	0.022
OGR1	-0.33	0.61	0.78	0.3842	-0.048	$\pm$	0.057
OPN1SW	-1.67	0.89	0.82	0.9891	0.005	$\pm$	0.006
OPN3	-2.59	0.72	0.94	0.1202	0.004	$\pm$	0.082
OPRL1	-0.14	0.71	0.76	0.6371	0.020	$\pm$	0.034
OXER1	-0.22	0.60	0.52	0.6752	0.001	$\pm$	0.006
OXGR1	-0.97	0.92	0.91	0.1643	0.001	$\pm$	0.038
P2Y ₁ R	-0.34	0.84	0.91	0.4072	-0.002	$\pm$	0.007
P2Y ₂ R	-0.38	0.94	0.69	0.7339	0.002	$\pm$	0.015
P2Y ₁₁ R	0.58	0.86	0.50	<0.0001	0.192	$\pm$	0.016
PAR1	-0.12	0.73	0.87	0.9750	-0.063	$\pm$	0.139
PAR2	-3.04	0.95	0.86	0.6374	0.023	$\pm$	0.016
PTAFR	0.71	0.81	0.85	0.0039	0.119	$\pm$	0.022
QRFPFR	-0.21	0.87	0.62	0.2332	0.023	$\pm$	0.016
S1P ₂	0.33	0.94	0.96	0.0163	0.061	$\pm$	0.022
S1P ₃	0.52	0.43	0.47	<0.0001	0.072	$\pm$	0.032
S1P ₅	0.66	0.93	0.95	<0.0001	0.058	$\pm$	0.021
SREB1	-0.91	0.73	0.66	0.0843	0.011	$\pm$	0.032
SSTR2	-0.14	0.90	0.77	0.1463	0.039	$\pm$	0.022
TP	-0.69	0.82	0.82	0.1425	0.022	$\pm$	0.014

Table 5.13: Type 1 and cBRET values obtained in this chapter. cBRET y -intercept values are from fit of data collected in the absence of competitor and are given with 95% confidence limits (student t test).

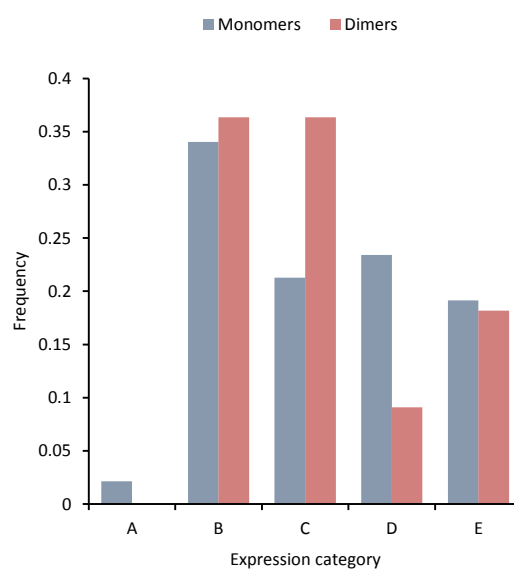


Figure 5.11: Apparent monomers and dimers demonstrate similar expression profiles. This indicates that apparent dimers are not artefacts of intracellular retention. C5R1, DP₂, and LTB₄R1 are not included.

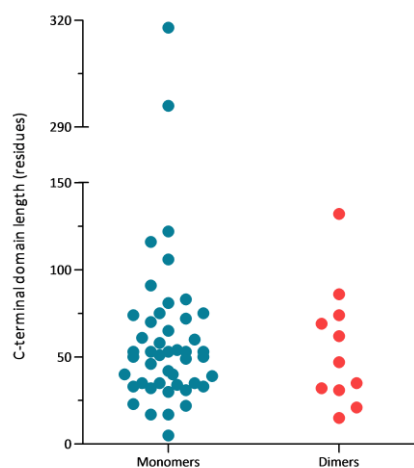


Figure 5.12: Apparent monomers and dimers have comparable lengths of their C-terminal domains. This suggests that the observed monomers are genuine and are not the mis-assignment of dimers with large C-terminal domains that preclude efficient energy transfer.

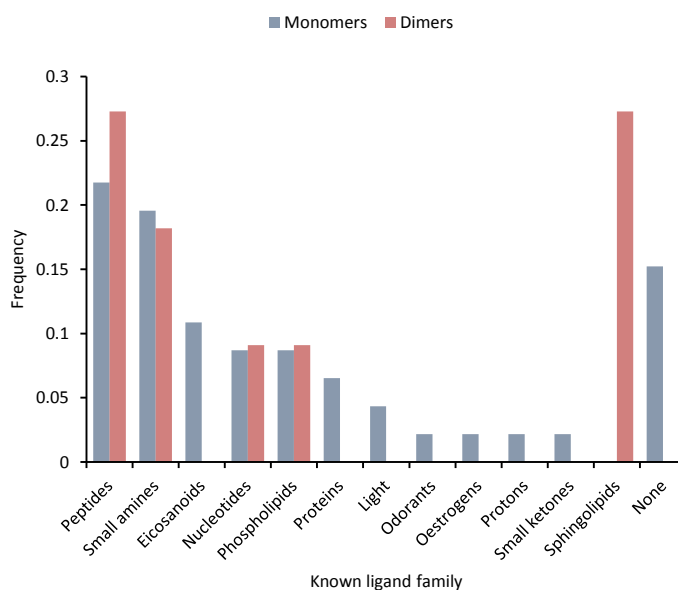


Figure 5.13: Frequency of known ligand selectivity by receptors reported as monomeric and dimeric in this chapter. Ligands are grouped into broad categories based on their size and chemistry. The common ligands that are exclusively recognised by monomeric receptors are the eicosanoids, whilst only sphingolipids are recognised only by dimers. Broadly, there are no obvious differences in the ligand selectivity of the monomeric and dimeric receptors.

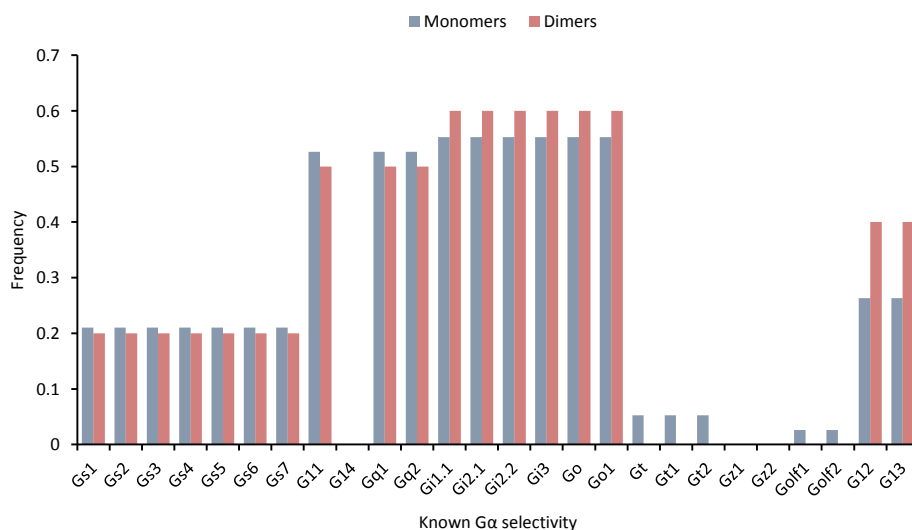


Figure 5.14: Frequency of known Gα protein-coupling by apparently monomeric and dimeric receptors. No significant preference is observed for a specific Gα subset by either group of receptors relative to the other. Information regarding the Gα selectivity of each receptor was obtained through the gpDB database (483). Receptors for which no information was available were excluded from the analysis.

α subfamily

28 α subfamily receptors were investigated in this study, 6 of which (α_2 cAR, H₁R, H₂R, S1P₂, S1P₃, and S1P₅) exhibited apparently dimeric behaviour. All of the α subfamily receptors exhibited equivalent behaviour in the type 1 and cBRET assays. Including β_2 AR and M₁, investigated in previous chapters, 20% of the α subfamily GPCRs studied have been suggested to be homodimers. The α subfamily is the most comprehensively investigated in this study, as ~31% of all known members were directly analysed, although some receptor clusters were more thoroughly covered than others. For example, no dopamine or cannabinoid receptors but all 3 LPA receptors were analysed.

β subfamily

The β -subfamily was the only subfamily to yield no evidence of dimerisation for any constituent receptor. All five β -subfamily receptors yielded data in each assay that was consistent with monomeric behaviour. Unlike the other subfamilies, the five β -subfamily receptors investigated share little conserved sequence identity (Fig. 5.15) and no ligands (Tables 5.9 & 5.10). In this sense they represent a broad cross-section of the larger β subfamily, of which there are 35 members. This makes the β subfamily the least comprehensively studied *Rhodopsin* subfamily in this analysis, with only ~14% of its members investigated.

γ subfamily

13 γ -subfamily members were investigated in this study, which, combined with CCR5 means that ~22% of the subfamily was investigated using the type 1 and cBRET assays. Only one γ subfamily receptor (CXCR4) yielded data consistent with dimeric behaviour, although 3 receptors (C5R1, DP₂, and LTB₄R1) could not be clearly assigned as either monomers or dimers (§5.3.4). After the β subfamily, therefore, the γ subfamily contains the next smallest proportion of known dimers, with only 10% of receptors exhibiting clear-cut apparently dimeric behaviour.

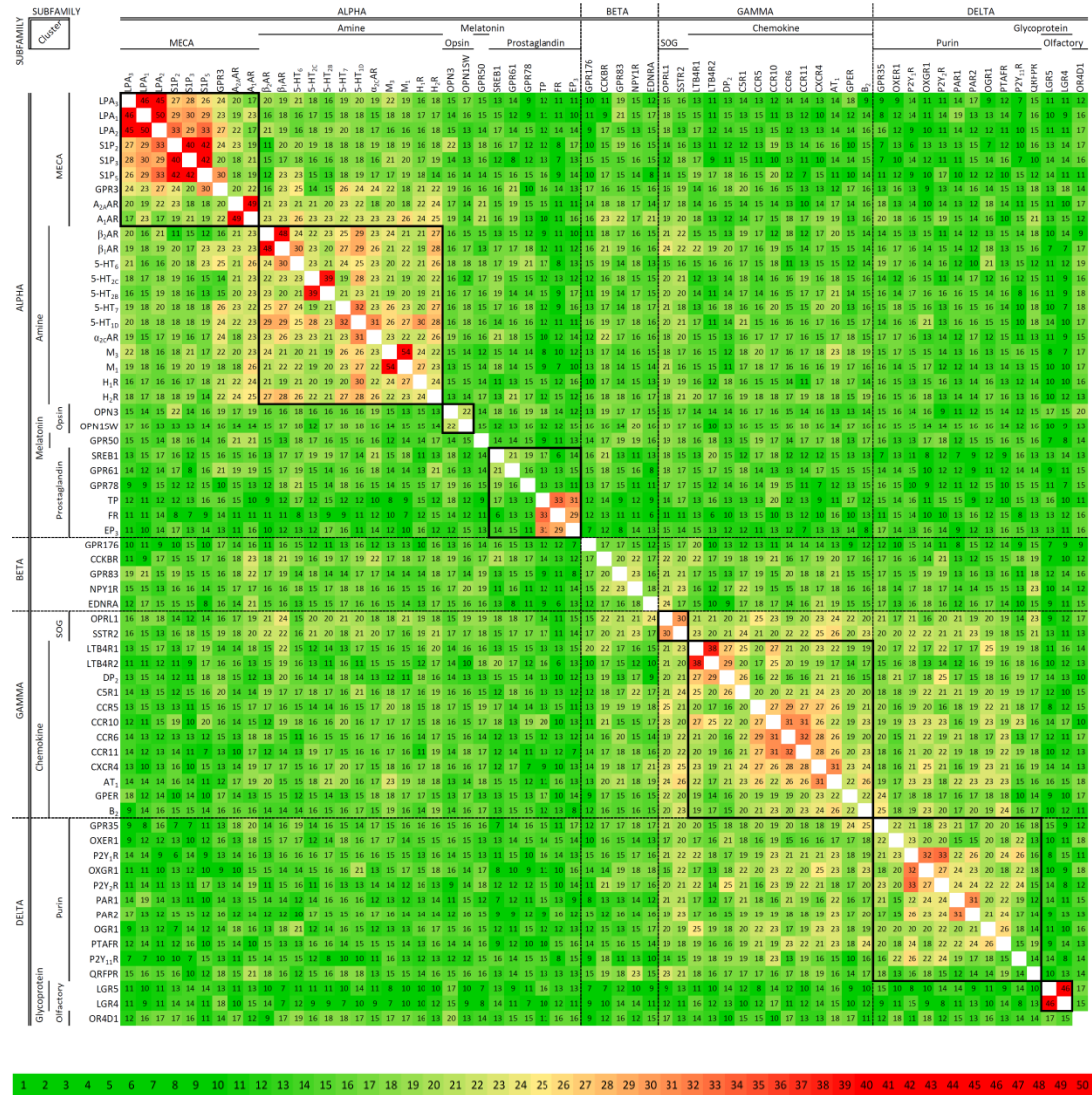


Figure 5.15: Percentage sequence identity between the *Rhodopsin* family GPCRs that have been investigated using type 1 and cBRET. Identity values were calculated by pairwise amino acid sequence alignment using the ClustalW 2.0.12 software (484) and indicate the percentage conservation between the two respective receptors. Values are coloured green to red in order of increasing sequence identity. Alpha, beta, gamma, and delta subfamilies are denoted by broken lines; receptor clusters are indicated by solid boxes.

δ subfamily

Stoichiometry was assessed for 14 members of the δ subfamily: 13 non-olfactory and one olfactory (OR4D1) receptor. There are 59 non-olfactory receptors within the larger subfamily, and so ~22% were investigated here. Of these, LGR4, LGR5, P2Y₁₁R, and PTAFR exhibited apparently dimeric behaviour whilst the remaining 10 receptors gave no evidence of dimerisation. Thus, type 1 and cBRET suggest dimerisation for ~28% of the analysed δ subfamily GPCRs, a larger fraction than for any other *Rhodopsin* subfamily.

Interpretation of cBRET y -intercept values

In most cases, receptors identified as monomers under type 1 and cBRET analysis also exhibited y -intercept values of BRET_{eff} vs. expression at or below zero when measured in the absence of competitor in cBRET (Fig. 5.16 & Table 5.13). This is analogous to a type 2 BRET assay, in which non-zero intercepts are taken to indicate dimerisation. Accordingly, all identified dimers had y -intercept values significantly greater than zero. This strengthens the conclusions regarding stoichiometry in each case. A number of monomeric receptors did, however, yield y -intercept values that were significantly above zero ($p=0.05$). Most of these values had lower confidence limits that were only very marginally above zero, *i.e.* <0.01 (5-HT₇, A_{2A}R, B₂, PAR2, QRFPR, and TB), although some were slightly higher (β_1 AR, CCR10, EP₃, GPR3, LPA₂, SSTR2) but below the y -intercept values typically observed for dimers (Table 5.13).

Interpretation of type 1 BRET_{max}

Whilst it is true that BRET_{max} is inappropriate as a direct measure of stoichiometry, it can nonetheless be informative in cases for which additional information regarding expression level and subcellular distribution is available. Type 1 BRET_{max} values observed for monomers were generally lower than those observed for dimers and also showed some correlation with expression level, such that more weakly expressed receptors also gave the lowest BRET_{max} (Fig. 5.17), as would be expected. However, a number of monomers exhibited BRET_{max} values far higher than the majority of receptors at similar expression levels, principally CCR10, OPN3, OPRL1, and OXGR1. These receptors also exhibit steeper slopes of BRET_{eff} vs. expression level in cBRET assays (Figs. A.6b, A.9b, A.12b, & A.13b).

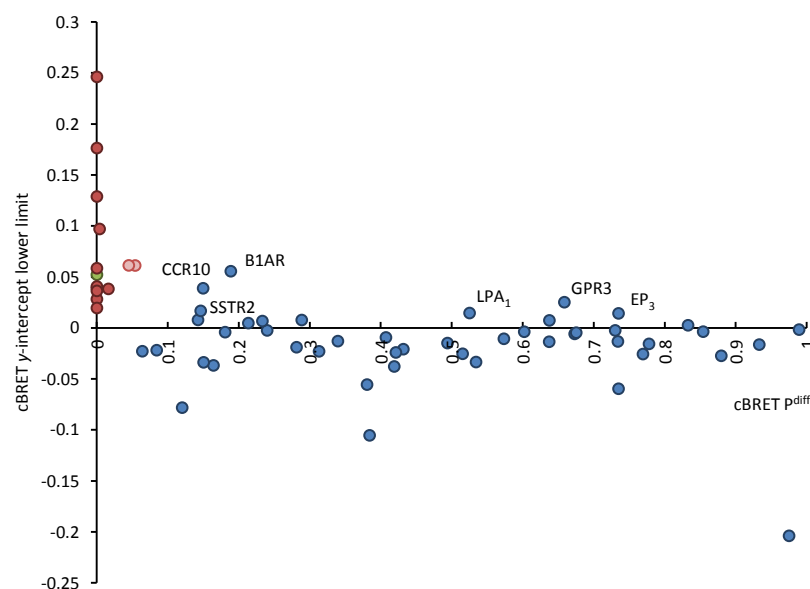


Figure 5.16: y -intercept values obtained using cBRET in the absence of competitor generally correlate with apparent stoichiometry. Receptors concluded to be monomers are shown in blue, dimers in red. C5R1 and DP₂ are shown in pink, LTB₄R1 in green. All apparent dimers yield a y -intercept value with a lower 95% confidence limit that is above zero, as do the ambiguous cases C5R1, DP₂, and LTB₄R1. The majority of apparently monomeric receptors have y -intercept values that are not significantly non-zero, although a small number have lower 95% confidence limits greater than zero. The six most significant of these (β_1 AR, CCR10, EP₃, GPR3, LPA₁, and SSTR2) are labelled.

BRET_{max} of dimeric receptors does not appear to show any correlation with expression level (Fig. 5.17), which is as expected because BRET_{max} for dimers is far more dependent on relative positioning of the acceptor and donor proteins than upon total receptor concentration. BRET_{max} values observed for dimers ranged from 0.1139 (H₁R) to 0.9930 (CXCR4), although most were within the range of 0.2-0.4 (Table 5.14).

5.3.4 Ambiguous cases

C5R1 and DP₂

C5R1 and DP₂ failed to give data corresponding to either monomeric or dimeric behaviour. Data obtained using the type 1 BRET assay for C5R1 fitted marginally better to a dimer model ($R^2=0.02$) than to a monomer (Fig. 5.18), though analysis of the residual BRET_{eff} distribution shows that neither model accurately describes the data (Fig. 5.19). A similar distribution is observed for DP₂, which yielded data fitting slightly better to a monomer (dimer $R^2=-0.06$), but without closely corresponding to either model. The data obtained for these receptors using the cBRET assay is similarly inconclusive as both receptors appear to show very marginal decreases in BRET_{eff} in the presence of competitor (Fig. 5.20b). For C5R1, the difference between data taken in the presence and absence of competitor protein is barely significant ($P^{\text{diff}}=0.0450$), whereas for DP₂ it is just outside 95% significance ($P^{\text{diff}}=0.0540$). In both cases it is difficult to confidently rule out experimental noise. The decrease in BRET_{eff} for both receptors, if it is genuine, is also only minimal.

LTB4R1

The type 1 BRET assay data for LTB4R1 fit far more closely to a constant value than to the hyperbolic relationship expected of a dimer (Fig. 5.18), however cBRET analysis revealed a BRET_{eff} decrease in the presence of competitor that is highly significant ($P^{\text{diff}}<0.001$; Fig. 5.20a). The receptor cannot therefore be unambiguously assigned as a monomer or dimer. In fact, the type 1 BRET data collected for LTB4R1 fit more closely to distributions predicted by Equation 1.2 for trimer ($R^2=0.38$) and tetramer ($R^2=0.31$) models than to the constant BRET_{eff} expected for monomers (Fig. 5.19).

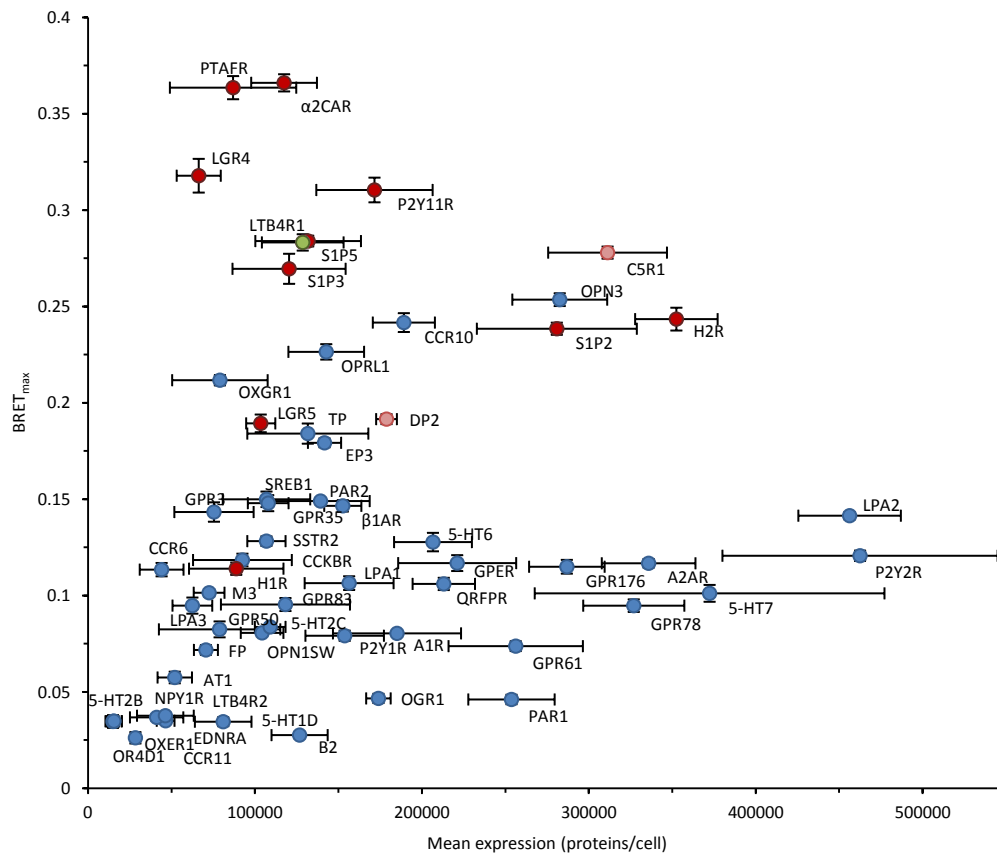


Figure 5.17: Relationship between BRET_{max} and expression level for HEK 293T *Rhodopsin* GPCRs. BRET_{max} values for monomers are generally lower than those of dimers at similar expression levels. Monomeric receptors are shown in blue; dimers in red. The ambiguous cases C5R1 and DP₂ are shown in pink. LTB4R1 is shown in green. Protein names are located as close to their respective points as possible. Error bars indicate SEM. of each parameter. CXCR4 is not shown for clarity. Absolute values are given in Table 5.14.

Protein	BRET _{max}	±	SEM	Proteins/cell	±	SEM
5-HT _{1D}	0.034	±	0.002	80960	±	16955
5-HT _{2B}	0.034	±	0.001	15480	±	4715
5-HT _{2C}	0.083	±	0.001	109109	±	9187
5-HT ₆	0.127	±	0.004	206648	±	23319
5-HT ₇	0.101	±	0.004	372375	±	104774
A ₁ R	0.080	±	0.001	185087	±	38365
A _{2A} R	0.116	±	0.002	335834	±	28060
α _{2C} AR	0.366	±	0.004	117431	±	19692
AT ₁	0.057	±	0.003	51951	±	10306
β ₁ AR	0.146	±	0.002	152616	±	11127
B ₂	0.027	±	0.001	126734	±	16815
C5R1	0.277	±	0.003	311236	±	35590
CCKBR	0.118	±	0.003	92481	±	29590
CCR6	0.113	±	0.003	44071	±	13121
CCR10	0.241	±	0.004	189197	±	18596
CCR11	0.036	±	0.001	41086	±	15983
CXCR4	0.993	±	0.013	76981	±	15371
DP ₂	0.191	±	0.002	178811	±	6214
EDNRA	0.034	±	0.003	14944	±	3517
EP ₃	0.179	±	0.002	141661	±	9939
FP	0.071	±	0.002	70604	±	7183
GP _{ER}	0.116	±	0.004	221139	±	35349
GPR3	0.143	±	0.005	75441	±	23750
GPR35	0.147	±	0.004	107918	±	12199
GPR50	0.082	±	0.004	78801	±	36367
GPR61	0.073	±	0.002	256216	±	40251
GPR78	0.094	±	0.003	326944	±	30287
GPR83	0.095	±	0.003	118252	±	38663
GPR176	0.114	±	0.003	286852	±	22627
H ₁ R	0.113	±	0.003	88773	±	28295
H ₂ R	0.243	±	0.005	352466	±	24714
LGR4	0.317	±	0.008	66295	±	13201
LGR5	0.189	±	0.004	103449	±	8729
LPA ₁	0.106	±	0.003	156402	±	26635
LPA ₂	0.141	±	0.001	456252	±	30709
LPA ₃	0.094	±	0.004	62491	±	11906
LTB ₄ R1	0.283	±	0.004	128616	±	24474
LTB ₄ R2	0.034	±	0.001	46471	±	5319
M ₃	0.101	±	0.001	72517	±	9218
NPY1R	0.037	±	0.001	46323	±	17001
O4RD1	0.026	±	0.002	28366	±	2276
OGR1	0.046	±	0.002	173963	±	7343
OPN1SW	0.080	±	0.001	104256	±	12777
OPN3	0.253	±	0.003	282591	±	28423
OPRL1	0.226	±	0.004	142698	±	22670
OXER1	0.034	±	0.001	15312	±	4902
OXGR1	0.211	±	0.002	78992	±	28608
P2Y ₁ R	0.079	±	0.002	153744	±	23515
P2Y ₂ R	0.120	±	0.002	462486	±	82421
P2Y ₁₁ R	0.310	±	0.006	171599	±	34847
PAR1	0.046	±	0.002	253673	±	25871
PAR2	0.149	±	0.001	139277	±	29441
PTAFR	0.363	±	0.006	86875	±	37848
QRFPR	0.106	±	0.003	213127	±	18690
S1P ₂	0.238	±	0.003	280852	±	47917
S1P ₃	0.269	±	0.007	120421	±	33884
S1P ₅	0.283	±	0.002	131847	±	31642
SREB1	0.149	±	0.004	106901	±	26190
SSTR2	0.128	±	0.002	106858	±	11452
TP	0.184	±	0.005	131662	±	36267

Table 5.14: BRET_{max} and receptor expression values of HEK 293T *Rhodopsin* GPCRs ± SEM.

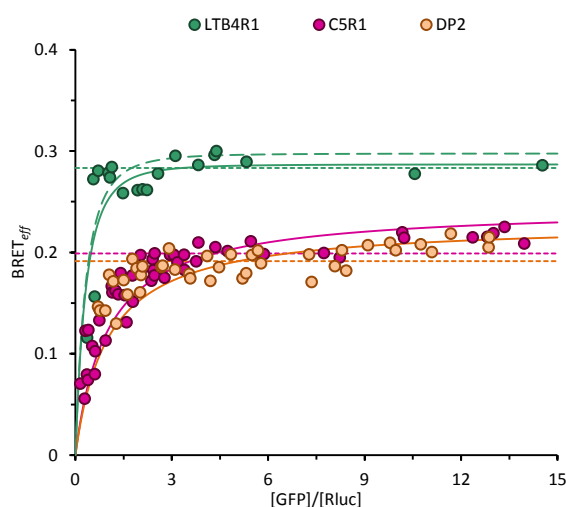


Figure 5.18: Type 1 BRET analysis of the non-classical chemokine receptors LTB4R1, C5R1, and DP2. The distributions of points for C5R1 and DP2 do not fit clearly to either a monomer or dimer model, but perhaps indicate weak dimerisation. LTB4R1 data fit well to a monomer model but also to predictions of trimeric and tetrameric behaviour described by Equation 1.2. Fits to monomeric models in all cases are shown as finely broken lines. Dimer models are shown for C5R1 and DP2 as solid lines. For LTB4R1, a trimer model is represented by a widely broken line, whilst a tetramer model is shown as a solid line.

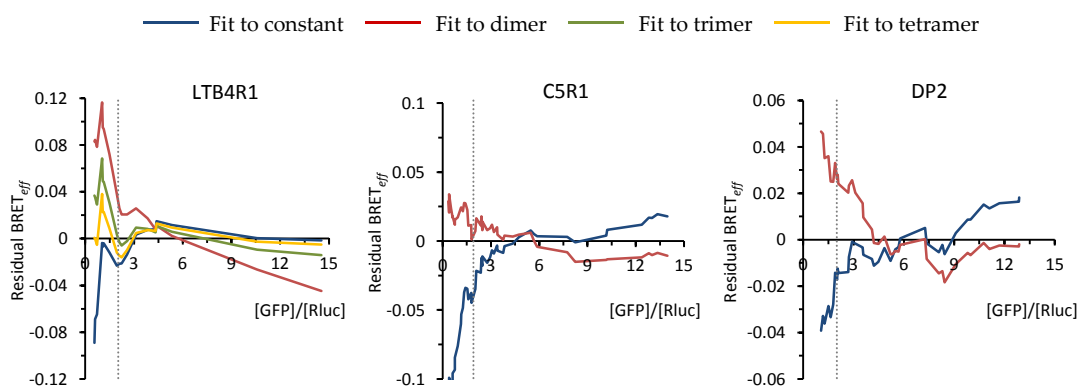


Figure 5.19: Residual BRET_{eff} plots of LTB4R1, C5R1, and DP2 fitted to models of varying stoichiometry according to Equation 1.2. Fits to constant (monomer), trimer, and tetramer models are similarly good descriptions of the data for LTB4R1, as all three reduce residual BRET_{eff} values to near zero. For C5R1 and DP2, neither model appears to adequately describe the data as both demonstrate poor minimisation of residual BRET_{eff}.

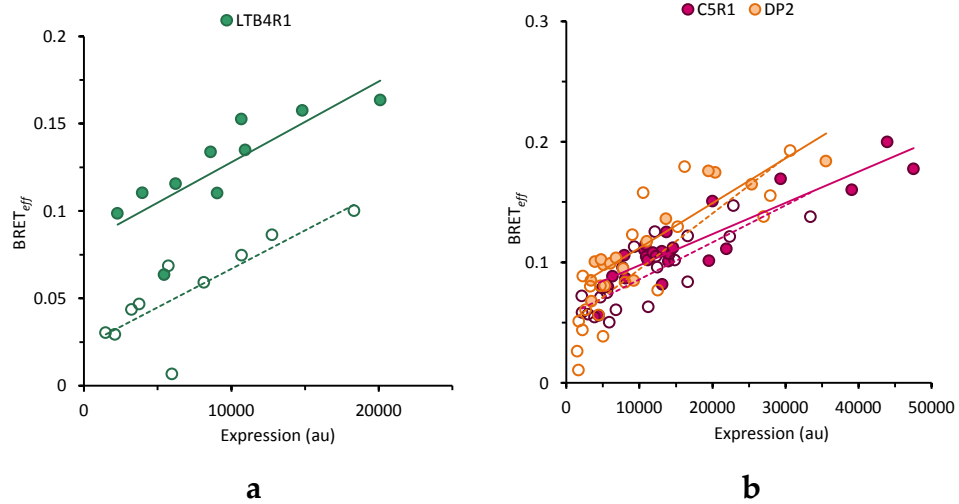


Figure 5.20: cBRET analysis of the non-classical chemokine receptors LTB4R1, C5R1, and DP2. **(a)** LTB4R1 demonstrates a significant change in BRET_{eff}-expression relationship in the presence of competitor, indicative of specific interaction. **(b)** C5R1 and DP2 exhibit marginal decreases in BRET_{eff} in the presence of competitor, although this is difficult to resolve unambiguously within the inherent experimental error. This could be indicative of very weak dimerisation or C5R1 and DP2. For monomers, data are plotted as best fit of all points, indicated by a solid line. For dimers, 'with' and 'without' competitor data are fitted separately as solid and broken lines, respectively.

5.4 Discussion

5.4.1 Dimerisation is not detected for the majority of *Rhodopsin* family GPCRs

Investigation of the stoichiometry of 60 *Rhodopsin* family GPCRs expressed by HEK 293T cells, using the complementary approaches of type 1 and cBRET, has revealed that 46 exhibit monomeric behaviour according to these assays. When combined with the previous observations that β_2 AR, CCR5, and M₁ are also monomeric (§3.4, §4.4), more than 80% of the *Rhodopsin* receptors directly investigated appear to behave as monomers. It is important to note, however, that the sensitivity limits of the BRET assays used mean that the existence of low levels of dimers cannot be ruled out. Although two distinct forms of behaviour have been observed within the *Rhodopsin* family, this could be explained by separate populations of monomers and dimers, or by the existence of two forms of dimers; one that is sufficiently extensive to be detected by type 1 and cBRET, and the other that represents a fraction of the total receptor population below the sensitivity limit of the assays (*i.e.* ~10-20%). For the purposes of brevity, those receptors that do not exhibit detectable levels of dimerisation are hereafter referred to as apparent monomers, though their absolute monomericity cannot be asserted from these data. There is no reason to suspect that the receptors studied here are not a representative cross-section of the *Rhodopsin* family as they bind to a diverse array of ligands and fulfil a broad range of physiological functions. As a group, the 60 *Rhodopsin* GPCRs natively expressed in HEK 293T cells have functions that might be expected for both endothelial and neuronal cells, consistent with previous observations of neuronal protein expression by these cells (371). Overall, the GPCRs studied here correspond to ~20% of the entire human non-olfactory *Rhodopsin* repertoire and correspond to a significant fraction of the family as a whole.

5.4.2 Examination of y -intercept and BRET_{max} values

y -intercept

In most cases, the y -intercept values of individual receptors correlated with their apparent stoichiometry as indicated by the type 1 R^2 and cBRET P^{diff} values – *i.e.* a zero intercept for monomers and non-zero for dimers. Although a small number of apparently monomeric receptors yielded non-zero intercepts, this does not suggest dimerisation on its own as y -intercept value is not the primary

read-out of the cBRET assay. This is because the cBRET assay is performed at higher expression levels than the type 2 BRET approach, which uses y -intercept as its only informative parameter. For this reason, the cBRET approach is less well suited to giving reliable y -intercept values as the slope of BRET_{eff} vs. expression can change at higher protein densities and so artefactually increase the y -intercept estimated by linear regression. Thus, despite the fact that in some cases non-zero intercepts are observed for otherwise apparently monomeric receptors, this does not undermine the conclusion that the relevant GPCRs are monomers. Nonetheless, the general correlation between stoichiometry and y -intercept suggests that for many cases the initial measurements were performed at sufficiently low expression levels to give reliable intercept values.

BRET_{max}

The majority of receptors yielded BRET_{max} values within the range expected of their apparent stoichiometry; *i.e.* higher for dimers than for monomers (Fig. 5.17). Nonetheless, a small number of apparent monomers did exhibit higher than expected BRET_{max} values given their respective expression levels. Nonetheless, BRET_{max} is dependent on a broad range of factors independent of stoichiometry, and so a high BRET_{max} does not in itself undermine an assertion of monomericity. The observation that many of these receptors exhibit steep slopes of BRET_{eff} vs. expression in the cBRET assay explains their high BRET_{max} values and suggests that the intramembrane distribution of these receptors may be different from that of monomers with less steep slopes and lower BRET_{max} values. In the case of OPN3, this may be due to partial receptor activation by ambient light during data collection, which might have led to internalisation and thus clustering in clathrin-coated pits. Such an effect may explain why both OPN3 and OPN1SW exhibited category D-type cellular fluorescence staining (Fig. 5.7), although this fails to explain the low BRET_{max} for OPN1SW.

The very high BRET_{max} of CXCR4 (0.993), which is comparable to that expected of covalent type 1 TM protein dimers, suggests that the N termini of the two CXCR4 protomers within the complex are positioned very close together, allowing high degrees of energy transfer. It also suggests that the receptor constitutively dimerises at a fairly high level, because a large monomeric fraction would be expected to preclude such a high BRET_{max} . An addition explanation, however, is that CXCR4 is highly clustered on the cell surface and so may experience a large degree of non-specific interactions in addition to the specific interaction within the dimer complex. If true, it would be expected that this would lead to a poor fit to a dimer model under type 1 BRET analysis. This is not observed, which

argues against this model. Nonetheless, the observation that CXCR4 overexpression induces a change in cellular morphology in HEK 293T cells (Fig. 5.6) indicates that the receptor may possess a significant degree of basal signalling activity, and so may undergo interactions with signalling or internalisation machinery that induce some degree of receptor clustering.

5.4.3 Interpretation of ambiguous cases

C5R1 and DP₂

C5R1 and DP₂ failed to yield data that fit closely to either the monomer or dimer models. In both cases, the data obtained under the type 1 assay seem consistent with a very weak dimer as BRET_{eff} is slightly increased at high [GFP]/[Rluc] values of ~12 compared to lower values of ~3, but the hyperbolic relationship expected of a dimer is not evident (Fig. 5.18). Data collected in the cBRET assay for each receptor also appear to reflect weakly dimeric behaviour as BRET_{eff} is marginally decreased in the presence of competitor (Fig. 5.20), but with a significance only very slightly above or below 95% confidence (C5R1 $P^{\text{diff}}=0.0450$; DP₂ $P^{\text{diff}}=0.0540$). The simplest and most likely explanation for these observations is that these receptors undergo only very low levels of dimerisation that are near the limits of detection sensitivity for the type 1 and cBRET assays.

LTB4R1

LTB4R1 was the only receptor that yielded contrasting conclusions in the type 1 and cBRET assays; exhibiting apparently monomeric behaviour under type 1 analysis but also yielding data in the cBRET assay that is consistent with specific receptor-receptor interactions. Unlike C5R1 and DP₂, however, both experiments give statistically significant results and so it is unlikely that LTB4R1 represents a weak dimer that is near the sensitivity limit of the techniques. It is also unlikely that these contrasting results are the product of aggregation as LTB4R1 was placed within expression category A. Instead, the most obvious explanation is that LTB4R1 forms higher-order oligomers. Oligomers would exhibit a decrease in BRET_{eff} in the presence of competitor in the cBRET assay, and would also fit poorly to a dimer model under type 1 analysis. The suggestion of LTB4R1 oligomerisation is supported by the fact that the type 1 BRET data for LTB4R1 fit more closely to trimer and tetramer models than to that expected of a monomer (Fig. 5.18). The fact that the data fit similarly well to both models could imply that the receptor exists within a dynamic equilibrium of stoichiometries, perhaps transitioning

between individual stable dimers and transient tetramers formed as dimers of dimers. Higher-order oligomerisation would also explain the high $BRET_{max}$ value observed for LTR4B1 compared to most apparently monomeric receptors.

