The Receptors

Katharine Herrick-Davis
Graeme Milligan
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G-Protein-Coupled Receptor Dimers

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The Receptors

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This book is dedicated to all the researchers in the GPCR field for their tireless efforts and to future generations who will continue the quest to solve the mysteries of this important class of cell membrane signaling proteins.

Foreword

On the Question of GPCR Oligomerization

Although evidence for the existence and role of G protein-coupled receptor (GPCR) oligomerization goes back as far as the 1970s [1], even before the genes or proteins forming their receptors were explicitly identified, the quaternary organization of GPCRs and the role of homo- or hetero-oligomerization in their functions remain a subject of intense debates [2–7] and vibrant research. The present book is a clear illustration of the quantity and quality of ongoing research programs aiming at probing the structural organization and physiological roles of GPCR oligomeric assemblies.

The current interest in the notion that GPCRs form dimers and higher oligomeric structures started in the late 1990s and early 2000s when biochemical, biophysical, and functional data using well-defined engineered systems supported the existence of family A receptor dimers [8, 9]. Soon after, the demonstration that the GABAb receptor, a prototypical member of family C GPCRs, existed as an obligatory heterodimer [10] brought additional support to the notion that GPCRs may form oligomers. These reports initiated a flurry of studies using a wide diversity of techniques assessing various aspects of the oligomerization going from the dynamics of assembly to the role of homo- and heterodimers in binding and signaling selectivity [11].

Despite this intense activity, healthy skepticism surrounding the notion of GPCR oligomerization has certainly been a trademark of the field. The resistance to accept family A GPCRs, which in contrast to family C do not require dimerization for function in purified systems [12, 13], can be explained by the fact that many of the data used to propose oligomerization stem from proximity-based assays such as bioluminescence and fluorescence resonance energy transfer [14–18], protein complementation [19–21], and imaging [22–25] that support the existence of oligomers but do not prove direct protein-protein interactions. Even co-immunoprecipitation and crystallographic evidence of dimers can be questioned as possible artifacts. At the functional level, distinguishing between the direct impacts of heterodimerization and downstream cross-talk regulation on binding and signaling properties of proposed heterodimers often remains a challenge.

Despite these legitimate limitations in the interpretation of some aspects of oligomerization studies, the abundance of evidence from multiple sources that are based on many complementary approaches overwhelmingly supports the notion that members of all GPCR subfamilies can form oligomers that have functional roles. Given that GPCRs function as complex allosteric machines, it should not come as a surprise that oligomerization among receptors and their signaling partners represents an intrinsic part of their structural mode of action.

Yet many questions remain partially or completely unanswered, in particular when considering family A and B receptors. Among these, whether ligands can change the oligomeric state of GPCRs or merely promote conformational rearrangements of preexisting oligomers is still the object of active investigations. Early evidence supported the notion that homodimerization occurred constitutively in the endoplasmic reticulum and is required for proper targeting of receptors to the plasma membrane [26–27]. This idea was reinforced by the observation that heterodimerization of GABABR1 and GABABR2 was required for the transport of an active receptor at the surface of the cells [28]. More recent evidence supports the notion that receptor assembly may be actively regulated by ligands once they have reached the cell surface [22, 29]. More studies are clearly needed to clarify the role of oligomerization in receptor trafficking and of the ligand-promoted regulation of assembly dynamics in receptor function. A better understanding of the interfaces involved in the oligomers' architecture will certainly provide tools to directly assess the regulation and the role of oligomerizaton in the various aspects of the receptor life cycle. Despite some advances in identifying interfaces and/or specific residues involved in the dimerization of specific receptors [30–33], no general rules have emerged yet. This will be essential to understand the processes underlying the specificity of interactions among GPCRs. This question is intimately linked with that of the stoichiometry of oligomerization. Although dimerization is often evoked as the simplest arrangement explaining the data, increasing evidence indicates that higher-order oligomers could be formed. In particular, the formation of tetramer has been proposed for members of class C GPCRs [34].

One aspect of GPCR oligomerization that has attracted considerable attention is the possibility that heterodimerization may lead to the formation of receptors with binding and signaling properties that are unique and different from each of the receptor protomers composing the complex [35]. This intriguing possibility takes a particular twist when considering the phenomenon of ligand-biased signaling and functional selectivity by which different ligands can direct the signaling of a given receptor toward different subsets of signaling pathways [36, 37]. The role of heterodimerization in this phenomenon remains completely uncharted territory.

Ultimately, the most important question will be to determine the significance of oligomerization in normal and pathological biology in vivo. Several studies have started to address this question [38, 39], and tools are being developed to determine whether or not the existence of family A or B homo- and heterodimers is required for specific biological function and could represent targets for the development of drugs with unique actions [40, 41]. Clearly, many questions remain open in the field of GPCR oligomerization, and considerable efforts are still needed to fully understand their structural organization and roles in biology. The present book offers a

number of interesting paths that have been followed to give us the level of understanding that we now have and, more importantly, that need to be pursued to provide an even clearer picture.

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Preface

G protein-coupled receptors (GPCRs) represent one of the largest families of cell membrane signaling proteins in the human genome. They are present on the surface of every cell, and they regulate the physiological functions of all the major organ systems in the human body. GPCRs modulate physiological responses to light, odorants, hormones, neurotransmitters, and therapeutic agents. A significant fraction of currently prescribed pharmaceuticals interact with GPCRs. While physiological processes regulated by GPCRs have been studied for decades, it remains uncertain whether the functional signaling unit is a monomer, dimer, or higher-order oligomer.

It is the purpose of this book to provide a detailed review of the current progress that has been made in advancing our understanding of the structure and function of this important class of cell membrane proteins. Dimer/oligomer formation has been reported to regulate all aspects of GPCR function including synthesis, ligand binding, and second messenger activation. This book focuses on the identification and functional significance of GPCR dimers and the role that they play in regulating human physiology in normal and pathological states. The book is made up of 20 in-depth chapters divided in four sections, Introduction, Receptors, Assembly and Trafficking, and Physiology and Therapeutic Potential. The purpose of different sections is to provide the reader with an overview over the vast range of current researches related to the role that dimerization plays in GPCR synthesis, folding, trafficking, and signaling. Pathologies associated with defects in these processes are reviewed along with the development of novel therapeutics in different chapters.

We thank all the authors for their timely and insightful contributions, the series editor Giuseppe Di Giovanni for suggesting this book as a part of his series and for his help in making this happen, and the neuroscience editor Simina Calin, at Springer New York, for keeping things moving along.

We hope that this book will provide direction for new avenues of research and will be a valuable resource to researches in the GPCR field.

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Contents

Part I
Introduction

Chapter 1
Historical Perspectives: From Monomers to Dimers and Beyond, an Exciting Journey in the World of G Protein-Coupled Receptors

Mario Rossi, Roberto Maggio, Irene Fasciani, and Marco Scarselli

Abstract G protein-coupled receptors (GPCR)s are the largest family of proteins in the human genome, and for a long time they were thought to be monomeric in nature. Nowadays, this belief seems rather eccentric, and the concept of lonely GPCRs wandering around the cell membrane has been replaced by a different view in which GPCRs have instead a very active social life, with promiscuous coupling among, but not limited to, their family members. This short chapter summarizes the major steps that have led scientists to change their convictions, from strong supporters of GPCR individualism to enthusiastic appreciators of GPCR camaraderie. A fascinating journey started more than 40 years ago that keeps and will continue to fascinate and excite the scientific community for years to come.

Keywords G Protein-Coupled Receptor • Dimerization • Radioligand Binding • Resonance Energy Transfer • Bivalent Ligand

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Abbreviations

BRET	bioluminescence resonance energy transfer
ER	endoplasmic reticulum
FRET	resonance energy transfer
GPCR	G-protein-coupled-receptor
PALM	photoactivated localization microscopy
PLA	proximity ligation assay
RET	resonance energy transfer
SMT	single-molecule tracking
TM	transmembrane
TR-FRET	time-resolved resonance energy transfer

1.1 Introduction

Sherlock Holmes, the fictional detective, made famous by the pen of the Scottish writer and physician, Sir Arthur Conan Doyle, quoted: "when you have eliminated the impossible, whatever remains, however improbable, must be the truth". This simple sentence holds also in science, especially when avant-garde ideas challenge pre-existing old concepts. In particular, because scientists are more likely to accept simpler ideas than complex-ground-breaking concepts, unconventional ideas have to go through a lot of scepticism, opposition and through intense validation processes before being fully accepted by the scientific community. The History of science offers numerous examples of this convoluted process [1] and one of the kind is certainly the concept of G protein-coupled receptor (GPCR) dimerization.

GPCRs, the largest family of cell surface proteins in eukaryotes, mediate the function of a remarkably large number of extra-cellular stimuli that range from light photons to large proteins. Importantly, about 4% of all human genes code for proteins of the GPCR family, and 30–40% of all the drugs currently in use target these receptors. Understanding the molecular mechanisms by which GPCR mediate signal transduction would therefore be crucial for the development of novel and more effective therapeutic drugs.

One of the earliest models of GPCR signal transduction, the "one ligand-one receptor interaction" model, identified receptor monomers as the "only" functional unit able to activate G-proteins. Indeed, experiments with GPCR single monomers incorporated into reconstituted lipid bilayers showed that single monomers of β_2-adrenergic, rhodopsin and μ-opioid receptors were able to directly interact with G proteins [2–4] validating the concept of monomers as the "only" functional unit responsible for GPCR signalling. However, during the past two decades, an increasing number of experimental evidence has shown that GPCRs form dimers and oligomers and have suggested that the "one ligand-one receptor interaction" model is too simplistic and could not accurately explain the complexity of GPCR signalling [5].

Nevertheless, because GPCRs have been considered to exist exclusively as monomers for a long time, the dimerization concept had to go through a laborious and exhausting scientific analysis before being widely accepted.

This chapter summarizes the most significant steps, from the early pioneer works to the most recent and sophisticated studies that have led scientists to the validation of the well accepted ground-breaking theory of GPCR dimerization as a crucial mechanism in GPCR-mediated physiological processes.

1.2 The Birth of the Dimerization Concept

The first indication that GPCRs could interact with each other goes back to 1975, when Lee E. Limbird, Pierre De Meyts and Robert J Lefkowitz, discovered a negative cooperative interaction among the β-adrenergic receptors [6]. The existence of such negative cooperativity was tested by a kinetic binding method, where the dissociation of receptor bound [^{3}H](−)alprenolol was studied either by the sole dilution of the ligand-receptor complex and by dilution in the presence of an excess of unlabeled (−)alprenolol. In particular, it was observed that the presence of the unlabelled (−)alprenolol increased the rate of [^{3}H](−)alprenolol dissociation, indicating that negatively cooperative interactions occurs among the β-adrenergic receptor binding sites. These data have become a milestone in the study of GPCR dimerization with radioligand binding assays [7]. However, in order to have more direct evidences of GPCR dimerization, we had to wait till the work of Claire M. Fraser [8] (1982), in which, analysis of mammalian lung membranes showed the existence, *in-vivo*, of β_2-receptor dimers with an apparent molecular weight of ~109,000 Daltons. Moreover, in photoaffinity labelling experiments, Sofia Avissar et al. (1983) demonstrated that muscarinic receptors, as well, could dimerize [9]. Strikingly, Avissar found that muscarinic receptor monomers go through a different degree of dimerization depending on the tissue from which these receptors were isolated. Specifically, muscarinic receptors were found as dimers (86,000 Daltons) in the cortex and hippocampus whereas as tetramers (160,000 Daltons) in the medulla, pons, cerebellum, and cardiac atria of rats. Taken together these data suggested that muscarinic receptors form dimers and/or oligomers in a "tissue-specific" manner indicating that "dimerization" was important for muscarinic receptor activity, *in-vivo*.

Even more intriguing evidences of GPCR dimerization came from the studies of [10] Roberto Maggio, Zvi Vogel and Jurgen Wess, (1993) with α_2/M_3 and M_3/α_2 chimeras, in which, the transmembrane (TM) regions 6 and 7 were exchanged between the α_{2C}-adrenergic and M_3-muscarinic receptors. Strikingly, even though these chimeras were no-longer functional, their responses were restored when co-expressed together. This clearly indicated that the chimeras regained their functionality through a dimerization process. In addition, data from epitope-tagged receptor techniques demonstrated that the TM regions of GPCRs were important for the interaction between GPCR monomers. In fact, a peptide corresponding to the TM6

region of the β_2-receptor was able to disrupt β_2-receptor dimerization and reduce β_2-receptor function [11]. Moreover, in 1996 another independent study showed the importance of TM regions for the interaction between dopamine D_2 receptor monomers. In fact, in this study, a peptide corresponding to the TM regions 6 of the D_2 receptor was able to completely prevent dimerization of this receptor [12] strongly suggesting that GPCR-trans membrane domain interactions were a crucial step for GPCR dimer formation.

While at the beginning it was proposed that GPCR dimerization might occur by a mechanism of domain swapping, soon it became clear that the most plausible mechanism of dimerization was by lateral contacts among external surfaces of GPCRs [13]. This became even more evident with the finding that GPCRs not only could form homodimers, but they could also form heterodimers; in fact, it is important to notice that the receptor domains involved in the heterodimerization process are substantially different from each other and therefore the chances of domain swapping very unlikely.

One of the first clear evidence of heterodimerization between two distinct wild type GPCRs came from three works that appeared simultaneously in the same issue of Nature in 1998 [14–16]. Remarkably, these studies showed that $GABA_{B1}$ and $GABA_{B2}$ receptors had to assemble into heterodimeric complexes in order to proper function, given that the two monomers alone were inactive. In addiction, a few years later, other studies have shown that the taste receptors go through heterodimerization processes in order to be functionally active [17, 18]. Strikingly, heteromerization was also demonstrated for other GPCR receptor subtypes, either within the same subfamily, such as between muscarinic M_2 and M_3 receptors [19], or across subfamilies such as between the distantly-related receptors dopamine D_2 and somatostatin SST_5 receptors [20]. Taken together these data began to raise the interest of the scientific community toward the concept of GPCR homo- and hetero-dimerization as important pharmaceutical targets for the development of new therapeutic strategies.

1.3 Establishing the New Concept

A great improvement in the field of GPCR homo- and hetero-oligomerization occurred with the introduction of techniques such as the resonance energy transfer (RET) approach and the single-molecule microscopy detection system. In fact, fluorescent microscopy *per-se* would not be able to resolve densely packed fluorescent or bioluminescent probes due to the physical resolution limit imposed by diffraction; therefore, scientists had to wait for the development of these new methodologies in order to actually visualize GPCR-GPCR interactions. The RET methods, the fluorescent energy transfer (FRET) and the bioluminescence energy transfer (BRET), are based on the principle that energy can be transferred from one probe to another probe by resonance. In particular, energy would be transferred from a donor to a light sensitive acceptor only if donor and acceptor are sufficiently close to each

other, less than 10 nm. Thus, for instance, only when two GPCRs, one carrying the donor and the other carrying the acceptor, are in close proximity, and therefore interacting with each other, the energy can be transferred, detected and GPCR dimerization process studied [21, 22].

One of the first interesting observations researchers made using RET methods, was that GPCR dimerization occurred during the maturation process in the endoplasmic reticulum (ER), which suggested oligomerization as a quality control system for newly synthesized receptors [23]. This hypothesis was clearly demonstrated for some obligatory heterodimers, such as $GABA_B$ receptors, and also proposed for other GPCRs. Therefore, GPCRs were thought to be organized in stable dimeric complexes before actually reaching the plasma membrane. However, this theory has recently been challenged by data obtained with the more sensitive single-molecule microscopy analysis. Nonetheless, improved RET methods such as the time-resolved FRET (TR-FRET) and the proximity ligation assay (PLA) indeed played a crucial role in validating the existence of GPCR complexes in primary cells and tissues [24, 25].

Today, the introduction of super resolution microscopy techniques such as the Photoactivated Localization Microscopy (PALM) has overcome the limit of resolution imposed by diffraction on fluorescence microscopy more effectively than RET approaches. In particular, PALM methods together with the single-molecule tracking (SMT) detection system have provided an extraordinary tool to visualize receptors at the single-molecule level. There is no doubt that these methods withhold the potential for understanding GPCR behaviors and for providing direct evidences of the existence of receptor dimers and oligomers in living cells. Importantly, PALM has permitted the visualization of single receptors highly expressed in fixed samples, while SMT has determined how GPCRs move and interact in living cells in the presence of different ligands [26, 27].

Strikingly, SMT has revealed the transient dynamic nature of dimer formation, where the GPCRs examined so far display a monomer-dimer equilibrium characterized by rapid association and dissociation [28]. At steady state, approximately 30–60% of receptors, depending from the subtype, are part of dimeric complexes. This is undeniably a breakthrough in the GPCR dimerization story; it proves that receptor monomers form transient dimers and at the same time it raises many questions about the molecular mechanisms involved in the dimerization processes and in the way dimer complexes function. In conclusion, the ability of GPCRs to form dimers *in-vivo*, has been supported by well-known direct visualization techniques, such as RET (as mentioned above), and more sensitive techniques like PALM and SMT.

1.4 Oligomers Make the Picture More Complicated

While evidences of GPCRs homo- and hetero-oligomerization were accumulating, it became clear that GPCRs could also assemble into higher-order oligomers. The first “direct” evidence of in-vivo GPCR oligomerization came from the seminal

work of the Palczewski' group [29], in which native membranes obtained from wild-type mouse retinal photoreceptors were analyzed by atomic force microscopy. In particular, this study gave direct experimental evidences that the prototypical member of class A GPCR rhodopsin receptor, formed paracrystalline arrays of dimers in rod outer-segment disc membranes of mouse retinal photoreceptors. Even though oligomerization could have been caused by the high density of rhodopsin receptors in the retina, the arrangement of dimers in rows strongly indicated that oligomer formations were the natural consequence of precise contacts between congruent interfaces of individual rhodopsin monomers. Afterward, Fotiadis showed that rhodopsin receptor oligomers, isolated with gentle detergents from native disk membranes, were able to activate G proteins more efficiently than rhodopsin-dimers or rhodopsin-single monomers [30, 31]. These data strongly suggested that tightly packed rows of rhodopsin-dimers were critical for proper light-mediated G-protein activation, in-vivo. The β_2 adrenergic receptor is another typical example of G-protein coupled receptor whose functions are finely regulated by oligomerization [32]. In particular, mutagenesis and co-expression experiments have shown that β_2 receptor oligomerization originates within the ER and is essential for the receptor maturation and trafficking to the plasma membrane [33]. The existence of β_2 receptor oligomers was also confirmed in cardiomyocytes by using super-resolution technique PALM [34]. An exciting hypothesis is that homo-oligomerization is a condition for GPCR compartmentalization, and therefore for confined increases of second messengers [35]. This hypothesis originates from a study with neonatal rat cardiomyocytes, in which β_1 adrenergic receptors were suggested to be co-localized with phospho-diesterase enzymes in specific areas of the cardiac myocyte plasma membranes. Specifically, Zaccolo and Pozzan [36] (2002) showed the formation of multiple micro-domains of stimulated β_1 receptors that produced cAMP accumulation in specific spots in cardiomyocytes, while co-localized phosphodiesterase prevented cAMP from diffusing. These cAMP "hot" spots would then activate a subset of protein kinase A molecules anchored in proximity to the T tubule and thus coordinate cardiomyocyte contractions [36].

However, in order to understand the role of oligomerization in GPCR functions, it is important to determine similarity and differences between oligomers and dimers in terms of GPCR-GPCR or GPCR- associated protein interactions that ultimately would affect GPCR signaling. For example, If we look at class C GPCRs, such as $GABA_B$ receptors, it would appear clear that $GABA_B$ heteromers are stable due to strong noncovalent interactions (same for GluR homomers), while GABA oligomeric complexes rely on weaker and transient interactions between heterodimers [27, 37]. Similar conclusions were also made for the class A GPCR M_3 muscarinic receptors, which might exist as either stable dimeric units or as tetramers [38]. Strikingly, another study demonstrated that the dopamine D_2 receptor formed tetramers and high-order oligomers using different TM domains [39]. These data further suggested that D_2 tetramer mediated signaling might dramatically differ from the signaling trigger by D_2 oligomer complexes [40]. Another interesting concept that would further emphasize the differences between dimer- and oligomer-mediated intracellular signaling is the spatial localization in specific "hot" spots of a cell type

that occurs for some higher-order oligomers (e.g. β_1 receptors co-localized with phospho-diesterase enzymes in cardiomyocytes). In fact, intracellular compartmentalization has been demonstrated to play a major role in generating physiologically-important cell-type specific signals" [40].

Other evidences of the importance of higher-order oligomers for GPCR function, in particular for hetero-oligomer complexes, came from the work of Bonaventura et al. (2015), with Adenosine A_{2A} and dopamine D_2 receptors [41]. They demonstrated that A_{2A} and D_2 receptors formed heterotetramers with unique pharmacological properties by showing a powerful allosteric type of interaction between A_{2A} and D_2 receptors, in which A_{2A} ligands decreased the affinity and intrinsic efficacy of agonists for D_2 receptors.

Moreover, another intriguing concept that must be mention about GPCR oligomer mediated signalling is the idea of "coincident-detection", in which, different protomers within the same hetero-oligomer are activated simultaneously to generate a "synergic" increase in second messenger productions, and thus their downstream effects [35].

Even though, during these years, studies on GPCR dimerization and olimerization have improved our knowledge on this fascinating field, many questions remain to be properly answered such as: What kind of interactions are responsible for the formation of dimers and eventually oligomers? What are the sizes of such oligomers? What functions do they serve? Which are the factors controlling their formation? Can we find new selective drugs active specifically on these receptor complexes? Notably, techniques like the single-molecule detection system described previously not only would play a crucial role in addressing these questions by deepening our understanding of the mechanisms involved in GPCR dimerization and oligomerization, but importantly, they would also help researchers to evaluate homo and hetero oligomer druggability.

1.5 Receptor Homo- and Heteromers as New Pharmacological Targets

One of the most intriguing consequences of receptor homo- and hetero-oligomerization is the possibility for the development of new pharmacological targets and new paradigms of allosteric regulations. In fact, receptor dimerization has open new ways to target GPCRs including the creation of bivalent ligands, molecules that consist of two pharmacophores linked by a spacer. A pioneer of this approach was Philip S. Portoghese who, even before the concept of receptor dimerization was born, constructed a bivalent ligand containing two beta-naltrexamine pharmacophores connected by oligoethylene glycol spanner that enhanced the ligand potency. Strikingly, these data laid the foundations for the concept of simultaneous occupation of proximal recognition sites for this type of ligands [42]. Such approach was then utilized in 2002 by Saveanu et al. [43] to target somastostatin SST_2 and

dopamine D_2 receptor heteromers. They demonstrated an enhanced potency of a chimeric somatostatin-dopamine molecule, BIM-23A387, in suppressing growth hormone and prolactin secretion from human pituitary somatotroph adenoma cells. Later on, the group of Portoghese developed an high affinity bivalent ligand for opioid DOR-MOR receptor heteromers [44] and for MOP-CB_1 receptor heteromers [45], both with potent analgesic effect but devoided of tolerance.