5.4.4 Dimeric receptors are clustered phylogenetically

Of the eleven GPCRs exhibiting dimeric behaviour in this analysis, seven have close phylogenetic relationships with other apparent dimers. Three distinct receptor clusters are evident for which the closely related receptors are exclusively dimeric: the S1P receptors S1P₂, S1P₃, and S1P₅; the histamine receptors H₁R and H₂R; and the leucine-rich repeat containing receptors LGR4 and LGR5. The phylogenetic distribution of the analysed GPCRs and their behaviour in the BRET assays are summarised in Figure 5.9, while the sequence identity between receptors is given in Figure 5.14. Both the S1P and LGR clusters share high degrees of sequence identity (>40%), which suggests that the mechanism of dimerisation in these cases is likely conserved between receptors. The histamine receptors are less similar, sharing only 24% identity. However, given the rarity of apparent dimers among the *Rhodopsin* GPCRs it seems likely that H₁R and H₂R emerged from a common dimeric ancestor rather than undergoing separate gain-of-dimerisation events. The remaining non-clustered dimers (α_2 CAR, CXCR4, P2Y₁₁R, and PTAFR) do not show substantial levels of sequence identity with other dimers, although several close relatives of these receptors exist that were not investigated here may dimerise, *e.g.* α_2 AAR, α_2 BAR *etc.*

Whilst the data derived from both the type 1 and cBRET assays concur in their conclusion of dimerisation for these eleven receptors, it is possible that the dimers observed are not physiologically relevant. In some cases dimers may only form under conditions of overexpression if the strength of interaction is weak. Alternatively, overexpression may cause a greater degree of misfolding in the expressed receptors that may lead to partial aggregation. Although confocal microscopy has eliminated large scale protein aggregation as a possibility for these receptors, it is possible that partially misfolded receptors may interact in a non-physiological manner that is not detectable by confocal microscopy. Although this is a possibility, it is unlikely as the expression profiles of the apparently monomeric and dimeric receptors are broadly similar (Fig. 5.11), and it is likely that low levels of misfolding would also translate to a greater degree of large scale aggregation. Thus, the simplest explanation of the collected BRET data is that these receptors form a significant fraction of dimeric complexes in a physiologically relevant manner.

It is also important to note that although apparent dimerisation has been identified in these cases, it is not possible to accurately quantify the extent of dimerisation in each case given the data available. To some extent, the relative degrees of dimerisation of some closely related receptors can be estimated based on their individual type 1 and cBRET profiles – for example, H₁R exhibits a lower type 1 BRET_{max} (Table 5.14) and a smaller relative change in BRET_{eff} in the cBRET assay (Fig. A.5) than H₂R, and so could be concluded to be less extensively dimeric. However, such interpretation is potentially inappropriate due to the numerous factors affecting the efficiency of transfer within dimeric complexes. Thus, except in the cases of the possible weak dimers C5R1 and DP₂, the outcome of these assays is broadly binary; indicating the presence or absence of apparent dimerisation but not the extent.

CHAPTER 6

Mechanisms of GPCR dimerisation

6.1 Introduction

6.1.1 Postulated mechanisms of GPCR dimerisation

Although the notion of GPCR dimerisation has existed for over twenty years, the possible means by which receptor-receptor interactions are achieved, where it occurs, are mostly unknown. It is only for the *Glutamate* family GPCRs that a widely accepted mechanism of dimerisation has been elucidated. As discussed previously (§1.2), large N- and C-terminal domains drive the dimerisation of these receptors without obvious contributions from the 7TM regions (133). A similar mechanism has been proposed for the *Frizzled* receptors (§1.2), which also have large N-terminal extensions that may be capable of inter-receptor interactions (121, 140). This raises the question of how GPCRs lacking large extra- and intracellular domains, such as the *Rhodopsin* and *Taste2* receptors, might mediate dimerisation. Crystallographic and RET studies suggest that the TM regions may drive dimer formation in *Rhodopsin* GPCRs. The most common regions mediating apparent dimerisation within receptor crystals are TM1, TM2, and H8, seen at the dimer interface for rhodopsin (301), κ -OR (155), μ -OR (156), and β_1 AR (302), though several other interfaces have also been observed, including TM5-TM6 in CXCR4 (77) and μ -OR (156), TM3-TM4 in CXCR4 (77), and TM4-TM5 in β_1 AR (302). Nonetheless, the potential for artefactual receptor oligomerisation during crystal formation makes interpretation of such structures difficult, and indeed the finding, in the present study, (§5.3) that *Rhodopsin* family GPCRs mostly behave as apparent dimers implies that many of these proposed interfaces may indeed be artefacts. Arguably, the most likely genuine mechanism of dimerisation yet identified for the *Rhodopsin* family GPCRs is that of CXCR4, where contacts involving TM3, TM4, TM5, and TM6 have been observed in 5 separate crystals (77) and correlates with type 1 and cBRET data

Some of the work detailed in this chapter was performed under supervision by Susan Tickner, Oriol College, and submitted as part of her M.Biochem, Trinity Term 2012. This constitutes the cloning of all *Frizzled* and *Taste2* family genes, with the exceptions of *FZD8* and *SMO*, and of the FZD10-TAS2R19 chimeras. She also collected much of the data included in Figures 6.11 and 6.14.

suggestive of CXCR4 homodimerisation (§5.3). However, whether other apparently dimeric *Rhodopsin* family GPCRs share the proposed mechanism is unknown. Determining the mechanism would help greatly in understanding the possible functional importance and evolutionary origins of *Rhodopsin* family GPCR dimerisation.

The identification, by type 1 and cBRET analyses, of eleven apparently dimeric *Rhodopsin* family GPCRs (§5.3) offers the possibility for the investigation of the means of dimerisation in these receptors. In this chapter, the sequence elements that contribute to dimerisation of one receptor identified as dimeric by type 1 and cBRET analyses, S1P₃, are investigated by systematic domain-swapping experiments. The postulated mechanism derived from this is then compared with other, non-*Rhodopsin*, GPCRs – the *Frizzled* and *Taste2* family receptors – in an effort to assess the ubiquity of such means of interaction across the wider GPCR family.

6.1.2 The S1P receptor family

Along with the closely related LPA receptors, the S1P receptors comprise half of the “endothelial differentiation” GPCRs, a subset of the *Rhodopsin* α subfamily. Humans possess five S1P receptors (S1P₁₋₅) that recognise the lysophospholipid S1P with nanomolar affinity, as well as its derivatives dihydro-S1P and phyto-S1P (446). S1P receptors are implicated in a variety of processes, including neuronal and vascular development (485), leukocyte migration (475), and regulation of heart rate (486). Interestingly, the structure of S1P₁ contains a three-turn α -helix that blocks the entrance to the typical *Rhodopsin* family ligand-binding pocket (164), indicating that the lipidic ligands may instead enter the receptor by insertion between TM helices. Otherwise, the structure of S1P₁ is very similar to that of other *Rhodopsin* family GPCRs.

The existence of S1P receptor homodimers was suggested previously on the basis of β -galactosidase complementation (487) and Co-IP (488) experiments, notwithstanding the problems of interpreting this discussed type of data (§1.4). The same investigations also reported heterodimerisation within the S1P receptor family, but reached contradictory conclusions regarding heterodimerisation with the LPA receptors, as S1P₁-LPA₁ interactions were reported using β -galactosidase complementation but not using Co-IP. In the present study, S1P₂, S1P₃, and S1P₅ behaved as dimers in both type 1 and cBRET assays, whereas LPA₁, LPA₂, and LPA₃ were apparent monomers (§5.3). The close relatedness of S1P and LPA receptors (Fig. 6.1) offers a way to investigate the potential mechanisms of S1P receptor dimerisation by making chimeric forms of the proteins.

6.1.3 *Frizzled* and *Taste2* family GPCRs

The *Frizzled* family in humans is comprised of 10 FZD receptors, which bind to secreted glycoproteins termed Wnt (117), and SMO, which apparently functions in a ligand-independent manner as the signalling component of the SMO-patched-sonic hedgehog complex (119). FZD/SMO signalling is extremely important in early embryonic development but is also influential in adults (118). All *Frizzled* family GPCRs possess a large, highly conserved N-terminal domain composed of 200-320 amino acids containing nine conserved cysteine residues, one of which is missing in SMO (26, 489). This is the binding domain for Wnt proteins and other glycoprotein ligands, *e.g.* R-spondin, in the FZD receptors, and is proposed to also mediate receptor dimerisation (121, 140).

The *Taste2* family in humans contains 28 full-length genes, more than 25 of which produce functional proteins, and 16 pseudogenes (98, 142). They are expressed primarily in the tongue and palate epithelium where they sense bitter-tasting compounds (143). Their topology is markedly different to that of the *Frizzled* receptors as they possess only extremely short N- and C-terminal domains (98), and recognise ligands primarily via residues in the interhelical loops (116).

The *Frizzled* and *Taste2* GPCRs therefore represent two non-*Rhodopsin* receptor families with very different functions and topologies. Since the *Frizzled* receptors are suggested to form dimers in a way that is intrinsically impossible for the *Taste2* GPCRs (*i.e.* through large N-terminal domains), these two families can be used to investigate the contributions of topology to receptor association. Thus, any apparent means of interaction identified for S1P₃ can be directly compared to mechanisms involved in dimerisation of non-*Rhodopsin* family receptors.

6.2 Materials and methods

6.2.1 Cloning of S1P₃-LPA₁ chimeras

Chimeras of the *S1PR3* and *LPAR1* genes were generated using multiple overlapping PCR reactions (§2.1.2) that in turn amplified the relevant segments of each gene and then extended them to produce the final construct. Domain boundaries for each gene were identified using the TMHMM v2.0 software from the Centre for Biological Sequence Analysis, Technical University of Denmark (Fig. 6.1). Oligonucleotide primers were designed as complementary to the domain boundaries within the final chimeras (sequences given in Table 6.1), and then used in PCR reactions in the strategy given in Table

6.2. Final PCR products were then inserted into the pGFP² vector using restriction digest of the *MluI* and *BamHI* sites, and their sequences confirmed using reversible terminator base sequencing. All constructs were then subcloned into the pRLuc and vector using the same restriction sites.

6.2.2 Cloning of *Frizzled* and *Taste2* family GPCRs

All eleven human *Frizzled* family GPCR genes, as well as the eight *Taste2* family genes expressed in HEK 293T cells, were isolated using PCR from cDNA prepared from mRNA extracted from HEK 293T cells (§2.1.2). This was done using the oligonucleotide primers given in Table 6.3. In all cases, the resulting PCR product was inserted into the pGFP² vector by ligation following digest with the relevant 5' and 3' restriction enzymes, as given in Table 6.3. The resulting constructs were confirmed by reversible terminator base sequencing. FZD8 and SMO were not able to be isolated in this manner and so were instead synthesised directly using the GeneArt[®] gene synthesis service (Invitrogen). All constructs were then subcloned into the pRLuc and pU vectors using the same restriction sites.

6.2.3 Cloning of FZD10-TAS2R19 chimeras

Chimeras of the genes *FZD10* and *TAS2R19* were generated using multiple overlapping PCR reactions that in turn amplified the relevant segments of each gene and then extended them to produce the final construct. The cloning strategy used is given in Table 6.4, and the oligonucleotide primers used are given in Tables 6.3 and 6.5. In all cases, the final PCR product was inserted into the pGFP² vector using restriction digest of the *MluI* and *BamHI* sites, and its sequence confirmed using reversible terminator base sequencing. All constructs were then subcloned into the pRLuc and vector using the same restriction sites.

6.2.4 Confocal microscopy and BRET data collection

Confocal microscopy (§2.4.2) of all constructs was used to assess protein expression and trafficking. In all cases, microscopy was performed 24 h after transfection of 6x10⁵ HEK 293T cells with 1µg DNA using GeneJuice[®] (Novagen), as per the manufacturer's protocols. Proteins were generally categorised into classes A-G based on the previously described criteria (§5.2.2).

No.	Target	Dir.	Primer sequence
4160	S3-N, L1-TM1	5'	GCCTCCGAGGGCAGCCTTGAATCACTGTT
4161	S3-N, L1-TM1	3'	AACAGTGATTCCAAGGCTGCCCTCGGAGGC
4162	S3-TM1, L1-IL1	5'	CTGATGGTTTTGATTGCGCGCTTCCATTTT
4163	S3-TM1, L1-IL1	3'	AAAATGGAAGCGGCGAATCAAAACCATCAG
4164	S3-IL1, L1-TM2	5'	TTTCACAACCGCATGATTTATTACCTAATG
4165	S3-IL1, L1-TM2	3'	CATTAGGTAATAAATCATGCGGTTGTGAAA
4166	S3-TM2, L1-EL1	5'	AAGGTCAACATTCTGTTCAACACAGGACCC
4167	S3-TM2, L1-EL1	3'	GGGTCCTGTGTTGAACAGAATGTTGACCTT
4168	S3-EL1, L1-TM3	5'	TCTCCACGGTCTGGTGGCTCCTTCGTCAG
4169	S3-EL1, L1-TM3	3'	CTGACGAAGGAGCCACCAGACCGTGGGAGA
3933	S3-TM3, L1-IL2	5'	CTGGCCATCGCCATCATCGAGAGGCACATT
3934	S3-TM3, L1-IL2	3'	AATGTGCCTCTCGATGATGGCGATGGCCAG
3935	S3-IL2, L1-TM4	5'	AACAAGAGGCACCGCGTAGTGGTGGTCATT
3936	S3-IL2, L1-TM4	3'	AATGACCACCACTACGCGGTGCCTCTTGTT
3937	S3-TM4, L1-EL2	5'	CCCATTCTGGGCTGGAAGTGTATCTGTGAT
3938	S3-TM4, L1-EL2	3'	ATCACAGATACAGTTCAGCCAGAAATGGG
3939	S3-EL2, L1-TM5	5'	CTCTACTCCAAGAAGTACTTAGTCTTCTGG
3940	S3-EL2, L1-TM5	3'	CCAGAAGACTAAGTACTTCTTGGAGTAGAG
3941	S3-TM5, L1-IL3	5'	CTCTACGCACGCATCTTTGGCTATGTTCCG
3942	S3-TM5, L1-IL3	3'	GCGAACATAGCCAAAGATGCGTGCGTAGAG
3943	S3-IL3, L1-TM6	5'	CTGCTGCGGACCGTGCTGAAGACTGTGGTC
3944	S3-IL3, L1-TM6	3'	GACCACAGTCTTCAGCACGGTCCGCAGCAG
3945	S3-TM6, L1-EL3	5'	ATCCTCTTCCTCATTCTAGACGTGTGCTGT
3946	S3-TM6, L1-EL3	3'	ACAGCACACGTCTAGAATGAGGAAGAGGAT
3947	S3-EL3, L1-TM7	5'	CCCATCCTCTTCAAGGTGCTGGCCTATGAG
3948	S3-EL3, L1-TM7	3'	CTCATAGGCCAGCACCTTGAAGAGGATGGG
3949	S3-TM7, L1-C	5'	TACACGCTGGCCAGCTCCTACCGCGACAAA
3950	S3-TM7, L1-C	3'	TTGTGCGCGTAGGAGCTGGCCAGCGTGTA
3951	L1-TM2, S3-EL1	5'	TACTTCTATCTCATGATGTCTGGCAAGAAG
3952	L1-TM2, S3-EL1	3'	CTTCTTGCCAGACATCATGAGATAGAAGTA
3953	L1-EL1, S3-TM3	5'	CTGACTGTAGCACATTCTCAGGGAGGGC
3954	L1-EL1, S3-TM3	3'	GCCCTCCCTGAGGAATGTGCTAACAGTCAG
3955	L1-TM3, S3-IL2	5'	TTACTGGCTATTGCAGAGCGGCACTTGACA
3956	L1-TM3, S3-IL2	3'	TGTCAAGTGCCGCTCTGCAATAGCCAGTAA
3957	L1-IL2, S3-TM4	5'	ATGAGCAACCGGCGGGTCTTCCTCCTGATC
3958	L1-IL2, S3-TM4	3'	GATCAGGAGGAAGACCCGCGGTTGCTCAT
3959	L1-TM4, S3-EL2	5'	CCCAGTGTGGGCTGGAAGTGCCTGCACAAT
3960	L1-TM4, S3-EL2	3'	ATTGTGCAGGCAGTTCAGCCACACTGGG
3114	<i>S1PR3</i>	5'	TAGTAGACGCGTGCCACCATGGCAACTGCCCTCCCGC
3115	<i>S1PR3</i>	3'	CTACTAGGATCCCCGTTGCAGAAGATCCCATTCTGAAGTGC
3116	<i>LPAR1</i>	5'	TAGTAGACGCGTGCCACCATGGCTGCCATCTCTACTTCCATC
3117	<i>LPAR1</i>	3'	CTACTAGGATCCCCAACCACAGAGTGGTCATTGCTGTGAACTC

Table 6.1: Oligonucleotide primer sequences used in the generation of S1P₃-LPA₁ chimeras. Target names denote the boundary between two protein domains that are joined by use of that primer. L1 = LPA₁; S3 = S1P₃; N = N-terminal domain; IL1-3 = intracellular loops 1-3; EL1-3 = extracellular loops 1-3; TM1-7 = Transmembrane helices 1-7; C = C-terminal domain. *e.g.* S3-TM7, L1-C forms the junction between TM7 on S1P₃ and the C-terminal domain of LPA₁.

Reaction		1 (R1)		2 (R2)		3 (R3)		4 (R4)	
Template	Primers	Template	Primers	Template	Primers	Template	Primers	Template	Primers
<i>S1PR3</i>	3114+4167	<i>LPAR1</i>	4166+3117	<i>R1+R2</i>	3114+3117	-	-		
<i>S1PR3</i>	3114+3938	<i>LPAR1</i>	3937+3117	<i>R1+R2</i>	3114+3117	-	-		
<i>S1PR3</i>	3114+3944	<i>LPAR1</i>	3943+3117	<i>R1+R2</i>	3114+3117	-	-		
<i>LPAR1</i>	3116+3952	<i>S1PR3</i>	3951+4169	<i>LPAR1</i>	4168+3117	<i>R1+R2+R3</i>	3116+3117		
<i>LPAR1</i>	3116+3954	<i>S1PR3</i>	3953+3934	<i>LPAR1</i>	3933+3117	<i>R1+R2+R3</i>	3116+3117		
<i>LPAR1</i>	3116+3956	<i>S1PR3</i>	3955+3926	<i>LPAR1</i>	3935+3117	<i>R1+R2+R3</i>	3116+3117		
<i>LPAR1</i>	3116+3958	<i>S1PR3</i>	3957+3938	<i>LPAR1</i>	3937+3117	<i>R1+R2+R3</i>	3116+3117		
<i>S1PR3</i>	3114+4167	<i>LPAR1</i>	4166+3954	<i>S1PR3</i>	3953+3115	<i>R1+R2+R3</i>	3114+3115		
<i>S1PR3</i>	3114+4169	<i>LPAR1</i>	4168+3956	<i>S1PR3</i>	3955+3115	<i>R1+R2+R3</i>	3114+3115		
<i>S1PR3</i>	3114+3934	<i>LPAR1</i>	3933+3958	<i>S1PR3</i>	3957+3115	<i>R1+R2+R3</i>	3114+3115		
<i>S1PR3</i>	3114+3936	<i>LPAR1</i>	3935+3960	<i>S1PR3</i>	3959+3115	<i>R1+R2+R3</i>	3114+3115		

Table 6.2: Cloning strategy used to generate *S1P₃*-*LPA₁* chimeras. Numbers correspond to oligonucleotide primers in Table 6.1.

No.	Target	Dir.	Primer sequence	Restriction
3336	<i>FZD1</i>	5'	TAGTAGACGCGTGCCACCATGGCTGAGGAGGAGGCGCC	<i>MluI</i>
3337	<i>FZD1</i>	3'	CTACTAGGATCCCCGACTGTAGTCTCCCCTTGTTC	<i>BamHI</i>
3322	<i>FZD2</i>	5'	TAGTAGGATATCGCCACCATGCGGCCCCGAGCGCCC	<i>EcoRV</i>
3339	<i>FZD2</i>	3'	CTACTAGGTACCCCCACGGTGGTCTCACCGTGTGCGC	<i>KpnI</i>
3341	<i>FZD3</i>	5'	TAGTAGACGCGTGCCACCATGGCTATGACTTGGATTGTCT TC	<i>MluI</i>
3342	<i>FZD3</i>	3'	CTACTAGGTACCCCCAGCACTGGTTCCATCTTCTTC	<i>KpnI</i>
3344	<i>FZD4</i>	5'	TAGTAGACGCGTGCCACCATGGCCTGGCGGGGCGCAGG	<i>MluI</i>
3345	<i>FZD4</i>	3'	CTACTAGGATCCCCTACCACAGT	<i>BamHI</i>
3354	<i>FZD5</i>	5'	TAGTAGACGCGTGCCACCATGGCTCGGCCTGACCCATC	<i>MluI</i>
3348	<i>FZD5</i>	3'	CTACTAGGATCCCCACGTGCGACAGGGACACCTGC	<i>BamHI</i>
3350	<i>FZD6</i>	5'	TAGTAGACGCGTGCCACCATGGAAATGTTTACATTTTGT GAC	<i>MluI</i>
3351	<i>FZD6</i>	3'	CTACTAGGTACCCCCAGTATCTGAATGACAACCACC	<i>KpnI</i>
3354	<i>FZD7</i>	5'	TAGTAGACGCGTGCCACCATGCGGGACCCCGGCGCGGCC	<i>MluI</i>
3355	<i>FZD7</i>	3'	CTACTAGGATCCCCTACCGCAGTCTCCCCTTGCTG	<i>BamHI</i>
3357	<i>FZD8</i>	5'	TAGTAGACGCGTGCCACCATGGAGTGGGGTTACCTGTTGG AAG	<i>MluI</i>
3358	<i>FZD8</i>	3'	CTACTAGGATCCCCGACCTGGGACAATGGCATCTG	<i>BamHI</i>
3360	<i>FZD9</i>	5'	TAGTAGACGCGTGCCACCATGGCCGTGGCGCCTCTGCGG	<i>MluI</i>
3361	<i>FZD9</i>	3'	CTACTAGGATCCCCGCGGTGTGTGGGGTTGGCTCCAAAG	<i>BamHI</i>
3363	<i>FZD10</i>	5'	TAGTAGACGCGTGCCACCATGCAGCGCCCGGGCCCCCGC	<i>MluI</i>
3364	<i>FZD10</i>	3'	CTACTAGGATCCCCACGCAGGTGGGCGACTGGGCAGGG	<i>BamHI</i>
3366	<i>SMO</i>	5'	TAGTAGACGCGTGCCAGGCTGGCCGCTGCCCGCCAGCG	<i>MluI</i>
3367	<i>SMO</i>	3'	CTACTAGGTACCCCCGAAGTCCGAGTCTGCATCCATGAG	<i>KpnI</i>
3370	<i>TASSR3</i>	5'	TAGTAGACGCGTGCCACCATGATGGGACTCACCGAGGGG	<i>MluI</i>
3371	<i>TAS2R3</i>	3'	CTACTAAAGCTTAGAGAAAATGGGTCCCTTGATC	<i>HindIII</i>
3372	<i>TAS2R4</i>	5'	TAGTAGACGCGTGCCACCATGCTTCGGTTATTCTATTCTC	<i>MluI</i>
3373	<i>TAS2R4</i>	3'	CTACTAGGATCCCCTTTTGTAAACAAAGAATCTTC	<i>BamHI</i>
3374	<i>TAS2R5</i>	5'	TAGTAGACGCGTGCCACCATGCTGAGCGATGGCCTAGG	<i>MluI</i>
3375	<i>TAS2R5</i>	3'	CTACTAGGATCCCCTGGGCCCCAGCATCTCC	<i>BamHI</i>
3376	<i>TAS2R10</i>	5'	TAGTAGACGCGTGCCACCATGCTACGTGTAGTGGAAAGGC	<i>MluI</i>
3377	<i>TAS2R10</i>	3'	CTACTAGGATCCCCTGTGACTCTGAGATTTTTCCTTTTC	<i>BamHI</i>
3378	<i>TAS2R13</i>	5'	TAGTAGACGCGTGCCACCATGGAAAGTGCCCTGCCGAGT ATC	<i>MluI</i>
3379	<i>TAS2R13</i>	3'	CTACTAGGATCCCCTCGTTTAGCCCATACCTTAGCTG	<i>BamHI</i>
3380	<i>TAS2R14</i>	5'	TAGTAGACGCGTGCCACCATGGGTGGTGTATATAAAGAGC AT	<i>MluI</i>
3381	<i>TAS2R14</i>	3'	CTACTAGGTACCCCCAGATGATTCTCTAAATTCTTTGTG	<i>KpnI</i>
3382	<i>TAS2R19</i>	5'	TAGTAGACGCGTGCCACCATGATGTGTTTCTGCTCATCAT TTC	<i>MluI</i>
3383	<i>TAS2R19</i>	3'	CTACTAGGATCCCCGCGTGTCTATCTGCCACAAAAC	<i>BamHI</i>
3384	<i>TAS2R20</i>	5'	TAGTAGACGCGTGCCACCATGATGAGTTTTCTACACATTG TTTT	<i>MluI</i>
3385	<i>TAS2R20</i>	3'	CTACTAGGATCCCCTGGAGTTGACTGGTTCTGTC	<i>BamHI</i>

Table 6.3: Oligonucleotide primer sequences used in the generation of *Frizzled* and *Taste2* family GPCR constructs.

Chimera	Reaction 1 (R1)		Reaction 2 (R2)		Reaction 3 (R3)	
	Template	Primers	Template	Primers	Template	Primers
FTT	<i>FZD10</i>	3363 + 3557	<i>TAS2R19</i>	3556 + 3383	R1+R2	3364 + 3383
TTF	<i>TAS2R19</i>	3382 + 3559	<i>FZD10</i>	3558 + 3364	R1+R2	3382 + 3364
TFF	<i>FZD10</i>	3560 + 3364	R1	3382 + 3364	-	-
FFT	<i>FZD10</i>	3363 + 3562	<i>TAS2R19</i>	3383 + 3561	R1 + R2	3363 + 3383
TFT	TFF	3382 + 3562	<i>TAS2R19</i>	3383 + 3561	R1 + R2	3382 + 3383
FTF	FTT	3363 + 3559	<i>FZD10</i>	3364 + 3558	R1 + R2	3363 + 3364

Table 6.4: Cloning strategy used to generate *FZD10-TAS2R19* chimeras. Numbers correspond to oligonucleotide primers in Tables 6.3 and 6.5.

No.	Target	Dir.	Primer sequence
3556	F10-N, T19-TM	5'	GACAAGCGCTTCGCATCAATTCTGGTAGTG
3557	F10-N, T19-TM	3'	CACTACCAGAATTGATGCGAAGCGCTTGTC
3558	T19-TM, F10-C	5'	ATCCTGATTATGGGAACTCTGCAGTCCTGG
3559	T19-TM, F10-C	3'	CCAGGACTGCAGAGTTCCTATAATCAGGAT
3560	T19-N, F10-TM	5'	TCTGCTCATCATTTTCAGTGGTCTGGCTGGCCA
3561	F10-TM, T19-C	5'	ATTTGGACCTCCAAGAGTAGGAAGCTAAAA
3562	F10-TM, T19-C	3'	TTTGTAGCTTCCTACTCTTGGAGGTCCAAAT

Table 6.5: Oligonucleotide primer sequences used in the generation of *FZD10-TAS2R19* chimeras. Target names denote the overlapping sections of the final chimera covered in each case: F10 = *FZD10*, T19 = *TAS2R19*, N = N-terminal domain, TM = 7TM domain, C = C-terminal domain. *i.e.* F10-N, T19-TM covered the junction between the *FZD10* N-terminal domain and the start of the *TAS2R19* 7TM region in chimeras FTT and FTF.

Transfection and data collection for type 1 and cBRET assays were performed as described previously (type 1, §3.2; cBRET, §4.2.3). Type 1 BRET was performed 24 h post transfections for all constructs with the exception of TAS2R19, which was performed after 48 h to allow for sufficient protein expression.

6.3 Results

6.3.1 TM4 influences apparent S1P₃ stoichiometry

In order to examine the mechanism of dimerisation in S1P₃, the sequence motifs involved were investigated using a domain-swapping approach utilising the apparently monomeric receptor LPA₁. These receptors were selected because the S1P and LPA receptors are the most closely related subfamilies yielding contrasting stoichiometries in the present BRET analysis of the HEK 293T-expressed *Rhodopsin* family GPCRs, and because S1P₃ and LPA₁ are amongst the most closely related of these receptors (Fig. 5.15).

Approximate identification of the dimer interface

Initially, the coarse identity of the potential dimer interface was assessed by the generation of three LPA₁-S1P₃ chimeras with varying contributions from each receptor. Chimera 1 consisted of LPA₁ with its N-terminal domain, TM1, IL1, and TM3 replaced by those of S1P₃. Chimera 2 contained the S1P₃ protein sequence from the N terminus to the end of TM4, followed by the LPA₁ sequence from the start of EL2 to the C terminus. Chimera 3 consisted of S1P₃ with its TM6, EL3, TM7, and C-terminal domain regions replaced by those of LPA₁. The boundaries of these domains were determined using the TMHMM v2.0 software from the Centre for Biological Sequence Analysis, Technical University of Denmark, and are shown in Figure 6.1. Expression of these chimeras was assessed by confocal microscopy (Fig. 6.2). Increased levels of retention within intracellular membranes was observed for all three chimeras compared to LPA₁ and S1P₃, though no evident protein aggregation was visible and so the constructs were considered suitable for use in BRET.

Type 1 BRET analysis of chimera 1 yielded data characteristic of that of a monomer (Fig. 6.3), which implies that the N-terminal domain, TM1, IL1, and TM2 of S1P₃ are not sufficient to induce dimerisation. Conversely, both chimeras 2 and 3 yielded BRET_{off} data expected of dimers in type 1 BRET assays (Fig. 6.3). There is no obvious, large difference between the data collected for chimeras 2

and 3, which implies that the additional S1P₃ sequence in chimera 3 does not greatly affect stoichiometry as measured by type 1 BRET. This suggests that the sequence motifs that mediate interaction in S1P₃ are likely predominantly located within EL1, TM3, IL2, and TM4.

Investigation of individual domains

To further probe the potential contributions of EL1, TM3, IL2, and TM4 to S1P₃ dimerisation, eight domain-swapped chimeras were generated in which a single LPA₁ region was replaced by the S1P₃ equivalent or *vice versa*. These were named 'LS', for LPA₁ containing an S1P₃ domain, or 'SL' for S1P₃ containing an LPA₁ domain. E1, T3, I2, and T4 suffixes are used to refer to EL1, TM3, IL2, and TM4, respectively. Thus, LSE1 consists of LPA₁ containing the S1P₃ EL1 sequence, whilst SLT4 consists of S1P₃ containing the LPA₁ TM4 sequence, for example. Confocal microscopy of these constructs expressed in HEK 293T cells demonstrated no evident aggregation for any chimera, although varying degrees of intracellular retention were apparent (Fig. 6.4).

Type 1 BRET analysis of SLE1, SLT3, and SLI2 produced BRET_{eff} data closely matching the predicted relationship for a dimer, whereas the same analysis of SLT4 gave a far weaker correlation to the dimer model although still marginally better than to the model for a monomer (Fig. 6.5 & Table 6.6). The most likely explanation for this is that SLT4 is only a weak dimer, whereas the other SL chimeras form more stable interactions. It is not possible to reliably interpret absolute BRET_{max} values for these dimers as a function of the extent of interaction because the total expression level varied between chimeras (data not shown). Thus, which of SLE1, SLT3, and SLI2 is the stronger dimer cannot be inferred from these data. However, these results correlate very well with the type 1 BRET analysis of LSE1, LST3, LSI2, and LST4. Of these, LST4 was the only construct to yield data consistent with dimeric behaviour (Fig. 6.6 & Table 6.6). TM4 thus appears to be sufficient, but not wholly necessary, for dimerisation in S1P₃.

This conclusion was tested further using cBRET analysis of the eight chimeras using wild-type S1P₃ as the untagged competitor. This allowed the investigation of whether the interaction domains within the chimeras were sufficient to dimerise with wild-type receptors. This analysis of the SL chimeras revealed significant decreases in BRET_{eff} at any given expression level in the presence of competitor, indicative of dimerisation (Fig. 6.7 & Table 6.6). However, consistent with the type 1 data, SLT4 demonstrated the smallest relative change upon introduction of wild-type S1P₃, which may again indicate that it is a weaker dimer than the other SL chimeras. Interestingly, SLT3 also yielded a

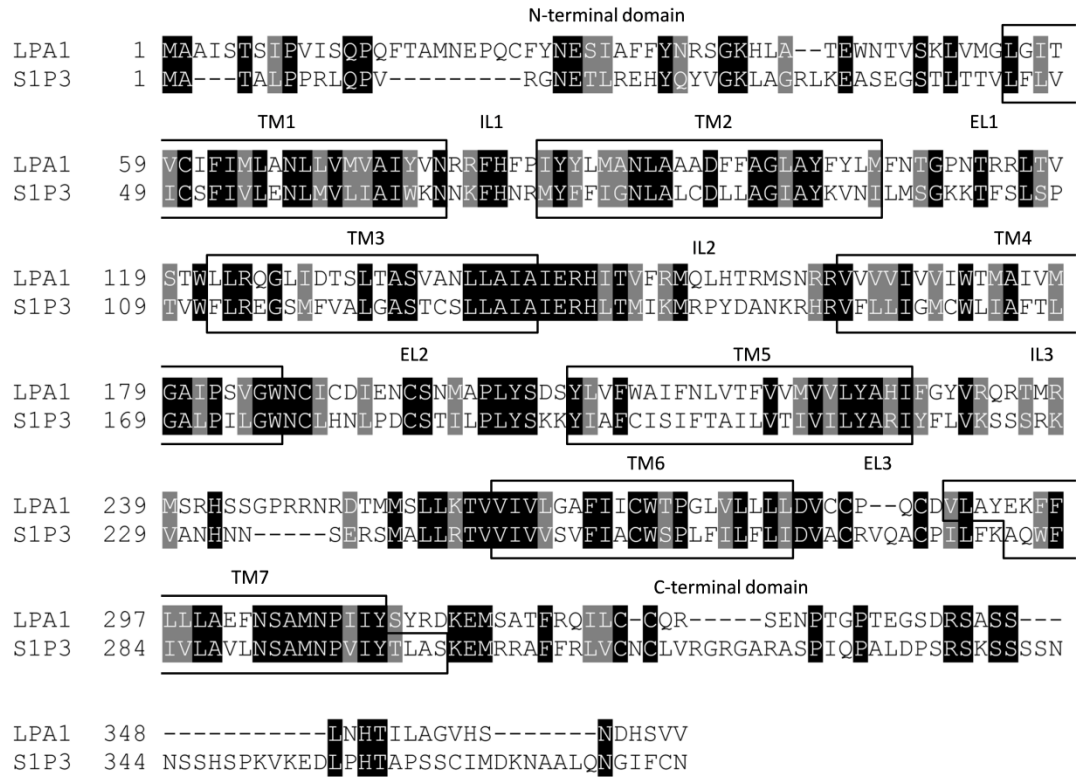


Figure 6.1: Alignment of LPA₁ and S1P₃ protein sequences. Conserved residues are shown in black. Chemically similar non-conserved residues are shaded grey. Boxes denote positions of TM helices as identified using the TMHMM v2.0 software from the Centre for Biological Sequence Analysis, Technical University of Denmark.

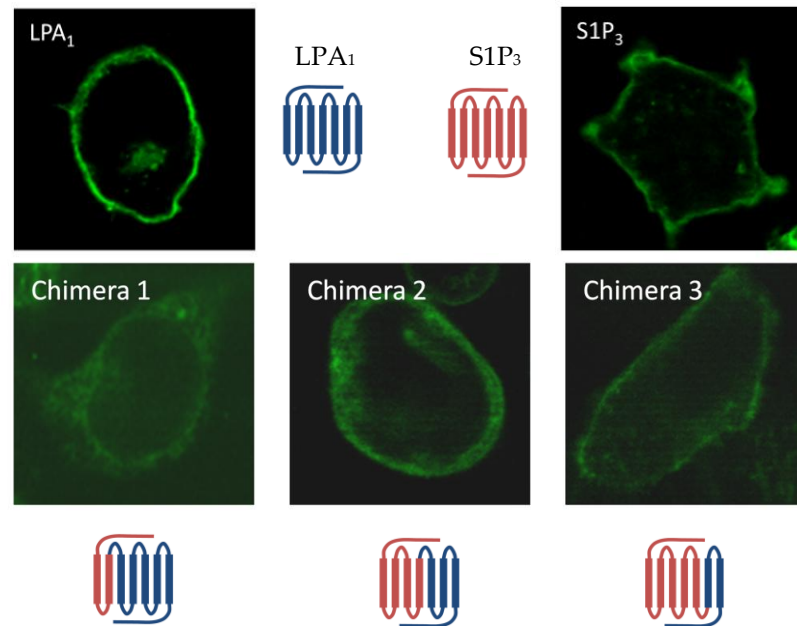


Figure 6.2: Representative confocal microscopy images of HEK 293T cells expressing the three LPA₁-S1P₃ chimeras from the pGFP² vector. All three constructs exhibit increased intracellular retention compared to the parent genes, but nonetheless aggregation was not observed and so all were suitable for use in BRET. Schematic representations present the LPA₁ (blue) and S1P₃ (red) composition of each construct.

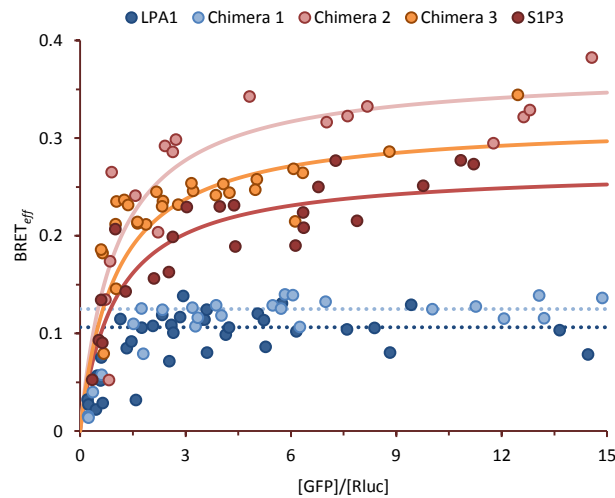


Figure 6.3: The observation of a type 1 BRET signature corresponding to a dimer is dependent on motifs between EL1 and TM4 in S1P₃. Type 1 BRET analysis of three LPA₁-S1P₃ chimeras as well as the parent receptors for comparison. Chimera 1 contains the N terminus, TM1, IL1, and TM2 of S1P₃ and exhibits an independence of BRET_{eff} upon [GFP]/[Rluc], indicative of a monomer. Fits of LPA₁ and chimera 1 data to a constant model are shown as broken lines. Chimeras 2 and 3 demonstrate hyperbolic dependences of BRET_{eff} upon [GFP]/[Rluc] that fit well to a dimer model (solid lines).

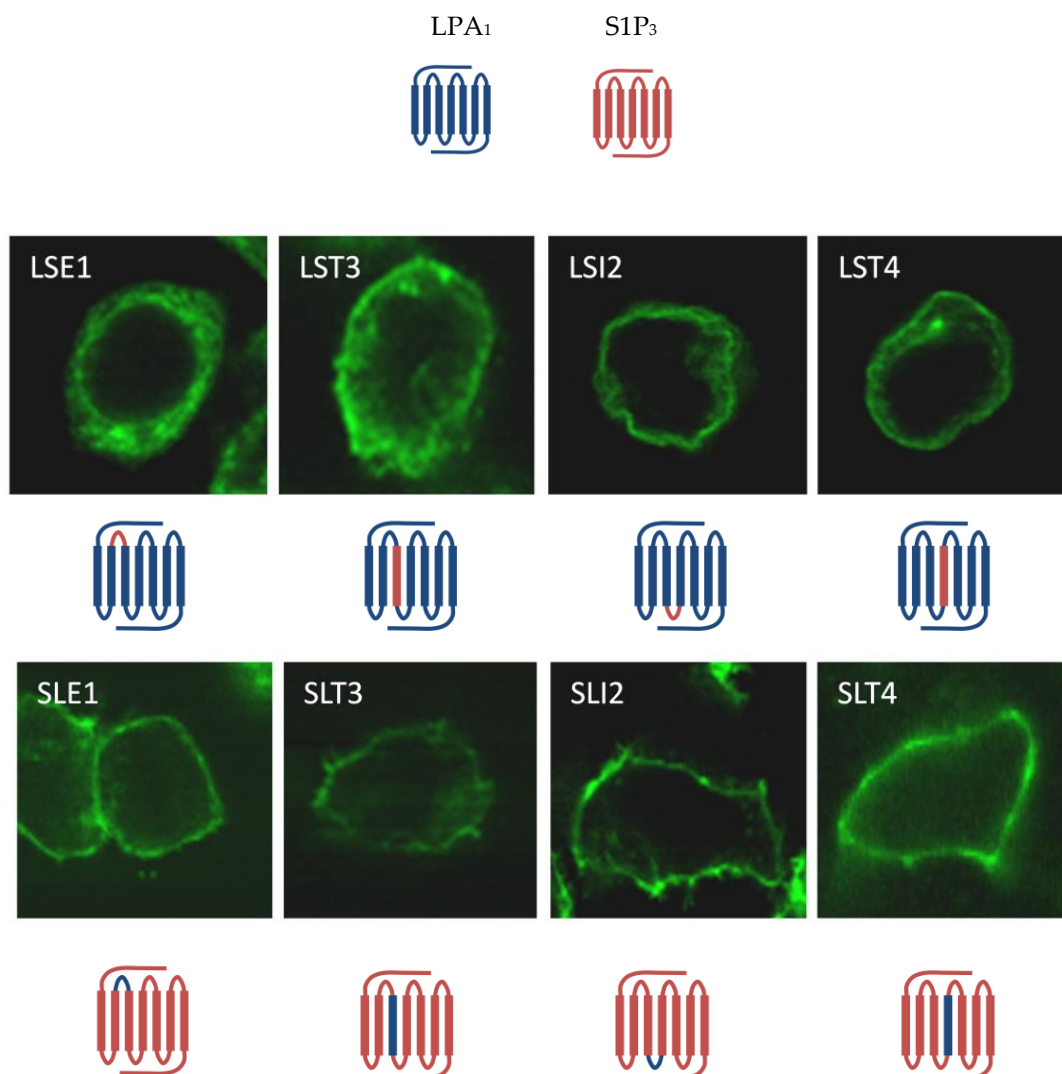


Figure 6.4: Representative confocal microscopy images of HEK 293T cells expressing the 8 LPA₁-S1P₃ individual domain chimeras as C-terminal GFP fusions. All 8 constructs exhibited suitable standards of expression for use in BRET, although varying degrees of intracellular retention were observed for different chimeras. Schematic representations present the LPA₁ (blue) and S1P₃ (red) composition of each construct.

smaller relative $BRET_{eff}$ change and y -intercept value than SLE1 and SLI2, indicating that it may also be more weakly dimeric than either of the others. cBRET analysis of the LS chimeras also appears to agree with the type 1 BRET data, insofar as only LST4 exhibits a change in the relationship between $BRET_{eff}$ and expression in the presence of competitor (Fig. 6.8 & Table 6.6), again suggesting that TM4 of S1P₃ is sufficient to induce dimerisation in LPA₁. Given the high sequence similarity between S1P₃ and the other S1P receptors, it seems likely that TM4 may be involved in dimerisation across the whole family.

6.3.2 S1P receptors can mutually influence one another's behaviour

An important corollary of a model of preserved interaction interfaces is that the receptors should be capable of forming heteromeric interactions that impede homodimerisation. This can be assessed using cBRET where the competitor is a different receptor to that expressed as the BRET pair. This approach was applied to S1P₃ using S1P₂ and S1P₅ as competitor proteins (Fig. 6.9a). The presence of untagged S1P₂ caused a significant ($P < 0.0001$) decrease in $BRET_{eff}$ at a given expression level, indicating that it inhibits S1P₃ homodimerisation. The introduction of S1P₅ as a competitor, however, did not have a significant effect on the $BRET_{eff}$ -expression relationship ($P^{diff} = 0.2434$; absolute R^2 and P^{diff} values for all hetero-cBRET experiments are given in Table 6.7). Similarly, cBRET using S1P₃ as the BRET pair and either LPA₁, LPA₂, LPA₃, or CCR5 as competitor showed no change in $BRET_{eff}$ at a given expression level in the presence of untagged receptors (Fig. 6.9b). Thus, of all 6 competitors used in this analysis, only S1P₂ alters $BRET_{eff}$ for S1P₃, possibly through direct heterodimerisation.

Application of the same hetero-cBRET strategy to S1P₂ and S1P₅ revealed a significant decrease in $BRET_{eff}$ at a given expression level for both receptors in the presence of any of the three S1P receptors as competitor (Fig. 6.9c & d). This suggests that all of the S1P receptors may influence the behaviour of S1P₂ and S1P₅, potentially via the disruption of homodimers by heterodimer formation.

6.3.3 *Frizzled* and *Taste2* receptors exhibit contrasting stoichiometries

Having established a possible means of interaction by S1P₃, the ubiquity of this mechanism was then investigated in non-*Rhodopsin* family receptors. Initially, the native stoichiometry of the *Frizzled* and *Taste2* GPCRs had to be established. Confocal microscopy of C-terminal GFP fusions of the 11 *Frizzled* and 8 *Taste2* receptors natively expressed by HEK 293T cells revealed that 11 undergo sufficiently

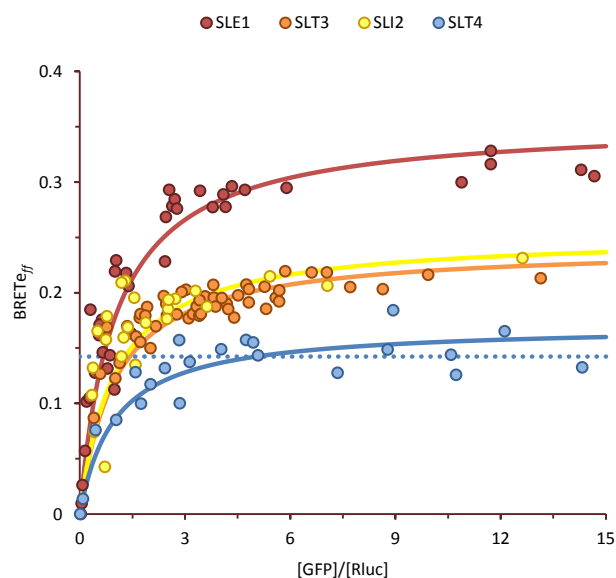


Figure 6.5: Type 1 BRET of S1P₃ chimeras containing individual LPA₁ domains. All chimeras yielded data consistent with dimeric behaviour, though SLT4 has a far weaker fit to a dimer model (solid line). A constant (monomer) fit is shown for comparison (broken line). Dimer fit R^2 values are given in Table 6.6.

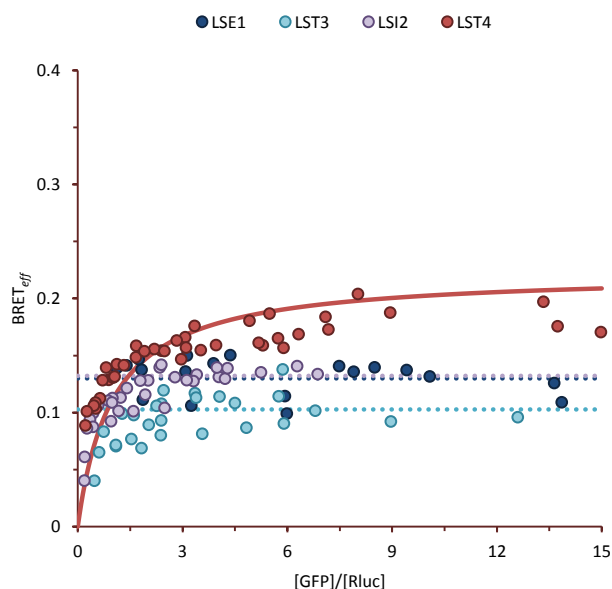


Figure 6.6: Type 1 BRET of LPA₁ chimeras containing individual S1P₃ domains. Chimeras containing the EL1, TM3, and IL2 domains of S1P₃ fit well to a monomer model (broken lines), whereas LST4 fits more closely to a dimer model (solid line). Dimer fit R^2 values are given in Table 6.6.

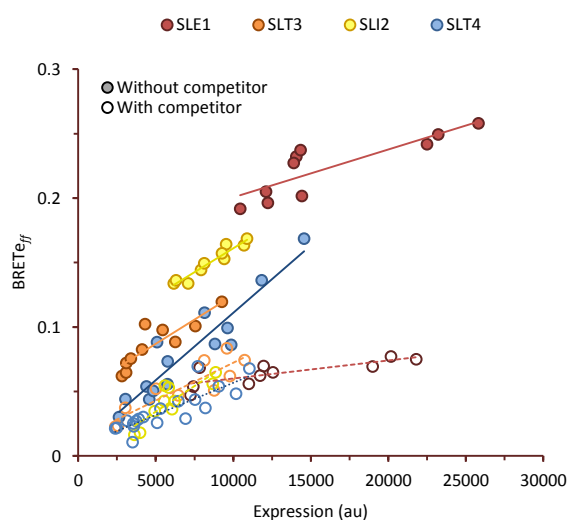


Figure 6.7: cBRET of S1P₃ chimeras containing individual LPA₁ domains. Wild-type S1P₃ was used as the competitor in all cases. A reduction in BRET_{eff} at any given expression level in the presence of competitor was observed for all chimeras though this effect was least significant for SLT4, indicating that this is the weakest dimer. Solid and broken lines indicate fits of the data obtained in the absence and presence of competitor, respectively. R^2 and P^{diff} values are given in Table 6.6.

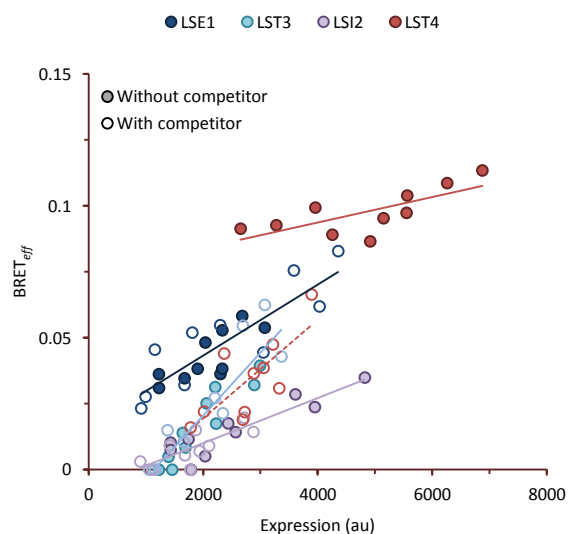


Figure 6.8: cBRET of LPA₁ chimeras containing individual S1P₃ domains. Wild-type S1P₃ was used as the competitor in all cases. LST4 is the only chimera to exhibit a change in the BRET_{eff}-expression relationship in the presence of competitor, indicating that this is the only construct to form dimers. Solid and broken lines indicate fits of the data obtained in the absence and presence of competitor, respectively. In cases of no significant difference between datasets, all points are fitted to a single line. R^2 and P^{diff} values are given in Table 6.6.

Chimera	Type 1 BRET Dimer fit R^2	cBRET R^2 (-competitor)	cBRET R^2 (+competitor)	cBRET P^{diff}
Chimera 1	-0.48	-	-	-
Chimera 2	0.49	-	-	-
Chimera 3	0.39	-	-	-
LSE1	-0.58	0.60	0.75	0.4122
LST3	-0.33	0.87	0.84	0.1280
LSI2	-0.57	0.82	0.57	0.7174
LST4	0.32	0.53	0.56	<0.0001
SLE1	0.24	0.72	0.61	<0.0001
SLT3	0.42	0.77	0.79	<0.0001
SLI2	0.69	0.92	0.73	<0.0001
SLT4	0.05	0.87	0.71	0.00036

Table 6.6: Type 1 and cBRET values obtained for LPA₁-S1P₃ chimeras in this chapter.

Receptor	Competitor	R^2	P^{diff}
S1P ₃	None	0.43	-
	S1P ₃	0.47	<0.0001
	S1P ₂	0.88	<0.0001
	S1P ₅	0.91	0.2434
	LPA ₁	0.41	0.9334
	LPA ₂	0.52	0.1139
	LPA ₃	0.92	0.4556
	CCR5	0.53	0.1231
S1P ₂	None	0.94	-
	S1P ₂	0.96	0.0163
	S1P ₃	0.76	<0.0001
	S1P ₅	0.85	<0.0001
S1P ₅	None	0.93	-
	S1P ₅	0.95	<0.0001
	S1P ₂	0.57	0.00012
	S1P ₃	0.73	0.00073

Table 6.7. Values obtained for cBRET of heteromeric S1P receptor interactions. cBRET of homomeric interactions obtained in §5.3 are included for reference.

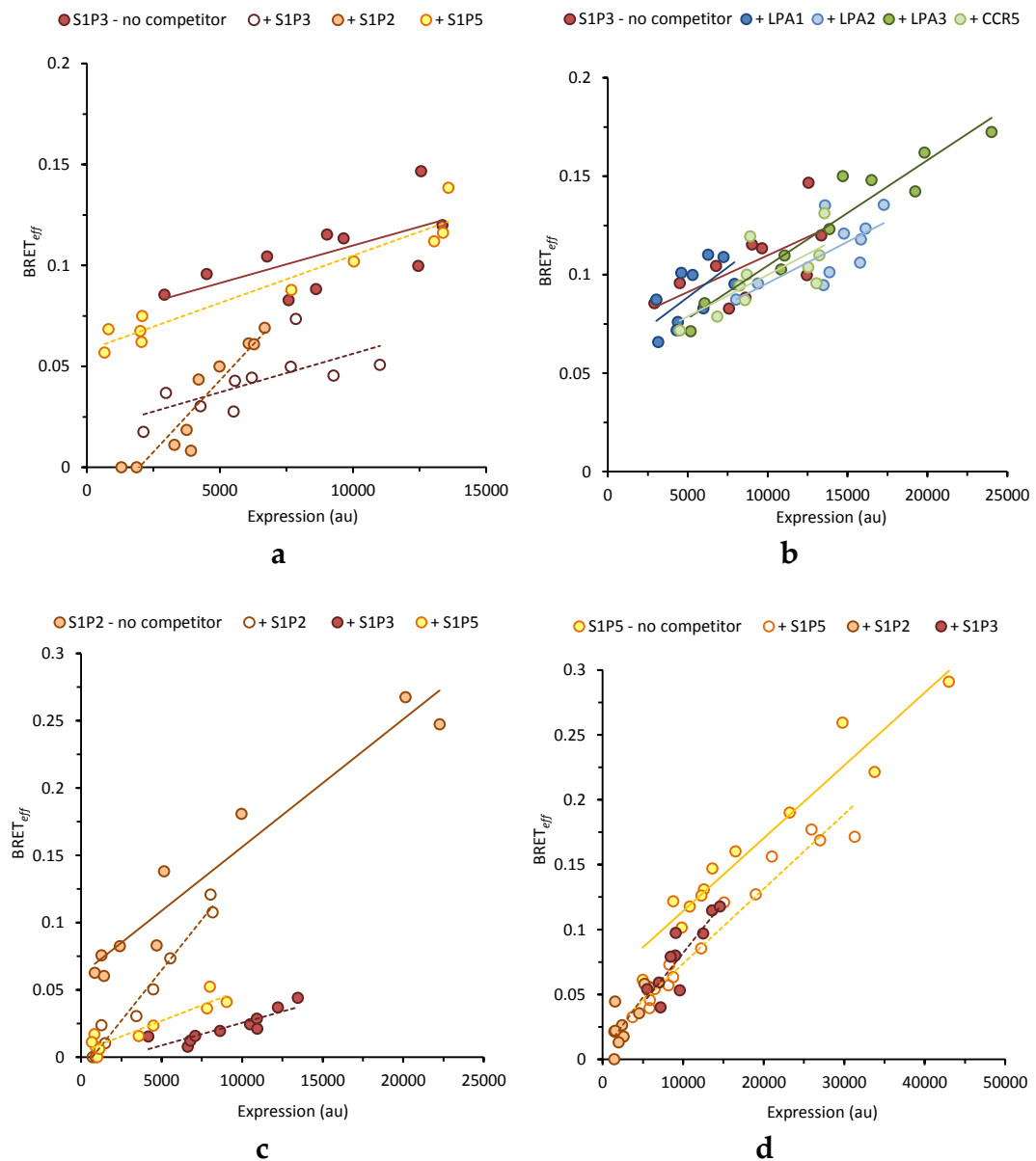


Figure 6.9: cBRET analysis of possible heteromeric interactions of S1P receptors. **(a & b)** BRET_{eff} between tagged S1P₃ receptors was significantly reduced in the presence of untagged S1P₂ and S1P₃ but not in the presence of S1P₅, LPA₁, LPA₂, LPA₃, or CCR5. **(c)** S1P₂ exhibited a decrease in BRET_{eff} when any of the three S1P receptors was used as competitor. **(d)** S1P₅ exhibited small but significant decreases in BRET_{eff} in the presence of untagged versions any of the three S1P receptors. In all cases, data are shown in the absence of competitor (*i.e.* “receptor – no competitor”) and then in the presence of varying competitor proteins (*i.e.* “+ receptor”). Solid and broken lines indicate fits of the data obtained in the absence and presence of competitor, respectively. Cases where the same protein is used as acceptor/donor and competitor have been shown previously (§5.3) and are given for reference.

efficient folding and trafficking to allow BRET analysis (Fig. 6.10). The quality of expression was assessed using the A-G classification system described in Section 5.3, wherein receptors placed into categories F and G are not subjected to further analysis. This was the case for 3 *Frizzled* and 4 *Taste2* family GPCRs. FZD8 could not be assigned a category as it exhibited extremely low levels of expression, which would also have precluded accurate data collection in the BRET assays, and so FZD8 was also left out of the analysis.

Type 1 BRET analysis of the *Frizzled* and *Taste2* receptors showed a clear difference in the behaviour of the two families (Fig. 6.11). Each of the 7 investigated *Frizzled* receptors exhibited a dependence of BRET_{eff} upon $[\text{GFP}]/[\text{Rluc}]$ that fitted the model for dimers better than that for monomers, suggesting that each of these receptors undergoes some degree of dimerisation. Conversely, all four *Taste2* receptors yielded BRET_{eff} values that were independent of $[\text{GFP}]/[\text{Rluc}]$ in the manner expected of monomers. cBRET analysis of the same receptors was consistent with the type 1 analyses: the *Frizzled* receptors exhibited a decrease in BRET_{eff} at a given expression level in the presence of competitor protein (Fig. 6.12a,b, & c), consistent with dimerisation, whereas the BRET_{eff} -expression relationship for the *Taste2* receptors was unaffected by the introduction of competitors (Fig. 6.12d), as expected for monomers. Absolute values derived from both type 1 and cBRET experiments are given in Table 6.8. Taken together, these data reveal two forms of behaviour that are shared in a family-specific manner.

6.3.4 Possible role of N- and C-terminal domains in FZD10 dimerisation

In order to investigate the mechanism of dimerisation within the *Frizzled* family receptors, chimeric constructs were generated containing sequences from both FZD10 and TAS2R19, which were selected as representative receptors from each family. Six chimeras were created wherein either the N- or C-terminal domain, or both, of one receptor was replaced with that of the other. Domain boundaries were identified using the TMHMM v2.0 software from the Centre for Biological Sequence Analysis, Technical University of Denmark. The FZD10 N-terminal domain was identified as M1-A227, and its C-terminal domain as T527-V581. For TAS2R19, the N- and C-terminal domains were identified as M1-S9 and S283-R299, respectively. These chimeras were given a three-letter designation based on their composition, in which F and T denote FZD10 and TAS2R19 components, respectively. The first letter indicates the origin of the N-terminal domain, the second indicates that of the 7TM region, and the third describes the C-terminal domain. Thus, FTF consists of the N- and C-terminal domains of

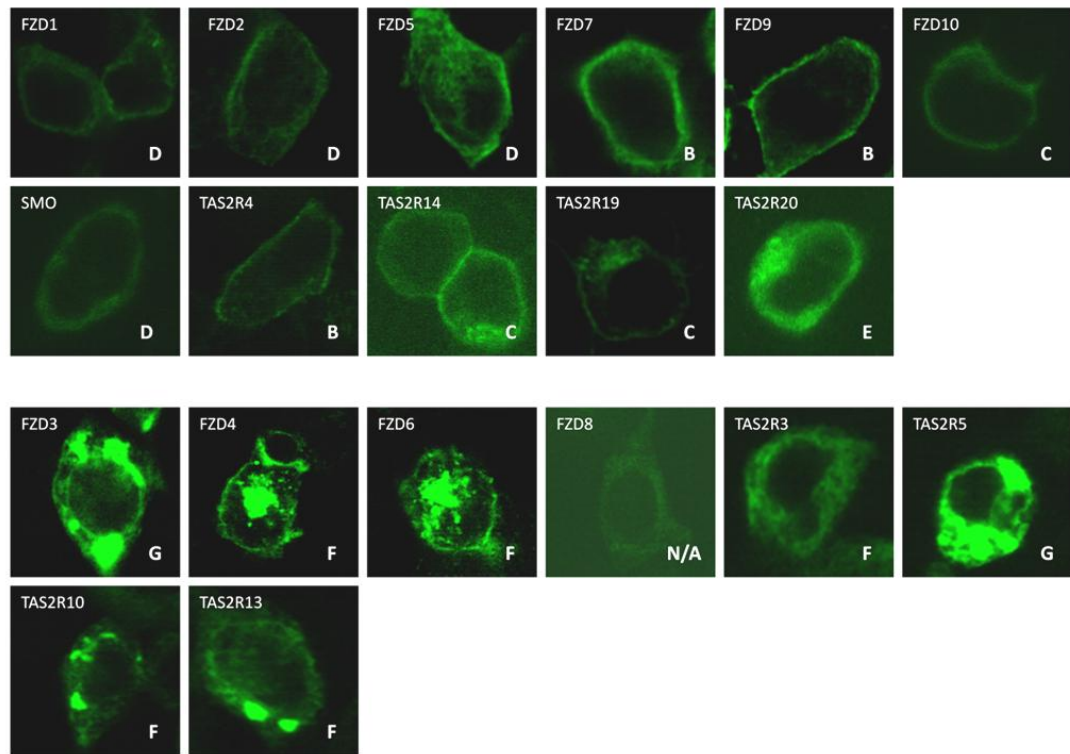


Figure 6.10: Representative confocal microscopy images of *Frizzled* and *Taste2* receptors expressed in HEK 293T cells as C-terminal GFP-fusions. Receptors were placed into categories A-G (Table 5.1) based on their subcellular localisation and degree of observable GFP aggregation. 7 receptors were not investigated by BRET as they were placed into categories F and G, indicating significant levels of apparent aggregation. FZD8 was not placed into a category as it exhibited extremely low levels of expression that made accurate inspection difficult. Such expression levels were too low to allow accurate measurements of $BRET_{off}$, and so this was also not investigated further.

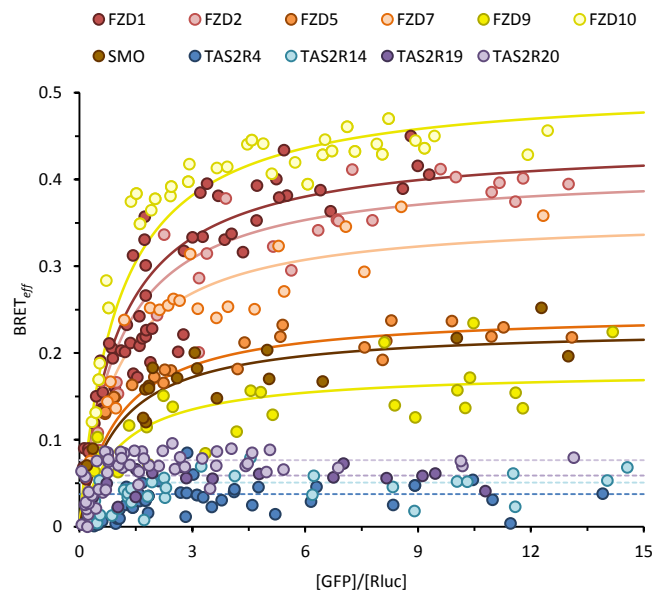


Figure 6.11: Type 1 BRET data of *Frizzled* and *Taste2* receptors suggests family-specific stoichiometries. Data collected for all 7 *Frizzled* receptors investigated fitted more closely to the distribution expected of dimers than that expected of monomers. Conversely, all 4 *Taste2* receptors investigated exhibited an independence of $BRET_{eff}$ upon $[GFP]/[Rluc]$, indicative of monomeric behaviour. For dimers, optimal fits to the dimeric model are shown by solid lines. For monomers, a broken line indicates the best fit to a monomer model. Dimer fit R^2 values are given in Table 6.8.

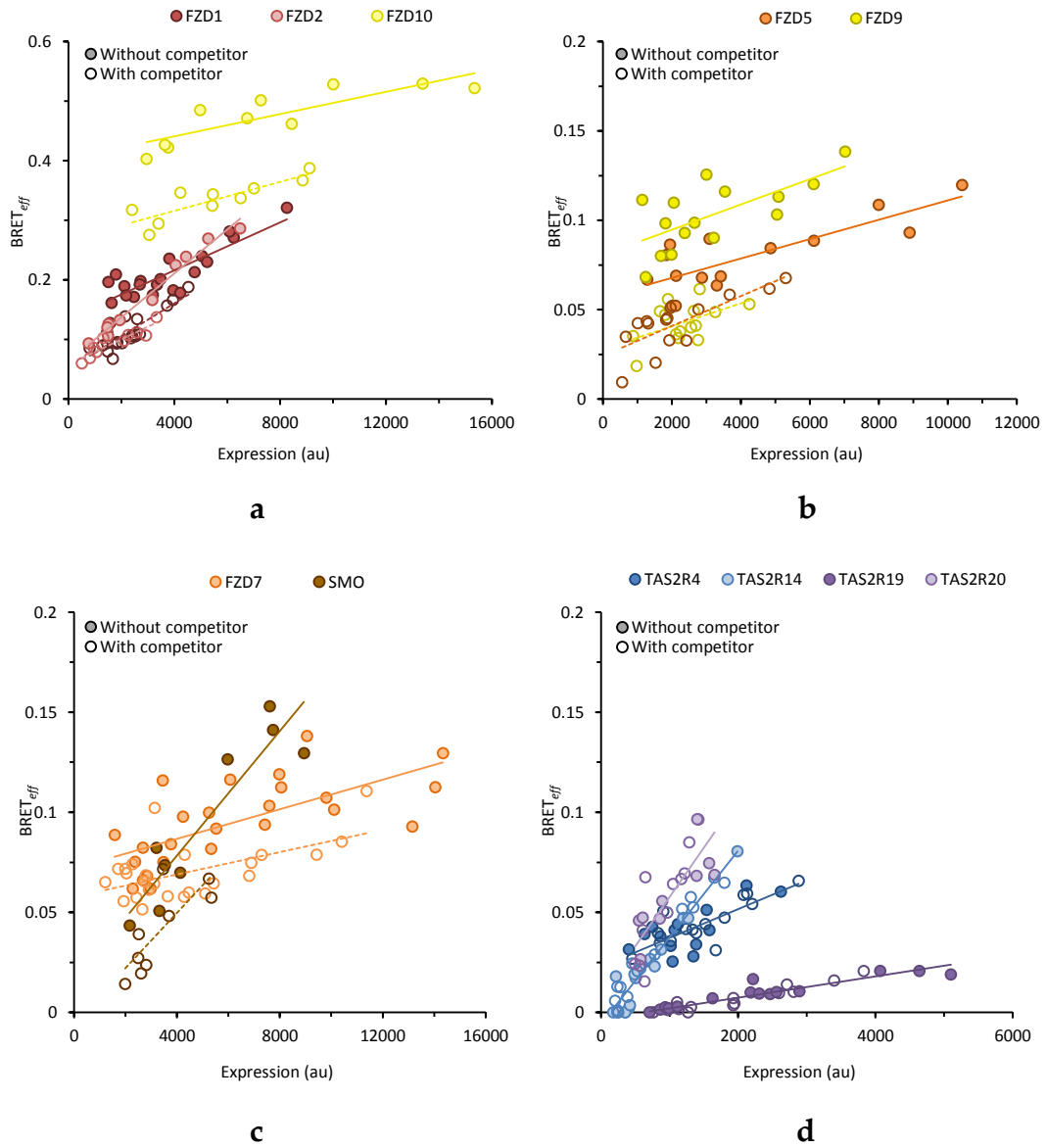


Figure 6.12: cBRET of *Frizzled* and *Taste2* receptors correlates with the type 1 BRET data. All 7 *Frizzled* receptors exhibit a decrease in $BRET_{eff}$ at a given expression level in the presence of competitor (**a**, **b**, & **c**). Conversely, no change in $BRET_{eff}$ -expression ratio is observed in the presence of competitor for any of the *Taste2* GPCRs investigated (**d**). R^2 values for linear regression of individual data sets and overall P^{diff} values are given in Table 6.8. In all cases, data collected in the absence of competitor is represented by filled circles, whereas those collected in the presence of competitor are shown as empty circles.

FZD10 flanking the 7TM region of TAS2R19. The six chimeras were all possible combinations of these components: FFT, FTT, TTF, TFF, FTF, and TFT. Five of the six chimeras expressed at sufficiently high levels for type 1 BRET analysis when transfected into HEK 293T cells; only FTT failed. Confocal microscopy suggested that none of the five well expressed chimeras aggregated, although the degree of intracellular retention varied (Fig. 6.13). The chimeras containing the FZD10 C-terminal domain (*i.e.* TTF, TFF, and FTF) appeared to undergo the greatest levels of retention, possibly due to interactions between this domain and components of the receptor-trafficking machinery.

In type 1 BRET assays these chimeras behaved differently from their parent proteins (Fig. 6.14). Firstly, replacement of the TAS2R19 C-terminal domain with that of FZD10 induced a change in the outcome of the type 1 BRET assay such that TTF exhibited a hyperbolic dependence of BRET_{eff} upon [GFP]/[Rluc], consistent with dimeric behaviour. This suggests that the FZD10 C-terminal is sufficient to induce the dimerisation of this construct. However, replacement of the C-terminal domain in FZD10 with that of TAS2R19 (*i.e.* chimera FFT) failed to abrogate apparent dimer formation, as FFT also yielded type 1 BRET data suggestive of dimerisation. This therefore implies that the C-terminal domain is sufficient but not necessary for FZD10 dimerisation. Replacement of both the N- and C-terminal domains of FZD10 with their TAS2R19 equivalents (*i.e.* TFT) appeared to abolish dimeric behaviour, yielding type 1 BRET data consistent with those expected of monomeric proteins. Thus the FZD10 7TM region does not appear to have any inherent dimerisation ability, and FFT instead appears to dimerise by its N-terminal domain. As FTT failed to express well, it was not possible to confirm that the FZD10 N-terminal domain was sufficient to mediate dimerisation. However, inclusion of this domain in FTF did not appear to impede dimerisation induced by the FZD10 C-terminal domain (Fig. 6.14). It would therefore seem that the behaviour of FZD10 and TAS2R19 in type 1 BRET assays is driven by differences in their N- and C-terminal domains, rather than in their 7TM regions. Given that these differing outcomes correspond to the expected behaviour of monomers and dimers, these data suggest that dimerisation in FZD10 is mediated by both its N- and C-terminal domains.

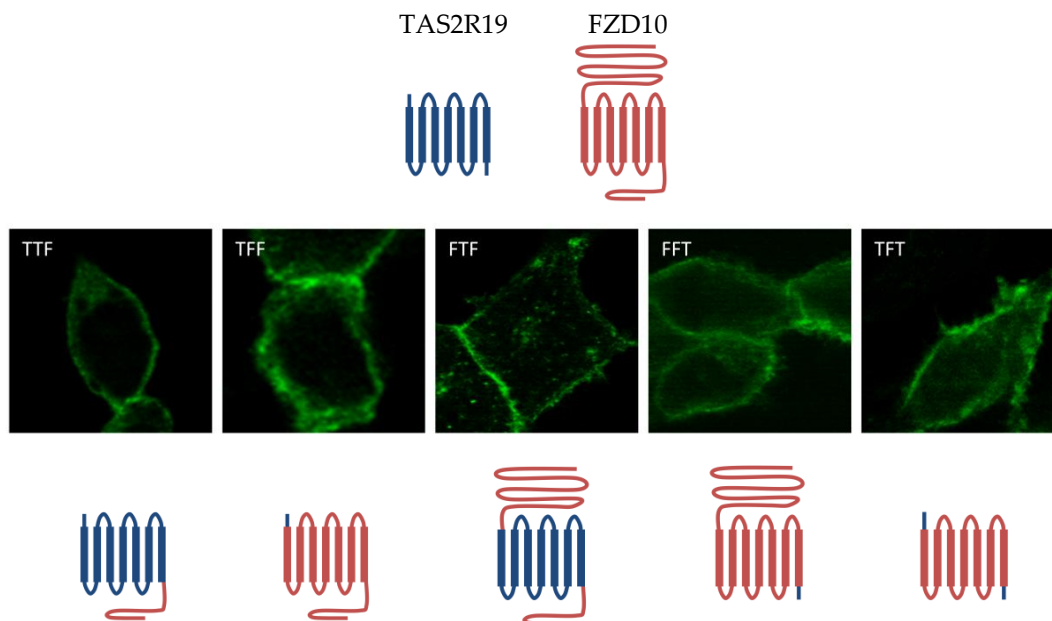


Figure 6.13: Representative confocal microscopy images of HEK 293T cells expressing the 5 successfully expressed TAST2R19-FZD10 chimeras from the pGFP² vector. Aggregation was not apparent in any case and so all were suitable for use in BRET. Schematic representations present the TAS2R19 (blue) and FZD10 (red) composition of each construct.

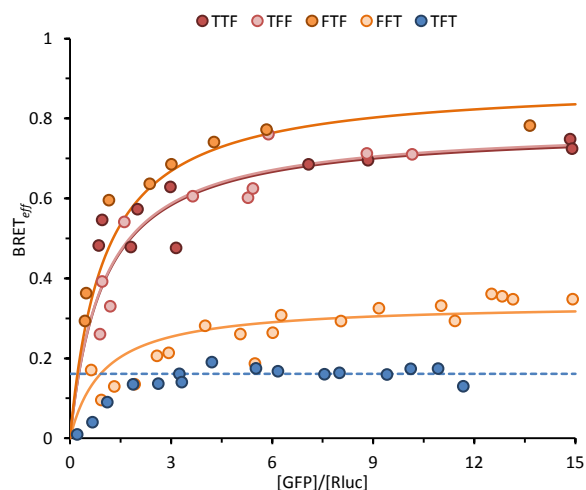


Figure 6.14: Type 1 BRET analysis of TAS2R19-FZD10 chimeras indicates a role in dimerisation of both the FZD10 N- and C-terminal domains. All chimeras containing either the FZD10 N- or C-terminal domains (TTF, TFF, FTF, and FFT) exhibited BRET_{eff} dependence upon [GFP]/[Rluc] in the manner predicted for a dimer. Replacement of the N- and C-terminal domains of FZD10 with those of TAS2R19 (chimera TFT) caused BRET_{eff} to become independent of [GFP]/[Rluc], which indicates monomeric behaviour.

Protein	Type 1 BRET Dimer fit R^2	Type 1 BRET $BRET_{max}$	cBRET R^2 (-competitor)	cBRET R^2 (+competitor)	cBRET P^{diff}
FZD1	0.44	0.443	0.74	0.71	<0.0001
FZD2	0.53	0.412	0.97	0.84	0.00147
FZD5	0.61	0.247	0.65	0.60	<0.0001
FZD7	0.50	0.358	0.45	0.32	<0.0001
FZD9	0.18	0.180	0.46	0.25	<0.0001
FZD10	0.13	0.508	0.72	0.73	<0.0001
SMO	0.32	0.229	0.84	0.62	0.0031
TAS2R4	-0.13	0.037	0.54	0.66	0.7544
TAS2R14	-0.14	0.050	0.91	0.95	0.1820
TAS2R19	-0.65	0.058	0.86	0.91	0.3675
TAS2R20	-0.20	0.076	0.66	0.67	0.9135
TTF	0.66	0.777	-	-	-
TFF	0.42	0.669	-	-	-
FTF	0.79	0.890	-	-	-
FFT	0.51	0.338	-	-	-
TFT	-0.19	0.160	-	-	-

Table 6.8: Type 1 and cBRET values obtained for *Frizzled* and *Taste2* GPCRs and TAS2R19-FZD10 chimeras in this chapter.

6.4 Discussion

6.4.1 Identification of a putative dimer interface in a *Rhodopsin* family GPCRs

The type 1 and cBRET data obtained for the *Rhodopsin* family S1P₃-LPA₁ chimeras in this chapter are consistent with a model of S1P₃ homodimerisation in which the dimer interface is organised around TM4, alongside other structural elements that contribute in a TM4-independent manner. This is in sharp contrast to the mechanism of dimerisation of the *Glutamate* family receptors, which does not appear to involve the 7TM region at all (133). The high sequence conservation within the S1P receptor family (Fig. 5.15) opens the possibility that a similar means of interaction is maintained across the whole family. If so, this could allow the formation of specific S1P heterodimers that interact via the same interface as homodimers.

6.4.2 Possibility of S1P receptor heterodimerisation via a conserved interface

The observation that BRET_{eff} for all three investigated S1P receptors is influenced by the presence of other members of the S1P receptor family (Fig. 6.9) suggests the formation of S1P receptor heterodimers. Thus, the S1P receptor population in the cell may exist as an equilibrium between homodimers and heterodimers as well as monomers, which may form either a significant fraction of the population or a brief, transient state adopted by receptors in the transition between homo- and heterodimerisation. cBRET is not capable of detecting heteromeric interactions that do not disrupt homodimerisation, and so if the effect observed in these experiments is genuine heterodimerisation then it must utilise the same or similar interface as homodimerisation.

Nonetheless, a number of alternative explanations for the observed hetero-cBRET results need also to be considered. One possibility is that the introduction of competitor receptors disrupted homodimer formation indirectly, perhaps by stabilising a monomer-prone conformation of the tagged receptor via signalling cross-talk or the depletion of a pool of dimer-stabilising third-party proteins. A similar effect could also cause apparent disruption of dimerisation through the promotion of an alternative dimer conformation in which the two C termini are more widely separated, reducing

BRET_{eff}. Thus, while heterodimerisation is indicated by these data, it is not the only explanation for the observed effects.

6.4.3 Differences in the apparent stoichiometry of *Frizzled* and *Taste2* GPCRs

The seven *Frizzled* and four *Taste2* family GPCRs behaved very differently in the type 1 and cBRET assays. Whereas the behaviour of the *Frizzled* family receptors corresponded to that expected of dimers, the *Taste2* GPCRs exhibited monomer-type behaviour. This implies that these two families have contrasting stoichiometries *in situ*. These observations are consistent with the foregoing analysis of *Rhodopsin* family stoichiometry wherein monomeric behaviour seemed dominant (§5.3), as the *Taste2* receptors are far more topologically similar to the *Rhodopsin* receptors than are the *Frizzled* GPCRs. Moreover, since it has been proposed that the *Taste2* family emerged from the *Rhodopsin* family ~300 million years ago (61), it would be reasonable to expect the behaviour of the progenitors to persist following divergence.