Interestingly, an important process that also characterized GPCR oligomers is the allosterism across homo- and heteromers that have been shown to play a crucial role in modulating receptor functions. Specifically, this mechanism has been shown to modify protomer conformations and eventually affinity for its ligands through conformational changes occurring in the other promoter of a dimer complex that bound a particular allosteric ligand [5]. One of the best characterized study of allosterism across dimers was by the group of Arthur Christopoulos [46]. They showed that the pure dopamine D_2 receptor allosteric modulator SB269652 [*trans*-1*H*-indole-2-carboxylic acid {4-[2-(cyano-3,4-dihydro-1*H*-isoquinolin-2-yl)-ethyl]-cyclohexyl}-amide] [47] mediated allosteric regulations on D_2 dopaminergic dimers by binding one of the promoter in the dimer complex and thus changing the binding properties of dopamine in the other associated protomer [46]. In particular, SB269652 is a bitopic compound able to engage both the orthosteric and allosteric sites of D_2 receptor promoters. This binding mode of SB269652, in fact, is typical of bifunctional compounds, that are called bitopic or dualsteric ligands [48]. Taken together, these data strongly suggest that the development of drugs targeting receptor homo- and heteromers would have tremendous implications for the development of new and more effective therapies.

1.6 Perspectives

The concept of GPCRs homo and hetero-oligomerization has now gone beyond the restricted circle of GPCR experts and it is accepted by the scientific community. Interestingly, studies on a relative large group of GPCRs have shown that the molecular mechanisms involved in the homo- and heterodimerization processes are not the same for all the GPCRs. In particular, these studies suggested that differences in the way GPCRs assemble together to form multimer complexes might withhold one of the key features responsible for tissue-specific, cell-specific, GPCR mediated signalling.

Indeed, there are many aspects of homo- and hetero-oligomerization that must be investigated thoroughly, and above all, their physiological and pathological implications.

One of the early studies that showed GPCR hetero-oligomerization as an important event for the pathogenesis of diseases came from the pioneering work by AbdAlla et al. (2001), in which preeclamptic hypertensive women showed a significantly clear positive correlation between the increase of angiotensin II AT_1/bradykinin B_2 receptor

heterodimerization and the angiotensin II hormone hypersensitivity associated with preeclampsia itself [49].

Therefore, taken together these data strongly suggest that in the near future GPCR dimers and oligomers will become extremely important pharmaceutical targets, given also the fact that GPCRs are virtually involved in every physiological process.

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Chapter 2
The Use of Spatial Intensity Distribution Analysis to Examine G Protein-Coupled Receptor Oligomerization

Richard J. Ward, Sara Marsango, John D. Pediani, and Graeme Milligan

Abstract Spatial Intensity Distribution Analysis (SpIDA) is a new approach for detecting protein oligomerization states that can be applied not only to live cells but also fixed cells and native tissue. This approach is based on the generation of pixel-integrated fluorescence intensity histograms from laser scanning fluorescence microscopy images. These histograms are then fit with super-Poissonian distribution functions to obtain density maps and quantal brightness values of the fluorophore that are used to determine the proportions of monomer and dimers/oligomers of the fluorophore-tagged protein. In this chapter we describe SpIDA and highlight its advantages compared to other biochemical or biophysical approaches. We provide guidelines that should be useful to readers who wish to perform SpIDA measurements and describe the application of SpIDA as a post-acquisition imaging histogram analysis software tool to investigate the oligomeric state of G protein-coupled receptors (GPCRs) at the surface of mammalian cells in order to define the steady-state proportion of monomeric and dimeric/oligomeric forms and how this may be regulated by cellular challenges such as ligand treatment.

Keywords SpIDA • GPCR • Quaternary structure • Dimerization • Image analysis • Ligand regulation

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Abbreviations

BG	O^6-BenzylGuanine
BRET	Bioluminescence Resonance Energy Transfer
CD86	Cluster of Differentiation Protein 86
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
FCS	Fluorescence Correlation Spectroscopy
FIDA	Fluorescence Intensity Distribution Analysis
FRET	Fluorescence Resonance Energy Transfer
GFP	Green Fluorescent Protein
GPCR	G-Protein Coupled Receptor
GUI	Graphical User interface
hD2	human Dopamine D_2 receptor
hD3	human Dopamine D_3 receptor
hM_1	human Muscarinic acetylcholine M_1 receptor
hM_3	human Muscarinic acetylcholine M_3 receptor
IU	Intensity Unit
mEGFP	monomeric Enhanced Green Fluorescent Protein
MEU	Monomeric Equivalent Unit
OX_1	human Orexin 1 receptor
PCH	Photon Counting Histogram
PM	Parmitoylation + Myristolation
PMT	Photo-Multiplier Tube
PSF	Point Spread Function
QB	Quantal Brightness
RET	Resonance Energy Transfer
RoI	Region of Interest
SpIDA	Spatial Intensity Distribution Analysis
TIRF	Total Internal Reflection Fluorescence
WN	White Noise
$5\text{-}HT_{2C}$	5-Hydroxytryptamine 2C

2.1 Introduction

G protein-coupled receptors (GPCRs) are the largest family of membrane proteins involved in cell signalling. Traditionally, they have been described as monomeric proteins able to form a ternary complex with ligands and downstream signalling proteins that bind the receptor at the extracellular and cytoplasmic sides of the plasma membrane respectively. Indeed, it has been shown directly that some receptors are able to activate G proteins in a ligand-dependent manner as monomers [1, 2]. Despite this, during the last two decades the view of GPCRs existing

and functioning only as monomeric entities has been challenged by a growing number of studies that support the hypothesis that GPCRs may also exist as dimeric and/or oligomeric complexes. In this context, it is abundantly clear from both biochemical and genetic studies that the formation of either homo- or hetero-complexes defines the pharmacology and function of members of the class C, metabotropic glutamate GPCR family [3] whilst, despite numerous molecular, biochemical and biophysical studies, for class A, rhodopsin-like GPCRs the situation is rather less certain in terms of both the basic concept of GPCR oligomerization and its functional significance. Indeed, as will be clear from other chapters in this volume, both of these remain controversial topics [3]. Traditional biochemical techniques used to address these issues, such as immunoblotting, cross-linking and co-immunoprecipitation, require prior solubilization of cells and tissue samples using non-physiological buffers and detergents that may cause aggregation or disruption of native biological interactions. Moreover, they do not provide any information about cellular and subcellular localization of potential interactions and little information about the interactions themselves that is not open to criticism and alternate interpretation [4].

Such limitations have been overcome in part with the introduction of biophysical methods based on Resonance Energy Transfer (RET) such as Bioluminesence Resonance Energy Transfer (BRET) and Fluorescence Resonance Energy Transfer (FRET) into studies on the quaternary organization of integral membrane proteins in intact cells. Although RET techniques are powerful approaches they generally require genetic manipulation of the proteins of interest and report close proximity (˂8 nm) between the reporters linked to the proteins rather than association *per se.* Furthermore, without integration of further specific techniques (e.g. [5].) they do not routinely give information about protein density or relative amounts of each oligomeric state in a mixture. Techniques based on temporal fluorescence fluctuations such as fluorescence correlation spectroscopy (FCS), fluorescence intensity distribution analysis (FIDA) or photon-counting histograms (PCH) [6] overcome some of these limitations as they allow quantitative measurement of protein concentration and oligomeric state. However, they do require that the density and the oligomeric state of the protein of interest remain constant during the acquisition time period.

In 2011, the Wiseman laboratory at McGill University, Montréal introduced a method described as Spatial Intensity Distribution Analysis (SpIDA) [7] which analysed pixel-integrated fluorescence intensity histograms generated from pre-defined regions of interest (RoIs) drawn on confocal laser scanning microscopy images of cells expressing a fluorophore-tagged receptor protein of interest. Histograms of the total number of pixels for each integrated fluorescence intensity value, within the selected RoI excited by the laser, are constructed and analysed by super-Poissonian distribution functions to obtain density maps and quantal brightness (QB) values of the fluorophore-tagged receptor protein. The profile shape of the histogram distribution provides specific information about the number and oligomerization state(s) of the tagged protein, thus enabling the determination of concentrations of monomer or higher-order oligomeric species within the specified RoIs.

SpIDA development was inspired by PCH [6] with the additional advantage that the analysis is applied to the spatial domain fluctuation, rather than the temporal one, allowing measurements of non steady-state receptor density and oligomeric state that occur under physiological conditions in sub-regions within single images of cells. Moreover, because SpIDA only requires a single input image to quantify the QB, the analysis is not as susceptible to the effects of cryptic/non-cryptic photobleaching or cell movement as it is for FCS and other temporal fluorescence fluctuations techniques; thus for SpIDA underestimation of brightness due to photobleaching is negligible and it is possible to detect a broader size range of protein oligomer species. SpIDA also provides a spatial map of local protein aggregation in the cell and more rapid image data acquisition and analysis over multiple fields is possible when compared to single point FCS experiments. Finally, SpIDA does not require an expensive, specialist microscopy setup and can be performed using any available commercial confocal microscope system [7–10].

The literature concerning SpIDA is, as yet, not very extensive; mostly derived from work of Godin and co-workers [7–10] who initially developed the method, whilst our own work with SpIDA [11–13] has provided the only examples to date of investigations of the organizational state of class A GPCRs. It should be noted, however, that Hamrang and co-workers have used SpIDA, as a proof of concept, to monitor the intracellular accumulation of Celltrace™ calcein red-orange AM, a cell permeant dye used to determine cell viability [14] and also to assess the expression of the P-glycoprotein [15]. They concluded that SpIDA is a rapid and user-friendly tool that, combined with live cell imaging and immunofluorescence staining, can be applied to the determination of pharmacological parameters or analysis of biopsies, providing a rapid prognostic technique. As such it is timely to consider the potential of SpIDA more broadly.

In the following sections we will consider the steps necessary to set up and perform SpIDA measurements, incorporating aspects that reflect our increasing experience of this approach. Using such underpinning information we will then describe the application of SpIDA to investigate the oligomeric state of class A GPCRs, in particular the 5-hydroxytryptamine 2C (5-HT_{2C}) and muscarinic M_1 and M_3 receptors, at the surface of mammalian cells and how this may be altered by treatment of cells expressing these receptors with appropriate antagonists and inverse agonist ligands.

2.1.1 SpIDA Procedure

A cartoon representation of the SpIDA strategy as applied in our group is shown in Fig. 2.1.

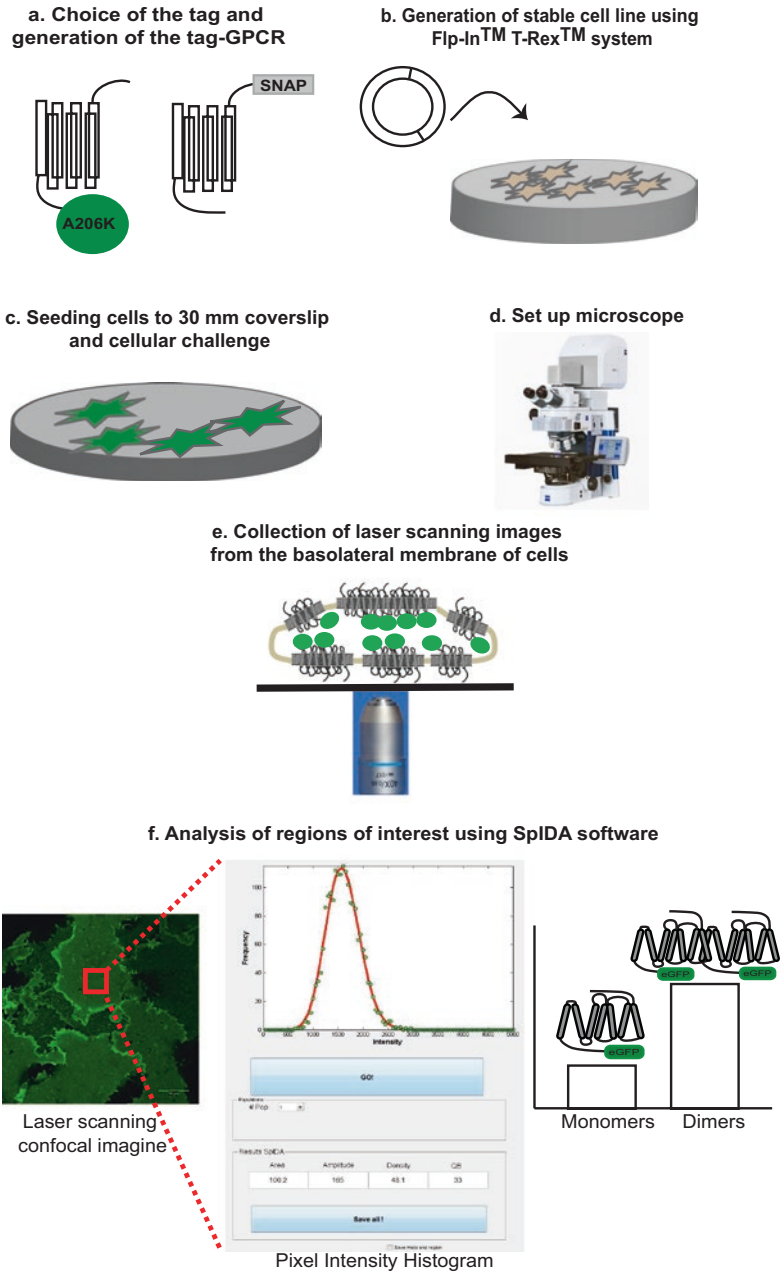


Fig. 2.1 Schematic representation of the procedure to perform SpIDA measurements as described in the text. The procedure involves (**a**) the generation of the plasmid construct containing the sequence coding for GPCR-mEGFP or SNAP-GPCR of interest, (**b**) the expression of the construct in Flp-In™ T-REx™ 293 cells, (**c**) the seeding of an appropriate number of cells to ensure an even monolayer on the coverslip, (**d**) setting up of the confocal microscope, (**e**) collection of laser scanner images from the basolateral membrane of cells, (**f**) selection and analysis of RoIs using MATLAB graphical user interface (GUI) programme

2.1.2 Choice of Fluorophore

SpIDA analysis requires that the protein of interest is modified to covalently incorporate or tightly bind a suitable fluorophore, or that a suitable high affinity antibody for the target protein is available, which itself can be labelled with a fluorophore. It is important that the fluorophore chosen exhibits a high quantum yield and, preferably, is extremely photostable. Moreover, if the selected fluorophore is an auto-fluorescent protein based upon the *Aequorea victoria* green fluorescent protein (GFP) [16] the point mutation, Ala206Lys, should be introduced in order to limit spontaneous GFP-GFP interactions [17]. This ensures that the auto-fluorescent protein is itself monomeric and exerts no aggregating effect upon the protein of interest to which it is fused. Clearly similar precautions are required if an alternative fluorescent tag is to be used. SpIDA has already been applied successfully to quantify mCherry and mGFP-tagged proteins and proteins visualized by immunofluorescence staining with antibodies conjugated to Alexa-Fluor 488, 633 and 647 [8].

Initially, we chose to modify transmembrane proteins of interest by addition of the monomeric version of the enhanced green fluorescent protein (mEGFP) at the intracellular carboxyl-terminal tail. As well as various GPCRs we have employed modified versions of the monomeric single transmembrane receptor Cluster of Differentiation 86 (CD86-mEGFP), the single transmembrane tyrosine kinase epidermal growth factor receptor (EGFR-mEGFP) [8–10] and the axonal guidance Roundabout receptor 1 (Robo1-mEGFP) [11] as proof of concept controls. GPCRs studied to date include the 5-HT_{2C} receptor (5-HT_{2C}-mEGFP) [12], the M_1 and M_3 subtypes of human muscarinic acetylcholine receptors (hM_1-mEGFP and hM_3-mEGFP) [13] and the human D_2 and D_3 dopamine receptors (hD_2-mEGFP and hD_3-mEGFP) (unpublished data).

As an alternative we have also begun to employ SpIDA in concert with 'SNAP'-tag technology [18, 19] and for this purpose we have modified proteins of interest by incorporating the SNAP-tag into the extracellular N-terminal domain of many of same receptors as above. The SNAP-tag is a modified version of the DNA repair protein O_6-alkylguanine-DNA alkyltransferase that displays faster reaction kinetics with O_6-benzylguanine (BG) substrates and no longer interacts with DNA. BG substrates can be conjugated with various fluorophores including Alexa-Fluor 488 which are then incorporated covalently into the SNAP tag, and we have used this to visualize constructs including SNAP-CD86, SNAP-EGFR and the human orexin 1 receptor (SNAP-OX_1), (unpublished data).

2.1.3 Selection of the Expression System

SpIDA can be applied to single images to measure protein density and interactions, not only in live cells, but also in fixed cells and tissue samples using fluorescent antibody labelling [7, 9]. This represents an important advantage of SpIDA when

compared to PCH, FCS or FIDA techniques. For these time-dependent approaches molecules of interest must diffuse or flow to be detected and, hence, these methods cannot be applied to fixed cells or tissue samples. To study aspects of GPCR signalling it is routine to express the receptors in a heterologous mammalian cell system and lines such as HEK293, COS-7 and CHO cells have been successfully employed to perform SpIDA measurements. We have routinely adopted the Flp-In™ T-REx™ system (Invitrogen Life Technologies). This has the ability to generate cell lines that stably express proteins of interest in an isogenic and inducible manner and where the expression level of the protein of interest can be regulated simply by varying the concentration of the inducing agent tetracycline/doxycycline [20]. This is of particular interest in assessing whether receptor density/expression levels can alter oligomeric organization simply on the basis of Mass-Action or whether the extent of dimerization of different receptors may vary at equal expression levels. For effective data collection cells are plated down onto poly-D-lysine coated 30 mm glass coverslips at an appropriate density, such that at the point of image collection the cells form a monolayer.

2.1.4 Establishing Imaging Conditions for SpIDA

There are several key steps to consider before beginning image collection for subsequent post-acquisition SpIDA analysis. These include definition of parameters which must later be input into the SpIDA software as well as careful and routine monitoring of laser power intensity, measurements of which should be made regularly to ensure consistency of results.

2.1.4.1 Laser Power Intensity Measurement

Due to the inevitable loss of power as lasers age, it is advisable to monitor the level of output on a bi-monthly basis so that any variations in output can be corrected for by, for instance, increasing the laser power used. This measurement requires the use of a laser power meter with an appropriate sensor head, which should be used according to the manufacturer's instructions.

2.1.4.2 Laser Spot Beam Waist Radius Size

The waist radius size of the laser spot beam can be quantified by imaging a z-stack of sub-diffraction-sized 100 nm Tetraspeck fluorescent microspheres (pre-mounted on a microscope slide, Invitrogen, Paisley, UK, Cat No T14792) [21, 22]. This procedure involves imaging the microspheres using the same laser and epifluorescence filter sets as to be used for recording the SpIDA images. Importantly, images should be acquired under conditions which avoid detector saturation. A RoI containing a single microsphere

is selected and a z-stack of images (z resolution = 100 nm, x, y resolution = 50 nm) acquired, at a pixel sampling frequency approximately three times smaller than the resolution of the objective lens in use (Nyquist sampling frequency). Recorded z-stack images are imported into the ImageJ software [23] and the MetroLoJ plugin [24] is used to generate a 3-dimensional microsphere image to quantify x and y (lateral) and z (axial) point spread function (PSF) values. Subtract 50 nm from each value (in order to measure the convolution of the beam) and then square either the x or y value and multiply by 3.14 to obtain the laser spot beam waist radius size.

2.1.4.3 Analog Detector Calibration

It is well established that the response to light of a photo-multiplier tube (PMT) is not constant and this variation is known as "shot" noise. Therefore to perform SpIDA accurately it is necessary to exclude noise fluctuations inherent to the PMT to ensure that only the fluorescent intensity fluctuations that are specifically emitted from the excited fluorescently labelled protein sample are measured.

To measure the shot noise a mirror slide is placed in the plane of focus of the microscope and a laser spot 1 pixel scan (scan speed = 12.8 μs per pixel) is conducted for ~10.5 seconds. This allows the laser line to uniformly illuminate the PMT sensor window so that spot mode fluctuation signals can be recorded. This should be repeated using a range of laser powers, until a point is reached at which the signal saturates the PMT detector (other microscope settings should be as described in Sect. 2.1.5). For each recorded fluctuation signal trace (generated from each test laser power intensity setting), the pixel intensity variance (standard deviation, squared) is calculated and plotted versus the mean pixel intensity to determine the measured intensity range over which the response of the detector is linear. An example of such a plot can be seen in Figure 6A of Zakrys et al. [11]. Linear regression is used to establish the best fit line through the data points and slope value of the linear part of the plot. The measured slope value (37.49 ± 0.08 intensity units (IU) in the example given above) can then be input into the SpIDA software. In addition, it should be noted that this value also defines the limit for the maximum intensity that can be analysed using the SpIDA software.

2.1.4.4 Assessment of White Noise Level

The white noise background signal can be calculated by measuring the mean fluorescent pixel intensity from a RoI drawn on an image where no fluorescent cells are present (e.g. area between cells). Alternatively, this measurement can be determined by repeating the region measurement process on an image collected in the absence of excitation laser light (i.e. zero laser power intensity, laser switched off). The mean fluorescent pixel intensity from such a region can be determined using the ImageJ program [23, 24], to give a value for white noise which can then be input to the SpIDA software.

2.1.5 Laser Scanning Confocal Image Acquisition

Images suitable for SpIDA analysis can be acquired using any conventional confocal laser scanning or Total Internal Reflection Fluorescence (TIRF) microscope. It must be emphasised that once settings are established they should be maintained through the collection of sets of data between which comparisons are to be made. Locally, confocal images have been acquired using a Zeiss LSM 5 PASCAL EXCITER laser scanning head coupled to a Zeiss Axiovert 200 M inverted microscope equipped with a 63x Plan Apochromat oil immersion lens with a numerical aperture of 1.4. Monomeric EGFP (or in the case of SNAP-tagged constructs SNAP-surface 488) is excited using the 488 nm line of the 25 mW multi Argon laser. Recommended image collection parameters with frame averaging switched off are as follows:

- set pinhole to 1 Airy unit and use unidirectional raster scan frame mode to collect each single optical section image (frame size 1024 × 1024; bit depth = 12).
- set the scan speed to a value which will be fast enough to "walk" along with the fluorescent receptor protein (e.g. pixel dwell time of 12.8 μs.pixel^{-1}). Ideally, the pixel dwell time chosen should be long enough to sample the fluctuation intensity signals, but also short enough so that they are not averaged/decayed out during the detection process.
- set the optical zoom to 1.5 or 2.2 which represents an x, y sampling size of 0.09 μm or 0.06 μm. Ideally oversample by a factor of 3, sampling size range of 0.03–0.09 μm, as this range is beneficial for optimizing detection of the fluctuation of the molecules within the confocal volume.
- optimize laser excitation intensity to minimise photobleaching and detector pixel saturation: values should be the same as those used to quantify the inherent 'shot' noise of the PMT detector. It is also vital to verify that the PMT offset and amplifier gain have been set to values of 0 and 1, respectively.
- select the secondary beam splitter and emission filter which will maximize detection of the emission signal.
- for each experimental image data group, ensure that all the imaging parameters (laser power intensity, pinhole diameter, PMT detection settings, filters, etc) remain constant to ensure accurate uniform quantification of monomeric/experimental QB values and subsequently derived receptor protein oligomerization state values.
- It is essential that on each day when experimental images are collected, a monomeric quantal brightness calibration control sample (see later) is imaged in parallel to be sure that (for instance) the QB value is not subject to drift.