The observation that the 7TM region of FZD10 does not appear to have any inherent capacity for dimerisation indicates that the *Frizzled* receptors undergo a mechanism of dimerisation much more similar to that of the *Glutamate* family GPCRs than of S1P₃ and other *Rhodopsin* family receptors.

Interpretation of the chimera BRET_{max} values

As with the *Rhodopsin* family GPCRs, BRET_{max} can be informative in instances where dimerisation is implied by type 1 BRET. Of the four FZD10-TAS2R19 chimeras whose type 1 BRET data corresponded to dimeric behaviour, three (TTF, TFF, and FTF) yielded similar BRET_{max} values of ~0.7-0.8, whereas FFT exhibited a substantially lower BRET_{max} of 0.34 (Table 6.8). This indicates either that TTF, TFF, and FTF form more stable dimers than FFT, or that their C termini are more closely positioned to each other within the receptor dimer. It is unlikely that any of the chimeras would undergo a greater degree of dimerisation than wild-type FZD10, and so considering that the BRET_{max} observed for FZD10 was 0.51, it is probable that at least some of the contribution to the increased BRET_{max} in these chimeras is due to the relative repositioning of their C termini relative to FZD10 and FFT. One possibility is that the C-terminal domain of FZD10 interacts with other areas of the intracellular region (*i.e.* IL1, IL2, IL3, and intracellular ends of TM helices) in a way that positions the C termini at a separation distance

giving the observed $BRET_{max}$ of 0.51 (Table 6.8). This interaction would then not be conserved in chimeras TTF and FTF, after the IL and TM regions were exchanged for those of TAS2R19. Thus, in these chimeras, the C termini could be more closely positioned to each other due to the mutual interaction of the C-terminal domains, increasing the $BRET_{max}$ to the observed values of 0.78 for TTF and 0.89 for FTF (see Fig. 6.15). The fact that chimera TFF also exhibits an increased $BRET_{max}$ relative to FZD10 (0.67) suggests that the FZD10 N-terminal region might also be important in ensuring correct dimer arrangement and/or a conformation that allows interactions of the C-terminal domain and the core IL/TM region. In this case, the interaction that causes separation of the C termini may be lost or destabilised. Since TFF yielded a lower $BRET_{max}$ than both TTF and FTF, it is possible that the interaction is still preserved to some degree, limiting the relative $BRET_{max}$ increase from wild-type FZD10.

An alternative explanation for the high $BRET_{max}$ value observed for some of the FZD10-TAS2R19 chimeras is that partial protein misfolding causes non-physiological association of the chimeric receptors such that low levels of aggregation occur but cannot be detected by confocal microscopy. Although this cannot be ruled out, it does not detract from the conclusion that the FZD10 7TM domain has no inherent dimerisation capacity, as aggregation would lead to high $BRET_{max}$ values and false oligomer signatures, whereas the chimera TFT exhibits behaviour consistent with that of a monomer, which cannot be an artefact of aggregation. Thus, the proposed difference in the contribution of 7TM regions to dimerisation in S1P₃ and FZD10 would still appear to be valid.

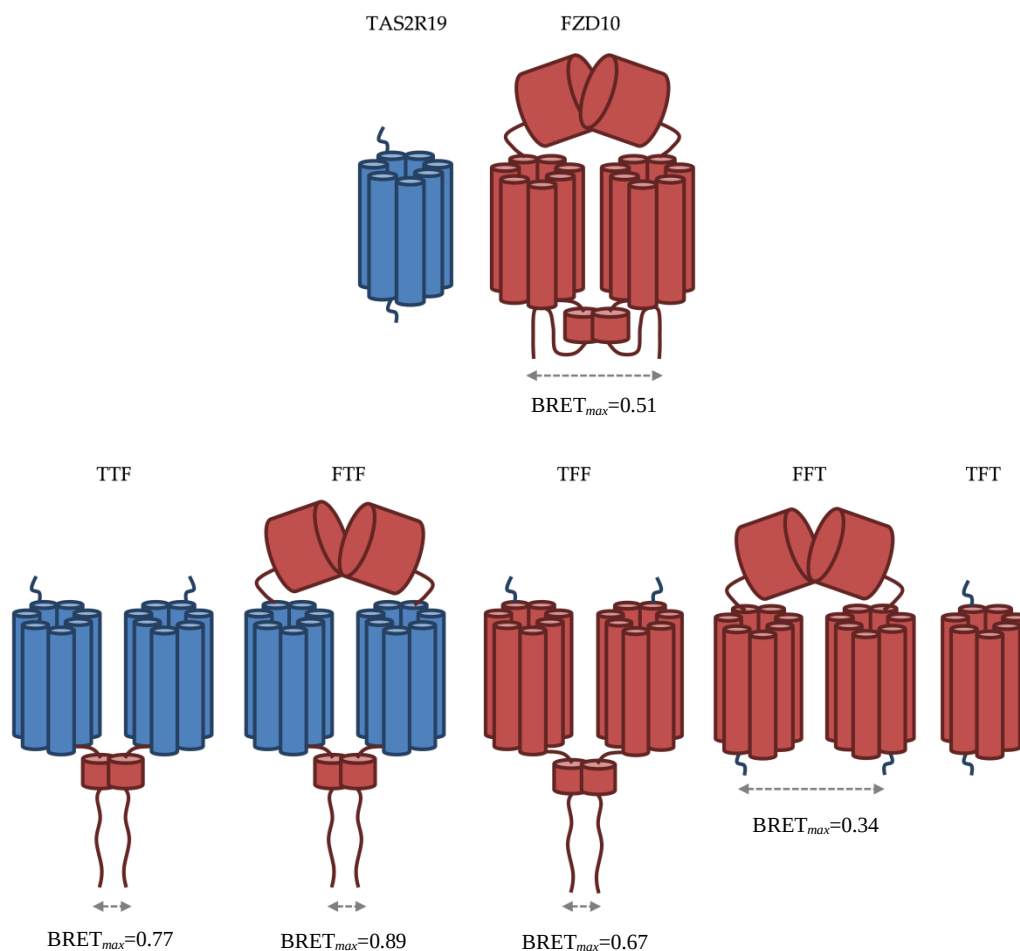


Figure 6.15: Schematic representation of possible domain arrangements within FZD10, TAS2R19, and chimeras of both. Both the N- and C-terminal domains of FZD10 appear to be sufficient to induce dimerisation in the chimeric receptors containing them, and both must be lost from FZD10 (*i.e.* TFT) in order to produce an apparently monomeric protein. The 7TM domains of both FZD10 and TAS2R19 do not appear to undergo significant levels of dimerisation on their own. The differing BRET_{max} values between chimeras and FZD10 indicate differences in the arrangement of the C-terminal domain. One possible model is shown in which the C-terminal domain of FZD10 interacts with the core region of the receptor but that this ability is lost in TTF, FTF, and TFF, such that BRET_{max} is increased due to the relative repositioning of the C termini. The BRET_{max} of FFT is lower than that of FZD10, which may be due to the lack of C-terminal domain dimerisation that brings the C termini closer together. This model assumes correct folding of the chimeras in question.

CHAPTER 7

A stimulated association BRET assay for the characterisation of GPCR heterodimers

7.1 Introduction

7.1.1 Current difficulties in heterodimer detection with BRET

The identification of putative S1P receptor heterodimers in cBRET assays (§6.3.2) could offer new insights into the nature of dimerisation within the *Rhodopsin* family. However, the data obtained using the cBRET assay are open to a number of possible interpretations, and so independent confirmation of the existence of S1P receptor heterodimers is ideally required. As discussed in Section 1.4.4, there is currently no technique capable of unambiguously identifying heterodimeric membrane protein complexes *in situ*. Type 1 and 2 BRET assays are reliant on equivalent rates of protein expression from acceptor- and donor-tagged constructs, and this cannot be guaranteed when investigating heteromeric interactions. The new cBRET assay can in principle be utilised to identify some heterodimers through the use of different proteins as the BRET pair and competitor, but is of limited utility. Firstly, this assay can only identify heterodimers formed by proteins that also undergo homodimerisation, otherwise the addition of competitor will have no effect on BRET_{eff} . Secondly, heterodimers that do not disrupt homodimerisation (*i.e.* that interact via a different interface) would also be undetectable by cBRET analysis because homodimeric complexes, and hence BRET_{eff} , will not be affected by the presence of competitor. Thirdly, observed changes in the BRET_{eff} -expression relationship in the presence of competitor in hetero-cBRET may also be attributable to factors independent of heterodimerisation, such as signalling cross-talk or depletion of a common interaction partner.

Some of the work performed in this chapter was performed under supervision by Alastair MacRae, Exeter College, and submitted as part of his FHS project BA Medical Sciences, Trinity Term, 2013. This constituted the cloning of all FKBP1-tagged constructs.

One technique developed specifically for the detection of GPCR heterodimers is GPCR-HIT (338), although even this is incapable of unambiguously identifying *bona fide* interactions. This is because the acceptor molecule in this case is fused to signalling proteins likely to redistribute upon signalling by the GPCR of interest. This has the potential to produce false dimer signals through increases in non-specific BRET_{eff} that arise from the recruitment of acceptor-tagged proteins into regions of the membrane with a higher local concentration of donor. This is discussed in more detail in Section 1.4.4. In order to rigorously analyse *Rhodopsin* family GPCR heterodimerisation, therefore, a new approach is needed.

7.1.2 A new BRET approach for identifying heterodimers

In this chapter, a new BRET technique is developed as a method of detecting heterodimerisation in membrane proteins, which is applied to the S1P receptors. This approach, termed stimulated association BRET (SABRE), is similar to GPCR-HIT but does not suffer the same problems of false dimerisation resulting from increased non-specific interactions. In the SABRE assay, putative heterodimeric interaction partners A and B are co-expressed in the same cells: protein A as a Rluc-/GFP-tagged BRET pair, and protein B as a fusion with FKBP. BRET_{eff} is measured in both the presence and absence of AP20187, which is a bivalent ligand of FKBP and so induces artificial dimerisation of protein B. If there is no interaction between proteins A and B, stimulated association of protein B will have no effect on BRET_{eff} as the distribution of protein A will be unaffected (Fig. 7.1a). Conversely, if specific interaction exists between the two proteins, dimerisation of protein B will increase the effective concentration of acceptor around each donor as protein A molecules will be indirectly recruited to one another (Fig. 7.1b). Thus, BRET_{eff} for heterodimers will be higher in the presence of AP20187 than in its absence. In principle, higher order hetero-oligomerisation should also be detectable by SABRE, as induction of protein B dimerisation should still cause a relative increase of the abundance of acceptor around each donor molecule.

The crucial difference between SABRE and other BRET approaches involving induced association, such as GPCR-HIT or third-party BRET (363), is that the acceptor- and donor-tagged proteins in SABRE are not directly affected by the change imposed upon the system, *i.e.* addition of AP20187. In both GPCR-HIT and third party BRET, the acceptor molecule is always inherently influenced by the imposed change (*i.e.* G protein-recruitment in GPCR-HIT), which makes interpretation of results more complicated. Thus, SABRE has the potential to be a simpler and more

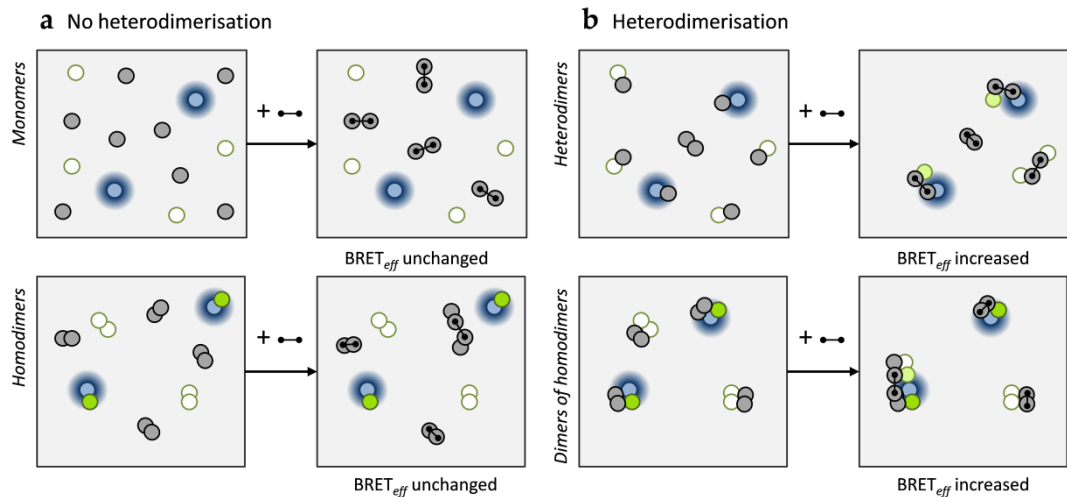


Figure 7.1: Overview of the SABRE technique for detecting heterodimerisation. Fluorescing and non-fluorescing acceptor molecules are shown as green and white circles, respectively, and donors as blue circles. The BRET-permissible area surrounding donors is represented as a blue halo. Proteins tagged with FKBP as shown as grey circles. AP20187 is represented as a black bar. (a) In cases of no heterodimerisation, induced association of FKBP-tagged receptors by binding AP20187 has no effect on the distribution of the acceptor- and donor-tagged proteins and so $BRET_{eff}$ is unchanged. (b) If specific heterodimerisation exists between the acceptor-/donor-tagged protein and the FKBP-tagged protein, introduction of AP20187 will recruit a greater number of acceptors into the local environment of each donor molecule, thereby increasing $BRET_{eff}$.

effective means of accurately identifying heterodimerisation. Moreover, although the most novel aspect of SABRE might be its ability to identify heterodimerisation, it can in principle also be applied to homodimers by using the same protein in the GFP-, Rluc-, and FKBP-fusion constructs. Used in this way, SABRE would complement analyses using type 1 and cBRET in the identification of homodimers.

7.2 Materials and Methods

7.2.1 Cloning of FKBP₁ constructs

A vector containing *FKBP* in place of *GFP*² was generated from the pGFP² vector by PCR amplification of *FKBP* from the pC₄-Fv1E vector (Ariad) using oligonucleotide primers 4016 + 4021 (Table 7.1). This produced an *FKBP* fragment with 5' *Bam*HI and 3' *Not*I restriction sites as well as a TAG STOP codon following the final codon of *FKBP* and a Gly-Ser-Gly-Ser-Gly-encoding linker between the *Bam*HI site and the start of *FKBP*. This fragment was used to replace *GFP*² in pGFP² by ligation following digestion with *Bam*HI and *Not*I to remove *GFP*². The integrity of this vector was confirmed by reversible terminator base sequencing (§2.1.2). All necessary genes were then subcloned into this vector using the relevant restriction sites within the multiple-cloning site of the pGFP² vector. The precise restriction sites used varied between constructs and correspond to those used in each case in previous chapters.

7.2.2 Cloning of FKBP₃ constructs

In order to generate constructs tagged with three FKBP proteins (FKBP₃) separated by flexible linkers of Gly-Ser-Gly-Ser-Gly, *FKBP*₁ was removed from the vector generated in Section 7.2.1 and replaced by *FKBP*₃. The *FKBP*₃ fragment was generated by PCR amplification of *FKBP* from the pC₄-Fv1E vector (Ariad) using oligonucleotide pairs 4016 + 4017, 4018 + 4019, and 4020 + 4021 (Table 7.1). This yielded three copies of *FKBP* with the following 5' and 3' restriction sites: *Bam*HI + *Sac*I, *Sac*I + *Xho*I, and *Xho*I + *Not*I. All copies were preceded by the sequence GGTTCGGATCGGGG, encoding the flexible linker, and the final copy was followed by a TAG STOP codon. These three copies were sequentially ligated together following digestion with *Sac*I and *Xho*I, in order to generate a complete *FKBP*₃ fragment with *Bam*HI and *Not*I 5' and 3' restriction sites. This was then used to replace *FKBP*₁ in the aforementioned

vector by ligation following *Bam*HI and *Not*I digestion. The integrity of the resulting vector was confirmed by reversible terminator base sequencing (§2.1.2). All necessary genes were then subcloned into this vector using the relevant restriction sites within the multiple-cloning site of the pGFP² vector. The precise restriction sites used varied between constructs and correspond to those used in each case in previous chapters.

7.2.3 Cloning of *Rluc8* constructs

Rluc8 was generated by site-directed mutagenesis of *Rluc* amplified from the pRluc vector using PCR. This was performed in a series of stages to generate the following mutations: A55T, C124A, S130A, K136R, A143M, M185V, M253L, and S287L. Initially, six segments of *Rluc8* were generated, each containing one or more direct mutations. This was performed using the following oligonucleotide primers as given in Table 7.1: (1) 1991 + 4138; (2) 4137 + 4139; (3) 4141 + 4143; (4) 4142 + 4145; (5) 4144 + 4147; (6) 4146 + 4035. These fragments were sequentially fused by chimeric PCR reactions as follows: (7) 1+2 using primers 1991 + 4139; (8) 4 using primers 4140 + 4143; (9) 7+8 using primers 1991 + 4143; (10) 4+5 using primers 4142 + 4147; (11) 9+10 using primers 1991+4147; (12) 11+6 using primers 1991+4035 to make final *Rluc8* gene. This was then used to replace *GFP*² in pGFP² by ligation following digestion with restriction endonucleases *Bam*HI + *Eco*RI. The integrity of the resulting vector was confirmed by reversible terminator base sequencing. All necessary genes were then subcloned into this vector using the relevant restriction sites within the multiple-cloning site of the pGFP² vector. The precise restriction sites used varied between constructs and correspond to those used in each case in previous chapters.

To generate a positive control suitable for use in *Rluc8* SABRE, a soluble GFP²-*Rluc8* construct was produced by PCR amplification of *GFP*² from pGFP² using oligonucleotide primers 4120 and 4513 (Table 7.1). This generated a *GFP*² gene with 5' *Mlu*I restriction site and Kozak sequence, and a 3' *Bam*HI site. This was then ligated into the pRluc8 vector following digestion with *Mlu*I and *Bam*HI and confirmed for integrity using reversible terminator base sequencing. The resulting vector encoded a soluble GFP² protein fused to *Rluc8* via its C terminus. This is analogous to the sGFP²-*Rluc* positive control used in previous BRET assays.

No.	Target	Dir.	Primer sequence	Restriction
4016	<i>FKBP</i>	5'	TAGTAGGGATCCTGGTTCCGGATCGGGGGGAGTGC AGGTGGAACCATC	<i>BamHI</i>
4017	<i>FKBP</i>	3'	CTACTAGAGCTCCCCTCCAGCTTCAGCAGCTCC	<i>SacI</i>
4018	<i>FKBP</i>	5'	TAGTAGGAGCTCGGTTCCGGATCGGGGGGAGTGCA GGTGGAACCATC	<i>SacI</i>
4019	<i>FKBP</i>	3'	CTACTACTCGAGCCCTCCAGCTTCAGCAGCTCC	<i>XhoI</i>
4020	<i>FKBP</i>	5'	TAGTAGCTCGAGGGTTCCGGATCGGGGGGAGTGCA GGTGGAACCATC	<i>XhoI</i>
4021	<i>FKBP</i>	3'	CTACTAGCGGCCGCTTTACTCCAGCTTCAGCAGCTC CACGTCG	<i>NotI</i>
4137	<i>Rluc (A55T)</i>	5'	GCAACGCCACTAGCAGCTACCTG	-
4138	<i>Rluc (A55T)</i>	3'	CAGGTAGCTGCTAGTGGCGTTGC	-
4139	<i>Rluc (C124A +S130A)</i>		GTAGGCGTAGTGGAAGGCCAGGGCGGC	-
4140	<i>Rluc (S130A + K136R)</i>	5'	TGGCCTTCCACTACGCCTACGAGCACCAGGACAGG ATCAAG	-
4141	<i>Rluc (K136R+ A143M)</i>	5'	CACCAGGACAGGATCAAGGCCATCGTGCACATGGA GAGCGTGG	-
4142	<i>Rluc (M185V)</i>	5'	GTGGAGACCGTGCTGCCCAG	-
4143	<i>Rluc (M185V)</i>	3'	CTGGGCAGCACGGTCTCCAC	-
4144	<i>Rluc (M253L)</i>	5'	CTGCCCCAAGCTGTTTCATCGAGAG	-
4145	<i>Rluc (M253L)</i>	3'	CTCTCGATGAACAGCTTGGGCAG	-
4146	<i>Rluc (S287L)</i>	5'	CCTGCACTTCCTCCAGGAGGACG	-
4147	<i>Rluc (S287L)</i>	3'	CGTCCTCCTGGAGGAAGTGCAGG	-
1991	<i>Rluc</i>	5'	TAGTAGGGATCCGATGACCAGCAAGGTGTACGACC	<i>BamHI</i>
4035	<i>Rluc</i>	3'	CTACTAGGATTCTGCTCGTTCTTCAGCACTCTCTCC	<i>EcoRI</i>
4120	<i>GFP²</i>	5'	TAGTAGACGCGTGCCACCATGGTGAGCAAGGGCGA GG	<i>MluI</i>
4513	<i>GFP²</i>	3'	CTACTAGGATCCCCCTTGTACAGCTCGTCCATGC	<i>BamHI</i>

Table 7.1: Oligonucleotide primers used in this chapter.

7.2.4 SABRE assay

Transfection for the SABRE assay was performed in 6-well plates containing $\sim 6 \times 10^5$ HEK 293T cells per well. Cells were transfected with 1.5 μg total DNA per well using the GeneJuice® (Novagen) transfection reagent as per the manufacturer's protocol. Unless otherwise stated, this DNA was a mix of pFKBP_{1/3}, pGFP², and pRluc/Rluc8 in a ratio 26:12:1. 24 h post transfection, cells were removed from 6-well plates and suspended in 200 μl PBS. This was separated into two wells of a 96-well OptiPlate (PerkinElmer). To one well, 10 μl of 50 μM AP20187 (unless alternative concentration is specified) was added, whilst 10 μl PBS was added to the other. Cells were then incubated for 45 min at room temperature before data collection. BRET_{eff} was measured as described in Section 3.2.4. For assays involving Rluc8, the PMT gain setting of the Fusion® Microplate Analyzer (given in Table 3.2) was reduced to 3 to account for the increased intensity of the luminescence relative to Rluc.

Statistical analyses

Data obtained by SABRE analysis were analysed using the Prism5 (GraphPad) software. Data were treated as paired sets corresponding the measurements taken in the presence and absence of AP29187 for a single well of transfected cells. Significance was determined using a Student *t* test to evaluate whether the mean difference in BRET_{eff} between the AP20187+ and AP20187- measurements significantly deviated from zero.

7.2.5 Flow cytometry and confocal microscopy

Surface expression of CD28 in the presence and absence of AP20187 was assessed via confocal microscopy. HEK-293T cells were transfected in an identical manner to that used for SABRE analysis (§7.2.4). Cells were incubated with 5 μM AP20187 or PBS for 45 min at room temperature before fixation and imaging as described in 2.4.2 using GFP-suitable excitation and emission filters.

Flow cytometry to determine relative construct expression was performed on CD2-Rluc, CD2-FKBP₁, CD2-FKBP₃, and CD2-Rluc8. HEK 293T cells were transfected with 1 μg of the relevant vector per 6×10^5 cells using GeneJuice® (Novagen) as per the manufacturer's protocol. 24 h post transfection, cells were labelled for flow cytometry analysis as described in Section 2.3.1 using 0.05 $\mu\text{g}/\mu\text{l}$ of either mouse anti-human CD2 antibody (eBioscience: 12-0029-42) or mouse IgG isotype

control (eBioscience: 12-4714-12). Cells were then assessed by flow cytometry using fluorescence in PE channel as a read-out.

7.3 Results

7.3.1 Proof-of-principle of the SABRE technique

Application of SABRE to homodimerisation in known type 1 TM monomer and dimer controls demonstrated that the approach successfully discriminates between the two stoichiometries. Cells were transfected with a 2:1 ratio of pFKBP:pGFP²/pRLuc and a 12:1 ratio of pGFP²/pRLuc – *i.e.* 26:12:1 pFKBP:pGFP²:pRLuc. Each well of transfected cells was split into two separate wells of a 96-well plate for BRET_{eff} measurement, to which either PBS or 5µM (final concentration) AP20187 was added. Measurement of BRET_{eff} for these two wells after 45 min incubation at room temperature generated paired data sets in which cells were identical other than the presence or absence of AP20187. For the known monomers CD2 and CD86, no significant difference in BRET_{eff} was observed between the two wells, whereas the known dimers CD28 and CD80 both exhibited significant increases in BRET_{eff} in the presence of AP20187 (Fig. 7.2). Although CD28 is a covalent homodimer (490), CD80 only dimerises in a non-covalent transient manner (491), and so it appears that the SABRE methodology is suitable for the study of partial dimers, at least for type 1 TM proteins.

Extension of this approach to the detection of homodimerisation in GPCRs also apparently validated the SABRE method. Application of SABRE to the known GPCR dimer GABABR1b yielded a significant increase in BRET_{eff} in the presence of AP20187 (Fig. 7.3), consistent with its dimeric behaviour. FZD1, S1P₂, and S1P₃ also exhibited significant increases in BRET_{eff} in the presence of AP20187, whereas CCR5 did not (Fig. 7.3). This is consistent with previously described type 1 and cBRET data suggesting dimeric behaviour for FZD1, S1P₂, and S1P₃, but monomeric behaviour for CCR5 (§3.3; §4.3; §5.3). This indicates that despite the larger hydrodynamic diameter of GPCRs compared to type 1 TM proteins, SABRE is still suitable for the detection of GPCR dimers *in situ*. The fact that apparent dimerisation was observed for GPCRs from three different families (*i.e.* *Glutamate*, *Frizzled*, and *Rhodopsin*) suggests that the flexible linker segment (Gly-Ser-Gly-Ser-Gly) between the C terminus of the GPCR and the N terminus of FKBP is sufficient to allow independent rotation of the two GPCR dimers within the FKBP-induced tetramer. This is because such distantly related proteins are unlikely to share the same topology within the respective dimer complexes, and so if the two

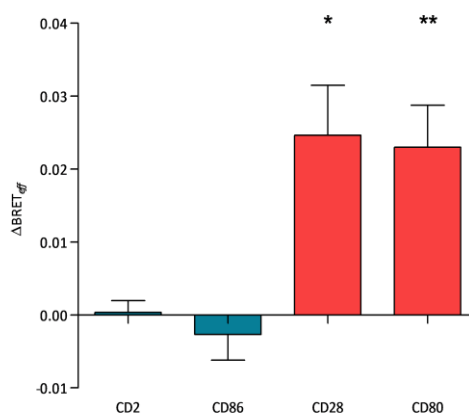


Figure 7.2: The SABRE principle is capable of distinguishing between type 1 TM monomers and dimers. Neither CD2 nor CD86 exhibited a significant increase in $BRET_{eff}$ in the presence of AP20187, which is consistent with their known monomeric behaviour. Conversely, $BRET_{eff}$ was observed to increase significantly upon addition of AP20187 for both CD28 and CD80, which is in line with dimerisation. Significance is shown for a one-sample t test of values against a $\Delta BRET_{eff}$ of zero (* $p > 0.05$; ** $p > 0.01$). Error bars represent SEM.

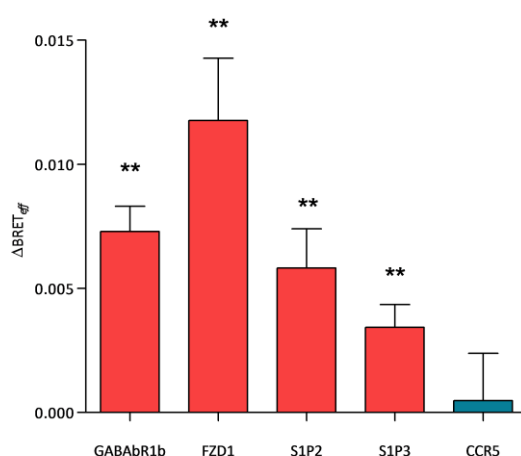


Figure 7.3: SABRE can also discriminate between apparent GPCR monomers and dimers. The well established GPCR dimer GABAbR1b yielded a significant increase in $BRET_{eff}$ in the presence of AP20187, consistent with dimeric behaviour. Three GPCRs that have been suggested to be dimers by type 1 and cBRET in this thesis (FZD1, S1P₂, and S1P₃) were also observed to increase $BRET_{eff}$ upon addition of AP20187. CCR5 did not exhibit a significant $\Delta BRET_{eff}$ in the presence of AP20187. Significance is shown for a one-sample t test of values against a $\Delta BRET_{eff}$ of zero (** $p > 0.01$). Error bars represent SEM.

dimers within the induced tetramer were rigidly fixed in their relative positions it is likely that at least one of the observed dimeric GPCRs would not have permitted BRET within the tetrameric complex. Nonetheless, the increases in $BRET_{eff}$ observed for the apparent GPCR dimers (Fig. 7.3) are smaller than those for CD28 and CD80 (Fig. 7.2), likely due to the difference in size between the two types of protein.

7.3.2 Improving the sensitivity of SABRE using FKBP₃

Although SABRE seems to reliably distinguish between monomers and dimers, the absolute increases in $BRET_{eff}$ observed for GPCRs are relatively small. This may make detection of weak dimerisation difficult, which is particularly pertinent if SABRE is to be used for the analysis of heterodimers, as these complexes may be less stable than their homodimer equivalents. Thus, in an attempt to maximise the observed increases in $BRET_{eff}$, a similar strategy was attempted using three copies of FKBP (FKBP₃) fused to the C terminus of the protein of interest rather than just one (FKBP₁). In principle, this should allow the formation of cross-linked networks of FKBP₃-tagged proteins in the presence of AP20187, which recruit GFP/Rluc-tagged proteins in the same way as above (Fig. 7.4). This would be expected to increase $BRET_{eff}$ more significantly than using FKBP₁-tagged proteins as the effective concentration of GFP/Rluc-tagged proteins recruited to an oligomeric cluster should be greater than those recruited to individual FKBP₁ dimers. Flexible linker sequences (Gly-Ser-Gly-Ser-Gly) were inserted between each FKBP in order to allow independent rotation of each copy and to maximise the likelihood of cross-linking between multiple proteins rather than the formation of FKBP dimers only.

Application of this approach to CD2, CD86, CD28, and CD80 yielded data similar to the FKBP₁-SABRE experiments, in that increases in $BRET_{eff}$ in the presence of AP20187 were only observed for the dimers CD28 and CD80 but not for the monomers CD2 and CD86 (Fig. 7.5). The absolute $\Delta BRET_{eff}$ observed for CD28 (0.053) was far higher than that observed using FKBP₁ (0.025), whilst CD80 also exhibited a modest improvement (from 0.023 to 0.033). The data obtained for both proteins were also more significantly non-zero than when using FKBP₁ ($p < 0.001$ rather than $p < 0.01$). Use of FKBP₃ in SABRE, therefore, appears to give a more sensitive assay than FKBP₁.

In order to ensure that the observed differences in the FKBP₁ and FKBP₃ approaches were not due to variation in the relative expression levels of the two constructs, flow cytometry was performed on HEK 293T 24 h post-transfection with 1 μ g of either CD2-Rluc, CD2-FKBP₁, or CD2-FKBP₃.

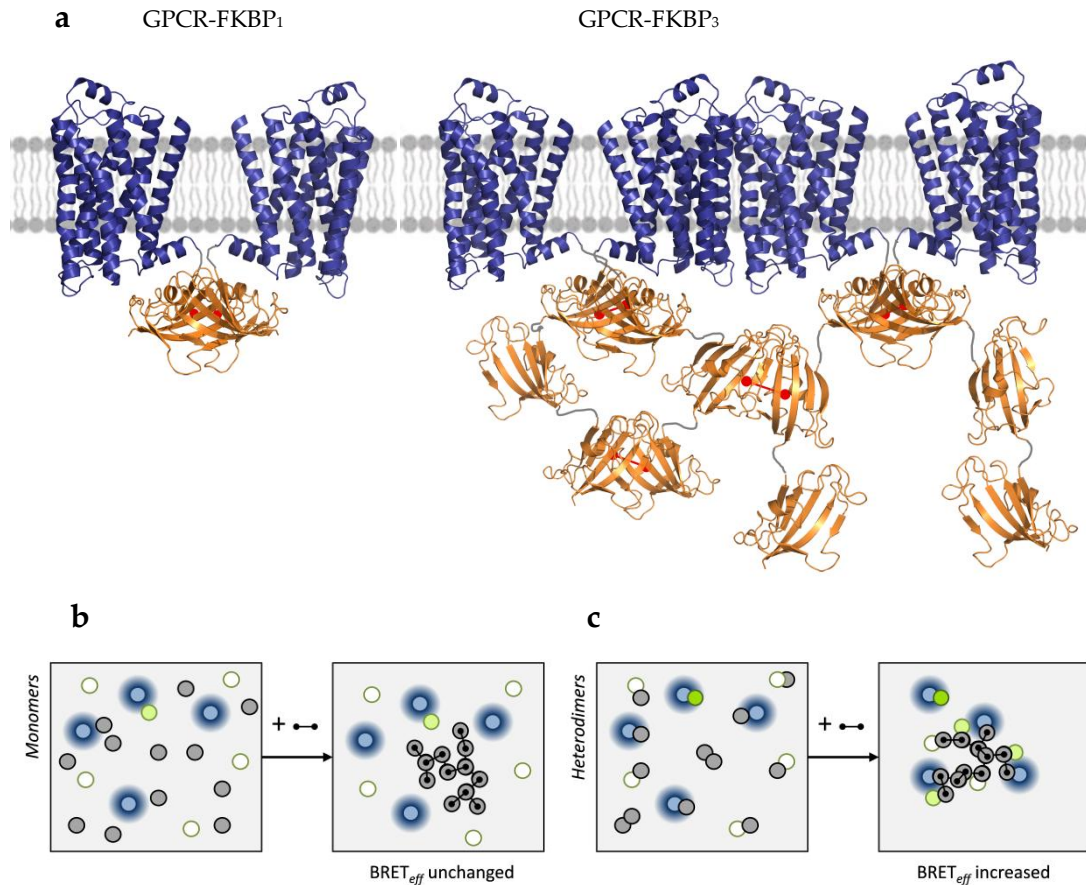


Figure 7.4: Comparison of SABRE using FKBP₁ or FKBP₃ GPCR fusions. **(a)** Use of FKBP₁ fusions can at most generate artificial dimers (left) whereas FKBP₃ should be capable of inducing oligomeric clusters of tagged GPCRs via cross-linking of different FKBP₃-tagged receptors (right). GPCR (PDB: 3NYA) is shown in blue; FKBP (PDB: 1D6O) is shown in orange; AP20187 is represented as a red bar; flexible linker regions are shown in grey. **(b & c)** Induction of FKBP₃-tagged GPCR clustering should have no effect on the BRET_{eff} of GFP- and Rluc-tagged receptors that do not interact with the clustered protein **(b)**. Conversely, BRET_{eff} should increase for GFP- and Rluc-tagged proteins that undergo specific interactions with the clustered FKBP₃-tagged receptor **(c)**. For **b** and **c**, fluorescing and non-fluorescing acceptor molecules are shown as green and white circles, respectively, and donors as blue circles. The BRET-permissible area surrounding donors is represented as a blue halo. Proteins tagged with FKBP₃ as shown as grey circles. AP20187 is represented as a black bar.

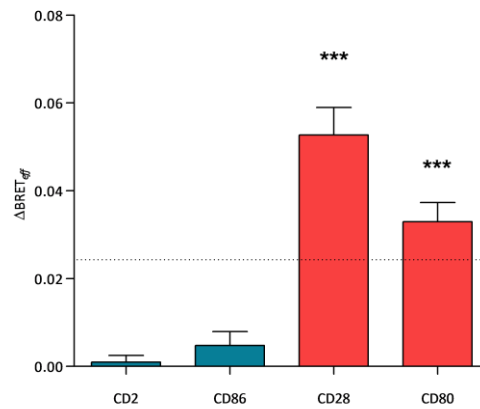


Figure 7.5: Using FKBP₃ for SABRE is more sensitive than using FKBP₁. Both CD28 and CD80 exhibited highly significant increases in BRET_{eff} in the presence of AP20187 that were larger than those observed using FKBP₁. Broken line denotes a $\Delta BRET_{eff}$ value of 0.025, which is equivalent to the values obtained for both CD28 and CD80 using FKBP₁. Neither CD2 nor CD86 exhibited a significant increase in BRET_{eff} in the presence of AP20187. Significance is shown for a one-sample *t* test of values against a $\Delta BRET_{eff}$ of zero (*** *p* > 0.001). Error bars represent SEM.

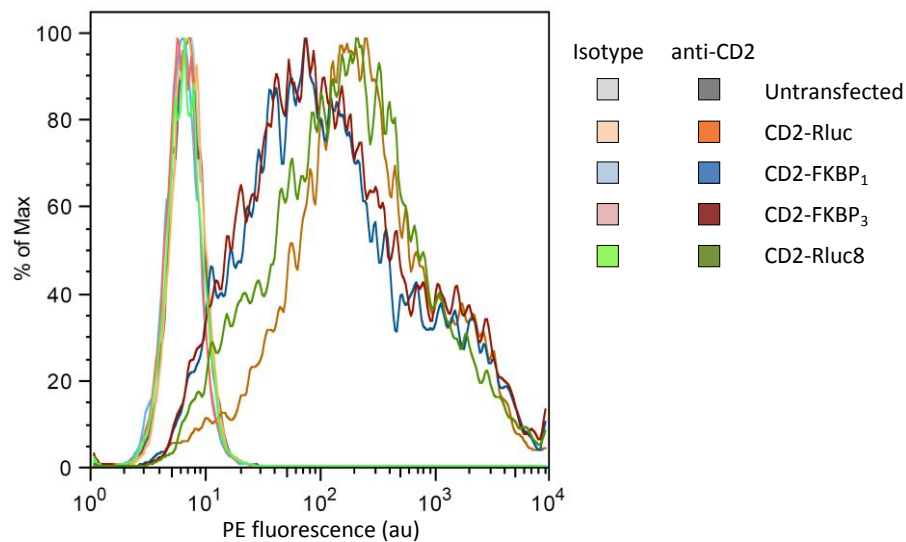


Figure 7.6: Equivalent expression is observed for the various constructs used in the SABRE assay. CD2 expressed to roughly equivalent levels when C-terminally tagged with either Rluc, FKBP₁, FKBP₃, or Rluc8. Flow cytometry is of HEK 293T cells transfected with 1 μ g of the relevant construct DNA per 6×10^5 cells and labelled with either anti-CD2 PE conjugate or PE-conjugated Isotype control. Equivalent expression from the pGFP² vector relative to pRluc has been established previously (Fig. 4.2).

Labelling with anti-CD2 PE-conjugated monoclonal antibody revealed comparable expression levels for all three constructs (Fig. 7.6). The increased $\Delta\text{BRET}_{\text{eff}}$ of FKBP₃ relative to FKBP₁ does not therefore appear to be due to differences in expression or surface targeting of the two constructs. It also does not appear to be caused by increased AP20187-induced protein internalisation, as no such internalisation was evident according to confocal microscopy of HEK 293T cells expressing CD28-GFP and either CD28-FKBP₁ or CD28-FKBP₃ (Fig. 7.7).

7.3.3 Incorporation of Rluc8 into SABRE

Given the increased quantum yield of the Rluc variant Rluc8 (311), its incorporation into the SABRE assay in place of original Rluc may help to broaden the scope of the assay to allow investigation of weakly expressed proteins. It was nonetheless necessary to confirm that the SABRE method worked when Rluc8 was used. Rluc8 was generated from Rluc using site-directed mutagenesis of the appropriate residues, and used to replace Rluc in all the control constructs. Flow cytometry revealed that Rluc8- and Rluc-tagged CD2 were expressed at equivalent levels (Fig. 7.6) and so the amounts of Rluc- and GFP-encoding DNA should also be suitable for Rluc8. SABRE performed using FKBP₃ and Rluc8 yielded similar outcomes for the type 1 TM controls CD2, CD86, CD28, and CD80 versus Rluc (Fig. 7.8). As Rluc8 has an improved signal-to-noise ratio than original Rluc, this variant was used hereafter.

7.3.4 Optimisation of the final SABRE assay

Thus far, the ratio of GFP/Rluc- and FKBP-tagged had not been systematically optimised for maximal $\Delta\text{BRET}_{\text{eff}}$ and so it was possible that the approach could be made more sensitive by using a different ratio. The optimum ratio is one in which the FKBP₃-tagged protein is in excess, in order to maximise interactions with GFP/Rluc8-tagged proteins, but not so high as to preclude BRET-productive oligomer formation upon addition of AP20187 (Fig. 7.9). If FKBP₃ expression is too low, the majority of GFP/Rluc8-tagged proteins will not interact with FKBP₃-tagged proteins and so only a small fraction will experience an increase in BRET_{eff} in the presence of AP20187. Conversely, if the FKBP₃-tagged protein is in large excess, any GFP/Rluc8-tagged proteins that are recruited into the induced oligomer will be unlikely to be within a BRET-permissive radius of each other as the majority of the oligomer will consist of FKBP₃-tagged proteins. Thus, the optimal situation is a compromise between

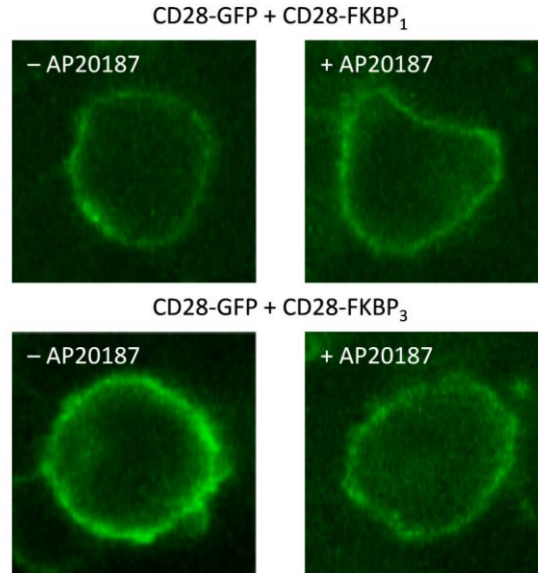


Figure 7.7: Confocal microscopy of HEK 293T cells co-expressing CD28-GFP and either CD28-FKBP₁ or CD28-FKBP₃ in the presence and absence of 5 μ M AP20187. Cells were incubated for 45 min after addition of AP20187 prior to fixation and imaging. Images shown are representative of the majority of cells. In all cases CD28-GFP appears to be largely localised to the plasma membrane, with only low levels of intracellular GFP fluorescence. The addition of AP20187 does not appear to induce internalisation of CD28-GFP.

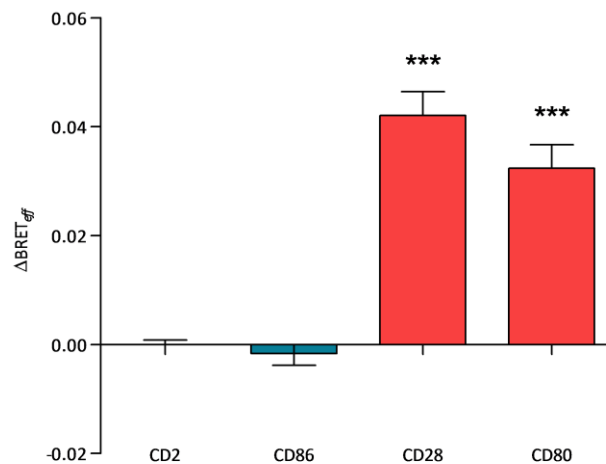


Figure 7.8: Application of the SABRE assay to type 1 TM controls using Rluc8 gives comparable data to when performed using Rluc. Significance is shown for a one-sample t test of values against a $\Delta BRET_{eff}$ of zero (*** $p > 0.001$). Error bars represent SEM.

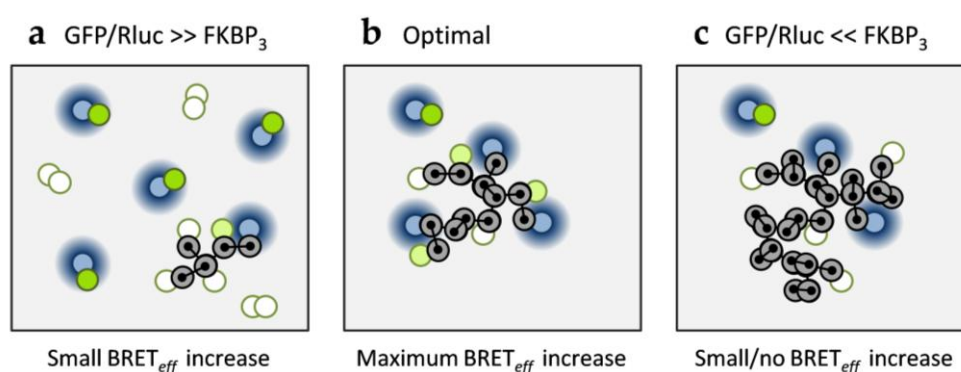


Figure 7.9: The relative abundance of GFP/Rluc- and FKBP-tagged proteins has a significant effect on the outcome of SABRE assays. **(a)** If the GFP/Rluc-tagged protein A is in large excess over the FKBP-tagged protein B then only a minority of protein A molecules will form heterodimers with protein B. Thus, only a small proportion of the protein A population will experience an increase in BRET_{eff} upon addition of AP20187. This may be difficult to resolve against the background of strong levels of BRET occurring within protein A homodimers. Conversely, if protein B is in large excess, addition of AP20187 may induce the formation of protein clusters in which the associated protein A molecules are too widely distributed to produce a measurable increase in BRET_{eff} **(c)**. The ideal scenario is one in which protein B is in sufficient excess to maximise interactions with protein A, but not so high as to dominate the AP20187-induced cluster and preclude an increase in BRET_{eff} **(b)**. Fluorescing and non-fluorescing acceptor molecules are shown as green and white circles, respectively, and donors as blue circles. The BRET-permissible area surrounding donors is represented as a blue halo. Proteins tagged with FKBP₃ as shown as grey circles. AP20187 is represented as a black bar.

maximising interactions between the two proteins and maximising the concentration of GFP/Rluc8-tagged proteins in the AP20187-induced oligomer.

In order to assess this, SABRE was applied to cells transfected with CD28 at varying ratios of pFKBP₃ and pGFP²/pRluc8 DNA, using a total of 1.5µg DNA per transfection reaction. The magnitude of $\Delta\text{BRET}_{\text{eff}}$ varied significantly with the ratio used (Fig. 7.10a). As expected, no increase in BRET_{eff} was observed when only pGFP²/pRluc8 was used, which indicates that the AP20187 is not having FKBP-independent effects on BRET_{eff} . Positive $\Delta\text{BRET}_{\text{eff}}$ values were obtained for all strategies including pFKBP₃, though this was only significant above a value of 0.75µg per transfection. The extent of $\Delta\text{BRET}_{\text{eff}}$ did not alter significantly above a level of 1µg pFKBP₃ per transfection. This is despite reduced absolute mean BRET_{eff} values in the presence and absence of AP20187 at higher pFKBP₃ levels (Fig. 7.10c). This is likely a result of increased competition from FKBP₃-tagged CD28 for position within homodimers, such that fewer BRET-productive dimers are formed. Thus, although $\Delta\text{BRET}_{\text{eff}}$ is fairly consistent at higher pFKBP₃ levels, the absolute BRET_{eff} observed is inversely proportional to the amount of pFKBP₃ used. Absolute Rluc8 emission levels also decrease as the amount of pFKBP₃ used increases (Fig. 7.10d), as less pRluc8 is included in each transfection reaction. At the highest amount of pFKBP₃ (1.4µg) total Rluc8 emission in a typical measurement is ~1000AU. At ~500AU, accurate BRET_{eff} determination becomes difficult as the level of Rluc8 emission that is detected in the GFP channel (~2% total Rluc8 emission) is of a magnitude similar to the background signal of the assay (~10AU). As CD28 is a type 1 TM protein, it expresses at higher levels than most GPCRs, and so it is likely that a transfection strategy using 1.4µg pFKBP₃ would yield too low a signal for BRET_{eff} measurement for many GPCRs. In light of this observation, and the considerations regarding $\Delta\text{BRET}_{\text{eff}}$, the optimal transfection strategy appears to be the use of 1µg pFKBP₃ and 0.5µg pGFP²/pRluc8.

Finally, the concentration of AP20187 required to maximise $\Delta\text{BRET}_{\text{eff}}$ was also determined. Too low a concentration of AP20187 might leave some FKBP-tagged receptors unable to incorporate into induced oligomers, whereas too high a concentration might saturate the FKBP proteins such that each binds its own AP20187 molecule and oligomerisation is not induced. Titration of AP20187 within the optimised SABRE assay applied to CD28 indicates that 5µM AP20187 yields the maximal $\Delta\text{BRET}_{\text{eff}}$ (Fig. 7.10b).

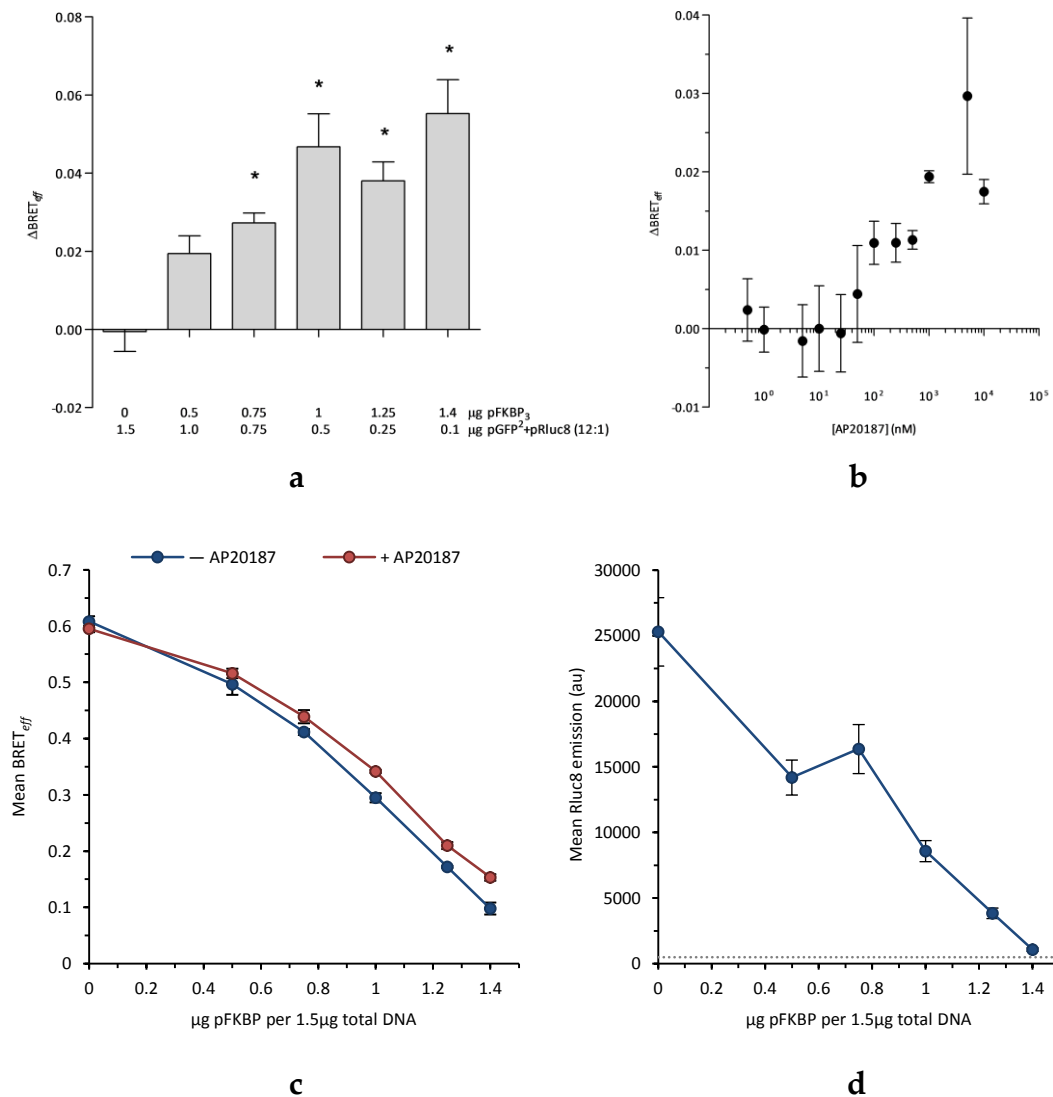


Figure 7.10: Optimisation of the SABRE assay. All experiments were performed using CD28 as a dimeric reporter. **(a)** Varying the ratio of pFKBP₃:pGFP²/pRluc8 used in transfection affects the $\Delta\text{BRET}_{\text{eff}}$ observed. Maximal $\Delta\text{BRET}_{\text{eff}}$ is achieved when 1 μg or more pFKBP₃ is used in the final 1.5 μg transfection reaction. **(b)** Titration of AP20187 indicates maximal $\Delta\text{BRET}_{\text{eff}}$ is reached using 5 μM AP20187. **(c)** As the amount of pFKBP₃ DNA used in the 1.5 μg total transfection increases BRET_{eff} in both the presence and absence of AP20187 decreases due to increased competition from FKBP₃-tagged proteins for position within CD28 homodimers. The difference between mean BRET_{eff} in the presence and absence of AP20187 is roughly consistent above a value of 1 $\mu\text{g pFKBP}_3$. There is no difference between BRET_{eff} with or without AP20187 when no pFKBP₃ is included. **(d)** As $\mu\text{g pFKBP}_3$ increases, absolute Rluc8 emission levels decrease due to reduced expression of both the GFP- and Rluc8-tagged proteins. At 1.4 $\mu\text{g pFKBP}_3$ detected Rluc8 emission is only around twice the level at which accurate measurement of BRET_{eff} becomes difficult (~500 au; indicated by a broken line). Significance is shown for a one-sample *t* test of values against a $\Delta\text{BRET}_{\text{eff}}$ of zero (* *p*>0.05). Error bars represent SEM.

7.3.5 Detection of apparent GPCR heterodimers

Application of the optimised SABRE assay to GABABR1b, FZD1, S1P₂, S1P₃, and CCR5, in tests for homodimerisation yielded data consistent with the proposed stoichiometry in each case: *i.e.* all receptors exhibited significant increases in BRET_{eff} in the presence of AP20187 with the exception of CCR5 (Fig. 7.11). This validates the ability of the final assay to distinguish between apparent monomers and dimers for GPCRs as well as type 1 TM proteins.

In order to investigate possible heterodimerisation within the S1P receptor family, the assay was then applied to these GPCRs using different proteins as the GFP/Rluc8- and FKBP₃-tagged species. All combinations of S1P₂, S1P₃, and S1P₅ were assessed, alongside LPA₁ and CCR5 as FKBP₃-fusions with S1P₃ tagged with GFP/Rluc8. The expression levels of all five GPCRs had previously been quantified and found to be broadly similar (S1P₂ = ~280,000 proteins/cell; S1P₃ = ~120,000 proteins/cell; S1P₅ = ~130,000 proteins/cell; LPA₁ = ~160,000 proteins/cell; CCR5 = ~100,000 proteins/cell; Table 5.14). This is an important consideration as proteins that undergo vastly different levels of expression from the same amount of DNA (*i.e.* different orders of magnitude) will not reach the optimal ratios of FKBP₃:GFP/Rluc8 if used jointly in the SABRE assay.

Data produced by the SABRE assay applied to these various combinations of GPCRs suggest the existence of S1P receptor heterodimers that are subgroup-specific (Fig. 7.12). Significant increases in BRET_{eff} were observed upon addition of AP20187 when S1P₃-GFP/Rluc8 was paired with either S1P₂- and S1P₅-FKBP₃, but not with LPA₁- or CCR5-FKBP₃. Similarly, S1P₂-GFP/Rluc8 paired with S1P₃-FKBP₃ also gave an increase in BRET_{eff}, although S1P₂-GFP/Rluc8 S1P₅-FKBP₃ did not yield a significant increase. S1P₅-GFP/Rluc8 only exhibited a significant increase in BRET_{eff} when FKBP₃ was fused to S1P₅, and not when fused to either S1P₂ or S1P₃.

7.4 Discussion

7.4.1 Observation of possible GPCR heterodimers

The SABRE assay developed in this chapter appears to be capable of faithfully reporting dimeric interactions of both type 1 TM proteins and GPCRs. This has allowed an independent assessment of the cBRET-derived indications that the S1P receptors are capable of heteromeric interactions within the family, but not with other GPCRs (§6.4.2). The results obtained using the SABRE assay

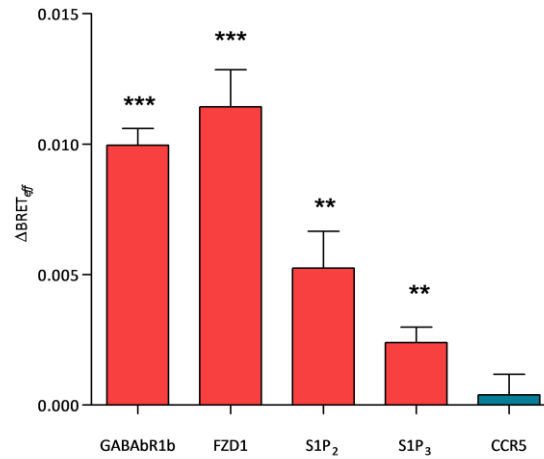


Figure 7.11: Optimised SABRE assay applied to GPCRs yields data correlating with the previously reported stoichiometry in each case. GABAbR1b, FZD1, S1P₂, and S1P₃ all exhibit a significant increase in BRET_{eff} upon addition of AP20187, consistent with homodimeric behaviour. Data collected for $\Delta BRET_{eff}$ for CCR5 are not significantly different from zero, consistent with monomeric behaviour. Significance is shown for a one-sample *t* test of values against a $\Delta BRET_{eff}$ of zero (** *p*>0.01; *** *p*>0.001). Error bars represent SEM.

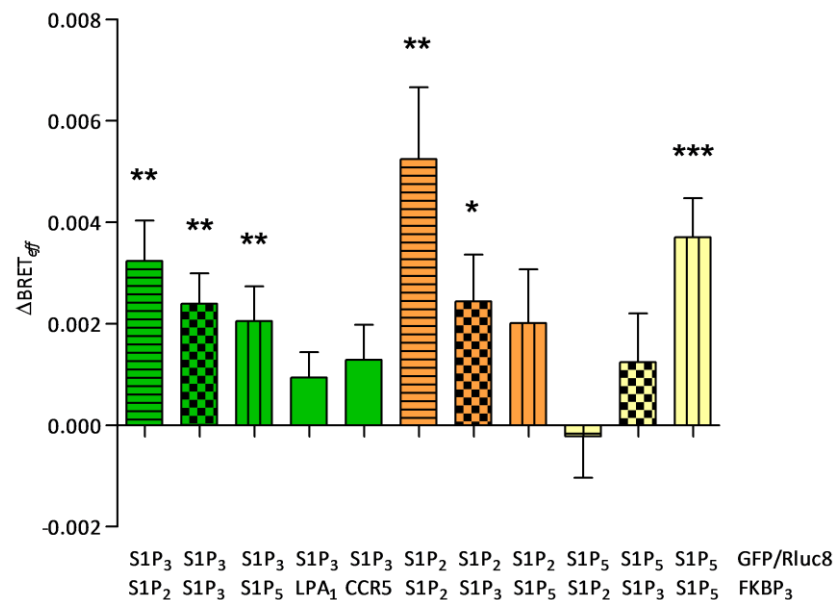


Figure 7.12: Apparent heterodimerisation within the S1P receptors is reported by the SABRE approach. Significant increases in BRET_{eff} are observed for some combinations of S1P receptors, but not for S1P₃ paired with either LPA1 or CCR5. Significance is shown for a one-sample *t* test of values against a $\Delta BRET_{eff}$ of zero (* *p*>0.05; ** *p*>0.01; *** *p*>0.001). Error bars represent SEM.

support this proposal, as positive $\Delta\text{BRET}_{\text{eff}}$ values are observed for some pairings of S1P receptors, but not with either of LPA₁ or CCR5. The observation of significant increases in BRET_{eff} does appear to depend somewhat on which protein is GFP/Rluc8-tagged, and which is FKBP₃-tagged. This is only the case with S1P₃ and S1P₅, which yield a significant increase when using S1P₅-FKBP₃ but not using S1P₃-FKBP₃ (Fig. 7.12). This could be due to varying relative dimer fractions between the two protein populations. For example, if S1P₅ is more weakly homodimeric than S1P₃ the monomeric fraction will be larger and so present a larger number of potential interaction sites to the GFP/Rluc8-tagged receptors than if S1P₃-FKBP₃ is used.

An additional observation is that none of the S1P receptors give a $\Delta\text{BRET}_{\text{eff}}$ value of the same magnitude as either GABABR1b or FZD1 in either homodimeric or heterodimeric SABRE assays. This indicates that these receptors are likely to undergo less stable dimerisation than the non-*Rhodopsin* family GPCRs, likely via a transient homodimer-heterodimer equilibrium.

7.4.2 Considerations for the use of SABRE

Potential for false dimer signatures

Although increases in BRET_{eff} in the presence of AP20187 are most simply explained by direct interaction between the GFP/Rluc- and FKBP-tagged proteins, there are several possible alternative explanations that must be considered. Firstly, in the case of FKBP₃, it is possible that the formation of large cross-linked oligomers traps GFP- and Rluc-tagged proteins within corralled areas of the membrane. This would increase BRET_{eff} if it caused a greater effective concentration of the GFP- and Rluc-tagged proteins. This does not seem likely, however, as in principle such corraling should affect only the diffusive behaviour, and not the effective concentration, of the trapped proteins. This is supported by the fact that the observed behaviour for monomers is equivalent using both FKBP₃ and FKBP₁, since the latter does not form oligomeric clusters. A second possibility is that induced association of the FKBP-tagged proteins causes their internalisation. This could artefactually increase BRET_{eff} if GFP- and Rluc-tagged proteins underwent partial non-specific co-internalisation along with the FKBP-tagged protein, thereby concentrating them in intracellular vesicles and potentially increasing non-specific BRET. Whilst this does not appear to apply to CD28 (Fig. 7.7), the extent to which this effect may exist may vary between proteins due to differences in their mode of internalisation.

Potential for false monomer signatures

A caveat of the SABRE approach is that only diffusing heterodimers can be detected. If either the GFP/Rluc- or FKBP-tagged proteins are immobile then BRET-productive induced dimers are unlikely to form upon addition of AP20178 as the FKBP proteins will at no point be near enough to bind the same ligand molecule. This is only a problem for proteins that are largely immobile rather than ones that are simply constrained in their diffusion, because if both proteins are simply held within a specific region of the membrane then BRET-productive induced dimers would still form. The monomeric SABRE signature observed for CCR5 (Fig. 7.11) is unlikely to be the result of immobile receptors as CCR5_{FKBP}GFP and CCR5_{FKBP}Rluc were shown to dimerise in type 1 (Fig. 3.8) and cBRET (Fig. 4.10) assays, which would not be possible if CCR5 was largely immobile. Nonetheless, this is an important consideration in the application of SABRE to other membrane proteins.

Differences in expression levels

The expression levels of all the proteins investigated for heterodimerisation by SABRE in this chapter had previously been quantified (Table 5.15) and so only suitable pairings of GFP/Rluc- and FKBP-tagged proteins were used. This is extremely important, as the relative expression levels of the two proteins will have a profound impact on the outcome of the SABRE assay. If the GFP/Rluc-tagged protein A is expressed at much higher levels than the FKBP-tagged protein B, then only a small proportion of protein A molecules will be able to form heterodimers with protein B, and so BRET_{eff} will only increase minimally upon addition of AP20187 (Fig. 7.9a). Conversely, if protein B is far more abundant than protein A then the majority of induced FKBP-FKBP dimers will not form BRET-productive pairs (Fig. 7.9c). This is not a problem when using SABRE to investigate homodimerisation, but it must be considered in assays of heterodimers.

CHAPTER 8

General discussion

8.1 Extension of the BRET method applied to GPCRs

The work in this thesis has sought to investigate general GPCR stoichiometry using assays that are reliable and unambiguous in their interpretation. Type 1 and 2 BRET had previously been shown to be capable of distinguishing between monomers and dimers (313), but had only been applied to two *Rhodopsin* family GPCRs, only one of which was a human receptor (β_2 AR), calling into question the extent to which these two receptors were representative of the family as a whole. This issue was addressed in this thesis by applying rigorous BRET assays to a larger range of GPCRs.

Type 1 BRET can reliably identify partial dimers

Due to the recent claims of transient GPCR dimerisation (346), it was initially important to test the ability of the type 1 BRET assay to detect dimers. Using a system of induced dimerisation of CCR5, which was otherwise reported as monomeric in type 1 and 2 BRET assays, it was determined that type 1 BRET is capable of reliably reporting dimerisation within mixtures of monomers and dimers (§3.3.2). The apparent sensitivity of the technique was found to be roughly 10-20% maximal dimerisation achieved by the inducible CCR5 dimer system, which at most equates to a population of 10-20% actual dimers, but most likely represents less than that as the system used is unlikely to reach a level of 100% dimers. The subsequent investigation of M₁ using the type 1 BRET assay failed to detect any partial dimerisation (§3.3.3), an observation that was supported by type 2 BRET analysis also suggesting monomericity of M₁. The observation of apparent monomeric behaviour of both CCR5 and M₁ contradicts the conclusions of some previous Co-IP- (378) and BRET-based (331, 379) analyses, but supports other studies that have failed to observe constitutive dimerisation of CCR5 and M₁ (346, 380).

A third rigorous BRET assay suitable for use in cells already expressing the receptors

To further probe the general behaviour of *Rhodopsin* family GPCRs, the stoichiometry of those receptors natively expressed in HEK 293T cells was investigated systematically using BRET. Using receptors in their native cell line maximised the likelihood of correct folding, trafficking, and signalling, and in this case HEK 293T cells afforded an opportunity to study a representative cross-section of the *Rhodopsin* family. However, of the previously used BRET assays, only type 1 BRET is suitable for such a study as type 2 BRET measurements are affected by native, untagged proteins expressed by the target cell line (§4.1.1). Thus, in order to complement the type 1 BRET approach, a new competition-based assay was developed that was not affected by the presence of native proteins, and also does not suffer the problems associated with traditional competition BRET assays. By measuring BRET_{eff} at varying expression levels in the presence and absence of competitor, this new approach discriminates between genuine dimers and apparent dimers arising due to reduced surface expression in the presence of competitor. Using the CCR5_{FKBP} inducible dimer system it was determined that the new cBRET approach also has an apparent sensitivity limit of ~10-20% maximal CCR5_{FKBP} dimerisation (§4.3.3). Its application to $\beta_2\text{AR}$, CCR5, and M₁ yielded data consistent with monomeric behaviour (§4.3.2), in line with type 1 and 2 BRET data obtained for each receptor.

Whilst this new cBRET assay has several potential applications not available to other BRET approaches (§4.4.2), within the scope of this study its most important asset is that it is a reliable technique and wholly independent from the type 1 BRET assay in terms of how the data are interpreted. Type 1 BRET relies on statistical comparisons with model behaviour predicted for different classes of stoichiometries, whereas cBRET simply measures the change in BRET_{eff} -expression relationship upon the introduction of competitor molecules. Because of this, the two approaches are unlikely to produce corresponding false results for the same protein. Type 1 BRET is highly unlikely to generate false-dimer artefacts but is potentially at risk of providing false-monomer results in cases of higher-order oligomerisation or very weak dimerisation. Conversely, cBRET is highly unlikely to give false-monomer results yet could produce false dimers in certain cases where addition of competitor proteins causes a relaxation in the localisation of tagged proteins within the membrane, reducing their effective concentration and, by extension, BRET_{eff} . The two techniques are therefore highly complementary since, in combination, they are more informative than either approach alone.

Application of the type 1 and cBRET assays to 60 diverse *Rhodopsin* family GPCRs expressed in their native HEK 293T cell line (§5.3) yielded data consistent with monomeric behaviour for 46

receptors, and data suggesting dimeric behaviour for 11 others (three receptors were ambiguous and may form very weak dimers or higher-order oligomers). In the vast majority of cases, the type 1 and cBRET produced matching outcomes. These observations are novel because, using the one pair of complementary assays, they have identified two types of behaviour within the *Rhodopsin* family. Previously, BRET saturation and competition assays had consistently reported GPCR dimerisation only (e.g. 328, 329, 330), as had most *ex vivo* studies such as Co-IP (e.g. 288, 293, 295).

Development of a “stimulated association BRET” assay for the study of GPCR heterodimerisation

The finding, within closely related receptor subfamilies, of consistent homodimerisation raised the possibility that these receptors might also heterodimerise via a conserved interaction interface. This was initially investigated using the cBRET assay for the S1P receptors (§6.3.2), and this suggested heterodimerisation may be genuine and restricted to closely related receptors. Nonetheless, these data were open to a number of different interpretations (§6.4.2) and so in order to confirm these conclusions independently the new SABRE approach was developed (§7.1.2) in an effort to circumvent the compounding issues associated with the principal technique currently used for identifying heterodimers, GPCR-HIT (§1.4.4). The data presented in Chapter 7 showed that SABRE readily distinguishes between known type 1 TM protein monomers and dimers, and that it detects two types of behaviour among GPCRs that correlate with differences in apparent stoichiometry. The identification of S1P receptor heterodimerisation using SABRE analysis confirms the observations obtained using the cBRET method.

8.2 Predominance of monomeric *Rhodopsin* family GPCRs

Investigation of the stoichiometry of 60 *Rhodopsin* family GPCRs expressed by HEK 293T cells, using the complementary approaches of type 1 and cBRET, failed to detect dimerisation for 46 receptors under these assays (§5.4). When combined with the previous observations that β_2 AR, CCR5, and M₁ are also apparently monomeric (§3.4, §4.4), more than 80% of the *Rhodopsin* receptors directly investigated here exhibit monomeric behaviour in the BRET assays. There is no reason to suspect that the receptors studied in this thesis are not a representative cross-section of the *Rhodopsin* family as they bind to a diverse array of ligands and perform a broad range of physiological functions. Overall, they correspond to ~20% of the entire human non-olfactory *Rhodopsin* repertoire and a significant fraction

of the family as a whole. Whilst the lack of extensive dimerisation in these receptors appears to be robust according to both type 1 and cBRET analysis, the formal possibility exists that these GPCRs form dimers at physiological expression levels but not when overexpressed in the BRET assays. This could be due to the stabilisation of receptor dimers by a third party, which is then insufficiently abundant to stabilise widespread dimerisation under conditions of GPCR overexpression. However, as no such stabilisation has been previously reported, it seems likely that the observations described in this thesis probably reflect the behaviour of the receptors at physiological densities.

These conclusions are strongly at odds with the early consensus that *Rhodopsin* GPCRs form constitutive dimers, initially established for other families, *e.g.* the *Glutamate* receptors (107, 134, 135), but then extended to the *Rhodopsin* family (*e.g.* 252). The new data are, however, in agreement with the growing body of single-molecule microscopy data that does not report constitutive dimerisation in many cases (251, 346, 351, 356). The stoichiometry of many of the GPCRs investigated in this thesis had not previously been investigated, although several receptors had been claimed to form dimers. Overall, twenty-seven of the 60 *Rhodopsin* GPCRs analysed in Chapter 5 were previously reported to form dimers (Table 8.1), of which 18 were assigned as monomers in the present investigation. This suggests that many previous studies may have been misinterpreted.

More generally, the data obtained in this thesis argue against the model of widespread allosteric regulation within GPCR homodimers founded, principally, on pharmacological analysis. Instead, they support a model in which cooperative effects observed between receptors could arise from indirect cross-talk within the GPCR population, as proposed elsewhere (263). This has ramifications for the development of novel receptor agonists/antagonists as it implies that attempts to exploit direct allostery may be ineffective in many cases. Nonetheless, as discussed above, there remains the possibility of low levels of dimerisation within the apparently monomeric population, which may still have functional consequences with regards to allosteric regulation.

Detectably dimeric *Rhodopsin* family receptors appear to be a small but significant minority

Although the 'typical' *Rhodopsin* family GPCR suggested by type 1 and cBRET in this thesis is monomeric, there appears to exist a significant minority of receptors that undergo dimerisation at levels above the sensitivity limits of the assays used; constituting ~20% of the non-olfactory *Rhodopsin* family receptors investigated. As only one olfactory receptor, OR4D1, was examined in this study and observed to be apparently monomeric, it is not possible to say with confidence what proportion of the total *Rhodopsin* family is likely to exhibit dimerisation. However, if OR4D1 is representative of the

olfactory receptors it is probably much less than 20%. Of the eleven receptors proposed to be dimeric according to type 1 and cBRET analysis, six had previously been reported to form dimers (Table 8.1). Nonetheless, the concurrence of the type 1 and cBRET results with the earlier experiments does not necessarily indicate that the previous studies were free of experimental bias. For example, S1P₂ and S1P₃ were previously characterised as dimers using a β -galactosidase complementation assay (487), consistent with the present BRET study. However, Zaslavsky et al. also claimed that LPA₁, LPA₂, LPA₃, and OGR1 formed dimers, whereas they each exhibited monomeric behaviour in the present study.

Lack of shared features among *Rhodopsin* family dimers

Amongst the receptors exhibiting dimeric behaviour in type 1 and cBRET assays, there does not appear to be any conservation of preference for G proteins (Fig. 5.14), which suggests that dimerisation most likely does not exist in order to allow interaction with specific signalling partners. This is consistent with previous studies that have demonstrated efficient G protein-coupling to monomeric receptors (e.g. 250, 251) but not with a model of a 2:1 GPCR:G protein signalling complex (e.g. 280). The small number of arrestins in humans makes it also seem unlikely that dimerisation facilitates specific GPCR-arrestin interactions, and instead it is probable that receptors within dimeric complexes would also be capable of signalling as monomeric units, perhaps with altered signalling properties. An alternative possibility, that dimerisation is a product of a need to recognise particular types of (possibly bivalent) ligands, is also not supported by the present study, as there is no clear difference in the ligand specificity of apparently monomeric or dimeric receptors in the *Rhodopsin* family (Fig. 5.13). When coupled with the observation that the apparently dimeric population does not share significant levels of sequence similarity (Fig. 5.15), it would seem that dimerisation within the *Rhodopsin* family is randomly assigned to some groups of receptors and not others. The question of why some receptors appear to be monomers while other form dimers is still, therefore, unanswered, and indeed it is even possible that dimerisation does not actually have any specific functional value.

Lack of higher-order oligomers

Although higher-order oligomerisation has been suggested for *Rhodopsin* family GPCRs in the past (e.g. 492, 493), the type 1 and cBRET data in this thesis failed to indicate higher-order oligomerisation for the vast majority of studied receptors. Only LTB₄R1 yielded data that could potentially correlate

Receptor	Technique used to support homo-oligomerisation
5-HT _{1D}	Co-IP (494), Western blotting (495)
5-HT _{2C}	Cysteine crosslinking (496), Co-IP (497), BiFC (498), FCS (498), FRET (497, 499), Radioligand binding (497, 499)
A _{2A} R	BRET (493), BiFC (493, 500), FRET (501)
<u>α_{2C}AR</u>	BRET (502), Co-IP (502)
AT ₁	BRET (316), Western blotting (503, 504)
β ₁ AR	BRET (328), FRAP (367), X-ray crystallography (302), Single-molecule microscopy (233)
B ₂	Co-IP (505), Western blotting (505)
C5R1	FRET (506)
CCKBR	BRET (507)
<u>CXCR4</u>	BiFC (508), BRET (509), Bivalent ligand crosslinking (510), X-ray crystallography (77)
EDNRA	Size-exclusion chromatography (424), FRET (511)
GPR50	Co-IP (512)
<u>H₁R</u>	Co-IP (513), FRET (513)
<u>H₂R</u>	Western blotting (492)
LPA ₁	β-galactosidase complementation (487)
LPA ₂	β-galactosidase complementation (487)
LPA ₃	β-galactosidase complementation (487)
<u>LTB₄R1</u>	Cysteine crosslinking (280), Radioligand binding (280), Cooperative ligand binding (281, 514)
M ₃	BRET (515)
NPY _{1R}	FRET (516)
OGR1	β-galactosidase complementation (487)
P2Y _{2R}	BRET (517), FRET (518)
<u>S1P₂</u>	β-galactosidase complementation (487), Co-IP (488)
<u>S1P₃</u>	β-galactosidase complementation (487), Co-IP (488)
SSTR2	FRET (519), Western blotting (519)
TP	Co-IP (520), ELISA (520)

Table 8.1: Previously published assertions of homo-oligomerisation by *Rhodopsin* family GPCRs investigated in Chapter 5. 26 of the 60 receptors investigated have previously been reported as homodimers in studies using a range of techniques. GPCRs found to be oligomeric using type 1 and cBRET are underlined. Only reports of homo-oligomerisation are included in this table; studies of heteromeric interactions are not shown. (ELISA = Enzyme-linked immunosorbent assay).

with higher-order oligomerisation: type 1 BRET data that fit well to oligomer models, and a decrease in BRET_{eff} in cBRET analysis that corresponds to direct interaction (§5.4). LTB4R1 has previously been reported to form homodimers that bind to single G proteins in a pentameric assembly, and to undergo cooperative conformational changes upon ligand binding (280, 281, 514). Higher-order oligomers have, however, not previously been reported. It is possible that the techniques used in these studies, *e.g.* SDS-PAGE and size-exclusion chromatography of cross-linked receptors (280), disrupted interactions within LTB4R1 oligomers. Alternatively, it is possible that the surface distribution of LTB4R1 is such that the receptors are highly concentrated within local areas of the cell surface or intracellular membranes, such that they behave as apparent oligomers in the type 1 and cBRET assays. In either case, the data in this thesis suggest that higher-order oligomerisation between *Rhodopsin* family GPCRs is at most a rare occurrence.

8.3 The various mechanisms of GPCR dimerisation

Although the notion of GPCR dimerisation has existed for over twenty years, for the vast majority of GPCRs there is no consensus on how dimers might form. The present suggestion that *Rhodopsin* family GPCRs are predominantly monomeric implies that there is no common dimerisation interface that is broadly conserved across the whole family. Instead, given that dimerisation appears to have emerged on several separate occasions within the *Rhodopsin* family (§5.4.4), it is possible that the mechanism of dimerisation is distinct in each case. In order to investigate this, the mechanism of S1P₃ dimerisation was investigated and compared to that of non-*Rhodopsin* GPCRs.