It is important to consider from whereabouts in the cell the images should be collected and also that membrane movement will not be an issue during image acquisition. In initial studies we selected images of sections through the plasma membrane. These were found to be unsatisfactory as they display as thin bands of fluorescence that are consequently difficult to analyse with the SpIDA software. Instead we now

focus on the basolateral surface of coverslip attached cells. In this way images can be collected with large homogeneous areas of fluorescence that are ideal for analysis by SpIDA [11–13].

2.1.6 Spatial Intensity Distribution Analysis of Laser Scanning Confocal Images

The laser scanning confocal images collected are analysed using MATLAB graphical user interface (GUI) programme to perform SpIDA analysis [7]. To open an image within the GUI program it is first necessary to enter values for the pixel size (dependent on the level of zoom used to collect the images, typical values would be 0.06–0.09 μm) and for the beam waist radius (see Sect. 2.1.4.2). Once the selected image has been opened, other values must be input, such as white noise (WN, see Sect. 2.1.4.4), slope variance (first click on "PMT noise" to open the dialog box, see Sect. 2.1.4.3), level of binning (the pixel intensity range to be incorporated into each point) and the expected range of values of fluorescent intensity (to prevent saturation of the image display and also to place the plot of intensity versus frequency in the middle of the graph, where all of the curve can be seen and its fit to the points assessed).

Once all the parameters have been set up, it is necessary to select RoIs within the laser scanning confocal image, which must be homogeneous and not contain regions which vary significantly in fluorescent intensity, such as very bright spots or "holes" with no fluorescence and then to analyse them by clicking "Go". GUI allows the results to be saved in the form of an image in which the selected RoI, the histogram and fitted curve are shown in addition to an Excel compatible file containing the various numerical outputs from the program [8]. Of these, the most useful for the analysis of GPCR oligomeric state are the QB and the mean fluorescent intensity values which give information about the oligomeric state and the density of the fluorophore tagged-protein when they are compared with the monomeric QB of the fluorescent label.

2.1.6.1 Determining the Monomeric Quantal Brightness Value

Determination of QB of the fluorophore label in its monomeric state represents a crucial step for accuracy of the analysis. The QB of the fluorescent label can vary dependent upon the microscope image collection settings and it is important to characterize this value for each set of experiments. Moreover, it is necessary that the fluorophore tag is monomeric and is in a location as close as possible to the one that will characterize the tagged-protein of interest (e.g. in the same subcellular compartment). For studies on GPCRs of interest fused to mEGFP strenuous efforts were made to determine QB of the auto-fluorescent protein. In initial studies a single mEGFP or a tandem of two mEGFPs linked by a short polypeptide were expressed as soluble proteins in the cell cytoplasm (Fig. 2.2a). SpIDA on RoIs from the

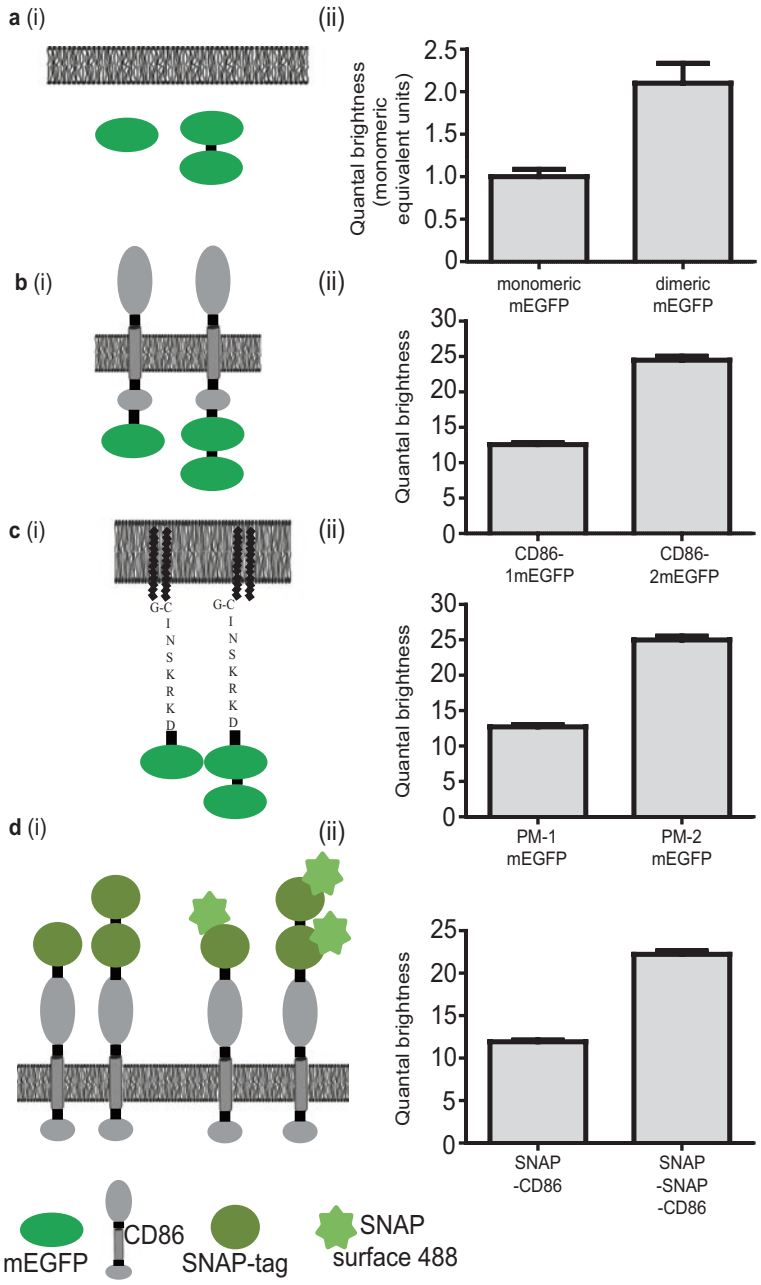


Fig. 2.2 Schematic representation of a single mEGFP or a tandem of two mEGFPs linked by a short polypeptide and expressed as soluble proteins in the cell cytoplasm (**a**(**i**)), fused at the carboxyl-terminal tail of CD86 (**b**(**i**))**,** or to the palmitoylation + myristoylation sequence, from the Lyn non-receptor tyrosine kinase (**c**(**i**)). SpIDA measurements performed on laser scanning confocal images of cells expressing either of these constructs indicated the QB (expressed as monomeric equivalent units in **a**), of the tandem to be twice that assessed for the single mEGFP (**a, b, c**(**ii**)). (**d**)**.** Representation of CD86 modified at the N-terminal by the incorporation of a SNAP-tag or a tandem of two SNAP-tags and labelled with SNAP-Surface 488 (**i**), QB assessed for SNAP-SNAP-CD86 was 1.86 times that assessed for SNAP-CD86 (**ii**)

cytoplasm of such cells showed that the QB value of the tandem mEGFP was very close to twice that of the single mEGFP [11]. As well as being anticipated, this confirmed that two molecules of mEGFP in very close proximity are identified as a 'dimer'. However, GPCRs are not routinely present in the cytoplasm and as integral membrane proteins it is essential to assess QB of the standard mEGFP at the plasma membrane. We have approached this in two ways. Firstly, we selected the CD86, a single transmembrane domain, integral membrane protein expressed on antigen presenting cells that is known to be monomeric [25, 26]. Modification of CD86 at the intracellular carboxy-terminal tail by in-frame fusion of either one mEGFP (CD86-mEGFP) or the tandem of two mEGFP (CD86-2mEGFP) and the production of stable, inducible Flp-In™ T-REx™ stable cell lines able to express these proteins upon addition of doxycycline initially showed both of these to be targeted efficiently to the plasma membrane. Imaging and analysis showed that QB for CD86-2mEGFP was almost exactly twice that of CD86-mEGFP (Fig. 2.2b) [11]. Secondly, as an alternate means of achieving membrane localization of this auto-fluorescent protein Ward et al., [12] modified mEGFP by attaching a short plasma membrane targeting palmitoylation + myristolation sequence [27] to the amino-terminal of both mEGFP (PM1-mEGFP) and the tandem mEGFP (PM2-mEGFP) (Fig. 2.2c). Expression of each of these in inducible Flp-In™ T-REx™ stable cell lines resulted in entirely plasma membrane localization. Images were collected and SpIDA analysis of these generated QB values which were very similar to those described using the CD86-based constructs [12]. Thus, either of these systems is appropriate for use in determining the organization of a plasma membrane associated protein of interest tagged with mEGFP.

The epidermal growth factor receptor (EGFR) has also been a vital "proof of concept" tool in our investigations using SpIDA. Although a single transmembrane domain tyrosine kinase rather than a GPCR, this receptor is well appreciated to exist largely in monomeric form until binding an activating ligand (e.g. epidermal growth factor (EGF)) [28]. With an mEGFP tag added to the carboxy-terminal tail and, again, stable expression in Flp-In™ T-REx™ cells, EGFR-mEGFP was, as expected, located at the plasma membrane, [11] and (data not shown). In the absence of ligand SpIDA indicated the construct to be predominantly monomeric, whereas after addition of EGF the construct rapidly dimerized [11] with a half-maximal effect at ~3 nM EGF [12]. Furthermore, we have shown using SpIDA that a non-dimerizing mutant of the EGFR (Tyr251Ala, Arg285Ser) [28], exhibits no re-arrangement of monomer to dimer upon treatment, an observation that we have confirmed using native polyacrylamide gel electrophoresis (data not shown). It should be stressed that the EGFR-mEGFP has not been used to determine the monomeric QB value in any of our experiments as it has been shown that a proportion of dimers (approximately 20%) is present at the surface of cells not treated with EGF [11, 12, 28].

Although much of the published work employing SPIDA has employed mEGFP or related auto-fluorescent protein tags there may be considerable benefits in developing the use of other fluorophores. The use of 'SNAP'-tagging as a possible alternative to auto-fluorescent proteins has been mentioned earlier [18, 19]. To this end we have added either single or a tandem pair of SNAP-tag sequences to the

N-terminal region of CD86 [11] (Fig. 2.2d). This reflects that the SNAP tag is highly flexible in the range of fluorophores that can be linked to it covalently. Moreover, in many situations heterologous expression of a protein of interest can result in a degree of inappropriate targeting and we wished to focus only on correctly targeted, cell surface-delivered proteins. By employing the cell impermeant reagent SNAP-Surface 488 (New England Biolabs, Cat No S9124) this allowed covalent incorporation of the fluorophore into only cell surface copies of SNAP-CD86, and allowed use of the same laser line and filter set as required for mEGFP tagged proteins. In this set of experiments the monomeric QB value (SNAP-CD86) was found to be 11.96 ± 0.18 (n = 446), whilst that for the tandem SNAP construct (SNAP-SNAP-CD86) was 22.25 ± 0.39 (n = 355). As such, QB of the protein labelled with two copies of the SNAP protein was less than twice (1:1.86) that of the single SNAP-modified form. The most likely explanation is that the tandem SNAP molecules were not labelled with the same efficiency as the single SNAP. This needs to be investigated further before SpIDA can be applied quantitatively without under-reporting the proportion of dimeric/oligomeric species.

Once established, the QB of the monomeric fluorescent label is used to normalize values of QB and mean fluorescent intensity obtained from the SpIDA study of tagged proteins of interest. In this way, monomeric equivalent units (MEUs) and the density of the receptor, expressed as number of receptors.μm^{-2}, can be calculated and used to present data such as shown in Fig. 2.3.

2.1.6.2 1 Population or 2 Population Mode

An option available whilst using the SpIDA software is that of utilizing either a 1 population or 2 population mode of analysis. Comprehensive details of the mathematical equations used for these are detailed in Godin's original publication [7]. The difference between these is that in the 2 population mode the fluorescence in the RoI is reported as the densities of monomer and dimer, monomer and trimer, monomer and tetramer or monomer and pentamer (depending upon which is selected), based upon a previously determined value for monomeric QB, rather than simply reporting average QB and mean fluorescence intensity for the RoI. We have made use of this in the analysis of the organization of Robo 1 [11] and also the EGFR, [11, 12]. However, in the case of GPCRs, where the existence of a wide range of possible oligomerization states has been reported [5, 26, 29–34] together with a possible rapid interchange between (at least) monomer and dimer [29, 35, 36] we have found that this option is perhaps less useful than the one population fit model, which can accurately quantify average QB and mean fluorescence intensity values in the presence of higher-order oligomeric populations.

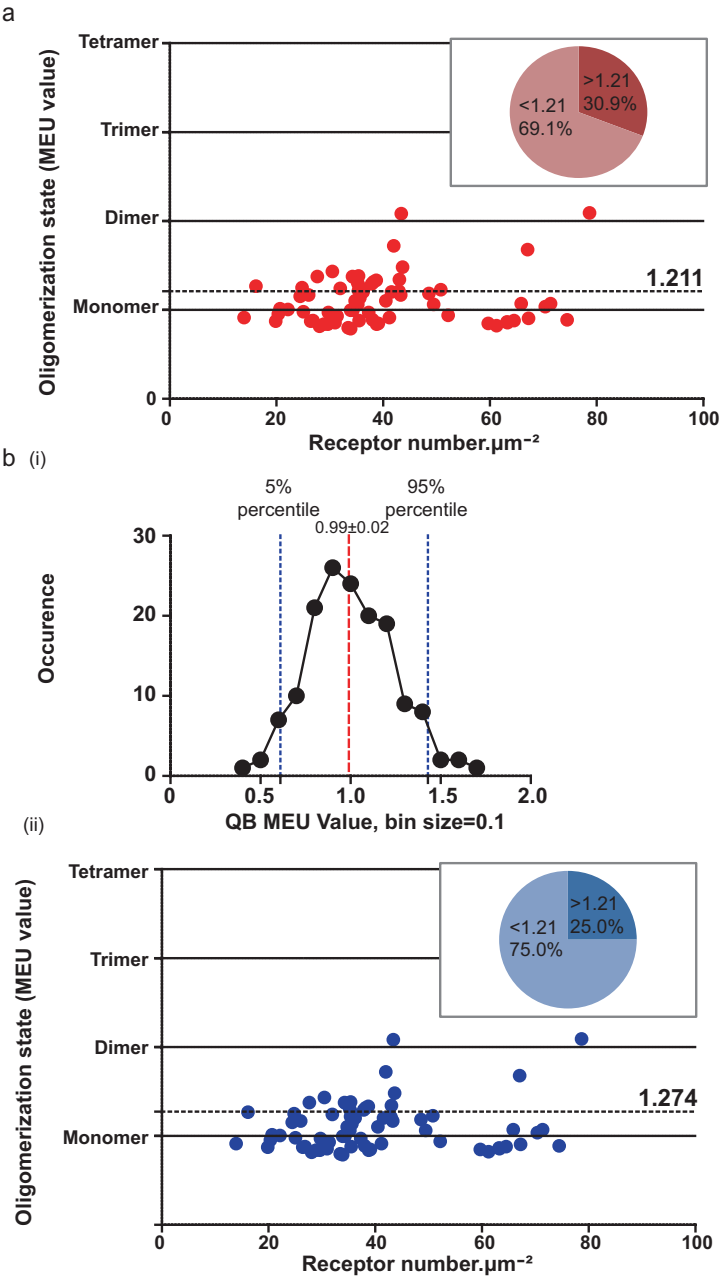


Fig. 2.3 Flp-In™ T-REx™ 293 cells harboring hM_1-mEGFP were treated with 100 ng.ml^{-1} doxycycline. 24 h after induction of receptor expression, confocal images across groups of cells were collected and analysed using the GUI program to perform SpIDA analysis. MEU QB assessed in individual RoIs was plotted against number of hM_1-mEGFP.µm^{-2} of the basolateral surface. The percentage of RoIs contacting predominantly monomers and oligomers within this dataset were defined using two different formulae. (**a**). The boundary between monomer and oligomer was defined using the formula "mean ± 3 × standard error" and, for this data set was 1.211 MEU QB.

2.1.6.3 When Does a QB Value Reflect Monomeric and When Dimeric/Oligomeric States?

When considering a plot of QB expressed as MEU (MEU QB) against receptor density, such as shown in Fig. 2.3, there are various ways to partition signals corresponding to monomers, dimers etc. Initially we defined this boundary using the formula "mean MEU QB + 3 × standard error" [12] and then considered that every RoI characterized by a MEU QB below this value contained, predominantly, species smaller then a dimer, whilst those characterised by a MEU QB above the cut off contained, on average, predominantly species larger than a monomer (Fig. 2.3a). More recently we have considered the distribution of QB values for a clearly defined monomeric protein. This should display Gaussian distribution if the protein is identified as monomeric across the range of expression levels studied. This is indeed the case for the PM1-mEGFP construct (Fig. 2.3b). For proteins displaying a mixture of monomers and dimers there is expected to be a skew of such distribution to higher MEU and this is indeed observed for GPCRs such as the M1 muscarinic acetylcholine receptor in its basal state [13]. Using mean ± 1.5 standard deviations of the Gaussian distribution as the boundary (1.274 MEU), values above this level were used to define RoI with predominant dimeric/oligomeric organization (Fig. 2.3b).

2.1.7 Determining the Quaternary Structure of GPCRs and How This May Be Affected by Ligand Binding

$GABA_B$ receptors are known to exist as constitutive $GABA_{B1}$-$GABA_{B2}$ heterodimers at the cell surface and both the mechanism and significance of the dimerization have been well described and accepted for this class C GPCR [3]. For this reason this receptor has long been considered to be an excellent model to test new approaches, SpIDA included [7]. Using SpIDA in the two population mode, Godin and collaborators measured the density and oligomerization state of the $GABA_B$ receptor in immunocytochemically labelled sections of rat spinal cord. The analysis

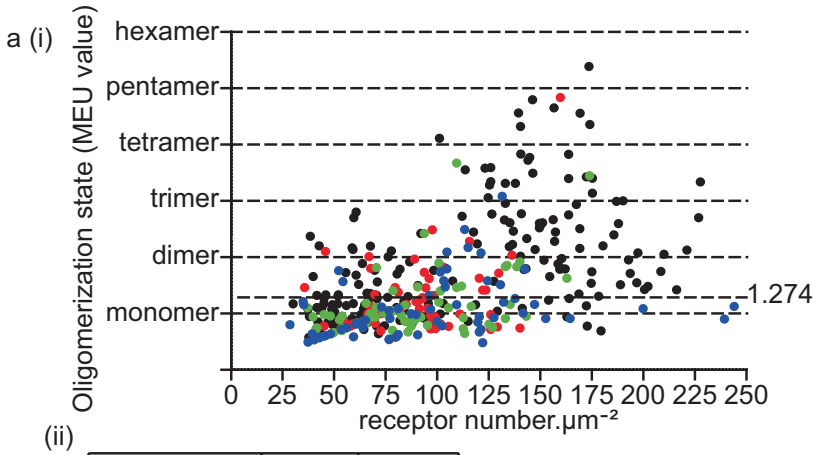
Fig. 2.3 (continued) RoIs characterized by QB MEU values greater than 1.211 were considered to contain a prevalence of hM_1-mEGFP in a dimeric/oligomeric state (30.9%); in contrast those with QB MEU values equal to or smaller than 1.211 contained a prevalence of hM_1−mEGFP in a monomeric state (69.1%). (**b**). The boundary QB MEU was defined by determining monomeric QB MEU. (**i**) Flp-In™ T-REx™ 293 cells harboring P-M-mEGFP were treated with 10 $ng.ml^{-1}$ doxycycline. 24 h after induction, confocal images across groups of cells were collected and analysed using GUI program to perform SpIDA analysis. QB values from individual RoIs were binned (bin size 0.1 MEU) and these displayed a symmetrical distribution. The boundary QB MEU was defined using the formula "monomeric QB MEU mean/((95%–5% percentile)/3)", that corresponds to mean ± 1.5 × standard deviation of the Gaussian distribution and had a value of 1.274. (**ii**) RoIs characterized by QB MEU values greater than 1.274 were then considered to contain a prevalence of hM_1-mEGFP in a dimeric/oligomeric state (25.0%), (Data are adapted from [11–13])

revealed the presence of mostly monomers in samples in which only one of the two $GABA_B$ subunits was labelled. In contrast however, a significant proportion of dimers were observed in samples in which both $GABA_B$ subunits were labelled with a subunit-specific primary antibody (and detected with the same secondary antibody conjugated to the Alexa488 fluorophore). These results indicated that SpIDA is able to provide useful information on GPCR density and quaternary structure and that it could represent a potent tool to investigate the phenomenon of the protein-protein interaction of class A GPCRs for which the question of oligomerization is more uncertain [3].

The 5-HT_{2C} receptor was the first class A GPCR for which quaternary structure was assessed using SpIDA [12]. Here the receptor was modified at the carboxy-terminal tail by in-frame fusion to mEGFP. Initial experiments were consistent with the receptor being a constitutive dimer, a set of observations in agreement with earlier studies [33, 37, 38]. However, detailed examination of SpIDA analyses revealed that the organization of 5-HT_{2C} receptor quaternary structure varied substantially with the density of the receptor at the cell surface: the size of the complexes and the percentage of dimer/oligomers clearly increased with receptor density. At modest expression levels a significant proportion of the 5-HT_{2C} receptor was detected as monomeric, whilst at higher expression levels dimers and, indeed, higher-order oligomers were predominant. This would be consistent with Mass-Action defining the balance between different organizational states.