S1P₃

Type 1 and cBRET assays of S1P₃-LPA₁ chimeras are consistent with a model of S1P₃ homodimerisation in which the dimer interface is organised around TM4, alongside other structural elements that contribute in a TM4-independent manner. The fact that TM4 of S1P₃ is sufficient to impose the type 1 and cBRET signatures expected of dimers on LPA₁ suggests that the TM4 helices of individual receptors within the putative S1P₃ dimer may form direct interactions with each another. This is because the conservation of surface exposed residues between LPA₁ and S1P₃ is limited and apparently randomly distributed (Fig. 8.1). It is therefore unlikely that a reciprocal dimer interface exists on LPA₁ that is capable of interacting with TM4 of S1P₃ but not LPA₁. This conclusion is

supported by the observation that no LS chimera other than LST4 gave a change in $BRET_{eff}$ in the cBRET analysis using wild-type S1P₃ as a competitor (Fig. 6.8). If non-TM4 regions of LPA₁ did interact with TM4 of S1P₃ then this would most likely result in a change in the degree of non-specific $BRET_{eff}$ as the receptors would have a greater effective hydrodynamic diameter as part of dimeric complexes with untagged S1P₃. Since no such change was observed, such interactions seem unlikely.

In light of these conclusions it is possible to postulate potential arrangements of S1P₃ within the putative receptor homodimer, using the structure of S1P₁ (164) as a substitute for S1P₃. Assuming direct interaction between the TM4 domains, two arrangements are possible in which TM4 of one protomer inserts between TM2 and TM4 (Fig. 8.2a&b) or between TM4 and TM5 of the other (Fig. 8.2c&d). In both instances, areas of S1P₃ other than TM4 would be positioned close enough to interact, explaining why TM4 was required for strong, but not weak, dimerisation. In both models there is notably little contribution to dimerisation from the non-TM portions of the receptors.

Nonetheless, the precise identity of the putative S1P₃ dimer interface is currently unknown as its structure has yet to be solved. The structure of closely related S1P₁ has, however, been solved by x-ray crystallography, and this did not form a dimer within the crystal (164). Whilst it was not studied here, its close relatedness to the other apparently dimeric S1P receptors would suggest that dimerisation is likely *in situ* for S1P₁. There are several possible reasons why an S1P₁ dimer was not observed in the crystal structure: (1) the use of a T4 lysozyme fusion in place of its native IL3 domain may have destabilised the dimer complex; (2) the S1P receptors may be highly transient dimers and so the dimer fraction may not be sufficiently large or stable enough to crystallise; (3) the capacity for dimerisation is not present in S1P₁, which would indicate that it was lost following its split from the dimeric S1P receptors, in which case dimerisation would appear to be dispensable for S1P receptor function. It seems unlikely that the position of T4 lysozyme relative to the S1P₁ 7TM region would directly disrupt dimerisation under either model proposed for S1P₃ (Fig. 8.3), although, it may still stabilise a conformation of S1P₁ that is less prone to dimer formation. It seems more likely that S1P₁ does not dimerise sufficiently (or indeed at all) for crystallisation of a dimer.

Rhodopsin family dimer interfaces

Except for S1P₃, the possible interaction mechanism(s) for other putative *Rhodopsin* family dimers was not directly studied in this thesis. The observation that these proposed dimers are generally not closely related offers the possibility that the dimer interface is different in each case. Of the *Rhodopsin* family

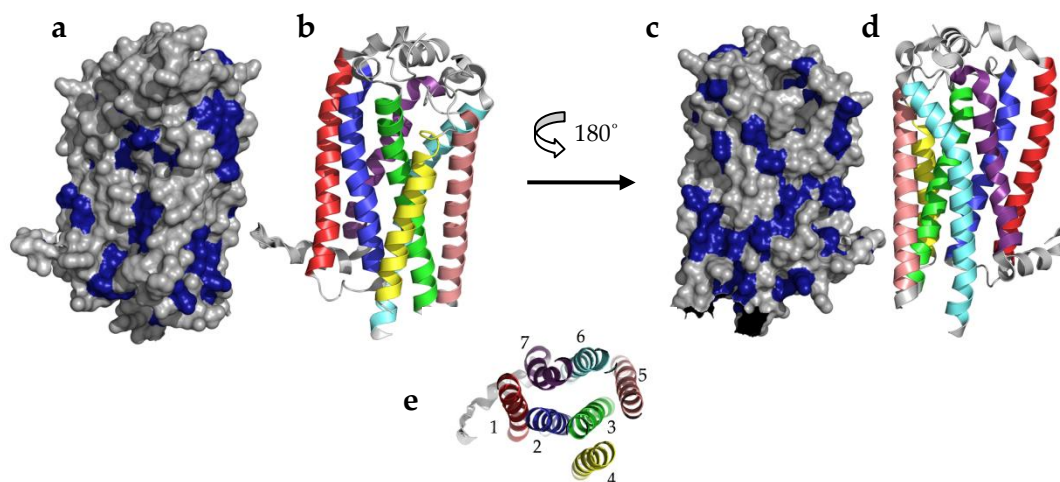


Figure 8.1: S1P₃ and LPA₁ share only minimal sequence conservation at the receptor surface. Conservation is shown here as grey (non-conserved) or blue (conserved) residues on a surface map of the closely related receptor S1P₁ (PDB code: 3V2Y). Views are provided of both the TM2/4 and TM6/7 faces of the receptor (a & c), and cartoon representations of the TM helical arrangement are given from corresponding viewpoints (b & d) or viewed from the extracellular side of the receptor (e). TM1 = red, TM2 = blue, TM3 = green, TM4 = yellow, TM5 = pink, TM6 = cyan, TM7 = purple. All non-TM regions are shown in grey.

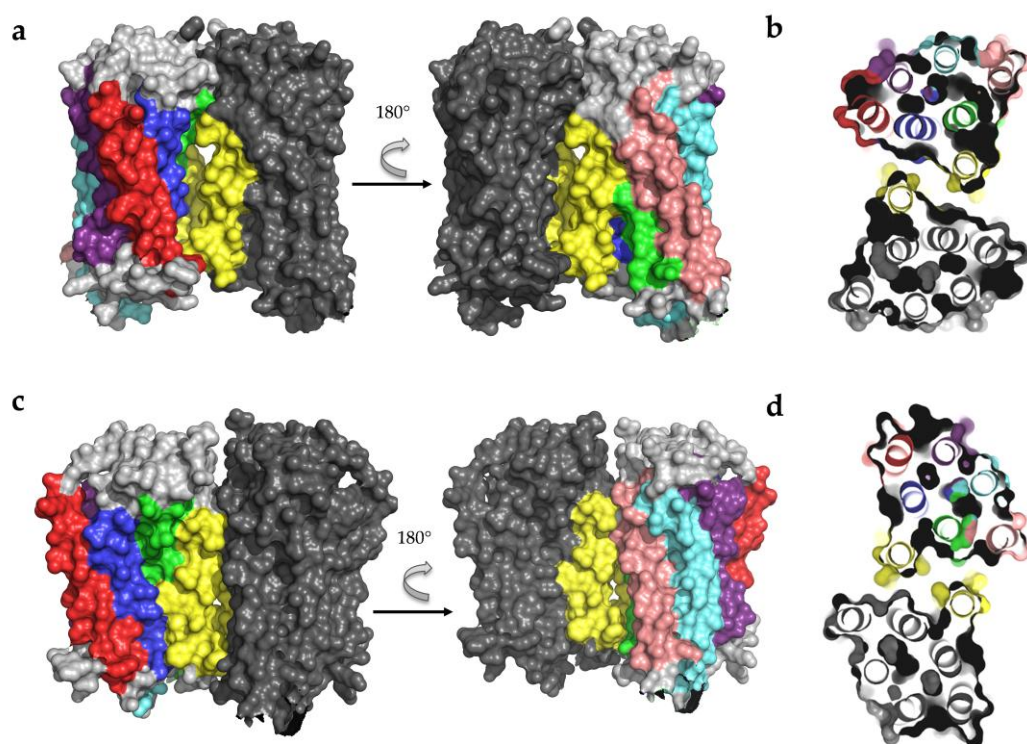


Figure 8.2: Possible arrangements of the putative S1P₃ dimer using the S1P₁ structure (PDB code: 3V2Y) as a substitute. (a) Surface representations of a possible S1P₃ dimer organised around contacts between TM2 and TM4 on opposite receptors. (c) Alternative arrangement in which dimerisation is mediated by interactions between TM4 and TM5 on opposite dimers. (b & d) Cross sections of a and c, respectively, as viewed from the extracellular side of the receptors. In each case, one receptor is colour coded as follows: TM1 = red, TM2 = blue, TM3 = green, TM4 = yellow, TM5 = pink, TM6 = cyan, TM7 = purple, non-TM = light grey. The other receptor is shown entirely in dark grey except for TM4, which is marked in yellow.

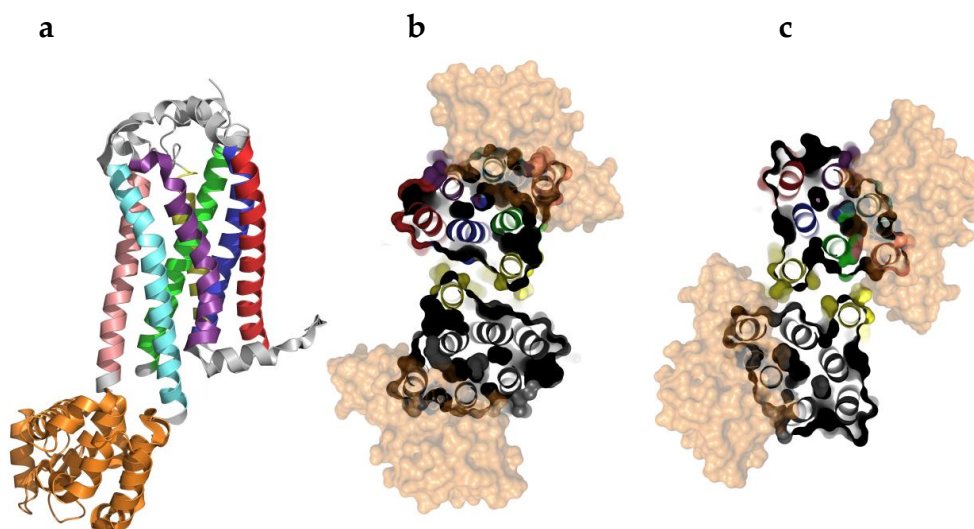


Figure 8.3: T4 lysozyme within the crystal structure of S1P₁ (PDB: 3V2Y). (a) Position of T4 lysozyme relative to the S1P₁ 7TM region. The position of T4 lysozyme is unlikely to cause direct steric hindrance to dimer formation in S1P₁ in the case of dimerisation by either the proposed TM2/4 interface (b) or the TMM 3/4/5 interface (c). Figures b and c are cross-sectional views of the putative dimer arrangements as seen from the extracellular side with the position of T4 lysozyme superimposed. T4 lysozyme is shown in orange. TM1 = red, TM2 = blue, TM3 = green, TM4 = yellow, TM5 = pink, TM6 = cyan, TM7 = purple. All non-TM regions are shown in grey.

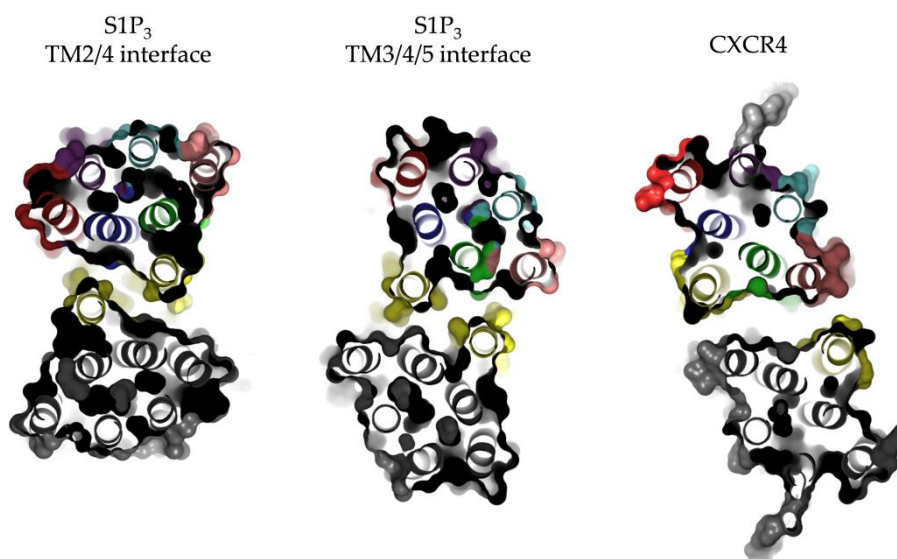


Figure 8.4: Comparison of putative S1P₃ dimer interfaces with that of CXCR4 in the available crystal structures (PDB: 3ODU). The interface of CXCR4 is somewhat similar to that proposed for the S1P₃ TM3/4/5 interface, except that the TM4 helices do not directly contact one another but rather form interactions with TM5 on the opposite receptor. Figures are cross-sectional views of the dimer arrangements as seen from the extracellular side. TM1 = red, TM2 = blue, TM3 = green, TM4 = yellow, TM5 = pink, TM6 = cyan, TM7 = purple.

receptors suggested to be dimers in this study, only CXCR4 crystallised as a dimer. This receptor formed the same dimer interface in five separate crystals and in the presence of two different ligands (77). The interface comprised interactions between side chains at the extracellular ends of TM4 and TM5 as well as, in some crystal lattices, a secondary interaction at the intracellular ends of TM3 and TM4. Considering the ubiquitous use of this interface (in the crystals) and the fact that CXCR4 was identified as a dimer by both type 1 and cBRET, it seems likely that this represents the genuine interface *in vivo*. Nonetheless, the high BRET_{max} value of CXCR4 reported in this thesis (Table 5.14) and elsewhere (315, 321) is at odds with the crystal structure, since the two C termini are on opposite sides of the complex rather than close together in the centre, which would have been expected to reduce BRET_{eff}. The last 26 residues of CXCR4 were truncated to allow crystallisation, however, so it is possible that they loop back towards the centre of the dimer *in vivo*. It is also possible that crystallisation stabilises a non-physiological arrangement of the CXCR4 dimer. Finally, although this receptor arrangement is similar to the TM3/4/5 interface proposed for S1P₃ (Fig. 8.4), in the CXCR4 structure the two TM4 helices do not directly interact but instead form contacts with TM3 and TM5. Thus, S1P₃ and CXCR4 appear to form different interfaces despite possibly similar receptor arrangements, which suggests that dimer interfaces may vary systematically across the *Rhodopsin* family.

Overall, the mechanism of interaction in the suggested dimers is still largely unknown, although there is some evidence that TM4 may be involved in some capacity for some receptors. Most of the 11 apparently dimeric receptors are classical *Rhodopsin* GPCRs, with only small intra- and extracellular domains and are therefore likely to interact in a manner similar to CXCR4, *i.e.* via small regions of the 7TM domain and interhelical loops. Notable exceptions to this are LGR4 and LGR5, which possess large N-terminal ligand-binding domains containing a distinctive leucine-rich β -sheet horseshoe structure (83). Ligand recognition in this way is highly unusual within the *Rhodopsin* family but similar to that seen in non-*Rhodopsin* GPCRs, *i.e.* the *Glutamate* and *Frizzled* receptors. Given that dimerisation in the *Glutamate* family is mediated by these large extracellular domains (107, 134, 135), and that a similar mechanism may apply to the *Frizzled* family GPCRs (§6.4), it seems highly likely that dimerisation in LGR4 and LGR5 is also mediated by their extracellular domains, distinguishing them from other proposed *Rhodopsin* family dimers. Indeed, the fact that the LGRs share common ligands with the *Frizzled* receptors, *i.e.* R-spondin glycoproteins (118, 443), strengthens this argument as it hints at a connection between dimerisation and ligand selectivity in these receptors. If this is the case, it implies that the other *Rhodopsin* family GPCRs possessing LGR-like N-terminal domains (*i.e.* LGR6,

LGR7, LGR8, FSHR, TSHR, and LHCGR) form dimers by similar means. This is supported by the observation that the FSHR N-terminal domain forms homo-oligomeric interactions in crystals (84), although whether this occurs *in vivo* is yet to be determined.

Nonetheless, it would appear that most *Rhodopsin* family GPCR dimers interact largely or wholly through their 7TM regions. Such a mechanism of association has been suggested as incapable of producing stable dimers on the grounds that interactions between hydrophobic residues within the 7TM region will not be sufficient to achieve the ~60kJ/mol binding energy required for stable dimerisation at physiological temperatures (133). This simple thermodynamic argument suggests that dimerisation in the *Rhodopsin* family involves only transient interactions.

Non-*Rhodopsin* family dimers

Given the apparent ability of *Rhodopsin* family GPCRs to interact via their 7TM regions, it is possible that such a mechanism also exists in non-*Rhodopsin* family receptors. This, however, has not been demonstrated, and the best understood GPCR dimers, members of the *Glutamate* family, are instead known to interact via their large N- and C-terminal domains (§1.2.4), and it has been suggested that the 7TM has no direct role in dimerisation in these receptors (133). In the present study, BRET was used to establish whether *Glutamate*-like or *Rhodopsin*-like dimerisation occurs in the *Frizzled* family receptors. The type 1 and cBRET assays suggested that seven *Frizzled* receptors exhibited apparently dimeric behaviour (§6.4), implying that it is a feature common to the family as a whole. Domain-swapping analysis between FZD10 and the apparently monomeric TAS2R19 suggested that dimeric behaviour is imposed upon TAS2R19 by the incorporation of either the FZD10 N- or C-terminal domains and that, conversely, BRET data corresponding to a monomer were only observed for FZD10 if both domains were replaced by those of TAS2R19. These data suggest that the 7TM domain of FZD10 is not sufficient to mediate dimerisation on its own, and that instead it is reliant on the N- and C-terminal regions, in a manner reminiscent of the *Glutamate* receptors.

The observation of *Frizzled* family N-terminal domain dimerisation is consistent with reports that the N-terminal domain of mouse FZD8 and the secreted FZD-related protein 3 form dimers in crystals (121), and that incorporation of the N-terminal domain of *Xenopus* FZD3 is sufficient to induce the dimerisation of the otherwise monomeric fibroblast growth factor receptor 1 (140). Given the highly conserved nature of the *Frizzled* family N-terminal domain, it seems highly likely that dimerisation through this domain is a feature of all eleven *Frizzled* GPCRs. In contrast, the C-terminal sequences of the *Frizzled* family are relatively poorly conserved between individual receptors (Fig. 8.5).

Thus, it is possible that the extent and nature of interaction between the two domains within the receptor dimer vary from one *Frizzled* receptor to the next. Nonetheless, the data obtained in this thesis clearly suggest that the 7TM region of the *Frizzled* receptors does not possess an inherent capacity for dimerisation. In light of the differences between this and the 7TM region-mediated dimerisation of *Rhodopsin* family GPCRs, it is possible that the role of dimerisation differs in each case – perhaps contributing to ligand recognition in the *Frizzled* and *Glutamate* family GPCRs, and to allosteric cooperative effects in the *Rhodopsin* family receptors.

8.4 GPCR heterodimerisation

GPCR heterodimerisation has become a prominent topic in recent years as many receptors have been suggested to form a broad array of different heteromeric interactions. Although its possible function has not been determined, it has often been suggested that cooperative signalling effects between distinct receptors are mediated by direct interactions between the GPCRs involved (*e.g.* 252, 253). Whilst this is a popular notion within the field, several technical issues and alternative explanations for the observed effects obviate the need for direct heteromeric interactions (§1.4.1). Heterodimerisation is well established among some *Glutamate* family receptors (138, 521), but is generally poorly understood for most GPCRs. The observation in this thesis that *Rhodopsin* family dimers are often closely related suggests that heterodimerisation is mediated by closely related interaction interfaces. This was examined for one such group of apparent dimers, the S1P receptors, three of which behaved as dimers in type 1 and cBRET assays.

S1P receptor heterodimerisation

Heterodimerisation within the S1P receptor family was studied using cBRET (§6.3.2) and SABRE (§7.3.5) assays. These assays suggested that heterodimerisation among the S1P receptors is receptor pair-specific. cBRET analysis of S1P₂, S1P₃, and S1P₅ suggested that all three receptors mutually affected one another's behaviour (Fig. 6.9), possibly through the formation of heterodimers that preclude homodimerisation. SABRE analysis also suggested that S1P₂ and S1P₃ directly interact, although it did not detect heterodimerisation between S1P₅ and the other receptors. The apparent discrepancy between the two techniques can be explained in two ways. Firstly, S1P₅ may only interact very weakly with S1P₂ and S1P₃, in which case it may only cause a slight disruption of homodimer

```

FZD1    1  GKTLSWRKFFYTRLTNSK-----
FZD2    1  GKTLSWRKFFYTRLTNSR-----
FZD3    1  KKTCFEWAASEFHGRRKKEIVNESRQVLQEPD---F--AQSLLRDPN-----
FZD4    1  -KTLHTWOKCSNRLVNSG-KVKR--EKRGN-----
FZD5    1  GKTLESWRRTTSRCCCRP-RRGH---KSGG-----
FZD6    1  KKTCTEWAGFFKRNKRDPISERRLVQESC--EFFLKHNISKV-----KHKKKHY
FZD7    1  GKTLSWRRTFYHRLSHSS-----
FZD8    1  GKTLESWRSLCTRCCWAS-KGAA---VGGG-----
FZD9    1  SKTFQWQSLCYRKIAAG-RARA-KACRAPG-----
FZD10   1  --TLQSWQQVCSRRLKKKSRKPPASVITS-----
SMO     1  KATLLTWRTTWRLTGQS-DDEPKRIKKSKMIAKAFSKRHELLQNPGQELSFMSHTVSHD

FZD1    19  -----
FZD2    19  -----
FZD3    42  -----TPIIRKSRGTSTQETSTHASSTQLAMVD-----DQRSKAGSI
FZD4    27  -----
FZD5    27  -----MAA-----
FZD6    49  KPSSHLKLVISKSMGTSTGATANHGTSAVAITSH-----DYLQGETLT
FZD7    19  -----
FZD8    27  -----AGATAAGGG-----GG
FZD9    30  -----SYGR-----
FZD10   28  -----G-----I
SMO     60  GPVAGLAFDLNEPSADVSSWAQHVTMKMVARRGAILPQDISVTPVATPVPPEEQANLWLW

FZD1    19  -----
FZD2    19  -----
FZD3    79  HSKVSSYHGSL-----HRSRDGR--YTPCSYRGM---EERLPHGMSRLTDHSRHS---
FZD4    27  -----
FZD5    31  -----
FZD6    92  EIQTSP-ETSMR---EVKADGA--STPR--LREQDCGEPASPAASISRLSGEQVDG---
FZD7    19  -----
FZD8    38  -----PGGGG-----
FZD9    34  -----
FZD10   31  YKKAQHPQ-----KTHHG-----
SMO     120 EAEISP-ELQKRLGRKKRRRKRKKEVCPL--APPELHPPAPAPSTIPRLPQLPRQKCLV

FZD1    19  -----
FZD2    19  -----
FZD3    125 -----SSHRL-NEQSRH---SSIR-----DLSNNPMTHTHTGT-----
FZD4    27  -----
FZD5    31  -----G-----DY-----PEAS-AA-LTGRTGP-P---G
FZD6    140 -KGQAG-SV-SESARSEGRISPKS-----DITDTG-LA-QSNNLQ---V
FZD7    19  -----
FZD8    43  -G--GG-PG-GGGGPGGGGSLY-----SDVS-TG-LTWRSQT-A----
FZD9    34  -----GTH-----CHY-----KAP-T--VVLHM-----
FZD10   44  -----
SMO     177 AA--GA-WGAGDSCRQ-GAWTLVSNPFCPEPSPQDPFLPSAP-AP-VAWAHGR-RQGLG

FZD1    19  -----QGETTV
FZD2    19  -----HGETTV
FZD3    154 -----SMN-RV-----IEEDGTS
FZD4    27  -----GWV-KP-----GKGSETVV
FZD5    49  P----AAT-YH-----KQVSLSHV
FZD6    176 P-----SSS-EPSSLKGSTSLLVHPVSGVRKEQGGGCHSDT
FZD7    19  -----KGETAV
FZD8    75  S----SVS-YP-----KQMPLSQV
FZD9    49  -----TKT-DP-----SLENPTH
FZD10   44  -----KYE--I-----PAQSPICV
SMO     230 PIHSRTNLMDE-----LMDADDF

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Figure 8.5: Alignment of *Frizzled* family member C-terminal domain sequences. Residues conserved in 6 or more sequences are shown in black. Chemically similar non-conserved residues are shaded grey.

formation detectable by cBRET, and cannot recruit enough S1P_{2/3} molecules to an induced oligomer to allow detection by SABRE. The alternative explanation is that there is genuinely no interaction between S1P₅ and the other receptors, and that the presence of S1P₅ within the cell causes a change in the behaviour of S1P_{2/3} that reduces BRET_{eff} in the cBRET assay, and *vice versa* when S1P₅ acts as donor/acceptor and S1P_{2/3} is the competitor. Neither of these possibilities can be discounted according to the current data. Nonetheless, the observation that, of the three S1P receptors investigated, S1P₅ has the smallest relative decrease in BRET_{eff} upon addition of competitor in the homo-cBRET analysis (Fig. 5.8) as well as the lowest projected *y*-intercept (Table 5.13), may indicate that S1P₅ is a weaker homodimer than either S1P₂ or S1P₃. This would be compatible with it forming weaker interactions with S1P₂ or S1P₃ as the same interface is likely to be employed in both the homo- and heterodimeric complexes.

Based on these considerations, a possible interaction scheme can be proposed in which the three S1P receptors may interact if present in roughly equal numbers at the cell surface (Fig. 8.6). In this, S1P₂ and S1P₃ are predominantly dimeric in either homodimers or heterodimers with one another. S1P₅, by contrast, has a larger monomeric fraction although is still capable of homodimerisation as well as interactions with the S1P₂ and S1P₃, albeit at relatively low levels. The predominant dimer state for S1P₅ would then be a homodimer, as the S1P₂ and S1P₃ receptors would for the most part be engaged in other interactions and so only have a relatively small monomer fraction free for interaction with S1P₅, producing a weak heterodimer. It is worth noting, however, that since absolute levels of dimerisation cannot be determined from the data provided in this thesis, it is possible that S1P₂ and S1P₃ also have large monomeric fractions and that the dimer species is in the minority. All that can be said is that S1P₅ most likely exhibits weaker interactions with itself and the other S1P receptors than they do with themselves and one another, but what this represents in real terms cannot be accurately judged.

Conservation of the putative S1P dimerisation interface

The possible role of TM4 in the S1P₃ homodimer interface, as described above, indicates that it may influence the stoichiometry of the entire S1P family. Moreover, the fact that apparent heterodimerisation is seen using cBRET analysis of the S1P GPCRs suggests that homo- and heterodimers of these receptors compete for the same or similar interface, as homodimerisation must be disrupted in order to observe heterodimers. TM4 is highly conserved between S1P₂, S1P₃, and S1P₅ (Fig. 8.7), although greater conservation is present in other regions of the receptors, most notably TM3,

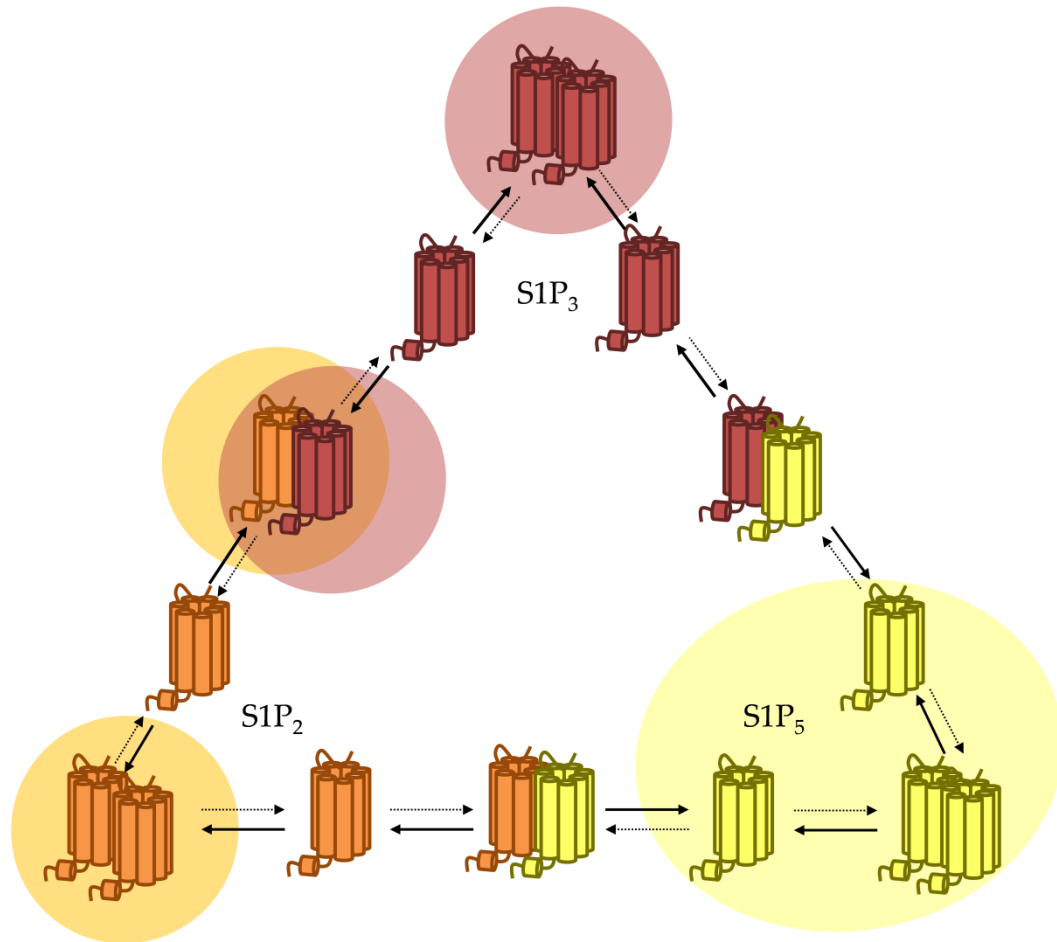


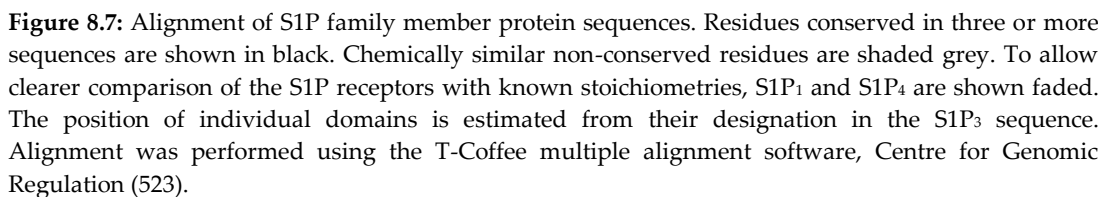
Figure 8.6: Possible interaction scheme of S1P receptors. S1P₂ is represented in orange, S1P₃ in red, and S1P₅ in yellow. Broken arrows indicate a slow transition; solid arrows represent a fast transition. The predominant species of each receptor are highlighted in the relevant colour of that receptor. In this model, the S1P₂ and S1P₃ receptors are largely homodimers or in heterodimeric complexes with one another, whereas the S1P₅ is more frequently monomeric and dimerisation is restricted largely to homodimerisation.

possibly due to its central role in the stabilisation of the 7TM core (158). Nonetheless, when mapped onto the known structure of S1P₁, the level of conservation between the S1P receptors is greater for the surface formed by TMs 2 and 4 than that formed by TMs 6 and 7 (Fig. 8.8), supporting a model of conserved receptor interaction mediated by this surface. S1P₄ is not included in this analysis as it diverged from the other receptors earlier in evolutionary history and so its stoichiometry cannot be presumed (26). Interestingly, S1P₅ shares less TM4 sequence conservation with S1P₂ and S1P₃ than they do with one another: *i.e.* 48% identity with S1P₂ and 35% identity with S1P₅, while S1P₂ and S1P₃ share 65% TM4 identity. This may explain why S1P₅ forms the weakest homo- and heterodimeric interactions. Indeed, TM4 of S1P₁ is more similar to that of S1P₂ and S1P₃ than is S1P₅, perhaps suggesting that this receptor is also likely to be a dimer *in vivo*.

Broader implications of GPCR heterodimerisation

The foregoing considerations regarding S1P receptor heterodimerisation perhaps offer some indication of how widespread heterodimerisation is among GPCRs. The observation that the same interface apparently mediates homo- and heterodimeric interactions in S1P receptors suggests that heterodimerisation results from a capacity for homodimerisation. That is to say, homodimerisation emerges during receptor evolution and subsequently develops into heterodimerisation upon duplication and divergence of the homodimeric receptor gene. This is supported by the observation that S1P₃ does not appear to interact with either LPA₁ or CCR5 in both the cBRET (Fig. 6.9) and SABRE (Fig. 7.12) assays, as it is predicted that only receptors that undergo the same mechanism of homodimerisation can interact as heterodimers. This has been observed for several members of the *Glutamate* family: the fact that all members of the mGluR-like group within the *Glutamate* GPCRs homodimerise via the same highly conserved N-terminal domain (§1.2.4) means that many are capable of heterodimerisation by the same mechanism (*e.g.* 521). If this is generally the case for all GPCRs, then it would suggest that heterodimerisation is as uncommon as homodimerisation beyond the *Frizzled* and *Glutamate* families. It would also suggest that heterodimerisation may occur in the closely related *Rhodopsin* family GPCRs reported to form homodimers in this thesis: for example, the histamine H₁R and H₂R receptors, or LGR4 and LGR5.

The model of homodimer-dependent heterodimerisation is in marked disagreement with widespread reports that *Rhodopsin* family GPCRs are broadly capable of forming heterodimers, often with distantly related partners (*e.g.* 255, 291, 522). Although GPCR heterodimerisation was not extensively investigated in this thesis, the conflicting observations concerning homodimerisation



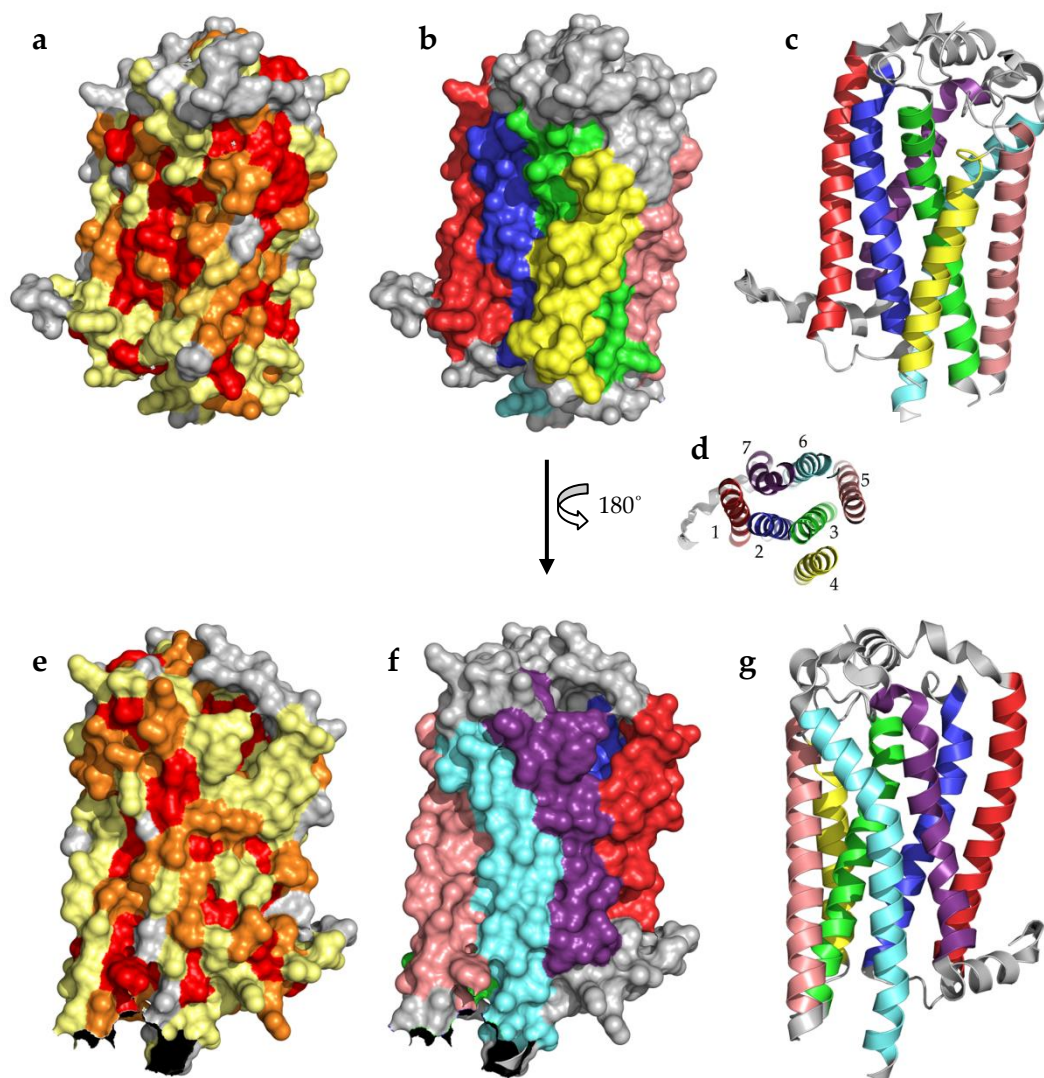


Figure 8.8: Representation of conservation of residues between S1P₁, S1P₂, S1P₃, and S1P₅. Structures shown are S1P₁, PDB code: 3V2Y. (**a** & **e**) Conservation of residues between the four receptors: grey indicates no conservation; yellow indicates conservation between two receptors; orange denotes conservation between three receptors; red residues are conserved between all four receptors. Conservation is greater on the TM2/4 face of the receptor (**a**) than the TM6/7 face (**e**). The position of TM helices within the receptor are shown as corresponding surface maps (**b** & **f**) and cartoons (**c** & **g**), and as viewed from the extracellular side of the receptor (**d**). TM1 = red, TM2 = blue, TM3 = green, TM4 = yellow, TM5 = pink, TM6 = cyan, TM7 = purple. All non-TM regions are shown in grey.

suggest that many of the concerns regarding the interpretation of the earlier studies of homodimers may be valid, and that these concerns may also apply to studies of heterodimerisation. Moreover, although it is possible that distantly related GPCRs may evolve mechanisms to interact that are entirely independent of any capacity to homodimerise, this seems unlikely in the context of the predominantly monomeric GPCR family apparently revealed by the present study. Had there been a biological imperative for receptors to be dimers, homodimerisation would be far more common. Thus, distantly related receptors would only develop heterodimerisation following convergent evolution of complementary interaction interfaces, which is unlikely to occur frequently without strong selective pressure being in its favour.

8.5 Evolutionary considerations

Independent emergence of dimers during evolution of the *Rhodopsin* family

The observations presented in Chapters 3 and 5 suggest a model of *Rhodopsin* GPCR evolution in which monomers are the predominant species. This is consistent with a model in which the GPCR signalling system is highly modular, with ligand-binding and signalling activity contained within an independently functioning unit, encoding by a single gene. It is also consistent with several previous studies reporting efficient signalling for single GPCR proteins (*e.g.* 197, 249, 251). The phylogenetic distribution of dimeric receptors within the *Rhodopsin* family (Fig. 5.9), and the lack of an overtly conserved mode of dimerisation, suggest that the capacity for dimerisation emerged from individual gain-of-dimerisation events that were retained in small phylogenetically-related clusters of dimeric receptors. This implies that the original ancestral *Rhodopsin* was most likely monomeric and that this behaviour has been retained in most family members throughout evolution. This proposal is supported by the observation of apparently monomeric behaviour for GPR50, a member of the melatonin cluster that shares the greatest sequence identity with the ancestral *cAMP* receptors (61). Inspection of the phylogenetic positions of the 63 *Rhodopsin* family receptors investigated in this thesis within the larger family (Fig. 8.9) suggests that most or all ancestral *Rhodopsin* GPCRs were monomeric and that this has been preserved throughout the expansion of the family during evolution, with dimerisation emerging on at least seven occasions, leading to the dimerisation of, for example, the S1P, histamine, and leucine-rich repeat-containing receptors. The distribution suggests that GPCRs that share common ligands but do not have close phylogenetic relationships will not necessarily have similar stoichiometries. For example, the adrenergic receptors, β_1 AR and β_2 AR are both apparently

monomeric whereas α_2 cAR seems to form dimers. It can be predicted that, even though H₁R and H₂R are dimers, H₄R is more likely to be a monomer as it is not closely related to either dimeric receptor and instead shares closer common ancestors with monomeric GPCRs *e.g.* the muscarinic acetylcholine receptors. Another consequence of this evolutionary model is that closely related GPCRs will have wholly different stoichiometries if a gain-of-dimerisation event occurred after the divergence of the two receptors in evolutionary history. This may have occurred during the evolution of the endothelial differentiation GPCRs, since the LPA receptors yield data consistent with monomeric behaviour yet the S1P receptors apparently form dimers despite the very high degree of sequence identity between members of the two groups (Fig. 5.15).

A model of independent gain-of-dimerisation events also suggests that a common structural element within *Rhodopsin* family dimers capable of mediating interactions is unlikely to exist that is comparable to, *e.g.*, the VFTM of *Glutamate* receptors. It may also mean that dimerisation has different roles in different receptors. Indeed, although dimerisation apparently occurs for some *Rhodopsin* family GPCRs, the functionality of the interactions have not been demonstrated and it is possible that it is simply the result of the chance evolution of complementary hydrophobic interfaces that do not have a functional effect on receptor behaviour.

Overall, a model of rare, independent dimer evolution explains many of the observations described in Chapter 5: (1) dimeric receptors represent only a small proportion of *Rhodopsin* family GPCRs; (2) dimers often exist in closely related phylogenetic clusters; (3) dimers do not appear to share ligand- or G protein-selectivity outside of their clusters; (4) closely related receptors can have different stoichiometries; (5) no monomers appear to be descended from dimeric ancestors that lost the capacity for dimerisation.

Possible constraints upon dimer evolution

The relative infrequency of dimer development in the *Rhodopsin* family suggests that there is no positive selective pressure for the evolution of dimeric receptors in order for the proteins to have basal functionality. Instead, the predominance of monomeric receptors may arise due to differences in the possible divergence rate of monomers and dimers owing to the different degrees to which newly evolved monomers and dimers can influence the activity of their original counterparts. If a gene encoding a monomeric receptor undergoes duplication, the products are genes encoding two distinct receptors that are able to undergo mutation and selection relatively independently as they do not directly interfere with one another's function. They would therefore be subject to the classical birth-



Figure 8.9: Phylogenetic relationships within the *Rhodopsin* family and of investigated receptors. Relationships are shown as a divergence tree based on the analysis of Fredriksson et al. (26). Names shown in grey denote receptors not assessed in this thesis. Coloured names indicate receptors concluded to be monomers (blue) or dimers (red). LTB4R1 is given in red due to the likelihood of oligomer formation. C5R1 and DP2 are shown in pink to denote possible weak dimerisation. Branches are coloured blue (monomers) and red (dimers) by predicted stoichiometric lineage assuming a monomeric model with individual gain-of-dimerisation events. Bold branches indicate known stoichiometries or those that are highly likely given the presence of multiple related nodes of consistent stoichiometry. Untested receptors flanked by two or more closely related receptors of known stoichiometry are deemed likely to share that behaviour and are shown in black with their predicted stoichiometry corresponding to their branch colour. Thin blue branches denote evolutionary lineages that lead to known monomeric receptors. Thin grey branches denote lineages for which the outcomes are unknown or that cannot be assigned with confidence as it is not known at what point dimerisation emerged.

and-death model of protein evolution, as described for some *Rhodopsin* family receptors (e.g. 51). In contrast, the product of gene duplication for a dimeric receptor is tightly functionally connected to its parent receptor as they will still undergo mutual interaction and regulation until they diverge sufficiently to lose the capacity for interaction. Whilst they are still able to interact, the two genes are not free to evolve independently as the mutation of one may affect the activity of the other (Fig. 8.10), in a manner equivalent to that of dominant negative mutations. To be selected, dimerisation would have to confer an increase in the fitness density of the receptor (37), which in turn imposes a constraint on the rate of protein evolution, as observed for several other protein families (e.g. 42, 43, 44). This is because mutations will tend to be deleterious and the rate of gene diversification will be accordingly reduced. Thus, over time, monomeric receptors become the predominant species within the family as they outcompete dimers to fulfil functional niches.

Although the apparently monomeric population identified in this thesis may still undergo levels of dimerisation below the sensitivity limits of type 1 and cBRET, the evolutionary argument discussed above is still valid. This is because receptors undergoing extensive dimerisation (i.e. above ~10-20% total receptor population) will experience a greater degree of evolutionary constraint compared to those that form less extensive dimers (i.e. below ~10-20% total receptor population), as the functional effects of mutation of a nascent gene on the parent will be more significant. In this manner, extensive dimerisation may be selected against due to constrained diversification, whilst monomers or low-level dimers are less restricted in their rate of expansion. Thus, the following discussion regarding the family-specific distribution of stoichiometry is still relevant even if the distinction is between high- and low-level dimers rather than between dimers and absolute monomers.

Broader implications for GPCR evolution

The existence of a constraint on the rate of dimer evolution relative to that of monomers may explain several features of GPCR evolution generally. The most striking feature of the GPCR family, is how asymmetrically distributed are its members. The extreme difference in the number of receptors in the *Rhodopsin* family compared to any of the other GPCR families in humans may be due, in part, to differences in stoichiometry between the various families and the different constraints on their rates of diversification and expansion as discussed above. It is well established that the *Glutamate* family GPCRs are capable of both homo- and heterodimerisation (§1.2.4), and a model of *Frizzled* receptor

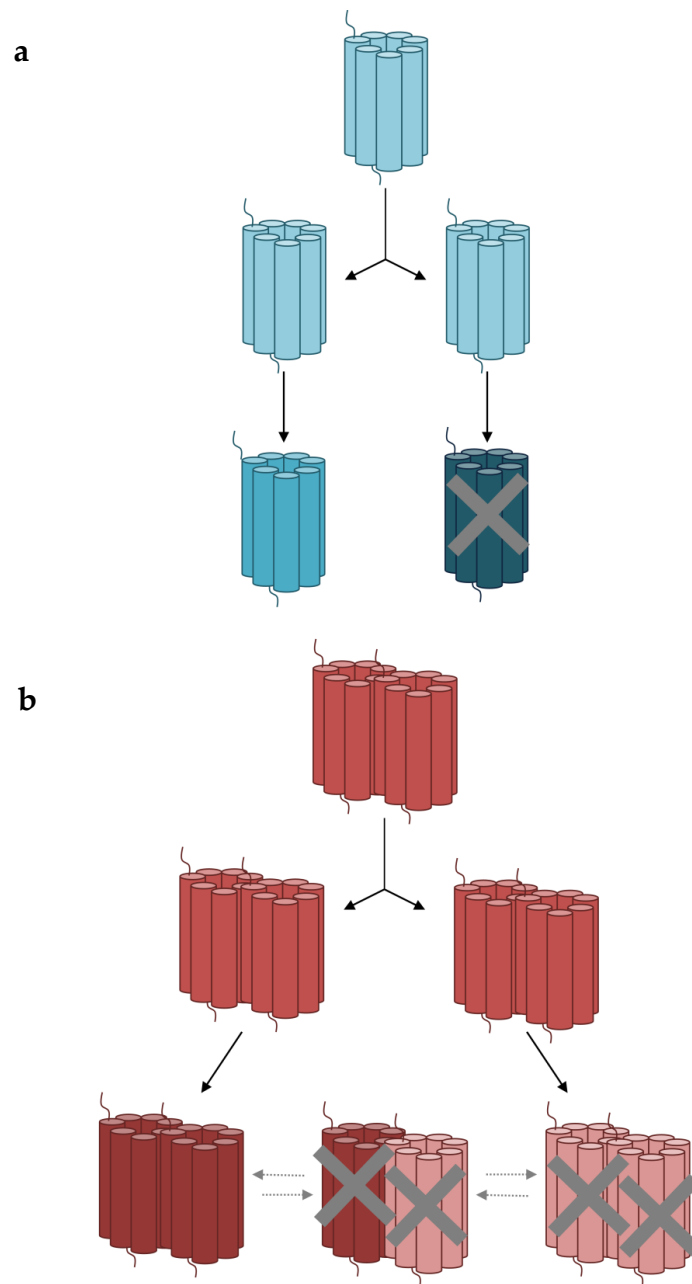


Figure 8.10: Possible influence of GPCR stoichiometry upon the rate of receptor evolution. **(a)** Products of gene duplication of a monomeric receptor are free to undergo relatively independent evolution. Detrimental mutations (indicated by a grey cross) will be unlikely to influence the behaviour of the other duplication product and so can degrade into pseudogenes over time. **(b)** Conversely, duplicated dimeric receptors that undergo detrimental mutation will interfere with their counterpart and so are more likely to have phenotypically lethal outcomes, thus constraining evolution of both duplicates.

homodimerisation mediated by their N-terminal domains is suggested by observations in this thesis (§6.4). The prevalence of dimerisation within these families may have constrained their diversification, explaining why the number of receptors in each of these families is small and largely conserved between species (Fig. 8.11; 61). This is in contrast to the apparently largely monomeric *Rhodopsin* family, which exhibits much more variable receptor numbers between species, suggesting more rapid rates of gene 'birth' and 'death'. The *Adhesion* and *Secretin* families also exhibit limited rates of expansion relative to the *Rhodopsin* family. The stoichiometry of these receptors was not examined in this thesis, however several previous studies claim dimerisation of both *Adhesion* (e.g. 524, 525, 526) and *Secretin* (e.g. 527, 528, 529) GPCRs. For the *Adhesion* family, there are conflicting reports of dimerisation occurring primarily via the 7TM region (530) or through the large N-terminal domains (526, 531), whilst there is evidence that in some *Secretin* family receptors dimerisation is TM4 dependent (532). Nonetheless, many of these studies employ the same techniques as have been used to investigate the *Rhodopsin* GPCRs and so may be open to misinterpretation for reasons previously discussed (§1.4). Even so, if dimerisation within these families is in fact genuine, it would explain their small size and slow rates of expansion relative to the *Rhodopsin* family.