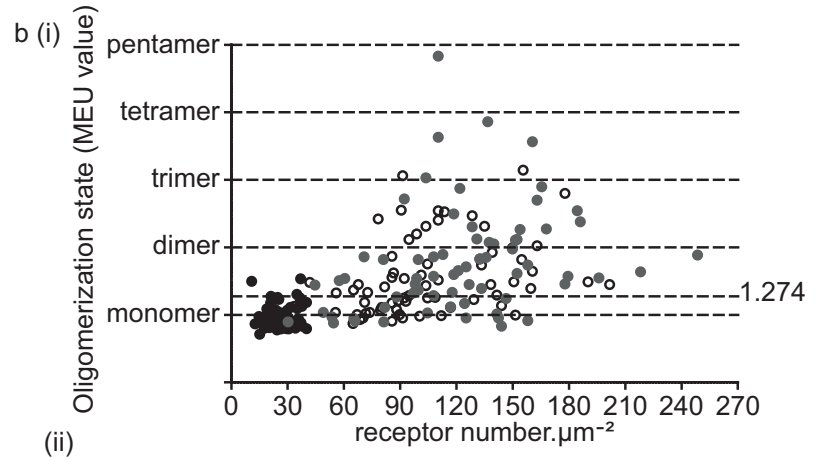
The question of whether ligand binding alters the quaternary structure of class A GPCRs remains contentious, and literature data is highly divergent [39–41]. Interestingly, in cells expressing 5-HT_{2C}-mEGFP, treatment with compounds from distinct chemical series that are antagonist/inverse agonists (SB242084 (6-Chloro-2,3-dihydro-5-methyl-*N*-[6-[(2-methyl-3-pyridinyl)oxy]-3-pyridinyl]-1*H*–indole-1-carboxyamide dihydrochloride), SB243213 (2,3-Dihydro-5-methyl-*N*-[6-[(2-methyl-3-pyridinyl)oxy]-3-pyridinyl]-6-(trifluoromethyl)-1*H*–Indole-1-carboxamide dihydrochloride) and RS102221 (8-[5-(2,4-Dimethoxy-5-(4-trifluoromethylphenylsulphonamido)phenyl-5-oxopentyl]-1,3,8-triazaspiro[4.5]decane-2,4-dione hydrochloride)) at this receptor resulted in a change in the quaternary structure of the 5-HT_{2C} receptor, to result in a mainly monomeric population [12] (Fig. 2.4a). This was concentration-dependent and consistent with receptor occupancy. Importantly, this was reversible. Washout of the ligands resulted in recovery of the original basal quaternary organization [12]. Although this recovery step appeared to be relatively slow, this may well reflect the high affinity and thus, slow dissociation of the ligands from the receptor after washout of ligand from the bulk solution. This requires further analysis but it would be useful to examine this feature using ligands with a rapid ‘off-rate’ from the receptor.

In a similar way, the quaternary structure of the human muscarinic M_1 (hM_1) and M3 (hM_3) receptors has been investigated [13]. When both receptors were modified at the carboxyl-terminal tail by the incorporation of the mEGFP and expressed in the inducible Flp-In™ T-REx™ 293 system, the greatest proportion of each of these receptors was found to be monomeric although, particularly for hM_1 a significant population of dimers was also observed in the basal state. Interestingly some



(ii)

	< 1.274 monomer	> 1.274 oligomer
untreated (●)	38.5%	61.5%
0.1 μM RS102221(●)	65.6%	33.3%
50 nM SB243213 (●)	74.2%	22.7%
50 nM SB242084 (●)	74.2%	22.7%



(ii)

	< 1.274 monomer	> 1.274 oligomer
untreated (●)	92.65%	7.35%
10 μM Pirenzepine (●)	39.71%	60.29%
1 μM Telenzepine (○)	26.47%	73.53%

Fig. 2.4 Flp-In™ T-REx™ 293 cells harboring the GPCR-mEGFP of interest were treated with 100 ng.ml^{-1} of doxycycline for 24 h to induce expression of the receptor construct. (**a**(**i**))**.** MEU QB assessed in individual RoIs plotted against number of 5-HT$_{2C}$-mEGFP.μm^{-2} of the basolateral surface of cells treated with the vehicle (*black circle*), 0.1 μM RS102221 (*red circle*), 50 nM SB243213 (*green circle*) or 50 nM SB242084 (*blue circle*). Treatment with the antagonist ligands at the receptor altered the quaternary structure of the receptor favouring the monomeric state (**a** (**ii**)) data are adapted from [12]. (**b**(**i**)). MEU QB assessed in individual RoIs plotted against number of hM$_1$-mEGFP.μm^{-2} of the basolateral surface of cells treated with the vehicle (*black circle*), 10 μM pirenzepine (*grey circle*), or 1 μM telenzepine (*open circle*). Treatment with the antagonist ligands altered the quaternary structure of the receptor favoring the dimeric/oligomeric state (**b**(**ii**)) (Data are adapted from [13])

years ago Ilien and co-workers [42] had noted that the M_1 receptor selective antagonist/inverse agonist pirenzepine (11-[(4-methylpiperazin-1-yl)acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*] [1, 4]benzodiazepin-6-one) promoted or stabilized M_1 receptor dimers. When assessed using SpIDA exactly the same conclusion was reached [13]. A feature frequently noted with sustained exposure of receptor-expressing cells and tissues to antagonist/inverse agonists ligands that bind the receptor is that this results in up-regulation of the receptor [43]. However, although, as noted above, higher levels of receptor expression can inherently promote oligomerization based on Mass-Action, in this case it was possible to demonstrate that the effect of pirenzepine to enhance the proportion of receptor dimers was separate from such an effect [13]. As anticipated the closely related drug telenzepine (4,9-dihydro-3-methyl-4-[(4-methyl-1-piperazinyl)acetyl]-10*H*–thieno[3,4-*b*] [1, 5]benzodiazepin-10-one) also promoted the proportion of observed M_1 receptor dimers (Fig. 2.4b). However, this was not simply a reflection of antagonist/inverse agonist occupancy of the receptor because both atropine ((*RS*)-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl) 3-hydroxy-2-phenylpropanoate) and N-methylscopolamine, two muscarinic antagonists that are chemically distinct from pirenzepine and telenzepine did not produce dimer stabilization. As with the $5\text{-}HT_{2C}$ receptor, washout of pirenzepine allowed hysteresis of the system back to the basal state [13]. Importantly, as assessed in this study, the treatment of cells expressing hM_1-mEGFP with the compounds listed above did not alter the spectral characteristics of mEGFP, confirming that the change in the QB observed did not reflect a change in the fluorescence properties of the fluorescent tag and clearly reflect changes in the oligomeric state of the receptor (Fig. 2.5).

Although selective for the M_1 receptor subtype, pirenzepine and telenzepine can also bind the M_3 muscarinic receptor, albeit with lower affinity. However, even at saturating concentrations neither of these drugs resulted in an increase in the proportion of M_3 receptor dimers [13].

An advantage of SpIDA is that it is a versatile technique and can be put to use to investigate various aspects relating to GPCR oligomerization, beyond those described above. It has the potential to be used to answer some of the many outstanding questions which remain unresolved. An obvious question, which follows on from above, is what effects do agonist ligands have upon the quaternary structure of GPCRs. The difficulty with investigating this is that many agonists cause clustering and internalization (often via a β-arrestin-dependent pathway) as a consequence of the activation of the receptor [44–46]. The combination of SpIDA with a system in which β-arrestin function is disabled should be able to resolve this question. In a similar way the importance of G protein coupling for the quaternary structure of the receptor could easily be assessed by performing SpIDA on cells in which the expression or function of selected G proteins has been impaired. Another interesting aspect that remains unresolved is the way in which GPCR monomers interact with one another, which residues for instance, define the interface of dimerization. SpIDA provides the possibility to study mutants, selected in combination with molecular modelling studies, which disrupt the interface of dimerization [47, 48]. It should also be noted that when analysing the effects of such mutants SpIDA has the impor-

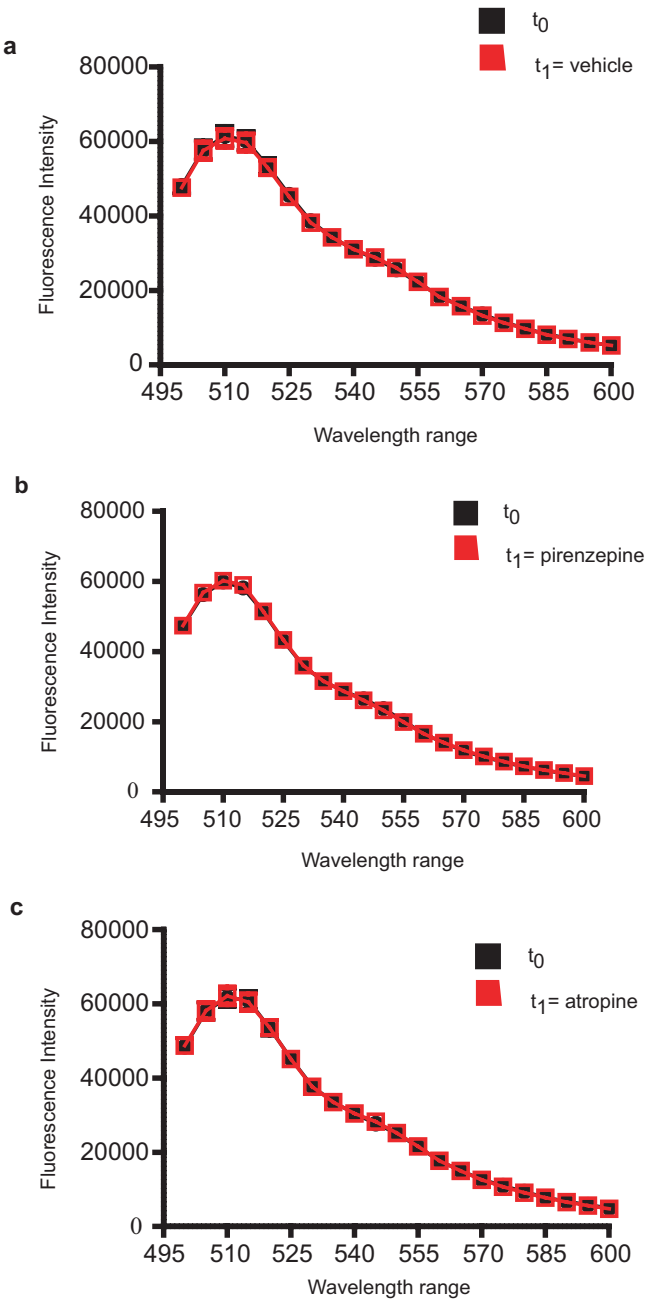


Fig. 2.5 Flp-In™ T-REx™ 293 cells were grown to 100,000 cells per well in 96-well black bottom plates (Greiner Bio-One) and treated with doxycycline to induce the expression of hM_1-mEGFP. After 24 h induction plates were read (**a**, **b** and **c**, $\mathbf{t_0}$) using a CLARIOstar fluorescence-compatible reader (BMG Labtechnologies). Specifically, cells were excited at 462 nm and the emission spectrum between 500 and 600 nm was collected at 5 nm intervals. The same process was repeated after the addition to each well of 100 μl HBSS supplemented with the vehicle (**a,** $\mathbf{t_1}$), pirenzepine (**b,** $\mathbf{t_1}$) or atropine (**c,** $\mathbf{t_1}$)

tant advantage of providing information regarding the expression level of the mutant receptors (receptors.μm^{-2}) which is of vital importance when comparing their effects to other mutants or "wild type" receptors. Similarly, the effects of other means of disrupting dimeric interfaces, such as the addition of peptides which mimic trans-membrane domains [49, 50], could be investigated by SpIDA.

Maybe the most exciting opportunity that SpIDA offers is the possibility to analyse RoIs selected from fixed tissues. This should allow SpIDA to define the quaternary structure of GPCRs *ex vivo* and potentially for this to be compared between normal and diseased tissues.

Thus far we have only discussed using images collected at the outer cell membrane, usually at the baso-lateral surface of the cell. However, when one considers that questions remain unresolved about the formation and trafficking of receptor oligomers through the cell [44–46], it follows that images could be collected from other regions and, so long as it is possible to select sufficiently homogenious RoIs, use SpIDA to interrogate the quaternary structure at other parts of the cell, at other moments during the "life-cycle" of a receptor.

Thus far we have only considered the study of homodimers by SpIDA. This leaves the question of heterodimeric interactions in abeyance. As with other techniques, dual or multi-color variations of SpIDA are being developed.

2.1.8 Future Perspectives of SpIDA

SpIDA can be used to extract much useful information from comparatively simply obtained laser scanning confocal images. As such its use is likely to grow and it is important to consider potential developments in both the application of SpIDA and also of further information which may be obtained.

Recently Godin and co-workers [51] described two interesting new developments which are likely to greatly extend the potential utility of SpIDA. The first of these addresses the question of incomplete labelling of the protein under investigation, a factor which will clearly cause an over-estimation of the density of lower oligomerization states and an underestimate of that of higher-order complexes if it occurs in a significant proportion of the labelled protein. This incomplete labelling could reflect various features depending upon how the protein of interest is labelled. For example, when a fluorescent protein is incorporated by genetically fusing it at the amino or carboxy-terminal of a protein of interest, a proportion of the fluorescent tag could be in a "dark state" [52, 53]. Where labelling is by chemical means (e.g. SNAP-tagging) or by labelled antibodies it is likely that the process will not fully efficient, as noted earlier for the labelling of a tandem SNAP construct. To correct for the fidelity of labelling Godin and co-workers describe a modification to SpIDA in which a labelling constant can be determined and then applied to correct the final result for the consequences of incomplete labelling. The second development is the ability to measure and assess three oligomeric populations at once rather than two as described previously (see Sect. 2.1.6.2) thus for instance, a mixture of

monomers, dimers and tetramers could be interrogated with respect to their individual densities. A potentially important aspect of the application of SpIDA is its use *in situ,* in native tissues, something that has already been described for both the $GABA_B$ receptor in rat spinal cord (see Sect. 2.3) and for the electrogenic sodium bicarbonate cotransporter (NBCel-A) in rat kidney [54]. In the latter case labelling was by a combination of anti-NBCel-A primary antibody followed by an Alexa 488 conjugated secondary. It was shown that NBCel-A is dimeric in the native kidney, though this was not found to be the case in heterologous expression systems (HEK293 and CHO-K1 cells), labelled in two different ways (EGFP, α-bungarotoxin Alexa 488), which suggested a predominantly monomeric arrangement. The results of this study are puzzling and there is a clear need for further work *in situ,* using native tissues, though great care will be needed to ensure proper labelling, by whatever means is chosen, whether by antibody or by making use of transgenic animals, modified to include a fluorescent label fused to a protein of interest.

Other exciting concepts which could evolve in the future are simultaneous cross-correlation quantification of the diffusion and oligomerization state of fluorescently labelled oligomer receptor species using TIRF stream imaging microscopy (which is ideal for imaging thin layers, such as the plasma membrane, with minimum background fluorescence) in combination with SpIDA. A final intriguing possibility is that of using 2-color SpIDA to explore heterodimeric interactions, a topic that, to date, has hardly featured in the literature [55].

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Chapter 3
Advanced Microscopy Techniques

Valerica Raicu and William F. Schmidt

Abstract Resolution of current controversies regarding the nature and functional roles of the oligomeric forms of G-protein coupled receptors (GPCRs) demands that experimental methods are both quantitative – i.e., they allow determination of size, geometry and stability of oligomers under varying experimental conditions – and applicable to receptors within their cellular milieu. Standard microscopy methods based on light do not provide the resolution necessary to resolve membrane receptor complexes, while techniques based on other contrast mechanisms (e.g., electron microscopy or atomic force microscopy) require sample destruction and fixation. Fortunately, techniques that exploit physical properties of fluorescent molecules, such as their ability to transfer excitations to an unexcited nearby fluorescent molecule (as in *FRET spectrometry*) and spatial or temporal fluctuations in the fluorescence intensity (*fluorescence correlation spectroscopies* and *photon counting histograms*) driven by aggregation and diffusion are capable of increasing the spatial and temporal resolution of all optical microscopies by several orders of magnitude. In this chapter, we overview the physical principles underlying such techniques, their comparative advantages and limitations, as well as their application to quantitative analysis of GPCR oligomerization in living cells.

Keywords G-protein coupled receptors (GPCR) oligomerization • Förster resonance energy transfer (FRET) • FRET spectrometry • Ensemble FRET • Fluorescence correlation spectroscopy (FCS) • Image cross-correlation spectroscopy (ICCS) • Photon counting histogram (PCH)

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3.1 Introduction

G Protein-Coupled Receptors (GPCRs) have been shown experimentally to form homo- or hetero-oligomeric complexes in vivo as well as in vitro [1–6]. While this concept enjoys broad acceptance for class C GPCRs and currently represents a majority's view for other GPCR classes [7–9], there have been suggestions that most GPCRs are not multimeric or that oligomerization is not essential for function [10–12]. It is still unclear whether the dearth of consensus is attributable to specific biases inherent in each technique used, data over-interpretation, or intrinsic structural and functional versatility of GPCRs.

Microscopic techniques not based on light, such as atomic force microscopy (AFM), could resolve complexes of proteins [13], though not with atomic resolution. Unfortunately, AFM-based images require extraction of receptor-bearing membranes from their natural environment and attachment to a solid substrate, which limits this method's applicability to receptor complexes in living cells.

It is well known that classical optical microscopy is not able to resolve proteins or their oligomeric complexes, due to the limit imposed by the diffraction of light. Optical microscopy's ability to reveal the localization of proteins inside the cells is usually enhanced by fusing fluorescent markers to the molecules of interest. Still, the structure of molecular complexes or even cellular features smaller than hundreds of nanometers may not be resolved using standard fluorescence microscopy.

Towards the end of the last century, new techniques, termed *super-resolution microscopy* or *nanoscopy*, were introduced, which circumvented limitations imposed by light diffraction, pushed the resolution limit well beyond the half-micrometer limit of standard wide-field optical microscopy and allowed visualization of small organelles and even molecular fibers inside the cells. These methods either employ carefully tailored illumination patterns, as in the case of stimulated emission depletion microscopy (STED) [14, 15] and structured illumination microscopy (SIM) [16], or increase the sparsity of the distribution of fluorescent molecules using certain photo-physical processes, as in the case of photoactivated localization microscopy (PALM) [17] and stochastic optical reconstruction microscopy (STORM) [18]. STED is based on two excitation beams – one exciting a small sample volume and another one depleting the same volume of excited molecules except for a minuscule region at its center – to simultaneously excite the molecules and reduce the excitation volume (through stimulated emission), regardless of the density of the fluorescent molecules in the sample. SIM adds sub-diffraction-limited resolution to wide-field microscopy by using a periodic illumination pattern. PALM and STORM, are based on determination of the center of the fluorescence spot (or point-spread function) in images acquired from distributions of molecules of which only some are in the "on" state (i.e. fluorescent) at any given time.

Nevertheless, spatial resolution commensurate with the distances on which protein interactions occur (~1 nm) remain out of reach for both fluorescence microscopy and nanoscopy techniques. Furthermore, in nanoscopy, image acquisition is often slow, leading to loss of dynamic information. Fortunately, the spatial resolution

of all optical methods may be enhanced by using innovative techniques that exploit physical properties of fluorescent molecules, such as their ability to transfer excitations to an unexcited nearby fluorescent molecule, or spatial and/or temporal fluctuations in the fluorescence intensity following diffusion of the molecules through the cellular milieu.

One of the most widely used methods for studying protein interactions in living cells is based on the detection of light from pairs of fluorescent tags attached to the protein of interest. If an "acceptor" (A) fluorescent molecule capable of absorbing light lies within ten nanometers of an optically excited "donor" molecule (D), it can extract energy from the donor through a non-radiative process called Förster Resonance Energy Transfer (FRET) [19–21]. Using FRET, it has been possible to determine intramolecular distances, probe molecular associations (or complexes), determine the spatial distribution of such complexes in living cells, and, under favorable conditions, determine the interaction energetics [2, 4, 22–26].

Bioimaging techniques based on fluctuations in the fluorescence signal, such as fluorescence correlation spectroscopy (FCS) and image cross-correlation spectroscopy (ICCS), have been developed to study fluorescent biomolecules [27–29]. Fluorescence intensity fluctuations are assumed to arise from spontaneous processes without disturbing the equilibrium of the biological system [30]. Physical processes giving rise to fluorescence intensity fluctuations allow determination of the local concentration of fluorescent molecules, the degree of colocalization and aggregation (or *oligomerization*), diffusion coefficients, and rate constants of molecular reactions between fluorescently-labeled molecules. The concepts of autocorrelation and cross-correlation of fluorescence intensity fluctuations, both spatial and temporal, are employed as means of describing the time-evolution and spatial distribution of fluorescent molecules. Two widely applied techniques involving related aspects of fluorescence correlation - fluorescence correlation spectroscopy (FCS) and image cross-correlation spectroscopy (ICCS) [29, 31] - yield insight into the spatio-temporal parameters which may be explored in bioimaging. These techniques have been proven particularly useful in the investigation of GPCRs [5, 32, 33] at low expression levels [5].

This chapter will introduce the principles underlying the family of experimental methods mentioned above and provide some specific examples of experimental techniques as well as their application to probing GPCRs oligomerization.

3.2 FRET-Based Microscopy

3.2.1 Definitions and the Principle of the Method

The quantity sought after in FRET experiments is the *efficiency of energy transfer* (or *FRET efficiency*), which represents the fraction of optical excitations transferred from donors to unexcited acceptors. If the molecular complexes to be probed by

FRET consist of several combinations of donors and acceptors (also called *oligomer configurations*) and/or are mixed with other oligomeric species or monomers, an *apparent FRET efficiency* (denoted by E_{app}) is measured, which incorporates contributions from all the donors and acceptors in the system.

Determination of the apparent FRET efficiency from fluorescence imaging measurements may be done using either fluorescence lifetime [34–37], or intensity [3, 38–44] measurements. However, oftentimes the number of lifetimes to be determined exceeds what may be extracted from experiments [3, 44]. Fluorescence intensity measurements of entire cells allow determination of an apparent FRET efficiency, E_{app}, which may incorporate fluorescence signals both from associated and un-associated proteins [20, 21].

Two of the more often used intensity-based FRET methods rely on monitoring the donor intensity before and after *acceptor photo-bleaching* and/or detecting the acceptor increased emission intensity (*acceptor sensitized emission*) [41, 45–49]. In spite of their usefulness, both acceptor photobleaching and sensitized emission pose significant challenges to investigators interested in mapping the distributions of protein complexes inside the cell or in the temporal changes of those distributions (as opposed to static averages over entire cells). In both methods, one successively scans the sample at two different excitation wavelengths. Because the time elapsed between such scans is relatively long, molecular diffusion and biochemical reactions occurring in the cell may cause the local composition of the sample to change from scan to scan.

Recent publications have demonstrated the feasibility of spectrally resolved fluorescence microscopy [50, 51] and demonstrated its use in quantitative FRET studies [43, 52–56]. The practical power of the spectral method comes from two unique features: its ability to exactly extract donor and acceptor signals from composite spectra, and the possibility to relate the experimentally measured intensities to other spectral and molecular parameters, which can then be used to determine the FRET efficiency. The spectral-FRET approach has evolved into two classes of methods for determination of protein complex geometry and/or stoichiometry: (i) the E_{app} distribution-based method [3, 4, 44], (ii) the ensemble FRET method [68], and (iii) hybrid methods combining both approaches [66, 67]. In the following paragraphs, we will provide an overview of the technology developments that have allowed introduction of these methods, as well as a brief description of the underlying theory.

3.2.2 Theoretical Basis of the Method

In this section, we will briefly review the kinetic theory of FRET in multimeric complexes of donors and acceptors [57] and introduce the main formulas used to both compute FRET efficiencies from experimental data and predict their values for different donors and acceptors configurations. The theoretically predicted efficiencies allow one to extract structural and stoichiometric information from the experimentally measured E_{app}.