The evolutionary success of monomeric GPCRs also offers a possible explanation for why the GPCR family is so large compared to families of other cell surface receptors. The sheer number of GPCRs and the diversity of their ligands suggest that they are uniquely suited to diversification compared to other receptor proteins. This may emerge because the ligand-binding pocket in GPCRs is well suited to allowing high-affinity interactions with ligand in a 1:1 binding ratio. Many other cell surface receptors interact with ligand in a 2:1 receptor:ligand ratio in order to augment the affinity of binding (e.g. immunoglobulin domain-containing proteins; 490) but this is not required for GPCRs due to the nature of the ligand-binding pocket. This has allowed GPCRs to undergo extensive diversification to produce a large number of receptors capable of binding to an extremely broad range of ligands that are nonetheless bound in a similar, ubiquitous manner. The evolutionary flexibility of the GPCR ligand-binding cavity may then be facilitated by the monomericity of the receptors themselves, which allows the receptors to signal as independent units and so does not negate the evolutionary success of the binding pocket. Thus, although monomeric behaviour may be an advantage to the rate of protein diversification, it might only have allowed such extreme family expansion in the case of GPCRs because of this inherent underlying advantage that GPCRs have over other cell surface receptors.

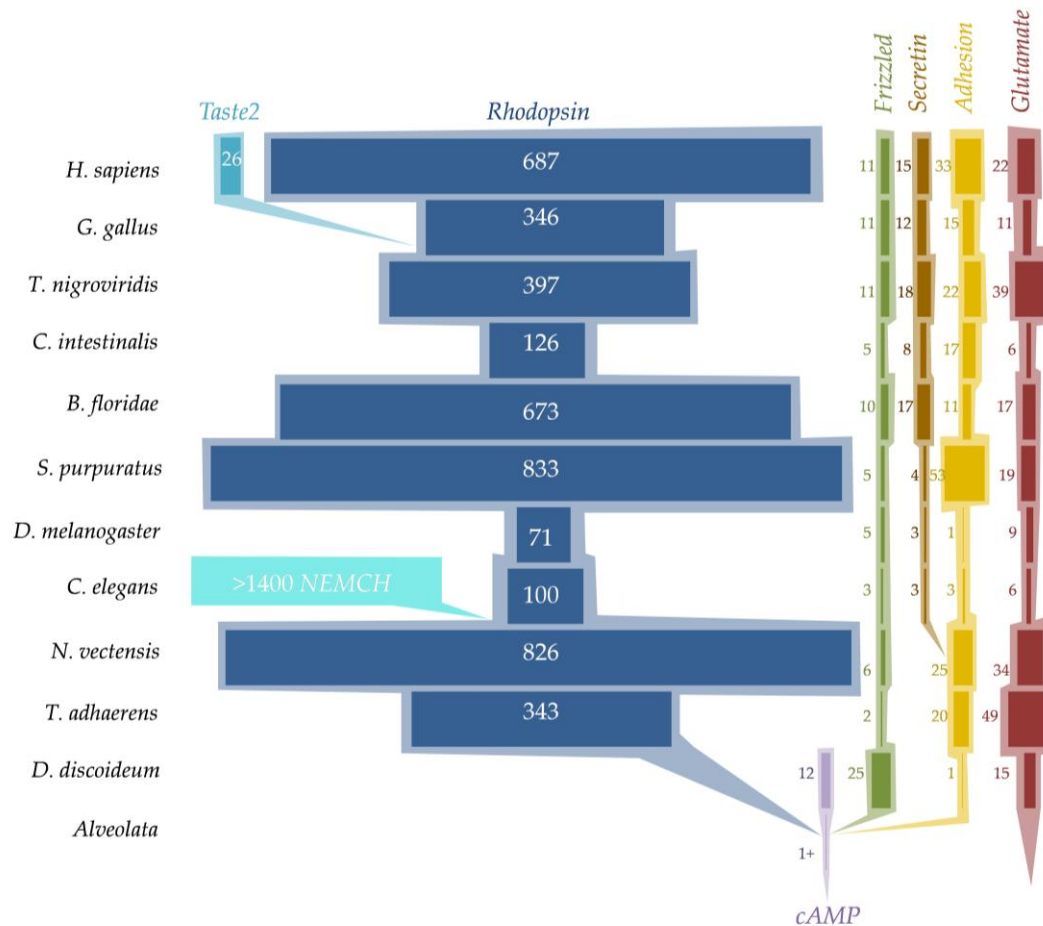


Figure 8.11: Overview of the size of the individual GPCR families within different species. Numbers indicate the number of known receptors in each family for the corresponding species, obtained from (61). The size of each block corresponds to the number of receptors within that family, with the exception of the NEMCH family of *C. elegans*. Branches between individual families indicate the emergence of one family from another. The Rhodopsin, Frizzled, and Adhesion families all emerged from the cAMP family, whilst the Glutamate family appears to have diverged earlier. The NEMCH and Taste2 receptors subsequently emerged from the Rhodopsin GPCRs, whereas the Secretin family emerged from the Adhesion receptors.

Undoubtedly, stoichiometry is not the only factor influencing the rate of evolution of individual GPCRs. Selective pressures arising from different functional properties will also contribute to the direction and rate of protein evolution in all cases. However, when GPCR families are viewed as a whole many of these effects would be diluted due to the breadth of functionality therein, and so the stark contrast in the rates of diversification between families is likely to be due to factors shared by all receptors within that family, such as stoichiometry. Nonetheless, important insights can perhaps be obtained by comparing receptors with contrasting stoichiometries that are likely to undergo similar selective pressures because they have similar functional roles.

Sweet/Umami vs. *Taste2*

Two groups of receptors active in similar functional niches that exhibit vastly different rates of diversification are the sweet/umami and bitter taste receptors. Both groups of receptors are expressed almost exclusively on the tongue and palate epithelium where they mediate the sensation of sweet and umami or bitter tasting compounds, respectively (143, 533). The sweet (or '*Taste1*') receptors are members of the *Glutamate* GPCR family known to dimerise via their large N-terminal domains in a manner that determines ligand specificity: a heterodimer of TAS1R1 and TAS1R3 recognises umami tasting compounds, *e.g.* glutamate and 5'-ribonucleotides, whereas a heterodimer of TAS1R2 and TAS1R3 senses sweet tasting compounds, *e.g.* sucrose and artificial sweeteners (521). The bitter-sensing type 2 receptors form the separate *Taste2* family. The analysis of the *Taste2* receptors in this thesis using the type 1 and cBRET assays suggests that these receptors, in marked contrast to the *Taste1* receptors, are entirely monomeric (§6.3.3).

A striking difference between the two groups of taste receptors is the number of genes encoding each family in the human genome. There are only three *Taste1* GPCR genes (534), yet there are 28 full-length genes as well as 16 pseudogenes for the *Taste2* receptors (98, 142). This is particularly remarkable considering that the *Taste2* receptors only emerged from the *Rhodopsin* family ~300 million years ago, whereas the most evolutionarily ancient *Taste1* receptors have been found in a pufferfish (*Tetraodon nigroviridis*), which diverged from the human lineage nearer to 500 million years ago (61). This indicates that the *Taste2* receptors have undergone far more rapid evolutionary diversification than the *Taste1* GPCRs. This is further supported by the fact that human *Taste2* receptors possess unusually high levels of allelic diversity compared to other genes, with a particularly large number of polymorphic amino acid substitutions (535). This may in part be due to relaxation of the selective pressure for bitter taste sensation in humans as a result of changes in diet, the development of

cooking, and other methods of toxin avoidance (536). It is, nonetheless, a striking demonstration of the rapid rate of evolution in the *Taste2* GPCRs. Indeed, even closely related species sometimes possess strikingly different *Taste2* genes. For example, both humans and chimpanzees exhibit intra-species variation of sensitivity to the bitter tasting compound phenylthiocarbamide due to polymorphisms in the *TAS2R38* gene, yet the polymorphic alleles that generate this variability are entirely different in the two species (537). The fact that the *Taste1* and *Taste2* receptors have such remarkably different rates of receptor evolution appears counter-intuitive given the similarities in their physiological location and function perhaps until the effect of their stoichiometry upon their evolution is considered. These receptors represent a key example of how differences in apparent stoichiometry correlate with differences in the relative rates of receptor diversification.

For these reasons, circumstances in which rapid receptor diversification is advantageous will likely preferentially promote the emergence of new receptors from the *Rhodopsin* family rather than from the predominantly dimeric GPCR families. This is because the dimeric families cannot evolve efficiently diversifying monomeric receptors as their mechanism of ligand-recognition is inherently linked to their ability to dimerise. Thus, whilst these families may be able to fill some of the need for new receptor function, they cannot generate a large range of receptors in a short time, and so the *Rhodopsin* family-derived GPCRs will always be more competitive. Rapid receptor diversification may be advantageous in cases of sensation of environmental ligands (*e.g.* odorants, taste compounds *etc.*) as the range of potential ligands is far larger and recognition of many such molecules may be more directly linked to the fitness of the organism in many cases (*e.g.* detecting toxins, identifying food *etc.*). The emergence of new sensory receptors may therefore be a phenomenon predominantly restricted to the *Rhodopsin* family, which would explain why the *Taste2* receptors emerged from the *Rhodopsin* GPCRs rather than from the *Taste1* receptors, even though the *Taste1* GPCRs already performed the role of taste sensation.

Other sensory GPCRs

Evidence for the pre-eminence of *Rhodopsin* family-restricted sensory receptors in evolutionary terms is most obvious for the olfactory receptors, which number ~460 in humans and are matched by a similar number of pseudogenes (85), but are even more numerous in other mammals (51). The fact that these, like the *Taste2* receptors, possess only very small intra- and extracellular domains (90), and have also exhibited apparently monomeric behaviour in type 1 and cBRET assays (§5.4), suggests that their emergence as a group within the *Rhodopsin* family is linked to the selective pressure for rapid

diversification in olfaction. This fits very well with the birth-and-death mechanism of evolution proposed for olfactory receptors (51, 93).

A final example of rapidly diverging sensory GPCRs emerging from the *Rhodopsin* family are the nematode chemosensory (NEMCH) receptors, which split from the *Rhodopsin* receptors ~1000 million years ago (61). These receptors represent the largest known family of GPCRs present in a single organism, with over 1700 genes and pseudogenes in *C. elegans* (56). These are divided into over 20 subfamilies of varying size, though the overall topology of the receptors is consistent across the whole family and very similar to that of typical *Rhodopsin* family GPCRs – *i.e.* without large N- and C-terminal domains (538). They are typically expressed on chemosensory neurons, where they mediate the detection of environmental chemical stimuli in a manner equivalent to vertebrate olfactory receptors (539). The sheer number of NEMCH receptors is likely to be partly due to the lack of alternative sensory mechanisms for nematodes, which lack olfactory or taste receptors and have no sense of sight. Analysis of the rates and mechanisms of evolution within the NEMCH family has revealed extremely rapid diversification of individual receptors that correlates strongly with a birth-and-death model of gene evolution (56). These considerations suggest that the broadly monomeric behaviour of the *Rhodopsin* GPCRs suggested by the experiments described in this thesis likely applies to most or all NEMCH receptors. This has not yet been assessed by the field as nearly all investigations into GPCR stoichiometry have focussed on human receptors, but if it were found to be the case it would strengthen the argument that the *Rhodopsin* family is predominantly monomeric, and that it is this that drove the evolution of the huge numbers of receptors found in eukaryotic genomes today.

APPENDIX

Type 1 and cBRET data of *Rhodopsin* family GPCRs

The data in this appendix constitute the original data for each of the 60 *Rhodopsin* family GPCRs discussed in Chapter 5. BRET_{max} , R^2 , y -intercept, and P^{diff} values given in Figures 5.9, 5.10, 5.16, and 5.17, and Tables 5.13 and 5.14 were obtained by analysis of these data.

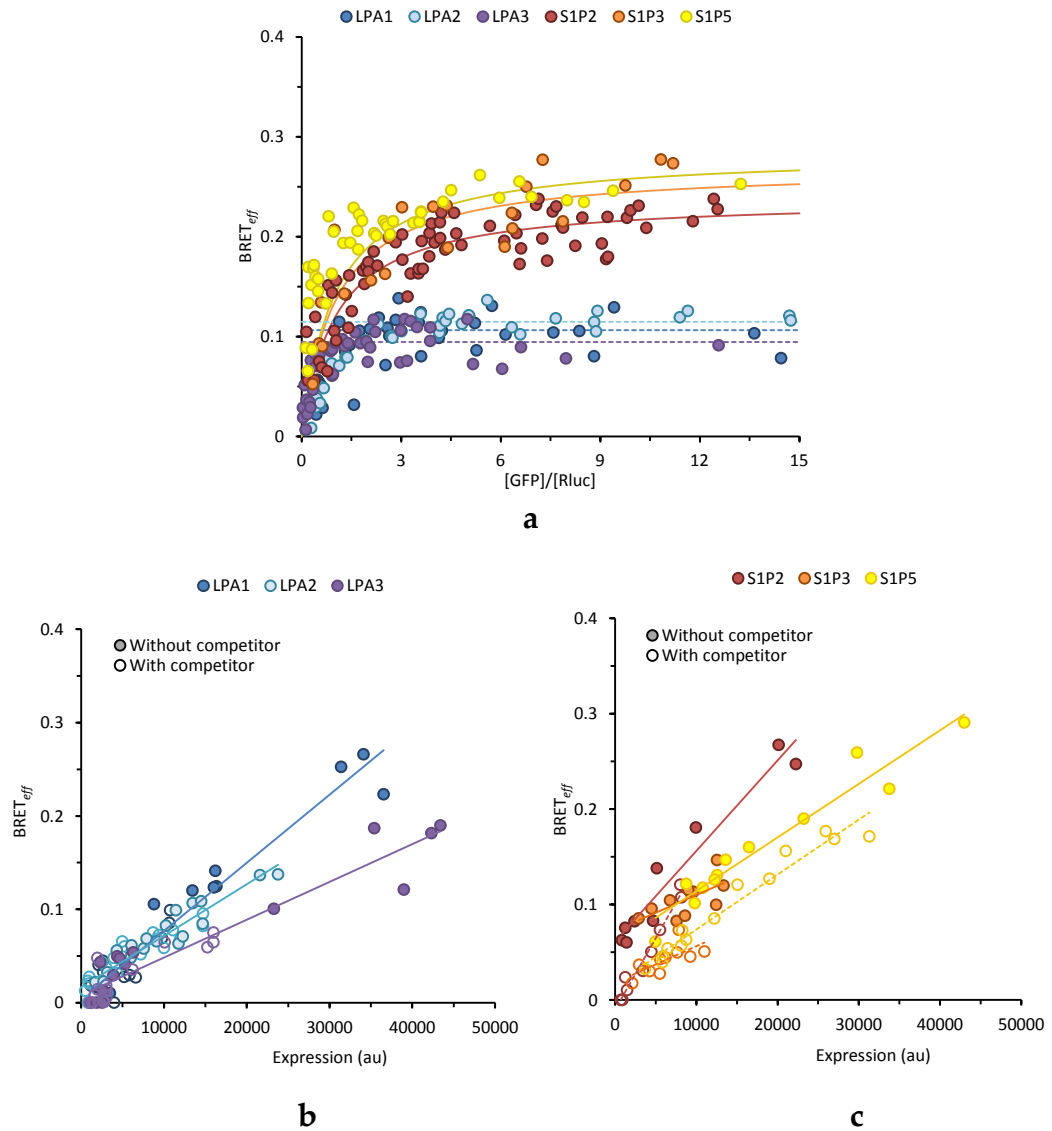


Figure A.1: The endothelial differentiation receptors show divergent stoichiometries. **(a)** All three investigated LPA receptors show type 1 BRET_{eff} values consistent with those of a monomer, whereas the three S1P receptors give data that fit well to a dimer model. Data fits to a dimer model are shown with solid lines; fits to a monomer model are shown with broken lines. The optimal fit is shown in each case. **(b)** cBRET of the LPA receptors displays no change in BRET_{eff}-density relationship, consistent with monomeric behaviour. **(c)** S1P receptors demonstrate a significant drop in BRET_{eff} relative to density in the presence of competitor receptors, indicating the presence of homodimers. For monomers, cBRET data are plotted as best fit of all points, indicated by a solid line. For dimers, 'with' and 'without' competitor data are fitted separately as solid and broken lines, respectively.

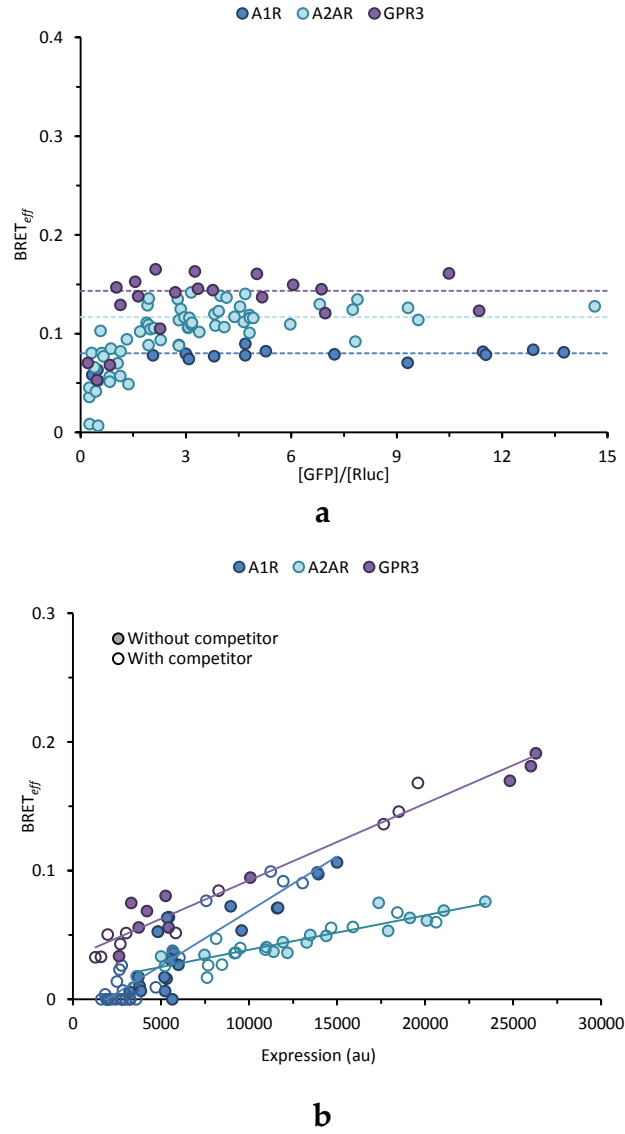


Figure A.2: The remaining non-S1P MECA receptors are apparent monomers. **(a)** Type 1 BRET of the adenosine receptors and GPR3 reveals an independence of $BRET_{eff}$ upon GFP:Rluc ratio, suggesting that all three receptors are monomeric. Fits of all data to their optimal, monomeric models are shown as broken lines. **(b)** Assessment of the receptors using cBRET is also suggestive of monomeric behaviour as no reduction of $BRET_{eff}$ is observed in the presence of competitor receptors. cBRET data are plotted as best fit of all points, indicated by a solid line.

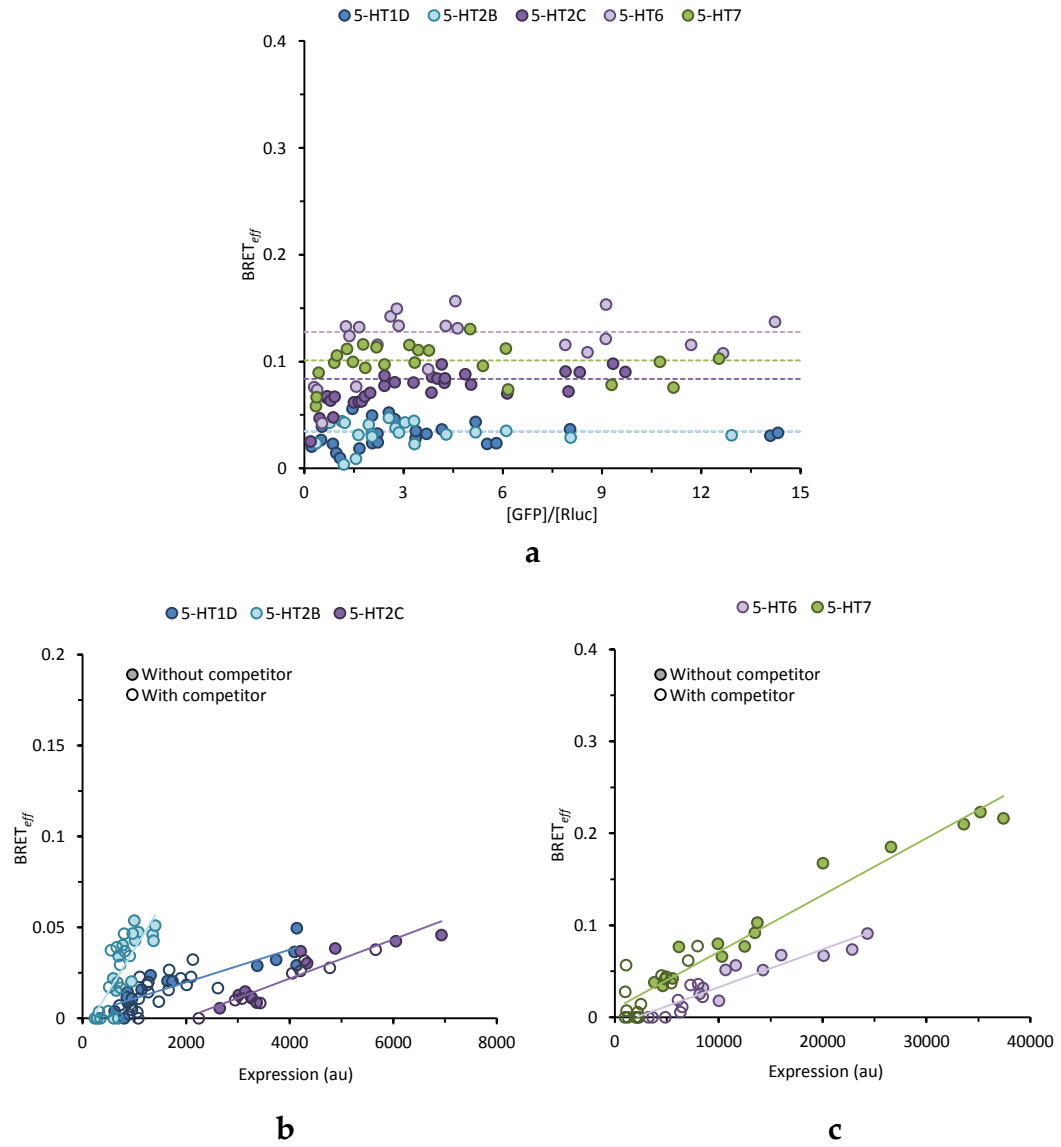


Figure A.3: The serotonin receptor subfamily does not display any apparent homodimerisation under type 1 or cBRET analysis. **(a)** Type 1 BRET of all serotonin receptors is consistent with monomeric organisation. Fits of all data to their optimal, monomeric models are shown as broken lines. **(b and c)** All five serotonin receptors failed to show a change in $BRET_{eff}$ -density relationship in the presence of competitor receptors, suggestive of monomeric behaviour. cBRET data are shown as separate figures for clarity and are plotted as best fit of all points, indicated by a solid line.

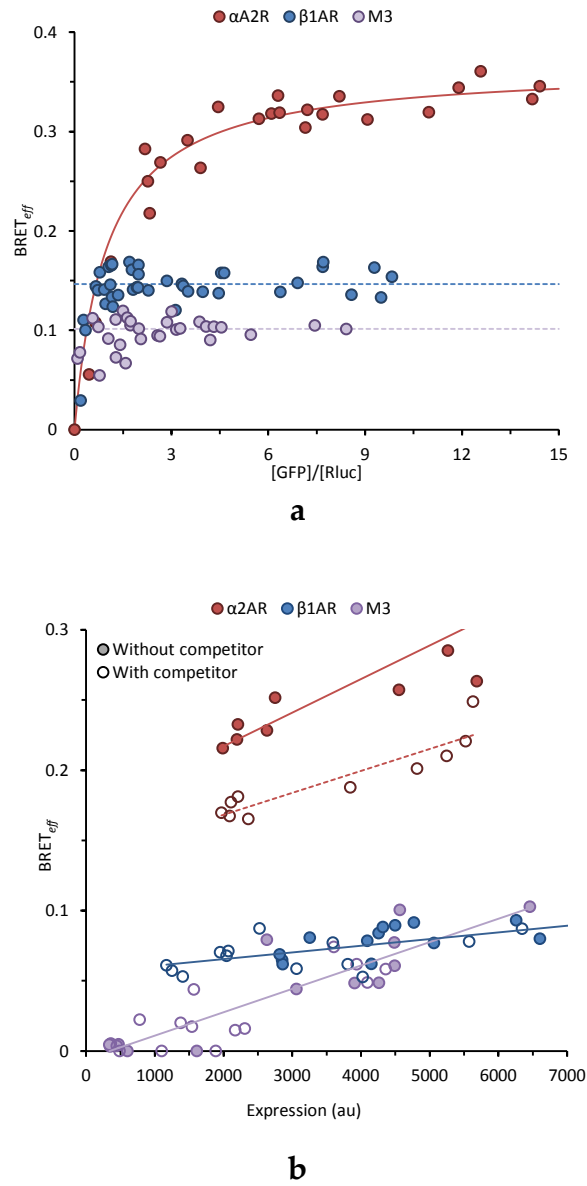


Figure A.4: Adrenergic and muscarinic receptors display varying stoichiometries. **(a)** Type 1 BRET analysis of the β_2 adrenergic and M_3 muscarinic receptors shows a good fit to a monomer model, whereas the α_{2A} adrenergic receptor displays a hyperbolic relationship characteristic of dimers. Data fits to a dimer model are shown with solid lines; fits to a monomer model are shown with broken lines. The optimal fit is shown in each case. **(b)** cBRET analysis of these receptors is consistent with the type 1 BRET data: β_1AR and M_3 show data distributions indicative of monomers whereas $\alpha_{2C}AR$ show significant competition from unlabelled receptors. For monomers, cBRET data are plotted as best fit of all points, indicated by a solid line. For dimers, 'with' and 'without' competitor data are fitted separately as solid and broken lines, respectively.

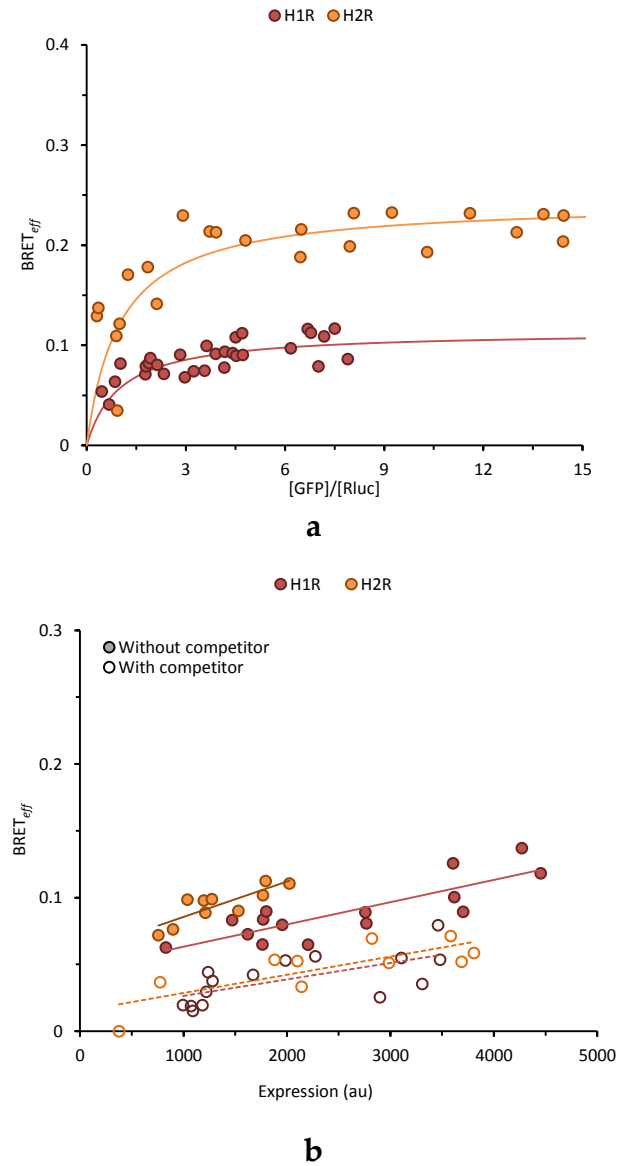


Figure A.5: The histamine H₁ and H₂ receptors form apparent homodimers. **(a)** BRET_{eff} values for H₁ and H₂ receptors in type 1 BRET were found to be dependent on GFP:Rluc ratio in a relationship that fits well to the expected relationship of a dimer model. Fits of all data to their optimal, dimeric models are shown as solid lines. **(b)** Both histamine receptors display significant decreases in BRET_{eff} in the presence of competitor receptors in cBRET, consistent with the conclusion that these receptors form homodimers. 'With' and 'without' competitor data are fitted separately as solid and broken lines, respectively.

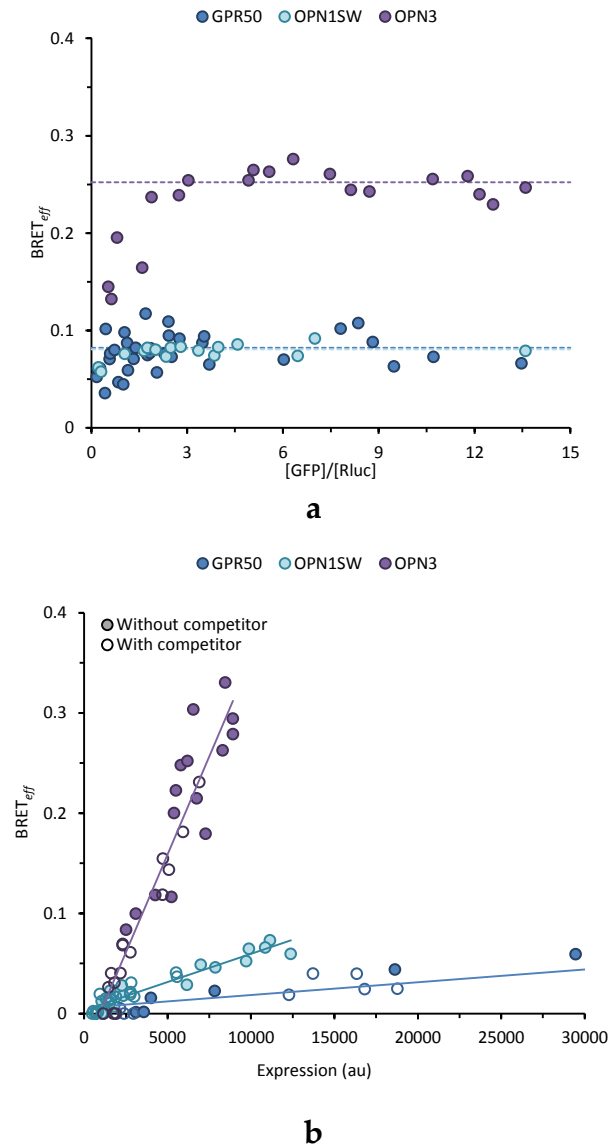


Figure A.6: Investigated opsin and melatonin subfamily receptors display behaviour consistent with monomericity. **(a)** Type I BRET of the two opsin subfamily receptors (OPN1SW and OPN3) and single melatonin subfamily receptor (GPR50) shows $BRET_{eff}$ distributions for all three receptors consistent with a monomer model despite highly different absolute $BRET_{max}$ values. Fits of all data to their optimal, monomeric models are shown as broken lines. **(b)** All three receptors displayed no change in $BRET_{eff}$ in the presence of competitor receptors in cBRET, in agreement with the type 1 BRET data. The steep slope of $BRET_{eff}$ vs. expression level observed for OPN3 explains its significantly higher type 1 $BRET_{max}$ value relative to OPN1SW or GPR50, and may indicate restricted distribution of OPN3 within the plasma membrane leading to an increased effective receptor concentration. cBRET data are plotted as best fit of all points, indicated by a solid line.

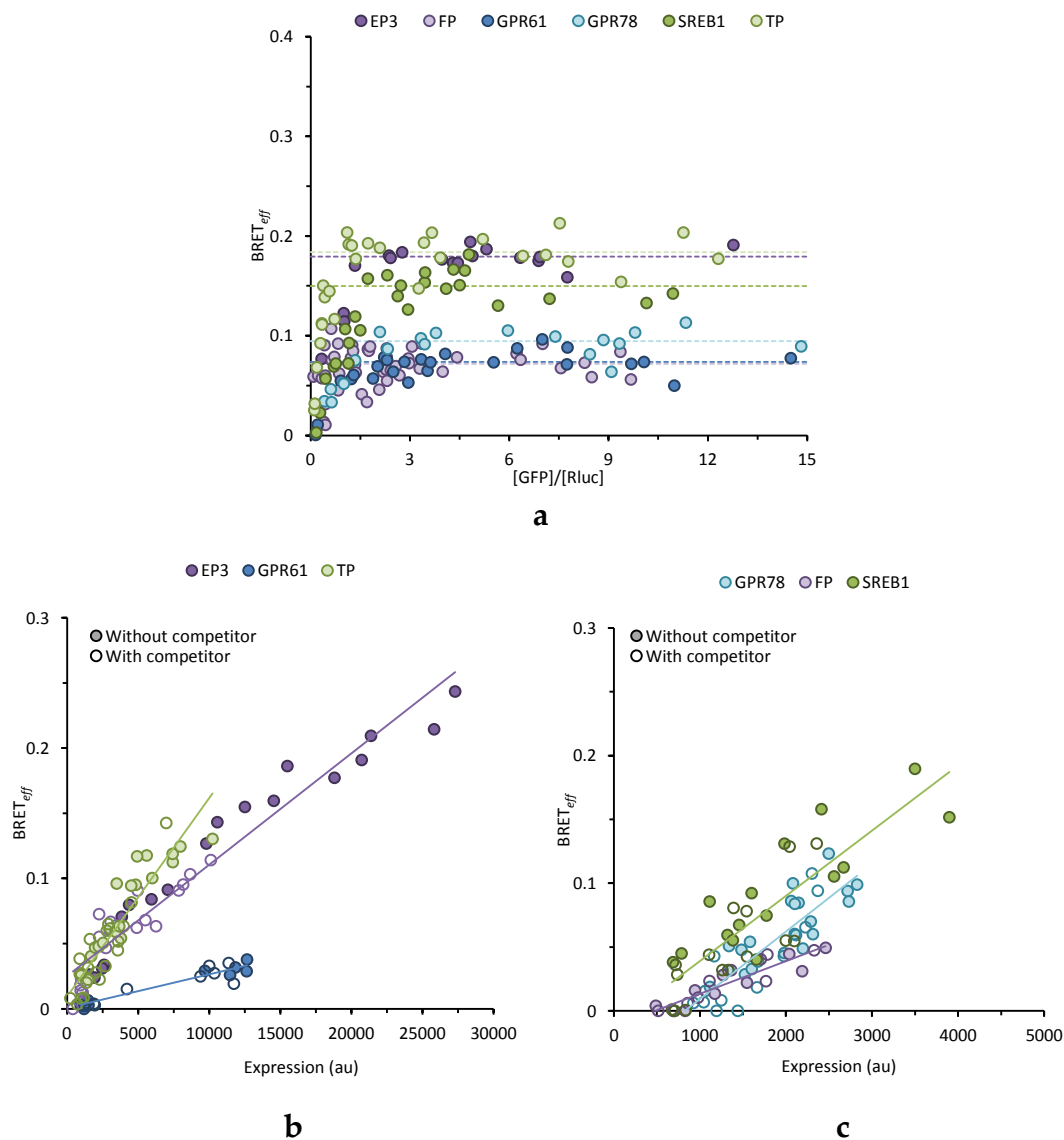


Figure A.7: The prostaglandin subfamily shows no apparent homodimerisation. (a) Type 1 BRET analysis of all prostaglandin subfamily receptors is consistent with monomeric organisation. Fits of all data to their optimal, monomeric models are shown as broken lines. (b and c) All prostaglandin subfamily receptors failed to show a change in $BRET_{eff}$ -density relationship in the presence of competitor receptors, suggestive of monomeric behaviour. cBRET data are shown as separate figures for clarity. cBRET data are plotted as best fit of all points, indicated by a solid line.

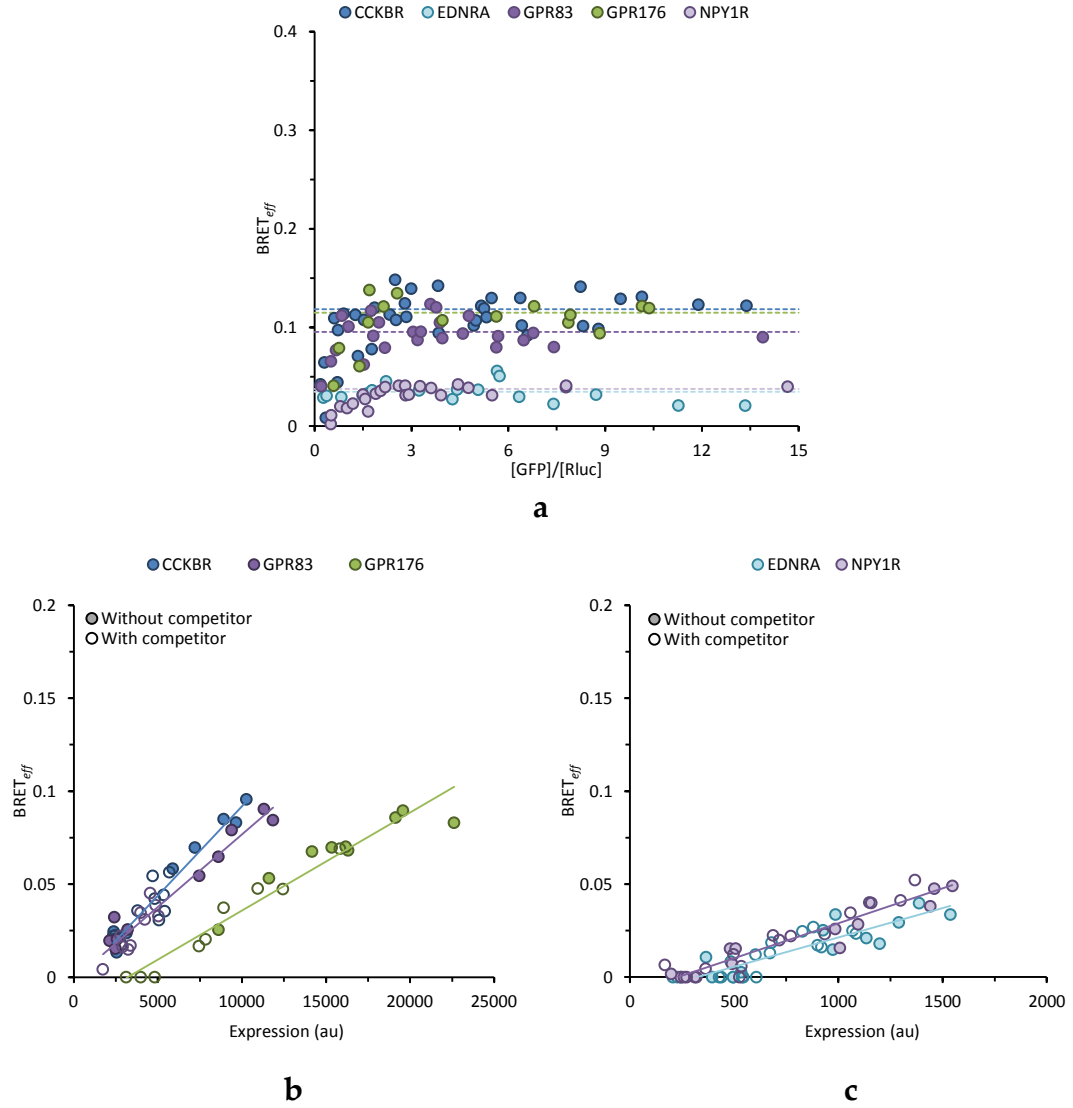


Figure A.8: The *Rhodopsin* β subfamily is entirely monomeric as reported by type 1 and cBRET analysis. **(a)** Type 1 BRET analysis of five β subfamily members shows an independence of $BRET_{eff}$ on $[GFP]:Rluc$ ratio, indicative of monomers. Fits of all data to their optimal, monomeric models are shown as broken lines. **(b and c)** All β subfamily members failed to show a drop in $BRET_{eff}$ in the presence of competitor receptors in cBRET. cBRET data are shown as separate figures for clarity and are plotted as best fit of all points, indicated by a solid line.

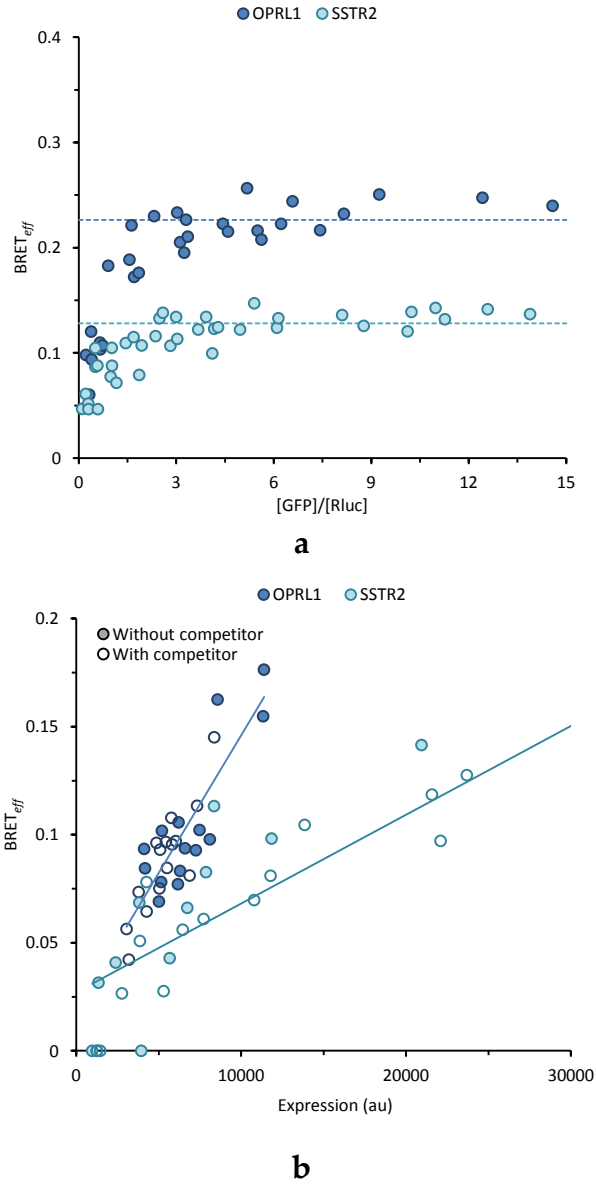


Figure A.9: The SOG subfamily does not exhibit apparent homodimerisation. **(a)** Type 1 BRET analysis of both OPRL1 and SSTR2 generates data that fit best to a monomeric model of BRET_{eff} dependence on GFP:Rluc ratio. Fits of all data to their optimal, monomeric models are shown as broken lines. **(b)** Neither receptor shows a significant change in the BRET_{eff}-density relationship when investigated using cBRET, consistent with their being monomers. cBRET data are plotted as best fit of all points, indicated by a solid line.

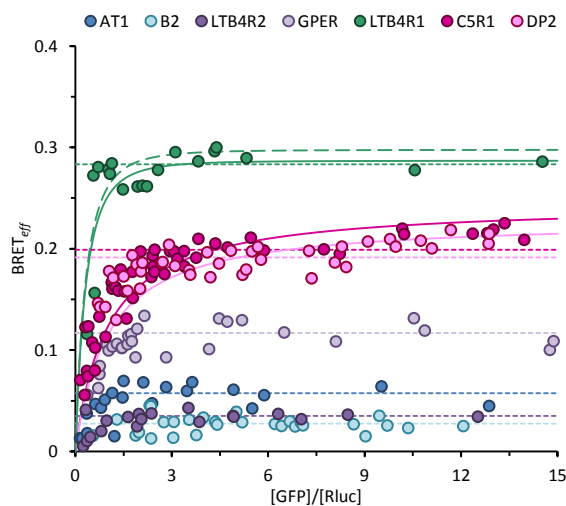


Figure A.10: Type 1 BRET analysis of the non-classical chemokine family does not reveal clear homodimerisation. AT₁, B₂, GPER, and LTB₄R₂ exhibit $BRET_{eff}$ signatures consistent with monomeric behaviour. The distributions of points for C5R₁ and DP₂ do not fit clearly to either a monomer or dimer model, but perhaps suggest weak dimerisation. LTB₄R₁ data fit well to a monomer model but also to predictions of trimeric and tetrameric behaviour described by Equation 1.2. Fits to monomeric models in all cases are shown as finely broken lines. Dimer models are shown for C5R₁ and DP₂ as solid lines. For LTB₄R₁, a trimer model is represented by a widely broken line, whilst a tetramer model is shown as a solid line.

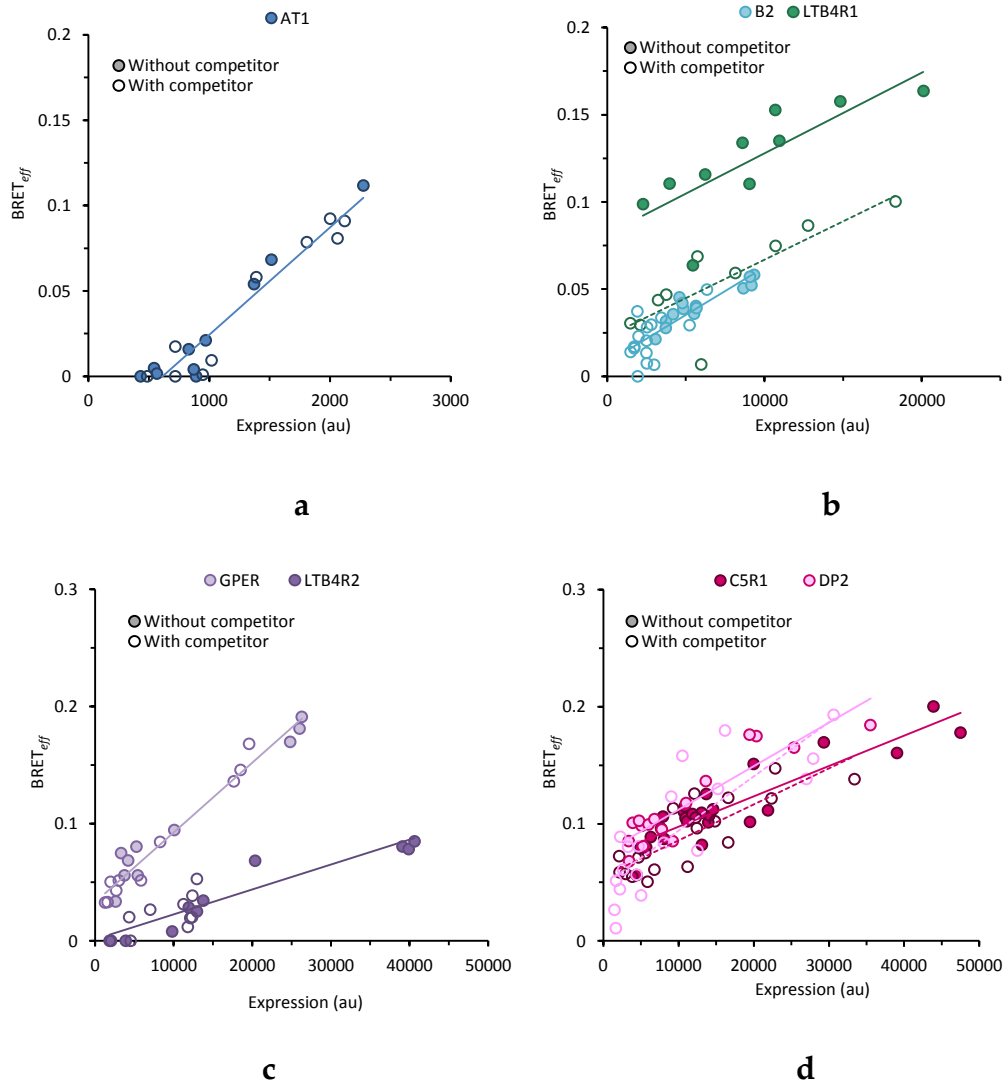


Figure A.11: cBRET analysis of the non-classical chemokine family. **(a, b & c)** AT₁, B₂, GPER, and LTB₄R₂ do not appear to undergo a reduction in BRET_{eff} at a given expression level in the presence of competitor, consistent with monomeric behaviour. LTB₄R₁ demonstrates a significant change in BRET_{eff}-expression relationship in the presence of competitor, suggestive of dimerisation. **(d)** C5R1 and DP₂ exhibit marginal decreases in BRET_{eff} in the presence of competitor, although this is difficult to resolve unambiguously within the inherent experimental error. This could be indicative of very weak dimerisation or C5R1 and DP₂. For monomers, data are plotted as best fit of all points, indicated by a solid line. For dimers, 'with' and 'without' competitor data are fitted separately as solid and broken lines, respectively.

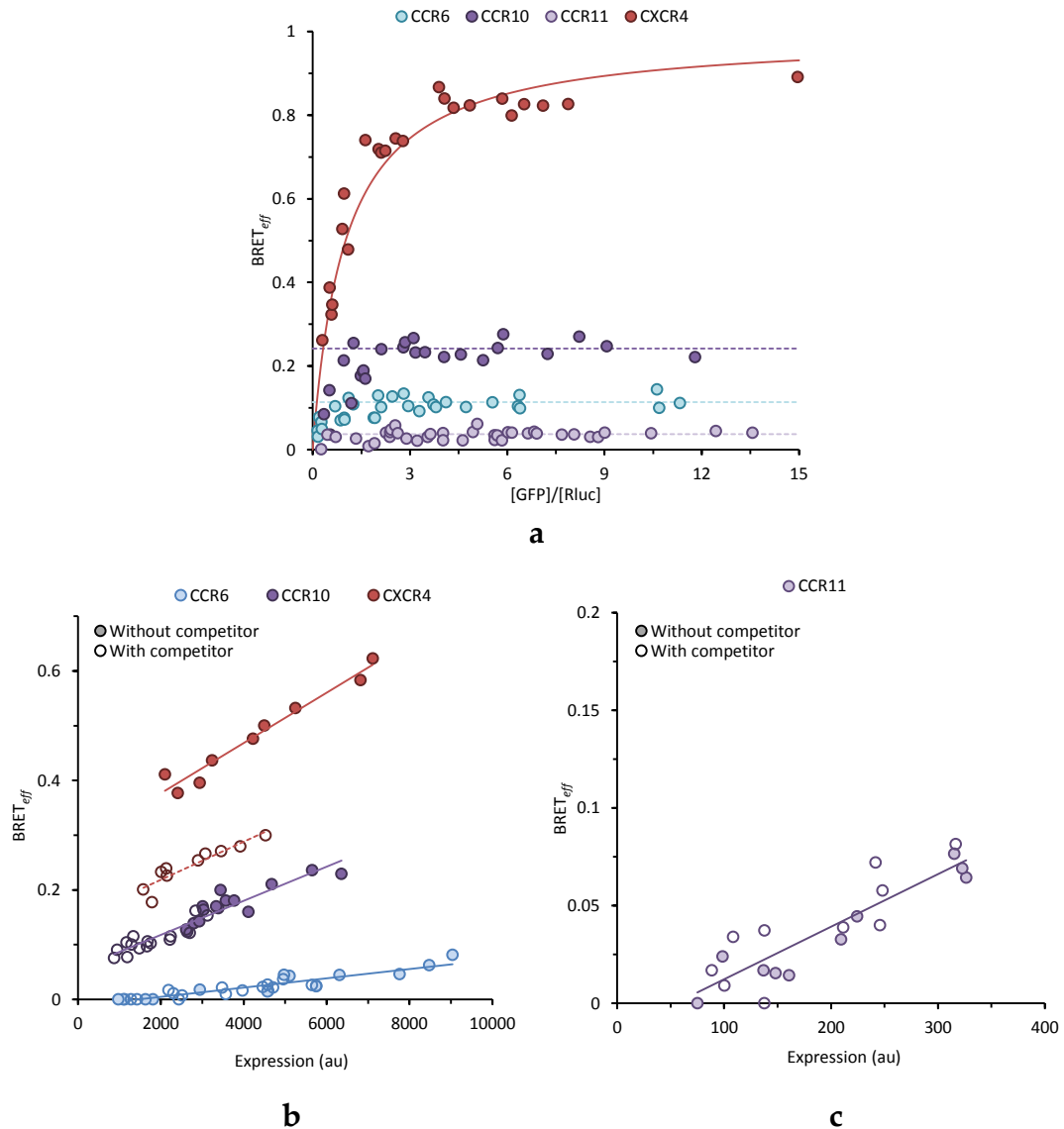


Figure A.12: The classical CC/CXC-motif chemokine subfamily demonstrates distinct stoichiometries. (a) Type 1 BRET analysis of the CC-motif chemokine receptors CCR6, CCR10, and CCR11 demonstrates a distribution of data characteristic of monomers, whereas CXCR4 shows a hyperbolic dependence of $BRET_{eff}$ upon GFP:Rluc ratio that fits well to a dimer model. The high $BRET_{max}$ of CXCR4 is also beyond a level that could realistically be achieved by non-specific interactions alone. Data fits to a dimer model are shown with solid lines; fits to a monomer model are shown with broken lines. The optimal fit is shown in each case. (b and c) cBRET analysis of these receptors agrees with the type 1 BRET conclusions, in that the CC-motif receptors fail to show a change in $BRET_{eff}$ -density relationship, whereas CXCR4 demonstrates a significant drop in $BRET_{eff}$ at any given density. For monomers, cBRET data are plotted as best fit of all points, indicated by a solid line. For CXCR4, 'with' and 'without' competitor data are fitted separately as solid and broken lines, respectively.

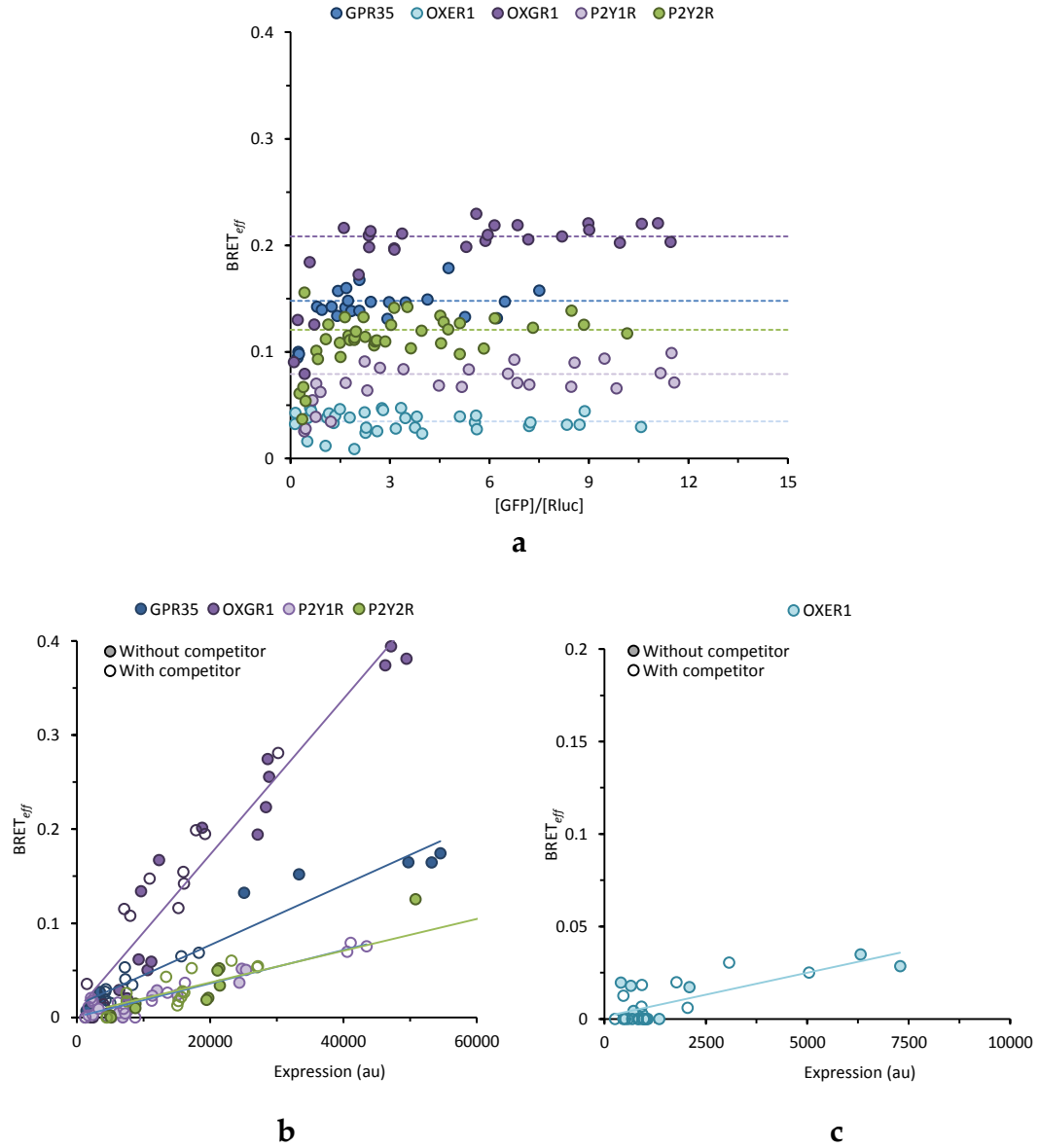


Figure A.13: The purin subfamily is predominantly monomeric as reported by the type 1 and cBRET assays. (a) Type 1 BRET of five purin subfamily receptors reveals an independence of $BRET_{eff}$ upon GFP:Rluc ratio, indicative of monomeric behaviour. Fits of all data to their optimal, monomeric models are shown as broken lines. (b and c) cBRET analysis of these receptors correlates with the type 1 data as none demonstrates a change in $BRET_{eff}$ in the presence of competitor receptors. cBRET data are shown as separate figures for clarity and are plotted as best fit of all points, indicated by a solid line.

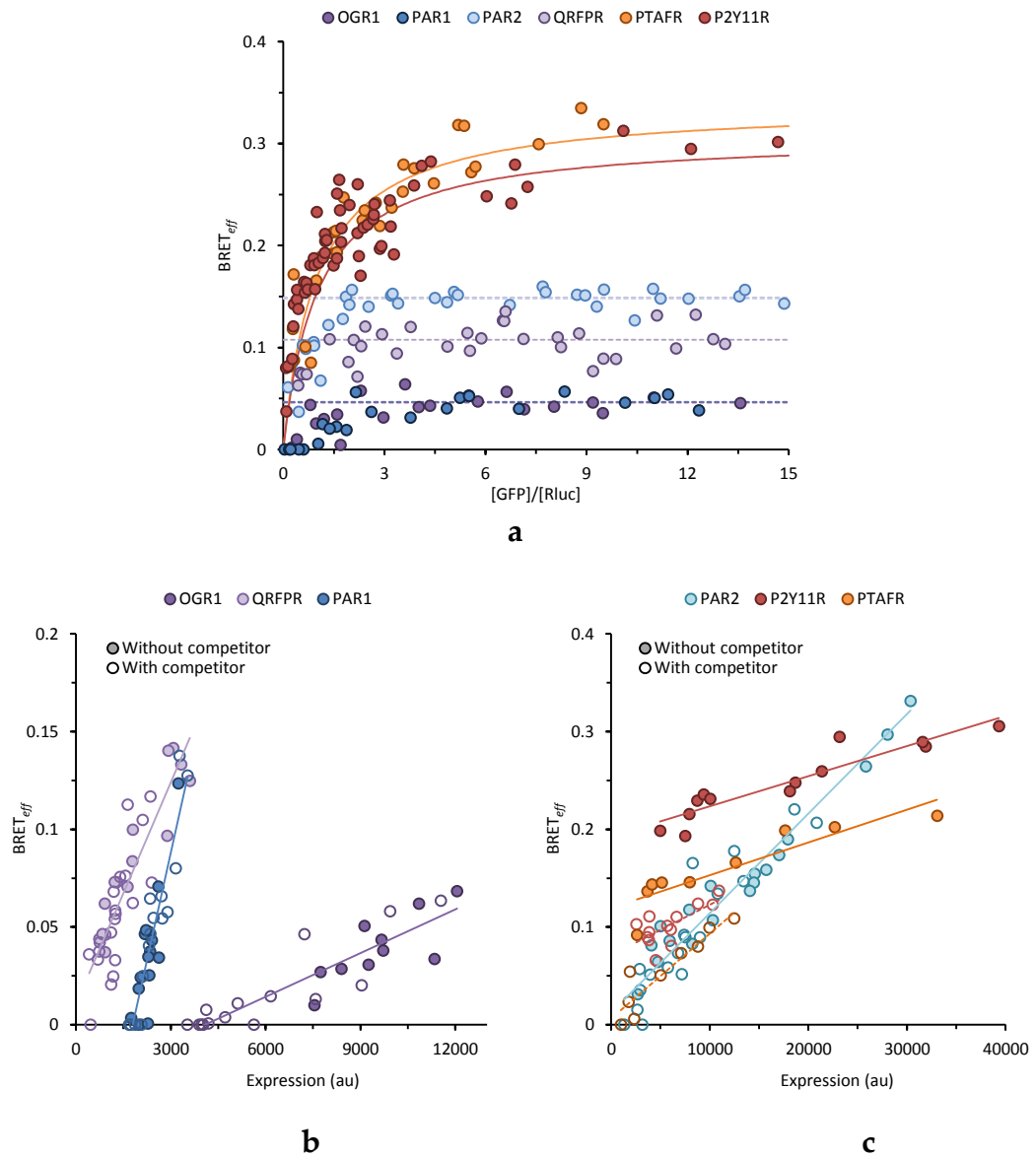


Figure A.14: P2Y₁₁R and PTAFR are apparently dimeric exceptions within the purin subfamily. (a) Type 1 BRET analysis of OGR1, PAR1, PAR2, and QRFPR shows a $BRET_{eff}$ -GFP:Rluc relationship that fits well to a monomeric model, however analysis of P2Y₁₁R and PTAFR reveals a hyperbolic dependence of $BRET_{eff}$ upon GFP:Rluc ratio that is consistent with a dimer model. Data fits to a dimer model are shown with solid lines; fits to a monomer model are shown with broken lines. The optimal fit is shown in each case. (b and c) cBRET analysis of these receptors is consistent with the type 1 BRET data: all receptors show data distributions indicative of monomers with the exceptions of P2Y₁₁R and PTAFR, which show significant competition from unlabelled receptors. For monomers, cBRET data are plotted as best fit of all points, indicated by a solid line. For dimers, 'with' and 'without' competitor data are fitted separately as solid and broken lines, respectively.

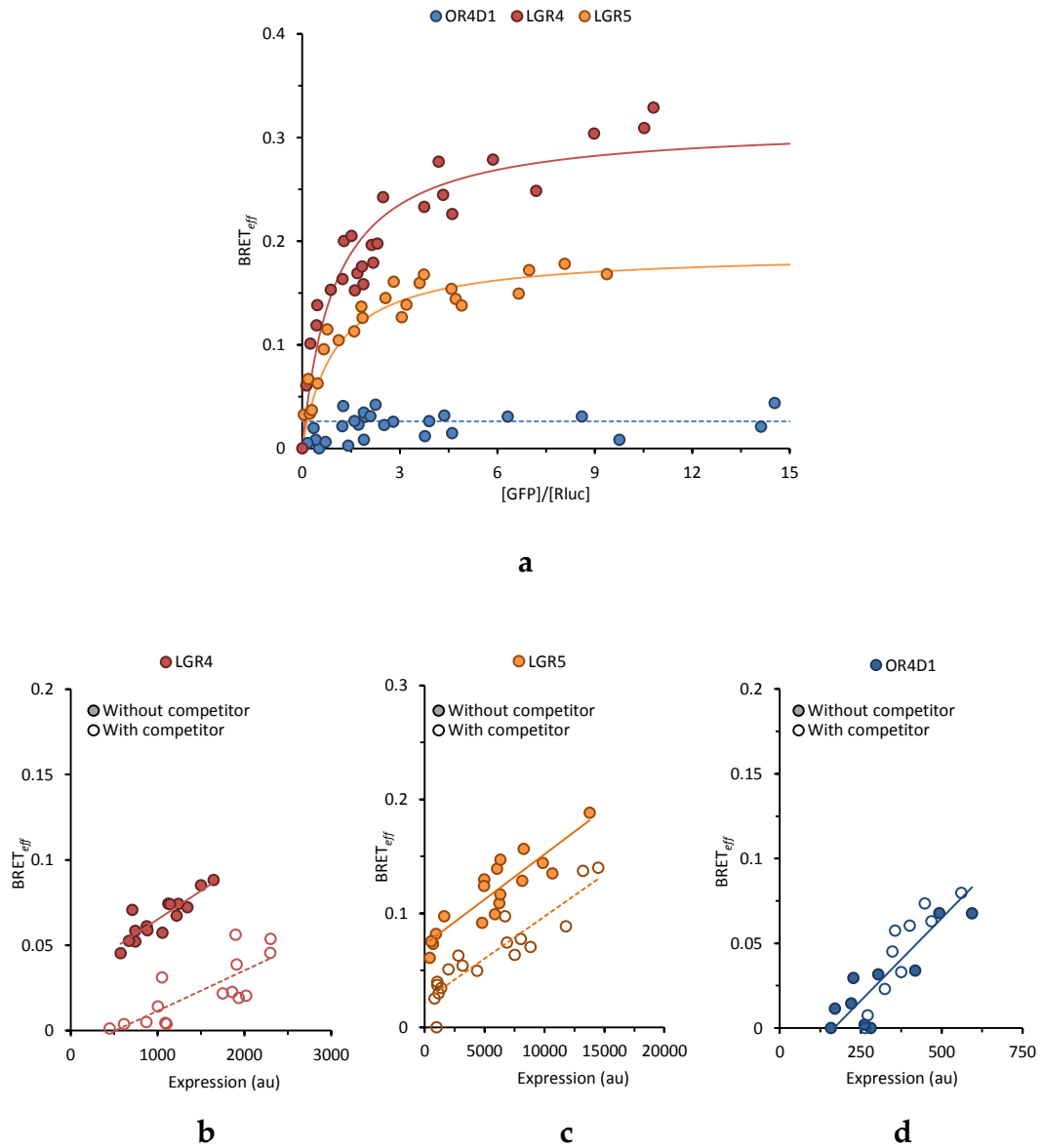


Figure A.15: The glycoprotein and olfactory subfamilies exhibit contrasting stoichiometries. (a) Type 1 BRET analysis of OR4D1 shows a $BRET_{eff}$ -GFP:Rluc relationship that fits well to a monomeric model, however analysis of LGR4 and LGR5 reveals a hyperbolic dependence of $BRET_{eff}$ upon GFP:Rluc ratio that is consistent with a dimer model. Data fits to a dimer model are shown with solid lines; fits to a monomer model are shown with broken lines. The optimal fit is shown in each case. (b, c, & d) cBRET analysis of these receptors is consistent with the type 1 BRET data: OR4D1 shows a data distribution indicative of a monomer, whereas LGR4 and LGR5 exhibit significant competition from unlabelled receptors. For monomers, cBRET data are plotted as best fit of all points, indicated by a solid line. For dimers, 'with' and 'without' competitor data are fitted separately as solid and broken lines, respectively.

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