Let us consider an oligomer or *multiplex* containing n fluorophore-bearing protomers, or subunits, of which k are identical donors and n-k are identical acceptors. We further assume that only one donor in a complex is brought into its excited state at a time. This is achieved by keeping the intensity of excitation light intensity low enough so that the donor excitation rate is much lower than all of their de-excitation rates. Each donor may transfer energy to the n-k acceptors with a probability that depends on the acceptor distance and orientation relative to the donor. There accordingly is one pathway for each donor to lose excitation energy through radiative emission of a photon, one pathway for non-radiative de-excitation via interactions with the environment, and n-k different ways to lose excitation energy via FRET with nearby acceptors. An example of all possible energy transfer pathways for a pentamer with two donors and three acceptors is shown in Fig. 3.1.

In the absence of FRET, the quantum yields, Q^D, of all donors are equal to one another, as are the excitation rates of all acceptors, $\Gamma^{A,ex}$. By definition, FRET does not modify the excitation rate of the donors through absorption of photons, $\Gamma^{ex,D}$. It also does not modify the quantum yield of the acceptors, Q^A, which only depends on de-excitation rates through various pathways. However, FRET reduces the quantum yield of the i-th donor in a certain oligomeric configuration q according to the following equation [3, 58]:

$$Q_{i,k,q}^{DA} = Q^D\left(1 - E_{i,k,q}\right), \tag{3.1}$$

where $Q^D = \dfrac{\Gamma^{r,D}}{\Gamma^{r,D} + \Gamma^{nr,D}}$ is the quantum yield of the donors in the absence of energy transfer; and $E_{i,k,q}$ is the FRET efficiency for the i^{th} donor. FRET also enhances the excitation rate of the j^{th} acceptor in the oligomer in the presence of donors, as given by the expression:

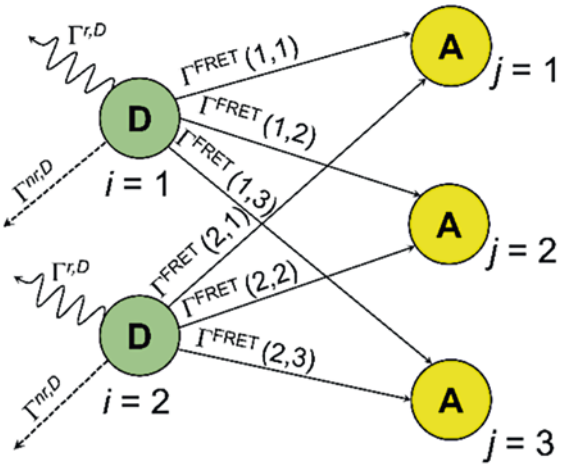

Fig. 3.1 Schematic representation of a particular configuration, q, of a fluorescent pentamer consisting of two donors (D, *green*) and three acceptors (A, *yellow*). Arrows indicate possible pathways for donor energy loss through: FRET (*solid line*, Γ^{FRET}), non-radiative loss (*dashed line*, $\Gamma^{\text{nr,D}}$), and radiative emission (*wavy line*, $\Gamma^{\text{r,D}}$)

$$\Gamma_{j,k}^{ex,AD} = \Gamma^{ex,A} + \Gamma^{ex,D} \sum_{i=1}^{k} E_{i,k,q}. \tag{3.2}$$

It may be shown [59] that, using these equations, the total number of photons emitted by the k donors in an oligomer in the presence of acceptors (i.e., FRET) may be expressed as.

$$F^{DA}\left(\lambda_{ex}\right) = F^{D}\left(\lambda_{ex}\right) - F_{D}^{FRET}, \tag{3.3}$$

while the total number of photons emitted by the n-k acceptors may be expressed as

$$F^{AD}\left(\lambda_{ex}\right) = F^{A}\left(\lambda_{ex}\right) + F_{A}^{FRET}, \tag{3.4}$$

where $F^{D}(\lambda_{ex}) = k\Gamma^{ex,D}Q^{D}$ and $F^{A}(\lambda_{ex}) = (n-k)\Gamma^{ex,A}Q^{A}$ denote the donor and acceptor emissions in the absence of FRET, $F_{D}^{FRET} = \Gamma^{ex,D}Q^{D}\sum_{i=k}^{k} E_{i,\,k,q}$ is the loss of emission from the donor due to FRET, and $F_{A}^{FRET} = \Gamma^{ex,D}Q^{A}\sum_{i=1}^{k} E_{i,\,k,q}$ is the corresponding gain in the acceptor emission. By comparing the expressions for F_{D}^{FRET} and F_{A}^{FRET}, we obtain a third relationship,

$$F_{D}^{FRET} = \frac{Q_{D}}{Q_{A}} F_{A}^{FRET}, \tag{3.5}$$

which has been proposed and used previously [3].

Further, by defining the apparent FRET efficiency of an oligomer as the fraction of excitations of donors transferred to acceptors, that is,

$$E_{app} = \frac{F_{D}^{FRET}}{F^{D}\left(\lambda_{ex}\right)}, \tag{3.6}$$

assuming that direct excitation of acceptors is negligible at the chosen wavelength (*i.e.*, $F^{A}(\lambda_{ex}) \approx 0$), and using Eqs. (3.3, 3.4 and 3.5) to solve for F_{D}^{FRET} and $F^{D}(\lambda_{ex})$, we obtain:

$$E_{app} = \left(1 + \frac{Q^{A}}{Q^{D}} \frac{F^{DA}}{F^{AD}}\right)^{-1}. \tag{3.7}$$

Equation (3.7) has been used to determine the FRET efficiency at each pixel in a fluorescence image following a single scan of the sample at a single excitation wavelength [3]. It circumvents difficulties associated with classical filter-based

methods for the determination of FRET efficiency using two or more excitation scans [58], provided the imaging technology meets the requirements listed in the next section.

3.2.3 Practical Implementation of FRET

3.2.3.1 General Requirements for Optical Microscopes Used in FRET Studies

In order to use FRET to determine the size, geometry, distribution and trafficking of protein oligomers inside the cell as well as the proportion of different types of such oligomers the imaging technology needs to meet the following criteria.

(i) Single-molecular-complex sensitivity. This is usually achievable by standard confocal microscopes but only if the molecular composition of the sample does not change on the time-scale of the measurements; that is the measurement of each pixel is faster than the molecular diffusion. Line-scan confocal or two-photon microscopes are usually best suited due to their simultaneous excitation of an array of pixels, which leads to much higher signal than for point-scan systems.

(ii) Image sectioning capability. Single molecule sensitivity needs to be coupled with an ability to isolate small numbers of molecules or molecular complexes in the field of view, particularly for FRET *spectrometry* approaches, where the distribution of values and not just the average over a region of interest is determined. This is ideally accomplished using instruments that present image sectioning capability, such as confocal and two-photon microscopes.

(iii) Ability to extract FRET efficiency values at image pixel level in a single sample scan. This requires that the instrument is capable of separating donor and acceptor signals at each pixel by using *spectral resolution* or some other physical property such as *fluorescence lifetime* or *polarization*. Currently, spectral resolution is the only known means to fully achieve this goal. Two-photon microscopes with spectral resolution meet this requirement, depending on their configuration, as discussed below. Confocal microscopes are significantly limited in this regard by their comparatively low signal-to-noise ratio, caused by the partial overlap between emission and excitation spectra which leads to discarding useful signal by the filters used to separate the emission from excitation. FLIM microscopes are limited to determination of oligomer size of two (i.e., dimers), due to the well-known fact that only a small number of distinct fluorescence lifetimes may be extracted from a fluorescence decay curve. As for wide-field fluorescence microscopes, full spectral resolution cannot be achieved with them.

(iv) Temporal resolution on the time-scale of milliseconds. Single-molecule sensitivity is not sufficient for determining single-complex-level FRET efficiency if

the integration time per pixel is not significantly shorter than the timescale of molecular diffusion (which scrambles the fluorescence information). This speed is easily achieved by confocal and two-photon microscopes with spectral resolution. Wide-field and spinning-disk microscopes equipped with filters (including tunable filters) are not fast enough for this purpose, because of the time it takes to switch filters.

3.2.3.2 Two-Photon Absorption Optical Micro-spectroscopy

A technique that meets all of the requirements for FRET spectrometry is two-photon microscopy (TPM) [60, 61] with spectral resolution, also known as *two-photon optical micro-spectroscopy*, as briefly reviewed in a publication by Biener et al. [62]. Standard TPMs serially excite individual voxels in the sample by raster-scanning a diffraction-limited spot across the sample, using a pair of galvanometric scanners, and passing the emitted fluorescence back through the scanner (to descan it) before detection using a photomultiplier tube (PMT). In most commercially available single-point-scan systems, spectral resolution is typically achieved through the use of a dispersive element, such as a prism or reflective grating, and projecting the spectrum onto an array of PMTs to resolve the fluorescence into several wavelength ranges. As the number of wavelengths is equal to the number of PMTs, spectral resolution in this arrangement is limited by the increased complexity and prohibitive costs associated with scaling up the number of detectors and associated electronics. In order to increase spectral resolution without increasing system complexity, a setup has been introduced which resolves the fluorescence into its spectral components via a transmission grating that projects light onto an electron-multiplying CCD (EMCCD) camera [5] (see Fig. 3.2). In this fashion, spectral resolution as high as 1 nm has been achieved. This resolution is necessary when more than two fluorescent species are present, such as when the sample itself also presents autofluorescence that is hard to distinguish from the FRET signal. High spectral resolution is also needed in various hyperspectral imaging applications (see, e.g., [32] and references therein).

The signal level in point-scanning single- or multi-photon microscopes is limited by the fact that only one voxel is excited at any given time, even when the beam is rapidly scanned along an entire line, which means that the sample at that voxel emits for only a fraction of the time it takes to scan the entire sample. When spectral resolution is added to such systems, the signal level decreases further due to the distribution of photons among multiple wavelength channels. To overcome this problem, one could increase either (a) the excitation power or (b) the scanning time. However, increasing excitation power leads to increased photobleaching or other undesired photophysical effects. Furthermore, in FRET studies, increasing the excitation power leads to simultaneous excitation of multiple donors within a protein complex, which causes the donors to compete for transferring the energy to the same acceptor. These effects can drastically alter the FRET efficiency data and result in an incorrect quaternary structure assignment. On the other hand, increasing

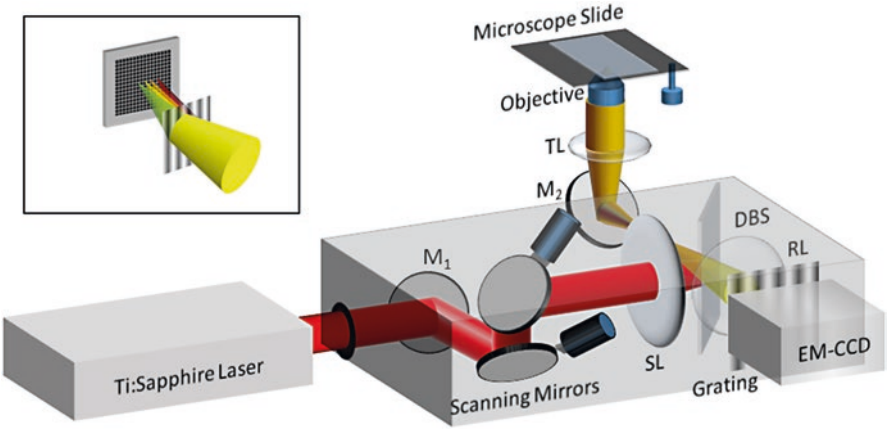

Fig. 3.2 (Color online) Two-photon excitation microscope with high spectral resolution using the line-scan excitation method. Significance of acronyms: *M* and M_1 plane mirrors, *SL* scanning lens, *TL* tube lens, *DBS* Dichroic beam splitter, *RL* imaging relay lens. *Inset*: Instantaneous spectrum measurement concept used by this type of microscope

the scanning time leads to blur in the image due to molecular diffusion, with consequent loss of information about the dynamics of the biological system of interest.

In a recent publication, a fluorescence micro-spectroscopic technique has been introduced in which all sample voxels along a line are excited simultaneously (or in parallel), thereby achieving increased scanning speed and sensitivity [62] (Fig. 3.3). The method relies on shaping the excitation beam into a line and using a camera to detect the signals emitted along the entire line. When integrated with the spectrally resolved TPM described previously [5], the method provided spatial resolution similar to that of the point-scan excitation modality [8], an acquisition speed comparable to that of commercial multiphoton and confocal microscopes that present no spectral resolution, and a sensitivity (*i.e.*, signal level for the same image acquisition time) that is more than two orders of magnitude higher than that of set-up shown in Fig. 3.7 [5].

3.2.3.3 Determination of FRET Efficiency from Optical Micro-Spectroscopy Data

In a spectrally resolved FRET experiment, the emission spectra at every pixel in the image of samples containing only donors or only acceptors are first obtained. These spectra may be described mathematically as:

$$I^t = \left(i_1^t \ \ldots \ i_l^t\right), \tag{3.8}$$

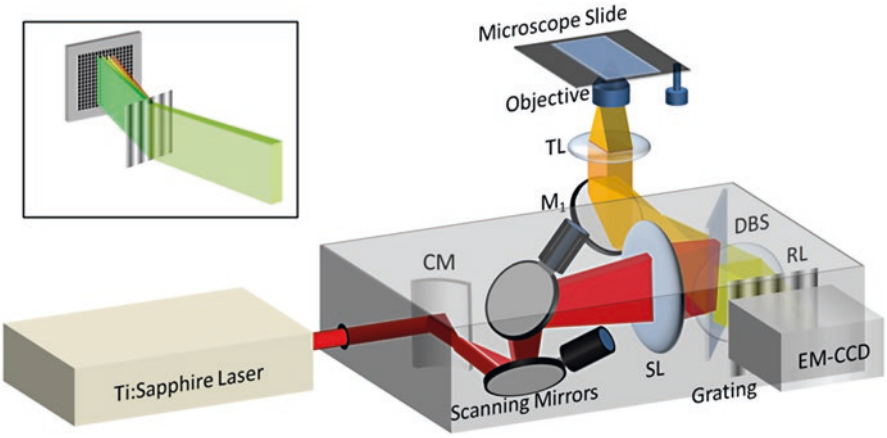

Fig. 3.3 (Color online) Two photon excitation microscope with high spatial and spectral resolution using the line-scan excitation method. Significance of acronyms: *CM* cylindrical mirror, *SL* scanning lens, M_1 plane mirror, *TL* tube lens, *DBS* Dichroic beam splitter, *RL* imaging relay lens. Inset: Instantaneous spectrum measurement concept used by this line-scan two-photon excitation microscope

in which t denotes either the donor or the acceptor, and $i_1^t \cdots i_l^t$ are the intensities at the wavelengths $\lambda_{em,1} \cdots \lambda_{em,l}$, respectively. Cells co-expressing donor- and acceptor-tagged proteins are then excited at a wavelength corresponding to the excitation maximum of the donor, and a composite spectrum consisting of signals from donors and acceptors is acquired for every pixel in an image.

The corresponding analytical expression comprising both spectral components is:

$$I^m = k^{DA}\left(\lambda_{ex}\right) I^D + k^{AD}\left(\lambda_{ex}\right) I^A, \tag{3.9}$$

in which $k^{DA}(\lambda_{ex})$ and $k^{AD}(\lambda_{ex})$ are the emission intensities of D in the presence of A, and of A in presence of D, respectively, at the excitation wavelength λ_{ex}.

Optimized values of $k^{DA}(\lambda_{ex})$ and $k^{AD}(\lambda_{ex})$ are determined according to a method of least-squares [43, 63], in which Eq. (3.9) is fitted to the measured intensity i^m at each pixel by minimizing the mismatch between experimental and simulated spectra. The values of $k^{DA}(\lambda_{ex})$ and $k^{AD}(\lambda_{ex})$ thus obtained are used to determine the total number of photons emitted by the donor [*i.e.*, $F^{DA}(\lambda_{ex})$] and the acceptor [$F^{AD}(\lambda_{ex})$], according to the following relationships [3]:

$$F^{DA}\left(\lambda_{ex}\right) = k^{DA}\left(\lambda_{ex}\right) \int i^D\left(\lambda_{em}\right) d\lambda_{em} = k^{DA}\left(\lambda_{ex}\right) w^D \tag{3.10}$$

$$F^{AD}\left(\lambda_{ex}\right) = k^{AD}\left(\lambda_{ex}\right) \int i^A\left(\lambda_{em}\right) d\lambda_{em} = k^{AD}\left(\lambda_{ex}\right) w^A \tag{3.11}$$

in which w^D and w^A are the integrals of the elementary emission spectra of the donor and acceptor.

These quantities are used to compute experimental values of the FRET efficiency of multimeric complexes containing donors and acceptors. Assuming that direct excitation of the acceptors is negligible at the wavelength of the incident light (*i.e.*, $F^A(\lambda_{ex}) \approx 0$), and using Eqs. (3.10) and (3.11), Eq. (3.7) becomes:

$$E_{app} = \left(1 + \frac{Q^A}{Q^D}\frac{k^{DA}}{k^{AD}}\frac{w^D}{w^A}\right)^{-1}. \tag{3.12}$$

Equation (3.12) has been used to determine the FRET efficiency at each pixel in spectrally resolved fluorescence microscopy following a single scan of the sample at a single excitation wavelength. It elegantly circumvents difficulties associated with classical filter-based methods for the determination of FRET efficiency using two or more excitation scans [58].

3.2.4 *Theoretical Models Used for Information Extraction from Experimental Data*

3.2.4.1 Prediction of FRET Efficiencies for Various Oligomer Configurations

The FRET efficiency, $E_{i,k,q}$, of the i^{th} donor in an oligomer such as the one illustrated in Fig. 3.1 is given by the following equation [3, 58]:

$$E_{i,k,q} = \sum_{j=1}^{n-k} \frac{\Gamma_{i,j}^{FRET} / \left(\Gamma^{r,D} + \Gamma^{nr,D}\right)}{1 + \sum_{j=1}^{n-k}\left[\Gamma_{i,j}^{FRET} / \left(\Gamma^{r,D} + \Gamma^{nr,D}\right)\right]}. \tag{3.13}$$

The quantity $\Gamma_{i,j}^{FRET} = \left(\Gamma^{r,D} + \Gamma^{nr,D}\right)\left(\frac{R_{i,j}^0}{r_{i,j}}\right)^6$ in this equation is the Förster rate of transfer from the i^{th} donor to the j^{th} acceptor through FRET, with $\Gamma^{r,D}$ and $\Gamma^{nr,D}$ being the donor radiative and non-radiative emission in the absence of FRET, $r_{i,j}$ the distance between the fluorophores, and $R_{i,j}^0$ the Förster radius [57, 58]. Note that because the orientation factor can differ between different donor-acceptor pairs in an oligomer [20, 64], the corresponding Förster radii also can differ.

To remove the explicit dependence on external calibration factors, such as spectral overlap between donor emission and acceptor excitation, $E_{i,k,q}$ may be written in terms of the pairwise FRET efficiency, E_p, corresponding to one of the D-A pairs in the oligomer, and the distance between them, denoted by r_1. Thus, we have:

$$E_{i,k,q} = \frac{E_p \sum_j \left(\frac{r_1}{r_{ij}}\right)^6}{1 - E_p + E_p \left[\sum_j \left(\frac{r_1}{r_{ij}}\right)^6\right]} \tag{3.14}$$

where the summation index j represents all acceptors.

By inserting the notations for F_D^{FRET} and $F^D(\lambda_{ex})$ introduced in Sect. 3.2.2 into the FRET efficiency definition (Eq. (3.6)), we obtain:

$$E_{k,q} = \frac{1}{k} \sum_{i=1}^{k} E_{i,\,k,q}. \tag{3.15}$$

This equation signifies that the FRET efficiency for an oligomer consisting of k donors and n-k acceptors, all arranged in a certain configuration, q, equals the average efficiency over all the donors in that particular oligomer. This provides means to compute expressions for the apparent FRET efficiencies of various configurations of donors and acceptors within an oligomeric complex of a certain size and geometry (such as DDAAA, DAAAA, DAAAD, AAAAD, etc., for the pentamer family illustrated in Fig. 3.1). Figure 3.4 provides several examples of possible oligomeric structures as well as the corresponding expressions for each configuration of donors and acceptors within the specific oligomeric geometry.

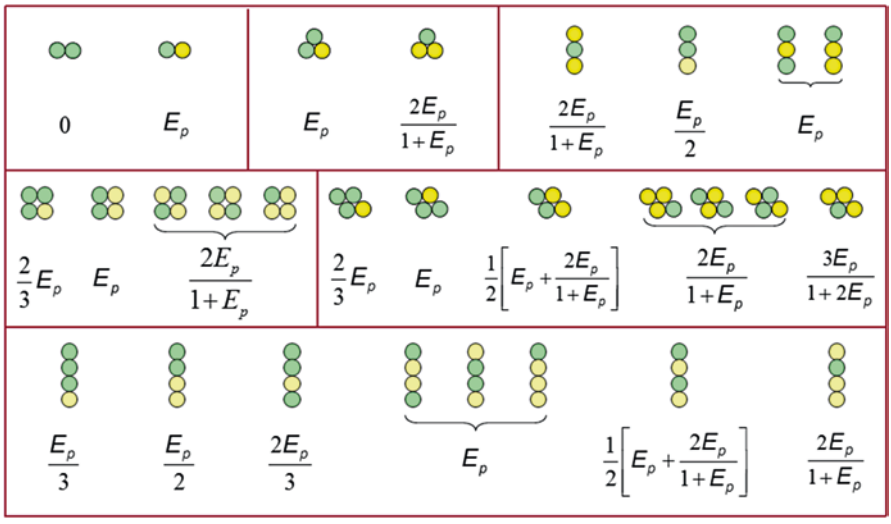

Fig. 3.4 FRET efficiencies predicted by Eq. (3.15) for some oligomers taking simple linear as well as 2D shapes. Only geometries that satisfy the equality $r_1/r_{ij} = 1$ for any non-diagonal r_{ij} distance are considered. In addition, the simplifying assumption is made that energy transfer along the diagonals is negligible

3.2.4.2 Computation of Average FRET Efficiencies for Mixtures of Oligomers

According to the kinetic theory of FRET, the expression for the apparent FRET efficiency for a population of oligomeric complexes in which each complex has the same size (n) and shape is [57]:

$$E_{app,n} = \frac{1}{n\left(1-X_A\right)} \sum_{k=0}^{n} \sum_{q} \left[\left(1-X_A\right)^k X_A^{n-k} E_{k,q} \right] \tag{3.16}$$

where $\frac{1}{n\left(1-X_A\right)}$ is the number of donors within a single complex when averaged over all complexes, X_A is the molar fraction of acceptors in the population of molecules (defined as $X_A = 1/\{1+[D]/[A]\}$, and $E_{k,q}$ is the average FRET efficiency for each oligomer characterized by a donor-acceptor configuration, q. The sum over q represents the sum over all possible configurations; hence there exist $\binom{n}{k} = \frac{n!}{k!(n-k)!}$ configurations for each particular value of k.

The average FRET efficiency for entire regions of interest containing mixtures of complexes with varying sizes and shapes is obtained by summing over the apparent FRET efficiency of each type of complex, $E_{app,n}$, contained within the mixture:

$$E_{app,theo} = \frac{1}{N_{D,tot}} \sum_{n} N_{D,n} E_{app,n} \tag{3.17}$$

where $N_{D,tot} = \sum N_{D,n}$ is the total concentration of donors in the mixture, and $N_{D,n} = n\mu_n (1-X_A)^n$ is the total concentration of donors in oligomers of size n, with μ_n being the total number of complexes of size n within the mixture.

3.2.5 Experimental Applications of FRET

3.2.5.1 FRET Spectrometry

Based on the above simple theoretical considerations, a strategy was proposed previously for determination, at every pixel in a fluorescence image [3], of the *apparent FRET efficiency* (E_{app}), i.e., the average fraction of energy transferred within a population of donor-tagged and acceptor-tagged molecules, some of which may be interacting. The method consists of generating distributions of FRET efficiencies (i.e., E_{app} *histograms*), rather than an ensemble average over a region of interest. Such distributions are then interpreted using models of molecular complexes (or oligomers) with certain *quaternary structures* (i.e., *sizes* and *geometries*) [3, 4], similar to those illustrated in Fig. 3.4. As depicted in that figure, each quaternary structure

(such as a square tetramer) entails multiple configurations of donors and acceptors (three for the square tetramer), each of which generates a specific peak in the E_{app} distribution. Taken collectively, the peaks represent a unique FRET fingerprint corresponding to a certain oligomeric structure, while the E_{app} histograms are veritable *FRET spectrograms*. In this manner, FRET becomes a spectrometric method, akin to that of mass spectrometry, for instance, which can be used for sorting out protein complexes according to their size and shape. FRET spectrometry allows for direct determination of the quaternary structure, especially if the complex is stable in time [4, 25, 26, 66, 67].

Figure 3.5 presents an example of FRET efficiency histogram obtained from a yeast cell co-expressing two sub-populations of the yeast pheromone receptor Ste2, each being tagged with a donor or an acceptor fluorescent protein. The analysis of that histogram using the model of a parallelogram (or rhombus) tetramer (see Fig. 3.4) is also included, which predicts five correlated peaks that best approximated the experimental data.

GPCRs investigated to date using histogram analysis (which include the S. cerevisiae pheromone receptor Ste2 as well as the M_3 muscarinic receptor) could be described as tetramers [3], which may dissociate into stable dimers at lower expression levels [4].

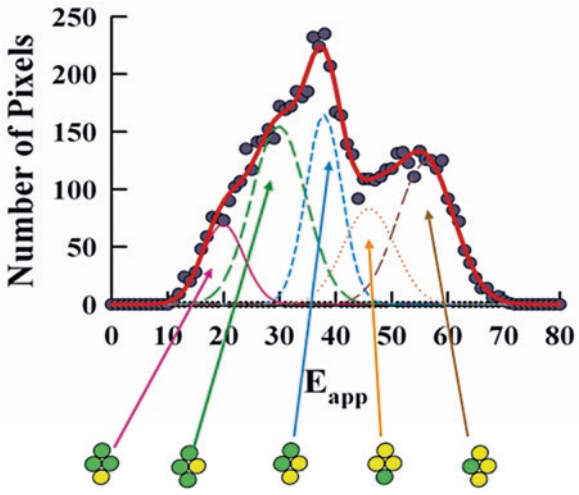

Fig. 3.5 Results obtained using spectral FRET from yeast (*S. cerevisiae*) cells expressing the fusion proteins Ste2p-GFP_2 and Ste2p-YFP. Spectral images acquired with a two-photon microscope with spectral resolution [44] were analyzed as described in the text to obtain pixel-level apparent FRET efficiency values (E_{app}), which were then used to generate the distribution of measured FRET efficiencies. Points represent experimental data, which were fitted to a sum (*thick red line*) of five Gaussian functions also shown individually (*thin color lines*). The position of each Gaussian peak was determined by an equation (see Fig. 3.4) corresponding to a structure indicated below the histogram

While broad histograms with distinctive peaks typically indicate the existence of different donor and acceptor configurations within oligomeric structures [3, 44], it is not always the case that narrower histograms correspond to lower order oligomers. Under certain conditions, GPCRs (or other receptors) may form higher order oligomers but produce rather narrow E_{app} histograms with a single dominant peak. This is particularly true when expression levels are higher than a few molecules per pixel, and or the interaction between dimers within a tetramer is weak and thereby leads to rapid homogenization of the distribution of tetrameric configurations across the field of view. To create a statistical ensemble of an entire population of cells, a method has been introduced whereby the positions of the dominant peaks in the individual E_{app} histograms are accumulated and binned to form *meta-histograms* of peak positions [65]. This method has been used recently to determine the quaternary structure of rhodopsin [66] as well as the Ste2 receptor in the absence and presence of its ligand, the alpha factor pheromone [67].

3.2.5.2 Statistical-Ensemble Approach to FRET

When the concentrations are so high that only an average FRET efficiency may be computed for each cell, the ensemble FRET approach may be used to determine an average oligomeric size upon making certain approximations [34, 43, 52, 68]. To this end, the average FRET efficiency, E_{ave}, and acceptor mole fraction, X_A (or the acceptor to donor concentration ratio) are computed for entire regions of a cell (e.g., the cell membrane or the cytoplasm), and then plotted against each other for a large number of measurements. The mole fraction or donor and acceptor concentration ratio are determined by exciting D and A at two different laser wavelengths, correcting their respective emissions for FRET and then performing a concentration calibration using pure solutions of donor and acceptor molecules [43, 68]. Fitting the experimental data with a theoretical model represented by Eqs. (3.14–3.17) and making certain approximations regarding the geometry of the complex, quaternary structure of the M_2 muscarinic receptor has been determined, which was a rhombus tetramer [34].

3.2.5.3 Combination Between the Two FRET Approaches

Recently it was proposed that the ensemble FRET approach may be used to also determine the relative abundance of the various oligomeric complexes, or the ratio of concentrations of protomers within tetramers and those within dimers, if all the geometrical parameters of the oligomeric species involved are known [66, 67]. In this method, the plot of the average FRET efficiency, E_{ave}, vs. acceptor mole fraction, X_A, is fitted with a theoretical model represented by Eq. (3.17) to the experimental data, with $E_{k,q}$ derived from Eq. (3.15) and the pairwise FRET efficiency, E_p, determined from the analysis of the meta-histograms, the concentrations of various oligomeric species are determined by minimizing a fitting residual. Using this

method, it has been determined that rhodopsin forms tetramers and higher order oligomers in equilibrium with dimers, depending on expression level [66], while Ste2 forms mostly tetramers and dimers whose relative proportion is regulated by ligand binding [67].

3.3 Spatial Fluorescence Correlation Spectroscopy

3.3.1 Principle of the Method

Image cross-correlation spectroscopy (ICCS) relies on the correlation of fluorescence intensity fluctuations measured from an observation volume defined by the diffraction-limited focal spot generated by an excitation scanning laser beam. Two different proteins are each labeled with a fluorescent molecule with different emission spectra. The excitation spectra are sufficiently far apart so that there is no overlap between any one emission and excitation spectrum, in order to avoid complications arising from FRET. A fluorescence microscope is used to acquire separate images of the two signals (see Sect. 3.3.2), denoted by, let us say, *A* and *B*. Grey-scale intensity maps of individual fluorescent contributions may then be correlated in order to determine the concentration, or number density, of both non-colocalized and colocalized fluorescent particles [29]. Next, we will introduce the necessary mathematical apparatus and then we will discuss what colocalization actually means in terms of the degree of aggregation of fluorescent particles.

3.3.1.1 Generalized Spatial Correlation Function

We define a generalized spatial intensity fluctuation correlation function which is a function of spatial lag variables ξ and η, applied to each image pixel (x, y) of fluorescent intensity maps *A* and *B*, given by:

$$C_{AB}(\xi,\eta) = \frac{\left\langle \left(I_A(x,y) - \widehat{I_A}\right)\left(I_B(x+\epsilon, y+\eta) - \widehat{I_B}\right)\right\rangle}{\widehat{I_A}\,\widehat{I_B}} \tag{3.18}$$

where $\widehat{I_A}$ and $\widehat{I_B}$ are the average intensities of spectral channels *A* and *B*, $I_A(x, y)$ is the fluorescence intensity of the pixel located at (x, y) in channel *A*, and brackets denote an ensemble average over all pixel positions (x, y) in the numerator. When image *A* and image *B* are the same, Eq. (3.18) yields a spatial *auto-correlation function*, while if image *A* and image *B* are taken from different spectral channels, Eq. (3.18) yields a spatial *cross-correlation function*.

3.3.1.2 Spatial Auto-Correlation

The generalized spatial correlation function takes advantage of the statistical relation between the variance in intensity measurements about their mean and the average number of fluorescent particles in the observation volume [69]. Assuming no interaction between fluorescent species, the number of fluorescent particles of species A within the excitation volume is equal to the inverse of the square relative intensity fluctuation of species A:

$$\langle N \rangle_A = \frac{\widehat{I_A}^2}{\langle I_A(x,y) - \widehat{I_A} \rangle^2} \tag{3.19}$$

Nevertheless, one cannot determine the concentration of fluorescent species directly from Eq. (3.19) without a method of filtering noise contributions (e.g., white noise from camera, cellular auto-fluorescence, nonspecific labeling, and out-of-focus signal) [70, 71] in order to correct the zero-lags amplitude (i.e., for $\xi = 0$, $\eta = 0$). It is evident from Eq. (3.18) that the inverse of the spatial auto-correlation function when there is zero spatial lag,

$$\langle N \rangle_A = \frac{1}{C_A(0,0)}, \tag{3.20}$$

is equivalent to Eq. (3.19).

Equations (3.19) and (3.20) provide the utility of the correlation function in practical applications; the limit of the correlation function as its spatial variables tend toward zero is equal to the variance of the fluctuations in the fluorescence intensity map. The connection of the spatial correlation function to relative brightness (akin to concentration in the case of fluorescent molecules) and size of image features is demonstrated in Fig. 3.6 using artificial images. We must keep in mind that direct determination of the number of fluorescent particles requires an estimation of the focal volume, and such concentrations are ensemble averages, which become less useful in heterogeneous cellular regions [31]. The correlation function in terms of fluorescence intensity fluctuations produces a Gaussian distribution [31] due to the number of molecules within the focal volume being governed by a Poisson distribution. If all fluorescence intensity fluctuations arise solely from fluctuations in the concentration of fluorescent molecules, the spatial distribution of the excitation energy is directly related to intensity fluctuations and thus the laser intensity profile will become a parameter in the correlation function [29].

The correlation of white noise will alter the average intensity of the image in the numerator of Eq. (3.19) and the auto-correlation of white noise will alter the intensity fluctuations in the denominator of Eq. (3.19) at the zero-lag position. The auto-correlation of randomly distributed and varying intensities on the camera results in a noisy zero-lag amplitude, which is demonstrated using artificial images in Fig. 3.7. When the average noise contribution and zero-lag white noise amplitude is compensated

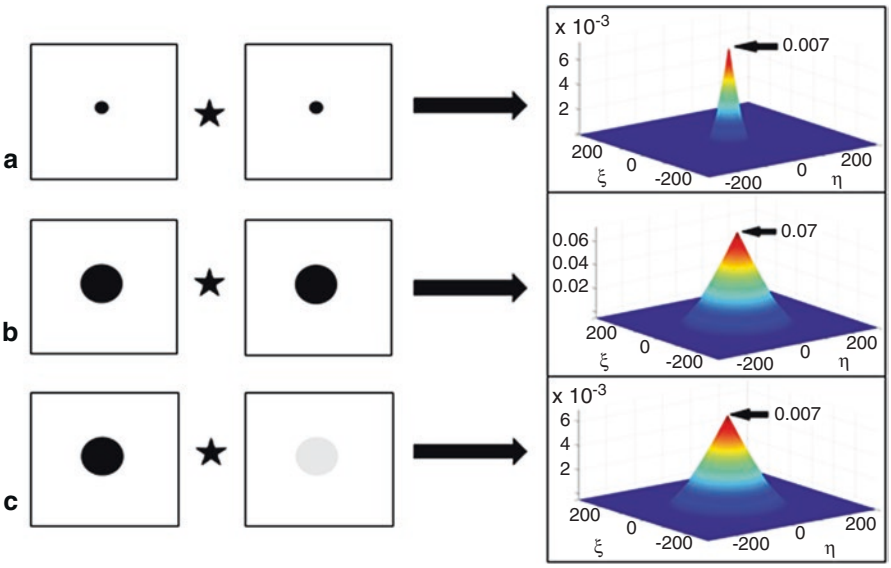

Fig. 3.6 Correlation functions of binary (i.e., *black* and *white*) images demonstrating the dependence of spatial correlation on image feature size and density (i.e., fluorescence intensity). The symbol ★ denotes correlation. (**a** and **b**), auto-correlation functions of images containing differently sized *circles*. The circle in (**b**) is ten times larger than the circle in (**a**), yielding a zero-lags correlation peak with ten times the amplitude and covering ten times the area on the spatial lag (ξ,η) plane; the ratio of the amplitude and area represent a constant "concentration" or brightness of the circles. (**c**) the larger circle is correlated with a circle of the same size but ten times dimmer or "less concentrated", and the amplitude of the correlation function becomes equal to that in (**a**) with an area on the spatial lag plane equal to that in (**b**), demonstrating the process of determining relative concentrations using cross-correlation

for via renormalization of the correlation function, the de-noised zero-lag amplitude of the function may be approximately fitted with a Gaussian function (via non-linear least-squares) [70, 71], or a localized approximation in the case of sufficiently large intensity maps. The Gaussian fit of the correlation function (which does not use the zero-lag position datum, but instead the de-noised correlation data surrounding the zero-lag position) requires the radius of the Gaussian laser beam intensity profile as a parameter, in addition to adjustments for misaligned laser lines and long-range correlations [29].

3.3.1.3 Spatial Cross-Correlation and Colocalization

While the zero-lag amplitude of the auto-correlation function of a single fluorescent species can yield information about its concentration, the zero-lag amplitude of the cross-correlation function between two fluorescent species with completely overlapping excitation volumes can yield information about the concentration of colocalized fluorescent species [29, 31]. When the point-spread functions of the

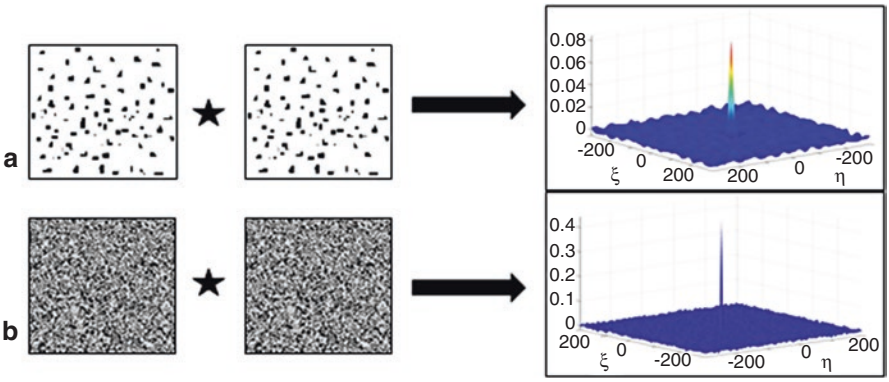

Fig. 3.7 Correlation functions of binary images demonstrating the white noise zero-lag amplitude. (**a** and **b**) demonstrate the formation of a white noise amplitude which approaches a δ-function at the zero-lags position, which must be removed from experimental images before a meaningful Gaussian amplitude can be determined. As image data are split into more and more uncorrelated pixels, the auto-correlation function at points other than the zero-lags position will approach zero, and the zero-lags amplitude will approach infinity as the image dimensions tend to infinity

fluorescent excitation and detection volumes of fluorescent species *A* and *B* are completely overlapping, and no cross-talk exists between spectral channels, an approximate relation may be written,

$$\langle N_{AB} \rangle = \frac{r_{AB}(0,0)}{r_A(0,0) r_B(0,0)}, \tag{3.21}$$

relating the number of colocalized fluorescent species *A* and *B* per beam area $\langle N_{AB} \rangle$ to the zero-lags amplitudes of the auto-correlation functions of *A* and *B* and the zero-lag amplitude of the cross-correlation function of *A* and *B*. The cross-correlation function need only be corrected for noise contributions to the average intensity, as fluctuations in intensity no longer correlate at the zero-lag position [31]. Combining Eqs. (3.20) and (3.21) will yield useful ratios representing the fraction of each fluorescent species that is colocalized with the other, known as *Mander's colocalization coefficients*:

$$M_A = \frac{N_{AB}}{N_A} = \frac{r_{AB}(0,0)}{r_B(0,0)}, M_B = \frac{N_{AB}}{N_B} = \frac{r_{AB}(0,0)}{r_A(0,0)} \tag{3.22}$$

Mander's coefficients yield a fractional representation of colocalization independent of intensity homogeneity [72]. Figure 3.8 illustrates the action of cross-correlation on images, demonstrating how spatial correlation is able to map the location of image features in addition to yielding information on image feature density and size (as in Fig. 3.1). Figure 3.8 also demonstrates a remarkable and perhaps

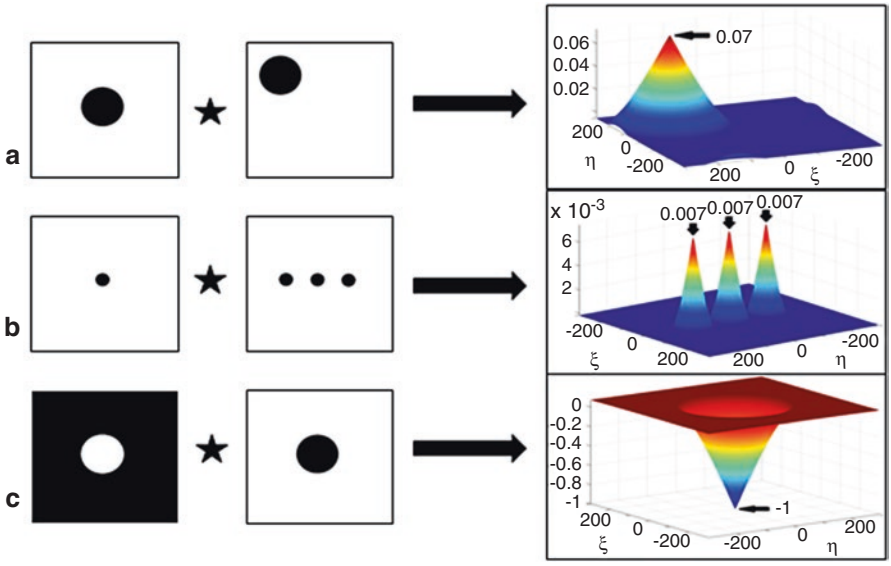

Fig. 3.8 Correlation functions of binary images demonstrating the dependence of spatial correlation on image feature relative position. (**a** and **b**) demonstrate the spatial mapping properties of correlation, and the importance of a zero-lags amplitude in indicating correlation between images; the overall images only correlate when a peak exists at the zero-lags position. (**c**) a negative correlation amplitude is obtainable with significant anti-correlation between images, indicating negative concentration within the theory and representing a practical limit to ICCS wherein intensity fluctuations from the mean tend to fall on opposite sides of the mean when comparing the two images

disconcerting result of Eq. 3.18, in terms of Eq. 3.20: if two images are sufficiently de-correlated, experimental assumptions break down and the correlation function yields a negative zero-lag amplitude.

Even with the high spatial resolution achievable with modern fluorescence methods, colocalization only indicates that the point-spread functions of individual fluorescent species are sufficiently overlapping so that fluorescent contributions in a localized space are indistinguishable from one another; thus a small amount of colocalization information can spill over to neighboring pixels [73]. The molecules are considered associated when their individual densities are equal to the density of the colocalized clusters [71].

3.3.2 Practical Implementation of ICCS

Image acquisition for ICCS may be accomplished with confocal and two-photon microscopes. In the simplest set-ups, the two signals from the fluorescent species *A* and *B* may be separated using dichroic mirrors and two separate cameras to collect

the emitted photons. Alternatively, spectrally-resolved pixel-by-pixel fluorescence intensity measurements, such as using a two-photon microscope with spectral resolution (as described in Sect. 3.2), may be used to detect both fluorescent species used in ICCS without loss of signal. The signals from the individual fluorescent species comprising the spectrally-resolved intensity maps are spectrally deconvoluted, or unmixed, into their respective spectral contributions via linear-least squares fitting algorithms, as described above for FRET (Sect. 3.2.3). To limit temporal decorrelation during scans of the observation volume, measurements should be taken with a high acquisition speed, such as by line excitation [62].

3.3.3 Experimental Applications of Spatial Fluorescence Correlation Spectroscopy

Researchers have taken advantage of the spatial dimension of fluorescence intensity fluctuations, developing a wide variety of commercially-viable techniques such spatiotemporal ICS (STICS) [28], k-space ICS (kICS) [74], raster ICS (RICS) [75–77], particle ICS (PICS) [78] and spatial intensity distribution analysis (SpIDA) [33] (presented in the next chapter), in addition to ICCS. These techniques originated from a spatial analog to FCS, image correlation spectroscopy (ICS), which was introduced in order to quantify the distribution and aggregation of plasma membrane components [69]. ICS uses the spatial and temporal correlation functions for auto-correlation processes alone, yielding concentrations and mobility coefficients but no information on colocalization. It was immediately shown to yield accurate number densities and states of aggregation in the analysis of fluorescent beads [69], and was later applied in order to study the distribution and aggregation state of Platelet-derived growth factor receptors on the surface of human dermal fibroblasts [79]. ICS has been applied to a wide range of biophysical investigations, including the quantification of dendritic spine densities in brain tissue of laboratory animals [80], the study of density, dynamics and interactions of alpha5-integrin in migrating cells [81], and the distribution and dynamics of Paxillin, an adaptor molecule involved in the assembly of focal adhesions [82].

ICCS was the natural next step in applying intensity fluctuation techniques to their fullest extent. It allowed the spatio-temporal analysis of both individual and colocalized fluorescent species, yielding concentrations and mobility coefficients of individual and colocalized species. The heightened sensitivity and detection limits of ICCS have facilitated novel applications in the study of plasma membrane receptor density, the fusion of virus particles to cell membranes, the movement of receptor aggregates to the plasma membrane, as well as the colocalization of biomolecules with spatial resolution beyond what confocal microscopy traditionally provides [70, 83]. ICCS has been applied in the determination of colocalization at the plasma membrane of influenza virus haemagglutinin mutant HA + 8 with macromolecular components of clathrin-coated pits [84], to probe new effects in polynucleotide

release from cationic lipid carriers [85], and to quantify the intracellular transport of polyplexes [86]. Recent ICCS studies have provided insight into the distribution, location and dynamic properties of epidermal growth factor [87], the organization and dynamics of SBD-bound lipid microdomains subjected to cholesterol removal and cytoskeleton disruption [88] and the intracellular dynamics of polystyrene nanoparticles in A549 Lung epithelial cells [89].

Application of fluorescence intensity fluctuation analyses to determine the concentration, colocalization and dynamics of biomolecules is particularly applicable to studying oligomerization of GPCRs at the plasma membrane. Spatio-temporal cross-correlation methods have been applied extensively in order to determine the affinity between recombinantly expressed GPCRs and their ligands (agonists or antagonists). Equilibrium thermodynamic parameters have been found for three distinct examples of human GPCRs: neurotensin receptor type 1 (NTR1), β_2-adrenergic receptor (ADRB2), and C-X-C chemokine receptor type 4 (CXCR4). The kinetic parameters have also been determined for fluorescently-labeled probes interacting with these GPCRs in addition to unlabeled compounds for a broad range of probe sizes [90].

3.4 Temporal Fluorescence Correlation Spectroscopy

3.4.1 Principle of the Method

Fluorescence correlation spectroscopy (FCS) relies on the temporal correlation of fluorescence intensity fluctuations measured from an observation volume over time defined by the diffraction-limited focal spot generated by a laser beam, wherein the level of intensity fluctuations for a single image pixel is measured and correlated over time as the fluctuations evolve in a static spatial domain [91]. ICCS (discussed above) is thus a spatial expansion of FCS to include every pixel within the observation volume. We will find that both spatial and temporal auto-correlation yield the number density of fluorescent particles, spatial auto-correlation yielding an average concentration over an image space and temporal auto-correlation yielding an average concentration over time at a single pixel, indicating localized concentration due to diffusion. Thus, in its practical implementations, FCS is more sensitive to the concentration of biomolecules than ICCS, relying on significant diffusive processes in order to determine spatial parameters. This transformation from the space domain (i.e., ensemble average) to a time domain (time average) is an example of application of the *ergodic principle*, wherein the occupation number should be determined equally well by a time or ensemble average given that the occupation number is a property of equilibrium [71]. The auto-correlation of fluorescence intensity fluctuations evolving over time will additionally yield the diffusion constant D from the time-evolution of intensity fluctuations. We will see that FCS can be applied to determine the oligomerization of fluorescently-labeled plasma membrane GPCRs

and other surface proteins with a spatial resolution generally only approachable with FRET [32].

3.4.2 Theoretical Background of the Method

3.4.2.1 Generalized Temporal Correlation Function

We define a generalized temporal intensity fluctuation correlation function of the temporal lag variable τ applied to a time-series of intensity maps A and B at pixel (x, y):

$$C_{AB}(\tau) = \frac{\left\langle \left(I_A(t) - \widehat{I_A}\right)\left(I_B(t+\tau) - \widehat{I_B}\right)\right\rangle}{\widehat{I_A}(t)\,\widehat{I_B}(t+\tau)}, \tag{3.23}$$

where $\widehat{I_A}(t)$ and $\widehat{I_B}(t+\tau)$ are the average intensities of channels A and B at times t and $t+\tau$ respectively, $I_A(t)$ is the fluorescence intensity of the pixel located at (x, y) in channel A at time t, and brackets denote an average over the image series at all values of τ. When image A and image B are the same, Eq. (3.23) yields a temporal *auto-correlation function*, and when image A and image B are taken from different spectral channels, Eq. (3.23) yields a temporal *cross-correlation function*. Note that Eq. (3.23) may be applied to many pixels in the region of interest, but this will require a separate analysis at each pixel.

3.4.2.2 Temporal Auto-Correlation

Similarly to spatial auto-correlation, auto-correlation as a function of temporal lag will quantify the self-similarity of the signal over time, reflecting the probability that the signal at different times still belongs to the same molecular event. The temporal correlation function is far simpler to visualize as a function of one variable (see Fig. 3.9 below) in comparison to spatial correlation as a function of two-variables Eq. (3.21). While the spatial lag variables in the spatial correlation function could be both positive and negative, decaying about the zero-lag position and forming a 2-D Gaussian, the time variable can only be positive and thus the temporal auto-correlation function will decay as a Gaussian. The rate and shape of this decay will reflect any dynamic processe which contributes fluctuations within the time-series, with the time lag τ being related to the residence time of fluorescence particles within the observation volume. Assuming no interaction between fluorescent species, the number of fluorescent particles of species A within the excitation beam area is equal to the inverse of the square relative intensity fluctuation:

$$\langle N \rangle_A = \frac{\widehat{I_A}^2}{\langle I_A(t) - \widehat{I_A} \rangle^2}. \tag{3.24}$$

In the case of auto-correlation, it is evident from Eq. (3.23) that the inverse of the spatial correlation function when there is zero temporal lag ($\tau = 0$) is equivalent to Eq. (3.24), and so we will again find an inverse relation between the concentration of fluorescent species *A* and the amplitude of the correlation function:

$$\langle N \rangle_A = \frac{1}{C_A(0)}. \tag{3.25}$$

In order to find the amplitude of the spatial auto-correlation function in ICCS we had to account for noise contributions and then fit the function to a Gaussian, as perfectly overlapping spatial correlation of white noise will produce a zero-lag amplitude unrelated to fluorescence fluctuations (see Fig. 3.7). White noise does not auto-correlate over time, and so concentrations can be directly determined from the auto-correlation curve. This seems to make temporal auto-correlation a mathematically simpler alternative to spatial auto-correlation when determining concentrations, but we must also note that this technique requires equipment designed for rapid temporal image acquisition and yields concentration information limited in its localization by the rapidity of fluid movement near the observation pixel [28, 92].

3.4.2.3 Diffusion and Transport Phenomena

The diffusion coefficient *D*, defined as the proportionality constant between the molar flux due to molecular diffusion and the concentration gradient within the observation pixel, can be derived from the characteristic decay time of the temporal correlation function τ_D, which is the average dwell time of the fluorescent species within the observation volume [5, 32, 93]. In actuality, any mobility coefficients which classify two-dimensional or three-dimensional transport phenomena of one of more fluorescent species can be determined by an analytic solution to the correlation curve specific to the case and the shape of the observation volume [28, 91, 92]. The most common transport phenomena are diffusion and flow due to internal dynamics, in addition to the rotation of fluorophores or photo-physical fluctuations [92, 93]. In the case of plasma membrane receptors transport phenomena may be simplified to a two-dimensional model. A good approximation for molecules undergoing free two-dimensional diffusion through a confocal observation volume is a decaying Gaussian profile, normalized by the zero-lags amplitude:

$$C_{AB}(\tau) = r_{AB}(0)\left(1 + \frac{\tau}{\tau_D}\right)^{-1} \tag{3.26}$$

where τ is the time lag variable. In this single-parameter auto-correlation curve the characteristic decay time τ_D is be determined when the auto-correlation curve has decayed to half its amplitude. The fitting of Eq. (3.26) to the correlation function will often require an offset parameter and can be accomplished with a non-linear least squares procedure in order to determine the characteristic decay time τ_D, which can then be related to the diffusion coefficient D:

$$\tau_D = \frac{w^2}{4D}, \tag{3.27}$$

where w is the e^{-2} radius of the Gaussian intensity profile of the focal spot, that is, the distance from the center to where the intensity decreases to e^{-2} of its central value. From the Stokes-Einstein equation we can further relate the diffusion coefficient to the hydrodynamic radius of the diffusing molecule R_H and the microviscosity of the medium surrounding the observation pixel η [5, 32, 92], that is,

$$D = \frac{k_b T}{6\pi\eta R_H}, \tag{3.28}$$

where k_b is Boltzmann's constant and T is the absolute temperature of the sample. Temporal auto-correlation can thus yield insight into molecular size which can be used to characterize structure and binding in biomolecules [93].

3.4.2.4 Fitting the Temporal Auto-correlation Function

Eq. (3.26) provides the auto-correlation curve with a single fitting parameter, τ_D. This serves as a simple first-order approximation which assumes that when the fluorophore's electrons are excited, they exist in the most probable singlet state of paired spins. The fluctuation of fluorescence intensity, or "blinking", is most impacted by the lower probability doublet and triplet electronic spin multiplicity states which are forbidden by quantum mechanics, and thus a transition to these states requires a long period of electronic relaxation in comparison to the singlet state. These transitions between electronic states with different spin multiplicity is known as *intersystem crossing*. The probability of intersystem crossing occurring and greatly impacting auto-correlation curves increases when the vibrational levels of the two excited states in the fluorophore overlap, since little or no energy must be gained or lost in the transition [92]. While these fast events are less likely, the large impact of fast decay parameters will often require a fit to these parameters in order to properly evaluate the auto-correlation curve [5, 32, 92]. Each excited state will contribute a characteristic decay component to the overall auto-correlation curve governed by a characteristic decay time τ_D unique to each state, found from the midpoint of each decay component [32].

The singlet state characteristic decay time τ_{D1} is the slow decay component, with decay rates on the order of milliseconds, characteristic of translational diffusion of fluorescent particles through the observation volume. Singlet state decay characterizes the temporal intensity fluctuations characteristic of transport phenomena, and thus the amplitude of the singlet decay component is the only parameter involved in determining concentration within a multiphasic fit, being equivalent to $r_{AB}(0)$ [5, 32]. The doublet and triplet state characteristic decay times τ_{D2} and τ_{D3} are the fast decay components, with decay rates on the order of microseconds, characteristic of the photophysical properties of the fluorescent probe resulting in reversible transitions between a bright fluorescent state and a "dark" state in which no photons are emitted [92]. In order to expand Eq. (3.26), we consider auto-correlation as a summation of contributions from each decay component:

$$r_{AB}(\tau) = r_{AB}(0)\left[A_1\left(1+\frac{\tau}{\tau_{D1}}\right)^{-1} + A_2\left(1+\frac{\tau}{\tau_{D2}}\right)^{-1} + A_3\left(1+\frac{\tau}{\tau_{D3}}\right)^{-1}\right] \quad (3.29)$$

where A_1, A_2 and A_3 are calibrated constants representing the decay fractions and diffusion times of each component. The triplet state component is often disregarded, as a biphasic fit is often sufficient in accounting for fast decay (see Fig. 3.9) [5, 32]. From the auto-correlation fitting procedure we may determine the important parameter τ_{D1}, which is related to the diffusion coefficient and molecular size by Eqs. (3.27) and (3.28) (see Fig. 3.9).

3.4.2.5 Temporal Cross-Correlation and Colocalization

While traditional FCS analysis does not deal with the consequences of temporal cross-correlation, the theory has been extended to quantify colocalization, in a method that is similar to ICCS. This extension of FCS is called dual-color FCS or fluorescence cross-correlation spectroscopy (FCCS). Temporal series of signals from two spectral channels, representing contributions from two individual fluorescent species, are cross-correlated as described by Eq. (3.23). The cross-correlation amplitude will be positive when a significant amount of fluorescent molecules interact and diffuse together through the observation volume. While the colocalization described in ICCS reflects colocalization over a large spatial domain, colocalization described by FCCS reflects colocalization over a tiny spatial domain as colocalized molecules diffuse [93]. Equations (3.21) and (3.22) may be applied to the generalized temporal correlation function in order to determine colocalized concentrations and colocalization ratios from temporal cross-correlation curve amplitudes, which will theoretically yield similar results to spatial analysis. Similar to FCS, FCCS cross-correlation curves may be analyzed and fit in order to determine mobility and reaction coefficients of colocalized molecules. Because the mass of a molecule may be related to its diffusion coefficient, FCCS allows insight into oligomerization based on the decay components of the cross-correlation curve [91].

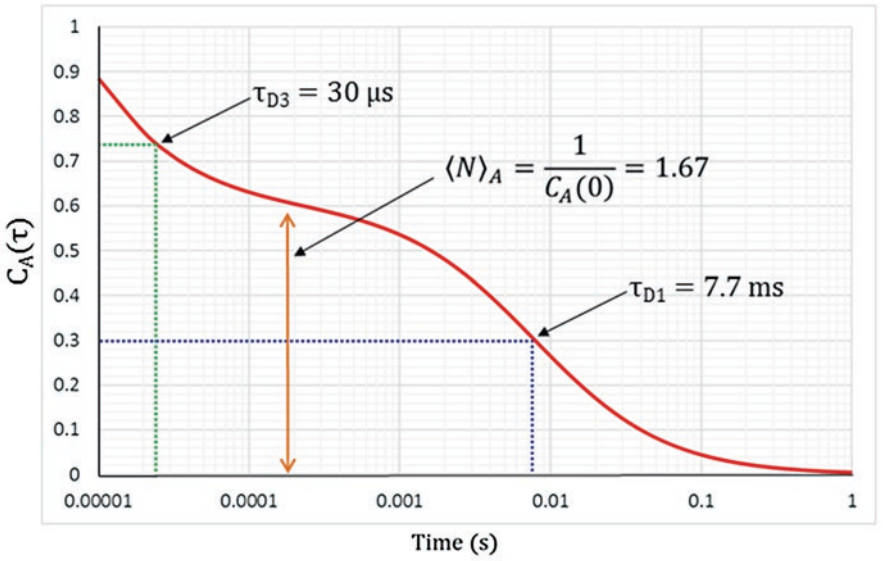

Fig. 3.9 Theoretical auto-correlation curve of a fluorescent system with fast triplet dynamics. Equation (3.29) was used to generate artificial data approximating experimental parameters with 1 μs time steps up to 1 s and plotted (*red*) on a logarithmic scale in order to demonstrate the characteristics of three-parameter temporal auto-correlation fitting. The decay component of the very fast triplet state is distinguishable from the slower decay components of the doublet and singlet states soon after initial excitation, with the primary contribution to the overall decay curve being the slow singlet state. The midpoint of the triplet (*green dotted line*) and singlet state (*blue dotted line*) decay curves yields their respective characteristic decay times. The amplitude of the singlet state decay curve (*orange solid line*) is the inverse of the concentration of fluorescent particles occupying the observation volume (Eq. 3.25)

3.4.3 Practical Implementation of FCS

The principle of measurement in FCS is simple (see [91] and references therein): a laser beam is coupled into a microscope to be either focused at a fixed point in the sample or rapidly scanned across an area. Commercially available single-photon confocal, spinning disk and TIRF microscopes, as well as multiphoton microscopes usually present the requisite temporal resolution for FCS. Detection of photons emitted by the sample is achieved using photo-multiplying tubes, avalanche photodiodes, as well as electron-multiplying cameras. Dispersive elements for providing spectral resolution are also incorporated in some experimental systems, which allow for two- or multi-color temporal cross-correlation measurements.

The relative independence of fluorescence intensity fluctuations from absolute intensity, allowing for low-concentration analyses, can be applied using known monomeric fluorescent properties in order to determine colocalization on a resolution which indicates oligomerization. With the knowledge that the quantum yield,

or molecular brightness, of a fluorescent protein is directly proportional to the number of fluorescent proteins in a molecular complex (with no quenching or fluorescent enhancement), it becomes a simple task to determine the number density of aggregations, and thus the oligomeric size [5, 32]. This is accomplished by applying Eqs. (3.20) or (3.25) for homo-oligomerization of a single fluorescent species and (Eq. 3.21) for hetero-oligomerization of two fluorescent species in order to find the total number of fluorescent molecules within the observation volume. The average fluorescent intensity, or count rate, is equal to the product of the number of each fluorescent species and the known molecular brightness of each fluorescent species. If the count rate is found from imaging and a control group is used to estimate the molecular brightness of a monomeric form of each fluorescent protein, the average molecular brightness of each aggregation can be found [5, 32]. This yields a statistical approach to quantifying oligomerization, which is often used in tandem with a co-analysis involving photon counting histograms or PCHs [94].

In an optical system with high temporal resolution, the observation period can be broken into segments, or "binned", yielding a histogram in which the number of temporal bins are plotted on the ordinate against the number of photon counts found in that bin on the abscissa. A constant intensity light source will theoretically produce a PCH that follow a Poisson distribution. As more fluorescent particles move through the Gaussian laser intensity profile, increased fluctuations in fluorescent intensity result in a broader PCH Poisson distribution [92]. The PCH curve is thus a function of the number of fluorescent molecules and their molecular brightness, with the curve approximating a Poisson distribution as the number of photon counts increases – for this reason often the "tail" of the PCH is the only data used in fitting [32] in order to determine molecular brightness of individual oligomeric species, even within a mixture of oligomeric species.

3.4.4 Experimental Applications of FCS

FCS was originally developed in the early 1970s from the theory of quasi-elastic light scattering (QELS) spectroscopy in order to expand upon perturbation techniques, taking advantage of spontaneous variations from equilibrium as opposed to forced variations (such as temperature-jump methods) [95]. The versatility of this technique was immediately realized and applied to fluorescent chemical kinetics in order to determine the chemical rate constant of the reaction between macromolecular DNA and the drug ethidium bromide to form a fluorescent complex [30]. Fluorescent intensity fluctuates not only in proportion to the changes in the number of fluorescent molecules as they move through the sample volume, but also as fluorescent molecules are created or eliminated by chemical reactions – both transport phenomena and chemical reactions are quantifiable through the same dynamic analysis. Ever-increasing improvements to optical and computational systems have

since allowed FCS to expand into further domains of biomolecular analysis, leading to improvements and expansions upon the theory and methodology.

Application of FCS to the study of membrane proteins became more widely spread as excitation lasers began to be raster-scanned across the sample as opposed to a point-excitation. Theoretical and experimental studies established that FCS could simultaneously study aggregation, chemical rate constants, concentrations and rotational dynamics –. This understanding led to advances in the understanding of lipid diffusion in plasma membranes [96]. Confocal optics allowed for single molecules and complexes to be studied with great depth sensitivity [97], and the application of temporal cross-correlation to two-photon microscopy allowed interaction dynamics to be mapped [98, 99]. The expansion of FCS into the realm of single molecules advertised its future utility and served as a landmark step in the analysis of spontaneous fluctuations. FCS was developed to take into account multiple excitations, yielding flow velocity and direction within microstructured channels from fluorescence correlations between two adjacent focal volumes [100]. This series of refinements in FCS have precipitated studies into oligomerization, molecular size and orientation within plasma membrane GPCRs [5, 32, 33], contributing to the biophysical protein-protein interaction toolkit of coimmunoprecipitation, fluorescence resonance energy transfer (FRET) and fluorescence lifetime imagine (FLIM). Temporal analysis with FCS continues to yield insight into cellular processes and biomolecular interactions; it has been recently applied to monitor diffusion and ligand binding for ion channels, tyrosine kinase receptors, and GPCRs [101] and in the study of oligomerization of various plasma membrane proteins and signal transducers using a FCCS/PCH approach [102–106].

The application of modern temporal fluorescence intensity fluctuation analyses in the high-resolution determination of GPCR oligomerization and oligomeric number has recently become a useful tool in the study of GPCR structure, binding and construct formation. An FCCS/PCH technique has been applied to the determination of the concentration, diffusion coefficients and oligomeric size of fluorescence-tagged serotonin 5-hydroxytryptamine 2C (5-HT2C) receptors diffusing within the plasma membrane of HEK293 cells and rat hippocampal neurons. The molecular brightness values of colocalized aggregations averaged to twice the value of fluorescent protein monomeric controls, indicating that 5-HT_{2C} receptors freely diffusing within the plasma membrane are dimeric without any significant monomeric or tetrameric expression [32]. This method has been applied in order to confirm the homo-dimerization of other class A GPCRs in the serotonin (5-HT2A), adrenergic (a1b-AR and b2-AR), muscarinic (M_1 and M_2), and dopamine (D_1) receptor families over a wide range of receptor expression and agonist treatment levels [5]. At the first glance, those results appear to be at variance with those obtained from FRET. Nevertheless, we will argue below that this need not be the case.

3.5 Advantages and Limitations of FRET, ICCS, and FCS

FRET has proven itself as a useful method that provides information on oligomer size and abundance concomitant with a unique ability to determine intermolecular distances within the oligomer (i.e., quaternary structure). The power of FRET, when carefully implemented into experimental techniques, stems from its sensitivity to distance changes, on length scales of one nanometer or smaller. No amount of statistical analysis could allow extraction of this distance-related information from fluctuations in fluorescence intensities alone or compensate for the lack of it. This unique property of FRET is exploited, for instance, in the method of FRET spectrometry discussed in Sect. 3.2. Nevertheless, this method works best at relatively low expression levels of the receptors. At higher concentrations, the ensemble properties of FRET must be used, which relate the FRET efficiency to statistical properties of the measured fluorescence intensities to provide information on the abundance of the various oligomeric species; this is somewhat akin to intensity-fluctuation-based methods, such as FCS and STICS.

One of main difficulties in using FRET on a broad scale is the need for relatively expensive equipment. Additionally, rigorous application of FRET requires development of rather sophisticated theoretical models to interpret the experimental data. Nevertheless, the latter feature is shared by many of the fluorescence fluctuation correlation techniques, including FCS, and it will most likely improved upon in the future as the method continues to be developed. Incorporation of such models in user-friendly computer programs will dramatically reduce the barrier to adoption of these fluorescence-based techniques.

The near-independence of ICCS from signal proportionality serves as an improvement over similar methods of quantifying colocalization. At the same time, this property also constitutes its practical limitation. Relative homogeneity within observation volumes may overemphasize colocalization despite vast differences in signal intensity (ICCS is thus a measure of "co-occurrence"), while relative heterogeneity of the molecular spatial distribution may lead to opposing intensity fluctuations about each image's mean intensity, yielding a negative correlation value – a result which bears no significance within the theory (see Fig. 3.8).

In comparison to the temporal intensity fluctuations analyzed in FCS, spatial intensity fluctuations allow for a wider application of spontaneous fluctuation techniques, as "fast" diffusion producing significant temporal intensity fluctuations from high concentration or diffusive environments is no longer required to quantify concentration and colocalization. While FCS remained the primary technique of analyzing spontaneous intensity fluctuations in fluorescence images for many years, molecules were often studied in solution or the cytoplasm in order to maximize diffusive fluctuations. Spatial analysis allowed the study of immobile plasma membrane proteins, protein clusters, or even chemically fixed cellular samples. Spatial methods such as ICCS have proven especially useful in experiments where the assumptions of FCS do not hold well due to the movement of macroscopic struc-

tures and low fluorescence concentrations, or where the relative locations of biomolecules become relevant to analysis.

Challenges continue to present themselves in the restriction of concentration ranges used in ICCS and FCS. Because relative fluctuations become smaller with increasing numbers of measured particles, a minimization of the number of particles is required for high accuracy. However, the fluorescence signal must be higher than the background signal – a certain balance must be achieved between the downsides of relatively high noise and the benefit of low concentration, especially in spatial ICCS where white noise auto-correlation peaks mask the true auto-correlation amplitude (see Fig. 3.7). Results can also be difficult to interpret, requiring fitting with theoretical models, the choice of which may be ambiguous. A particularly significant difficulty is for FCS to distinguish between contributions from monomers, dimers and higher order oligomers; the mass must differ by at least a factor of four [91] in order to distinguish respective diffusion coefficients from decay components. That is equivalent to being able to detect monomers and tetramers, but nothing in-between. This problem may be alleviated in the case of single oligomeric species, by using external oligomeric standards to calibrate the photon counting histograms (PCHs, see Sect. 3.4.2.). Recent developments in the field will also allow the possibility of access to lower and higher concentrations, improved selection of theoretical models without over-interpretation, and mass parallel computation which will quickly and cheaply yield combined spatial and temporal analyses [107].

3.6 Reconciling Experimental Results from FRET and FCS

The quantitative FRET studies mentioned above indicated that GPCRs form tetramers, at equilibrium with dimers and sometimes higher order oligomers. In fact, the broader literature on FRET-based methods has provided a wider variety of results, in which some of the same receptors exhibited different oligomeric sizes as reported by different investigators (see, e.g., [4, 5, 7] and references therein). These studies taken together seem to indicate concentration-dependent oligomeric size and appear to be at variance with recent fluorescence correlation spectroscopy studies that were only able to detect stable dimers over a relatively wide concentration range (see above) [5]. While it has been suggested in informal settings that such a wide variety of results may be due to different systematic errors introduced by the varying techniques used, it is also true that the champions of the differing results have used carefully chosen controls in order to reduce experimental errors and avoid trivial and not-so-trivial artefacts. Therefore, herein we explore the possibility that more fundamental physical and/or biological processes ought to be at play, which should explain away any significant discrepancy.

As is implicit in the theoretical treatment presented above, the FRET signatures of dimers or higher order oligomers are more obvious when the complex is stable over periods of time equal to or greater than the acquisition time of the fluorescence signal; depending on the fluorescence intensity level, acquisition times range from

100 μs to several milliseconds per image pixel. From investigations thus-far, it seems that GPCR dimers are relatively stable structures (at least relative to the acquisition times). Higher order oligomers should contribute more significantly if strong binding forces are involved between dimers (i.e., high binding affinity) or when the number of larger oligomers increases, so that during each integration time there is a significant fraction of receptors in large oligomer form. The latter implies, as already mentioned above, concentration-dependent oligomerization, which does not require a huge stretch of imagination.

In its turn, FCS relies critically on the fact that fluorescently tagged receptors diffuse in the plane of the membrane on the investigated timescales. Therefore, if an oligomeric species is not "seen" in the data, that is not necessarily equivalent with that particular species being physically absent from the membrane. In fact, it could be said in that case that the particular oligomeric species is either (***i***) short-lived (compared to the diffusion time-scale) or (***ii***) has very low mobility, thereby contributing mainly a constant vertical shift to the correlation curve [91]. Depending on the concentration of the large oligomers in the membrane, there may be few if any of them at the position of the laser beam used to excite the sample, which would make the constant contribution virtually negligible. In addition, fluorescent molecules are prone to photobleaching (with fluorescent proteins being more so than organic dyes) – a process which occurs after a certain average number of excitations. Since very slow oligomers resident within the focal volume of the laser beam would be excited more often, they would photobleach more severely. This would reduce the already small contribution of large oligomers to the FCS curve even more. An argument could be made, therefore, that FCS detection is biased towards dimers, since dimers are both more stable than monomers and more mobile than the tetramers or higher order oligomers. This is conceivable, especially in light of recent studies suggesting that functional GPCRs form complexes with G-proteins, adenylyl cyclase, and possibly other macromolecules in the signaling pathway [8], in a manner that involves cooperativity and, hence, receptor oligomerization [108–110]. Specifically, tetrameric GPCRs could facilitate binding to other molecules, the latter rendering the former even less mobile than would be expected for a molecular species with fourfold the mass of a monomer. It is also known that the lateral motion of membrane components is constrained significantly by interactions with the cytoskeleton and membrane microdomains, in which GPCRs seem to accumulate [8]. All these ideas point in the same direction of dramatically reduced mobility of tetrameric receptors compared to dimeric forms (or monomeric, if they exist as such).

It should be stressed that the above paragraph is not at all meant as a criticism directed at FCS techniques. In fact, in our opinion, the FCS ability to detect mobile vs. immobile fractions of oligomers in a membrane should be regarded as an opportunity for GPCR research. Such a feature, when complemented with other assays, such as those based on FRET, may be used to probe functional aspects of higher order oligomers vs. monomers or dimers. Detailed studies will need to be performed in the future in order to test this hypothesis and possibly put it to practical use.

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Part II
Receptors

Chapter 4
Class A GPCR: Light Sensing G Protein-Coupled Receptor – Focus on Rhodopsin Dimer

Beata Jastrzebska

Abstract Rhodopsin is a fundamental molecule of the visual system and a prototypical G protein-coupled receptor (GPCR) sensitive to light stimulus. Rhodopsin, like other GPCRs, is comprised of seven transmembrane helical domains embedded in the phospholipid bilayer where it responds to a light signal that ultimately results in a neurological response in the brain. It is highly expressed in specialized post mitotic neuronal cells (photoreceptors) in the retina, localized in internal discs called rod outer segments (ROS). Its structure, function and supramolecular organization within the membrane have been in the center of active studies for decades.

Keywords Rhodopsin • G protein-coupled receptor • Phospholipid bilayer • Post mitotic neuronal cells • Rod outer segments • Dimer interface heterotrimeric G protein • Membrane proteins • Photoreceptor • Rhodopsin • Transducin

Abbreviations

AFM	atomic force microscopy
Cryo-ET	cryoelectron tomography
EM	electron microscopy
GMD	coarse-grained molecular dynamics
GPCR	G protein-coupled receptor
PIE-FCCS	pulsed-interleaved excitation fluorescence cross-correlation spectroscopy
ROS	rod outer segments
TM	transmembrane helix

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4.1 Rhodopsin Structure and Activation

Rhodopsin consists of an apoprotein opsin and an inverse agonist, the 11-*cis*-retinal chromophore, which is covalently bound through a Schiff base linkage to the side chain of Lys296 of opsin protein. In the dark, this Schiff base is protonated, which results in a 498 nm absorption peak. Light activation, however, stimulates chromophore isomerization from 11-*cis*-retinal to all-*trans*-retinal and Schiff base deprotonation, accompanied by a spectral shift towards shorter wavelengths, changing the λ_{max} from 498 nm to 380 nm (Fig. 4.1a). Retinal isomerization also initiates structural changes in the rhodopsin polypeptide chain leading through several intermediate states including Batho, Lumi and Meta I rhodopsin to an active state conformation called Meta II. These changes include rearrangements in several structurally important microdomains that are conserved in class A GPCRs such as the D[ERY] motif located in TM3 and the NPxxY motif in helix TM7. Changes in the D[E]RY motif lead to the disruption of an "ionic lock" between Arg135 on TM3 and Glu247 on TM6 and to rearrangements in the NPxxY motif. These atomic alterations result in the movement of transmembrane helix TM5 towards TM6 and the outward tilt of TM6 that opens up the cytoplasmic surface and provides a binding site for its prototypical G protein, transducin, allowing signal transduction to occur. Based on lower-resolution structural mass spectrometry techniques, such as hydrogen-deuterium exchange and hydroxyl radical footprinting, it was found that light stimulated transition from dark state rhodopsin to its activated state causes a structural relaxation upon retinal isomerization, which then tightens upon transducin binding. The atomic structure of rhodopsin coupled with radiolytic footprinting led to the identification of ordered waters within transmembrane helices located close to highly conserved and functionally important receptor residues. Photo-stimulated structural rearrangements of the protein backbone and side chains are accompanied by reorganization of these structural water molecules that provide a hydrogen-bond network linking the ligand binding site to the effector (G protein, GRK, arrestin) binding site, indicating that these waters are essential not only for structural stabilization of the receptor but also for the activation process [1, 2].

Ultimately, isomerized all-*trans*-retinal releases from the chromophore-binding pocket with activated rhodopsin decaying to opsin and free retinal. Released all-*trans*-retinal is regenerated back to 11-*cis*-retinal through transport to the retinal pigment epithelial (RPE) cells in a series of enzymatic events called the visual (retinoid) cycle. Then 11-*cis*-retinal is transported back to the ROS and reused for regeneration of visual pigment restoring its sensitivity to light (Fig. 4.1a).

Availability of higher and lower resolution structural data of rhodopsin unraveled many details of this receptor activation process. (For a more comprehensive description of rhodopsin activation events the reader is directed to the recent reviews [3, 4]). Instead, this chapter will focus on knowledge accumulated for over a decade on rhodopsin dimerization and its potential functional implications.

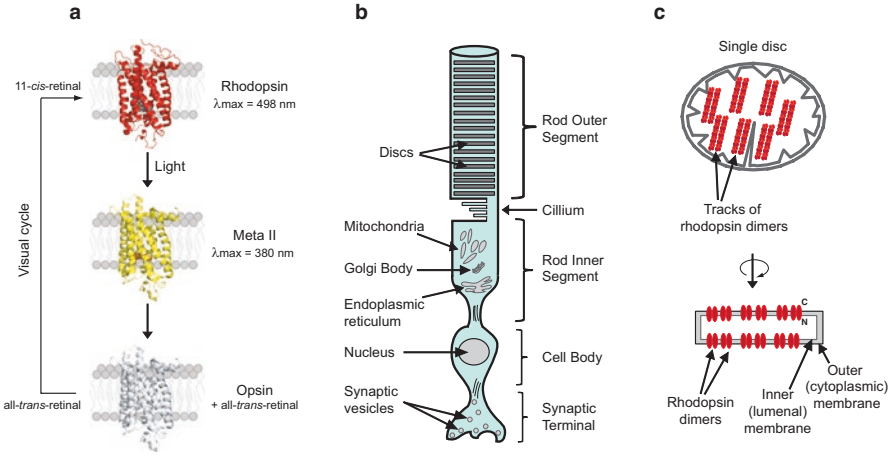

Fig. 4.1 Schematic organization of the retinal rod photoreceptor cell. (**a**) Different photo-states of rhodopsin. (**b**) Rod photoreceptor cell. Important cell segments and cellular components are indicated. (**c**) Schematic organization of rhodopsin dimers in a single rod outer segment disc

4.1.1 Rhodopsin Supramolecular Assembly – Visualization of Rhodopsin Dimer

Rhodopsin is expressed in the outer segment discs of rod photoreceptor cells in extremely high abundance. Each mammalian ROS consist of a stack of 1000–2000 distinct pancake-like discs enclosed by the plasma membrane (Fig. 4.1b). Molecules of rhodopsin occupy more than 50% of the space within the discs with density of ~24,000 molecules/μm^2 [5]. This unusually high protein concentration has enabled not only detailed studies of rhodopsin's biophysical and biochemical properties but also allowed initial insights into GPCR structure. Besides atomic structural details, the first 2D crystals of bovine, frog and squid dark state rhodopsin revealed a protein arrangement that was interpreted as rows of rhodopsin dimers [6, 7]. Such dimeric organization was also observed subsequently in 2D and 3D crystals from several other rhodopsins in both inactive and activated states. The first X-ray crystal structure of dark state rhodopsin determined in 2000 by Palczewski *et al.* also demonstrated dimeric packing, although the dimer was in an anti-parallel orientation [8]. Later, rhodopsin dimers in a membrane-like 'head to head' orientation were found in 2D crystals of Meta I [9] with the contacting surface involving TM1 and cytoplasmic helix H8. Interestingly, parallel dimers with the same interface formed by TM1, H8 and additionally helix TM2 were also shown by the crystal structure of photoactivated rhodopsin [10, 11]. These results achieved by several independent research groups clearly demonstrated a propensity of rhodopsin for self-association. Although such organization of receptor molecules could be driven by the crystallization conditions, it seems to be a common characteristic of other family A GPCRs as well [12–15].

Development of high-resolution imaging techniques and the advantageous high abundance of rhodopsin in the ROS discs allowed visualization of single rhodopsin molecules and their molecular organization in the native membranous environment. In 2003, imaging of the isolated murine disc membranes by atomic force microscopy (AFM) revealed organization of rhodopsin into nanodomains containing tightly packed rows of dimers [16]. Comparison of the rhodopsin organization in disc membranes isolated from wild-type mouse photoreceptors with the organization of opsin in the ROS discs obtained from the photoreceptors of Rpe65$^{-/-}$ mutant mice lacking production of 11-*cis*-retinal chromophore also showed a similar presence of structural dimers organized in paracrystalline arrays, indicating that rhodopsin's supramolecular organization is independent of the chromophore-binding status [17, 18]. These results were somewhat controversial with the suggestion that they were an artifact obtained upon adsorption to the mica [19]. Conversely, they have been supported by various biochemical and biophysical experiments, including the demonstration that such rows of dimers can be isolated from the disc membranes with mild detergents [20, 21]. Most definitively, 12 years later in 2015, the dimeric organization of rhodopsin in disc membranes of ROS was demonstrated by cryoelectron tomography (Cryo-ET) in the intact rod cell [22], supporting earlier AFM imaging. The rapid cryo-fixation of freshly isolated retina employed in these studies preserves the retina structure under close to native, artifact free conditions. Cryosection of the rod cells in two directions: parallel to the disc membrane and along the axis of the rod cells integrated information from both views, revealing the three-dimensional arrangement of rhodopsin molecules in the disc membrane. Analysis of cryo-ET tomograms identified tracks comprised of rows of dimers aligned parallel to the incisures of the photoreceptor disc (schematically depicted in Fig. 4.1c).

4.1.2 Packing of Rhodopsin Dimer and the Dimer Interface

Based on the supramolecular architecture of rhodopsin captured by the AFM images (Fig. 4.2a) and available crystallographic data, in 2003, the first model of higher-ordered organization of a GPCR in its native membrane was derived (Fig. 4.2b). It implicated the involvement of transmembrane domains in contact formation between neighboring rhodopsin molecules within the dimer as well as between distinct rows of dimers [17]. This model suggested that the intradimeric contacts that define the primary dimer interface involve transmembrane helices TM4 and TM5, whereas the formation of rhodopsin dimer rows is facilitated by the interaction between helices TM1, TM2, and H8. A structure of squid rhodopsin, solved soon after, agreed with a TM4/TM5 dimer very well [23]. Likewise, an earlier rhodopsin dimer with an interface formed by TM1, TM2 and H8 was found in the crystal structures of photoactivated rhodopsin [10, 11].

Different experimental strategies have been applied to identify the rhodopsin dimer interface to validate this semi-empirical rhodopsin-packing model. First, site-directed mutagenesis of specific amino acids predicted to be involved in the

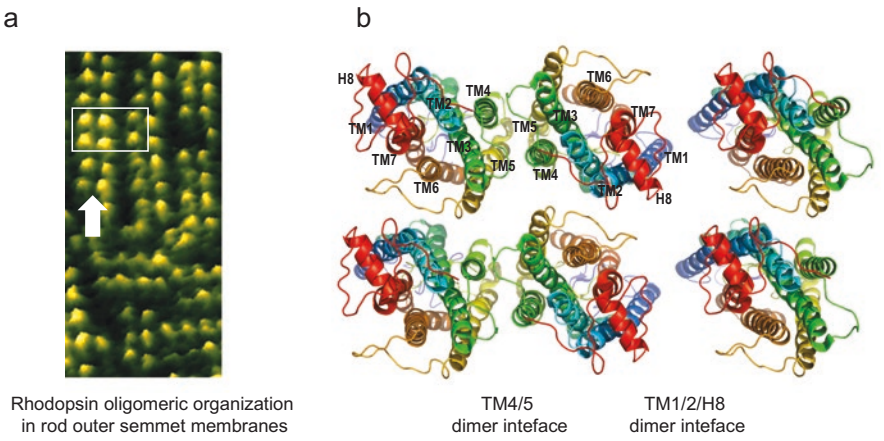

Fig. 4.2 Oligomeric organization of rhodopsin in murine disc membranes. (**a**) Rows of rhodopsin dimers imaged by AFM. An *arrow* indicates single rhodopsin dimer. In the *box*, two rhodopsin dimers from one row and two single rhodopsin molecules from the neighboring row (half of the second row) were selected. (**b**) Model of rhodopsin oligomer derived based on the rhodopsin organization in the rod outer segment discs imaged by the AFM. Two interacting interfaces were found: 1) an interface involving transmembrane domains TM4/TM5 is an internal dimer interface; 2) an interface involving TM1/TM2 and H8 is an interface between adjacent rows of dimers. TM helices are colored as follow: TM1 – *dark blue*, TM2 – *light blue*, TM3 – *dark green*, TM4 – *light green*, TM5 – *yellow*, TM6 – *orange*, TM7 – *light red*, H8 – *red*

formation of the dimer-contacting surface followed by intermolecular crosslinking let to the identification of Trp175 and Tyr206 at the dimer interface of opsin heterologously expressed in membranes of COS1 cells [24]. Trp175 is present in the extracellular loop connecting TM4 and TM5, and Tyr206 is located on the extracellular side of TM5 in agreement with the proposed model of rhodopsin supramolecular organization. Second, an elegant study involving cysteine Cys316-Cys316 crosslinking approach of rhodopsin in the rod outer segment disc membranes in combination with mass spectrometry probed the proximity of helix H8 between adjacent monomers, demonstrating the possibility of the TM1/H8 rhodopsin dimer interface in the native disc membrane [25]. All together, both of these studies support existence of two rhodopsin dimerization modes in agreement with the AFM-derived model. The same two receptor-receptor interaction interfaces involving TM4/TM5 and TM1/H8 also emerged from coarse-grain molecular dynamics (CGMD) simulations of spontaneous rhodopsin assembly [26]. Although TM2 was not mentioned in both these studies, its involvement in the formation of TM1/H8 dimer interface cannot be ruled out.

Furthermore, these early predictions of rhodopsin dimer assembly agreed not only with the structural details derived from later structural studies of rhodopsin [9, 10] but also with crystallographic packing of several GPCRs other than rhodopsin, in which both contacting surfaces TM1/TM2/H8 and TM4/TM5 or TM5/TM6 were found [12–15]. The existence of two distinct dimeric interfaces has also been sug-

gested by crosslinking studies of another family A GPCR, the D2 dopamine receptor, heterologously expressed in HEK-293 T cells at physiological densities [27–29]. Therefore, higher-ordered organization could be a common feature of many if not all class A GPCRs.

4.1.3 *Effect of Rhodopsin Packing on the Structure of ROS Disc Membranes*

Tight packing and higher-ordered organization of rhodopsin most likely is essential in development of ROS. Decrease in the number of rhodopsin molecules available for incorporation into the ROS membranes has significant impact on the structure of ROS discs. For example, mutations resulting in misfolded rhodopsin reduce the quantity of properly folded rhodopsin incorporated to the disc membranes and lead to severe degeneration of ROS [30]. In rhodopsin knockout mice ($Rho^{-/-}$) the ROS do not develop at all. In heterozygous mice ($Rho^{+/-}$) expression of rhodopsin is reduced by half compared to the wild-type mice and ROS are shortened by half as well [31, 32]. These structural alterations are accompanied by changes in the responses to light stimulus [32, 33]. Interestingly, even in the case of 50% reduced expression of the light receptor, a constant density in the individual discs is still maintained after the expression level of rhodopsin is stabilized [34]. Although mechanistic details of this phenomenon are not clear, specific constant density of rhodopsin in the ROS disc membrane might be required for efficient signaling and sensitivity of rod photoreceptor cells [35].

4.1.4 *Dynamic Properties of Rhodopsin Dimer*

While snapshot images of rhodopsin molecules derived from high-resolution state of the art imaging techniques provide clues about its membrane supramolecular architecture, they lack thermodynamic characterization of the receptor-receptor self-association essential to delineate the functional role of these rhodopsin clusters in vision. To address this problem, clustering and mobility of rod opsin has been measured in the live cell membranes by using time-resolved pulsed-interleaved excitation fluorescence cross-correlation spectroscopy (PIE-FCCS) [36]. PIE-FCCS quantifies the population of receptor-receptor dimers/oligomers that co-diffuse as stable complexes in and out of a small area defined by the laser focus while concurrently quantifying the total population of receptors. Therefore, receptor mobility can be measured with a sampling rate close to single–molecule tracking. These studies revealed that opsin expressed in the membrane of COS-7 cells exhibited concentration-dependent dimerization. At low expression levels, it was found to be in a monomer-dimer equilibrium with estimated dissociation constant $1/K_{eq}$ of 1010

molecules/μm^2 suggesting that at high concentrations like those in ROS membrane discs, more than 80% of the total rhodopsin population would be clustered into dimers and/or higher-ordered oligomers. Further, the unique structure of the pancake-like stacked disc membranes of ROS could potentially enforce additional stabilization and further self-association of rhodopsin molecules [5] in its native environment. On the other hand, the modest dimerization K_{eq} would allow the rhodopsin monomers to be present in the ROS membranes as well.

4.1.5 Effect of Lipids on Rhodopsin Oligomerization

Distinct phospholipid composition of biological membranes can promote the propensity of GPCRs to self-associate [37–39]. The lipid composition of ROS is complex and includes a variety of lipid head groups, lipid carbon chains and various concentrations of cholesterol depending on the maturity of ROS [40, 41]. Particularly, in ROS membranes, enrichment in phospholipids with long polyunsaturated acyl chains like docosahexaenoic acid (DHA) most likely influence rhodopsin's rigidity and higher-ordered organization [42]. Experimental support for this deduction also derives from studies of rhodopsin reconstitution into lipid bilayers. Purified rhodopsin self-associates into dimers and/or oligomers during reconstitution into lipid vesicles [37]. Rhodopsin oligomerization is concentration-dependent and affected by the thickness of the membrane bilayer [43]. In a more fluid bilayer composed of phosphatidylcholine (PC) with shorter acyl chains of (C12:0)PC and (C16:1)PC rhodopsin associates nonspecifically with a greater number of contacting surfaces than in a thicker lipid bilayer. In a bilayer of (C20:0) and (C20:1)PC, rhodopsin mobility significantly decreases with selective protein-protein contacts signifying more specific dimerization interfaces including helices TM1/TM2/H8 and TM4/TM5 [26, 38, 43, 44]. Therefore, rhodopsin oligomerization is highly influenced by the lipid matrix that helps to arrange this membrane receptor.

4.1.6 Disruption of Rhodopsin Dimer and Its Functional Consequences

Despite information about the helical arrangement and membrane topology of rhodopsin dimer, the physiological role of rhodopsin dimerization has been unclear. Recently, Jastrzebska *et al.* used TM domain-derived synthetic peptides as a tool to disrupt rhodopsin dimerization to investigate its function [45]. TM1 and TM2 as well as TM4 and TM5 peptides interfered with the formation of rhodopsin dimers as determined using multiple experimental approaches such as size exclusion chromatography, crosslinking of rhodopsin in the bovine ROS membranes with short (7.7 Å spacer arm) disuccinimidyl glutarate (DSG) crosslinker, and bioluminescence

resonance energy transfer (BRET) in HEK-293 cells stably expressing opsin or isorhodopsin regenerated with 9-*cis*-retinal. The TM4 and TM5 peptides inhibited rhodopsin self-association with EC_{50}s of 0.28 μM and 0.36 μM respectively, while the TM1 and TM2 peptides with similar EC_{50} values of ~0.5 μM. The TM3, TM6 and TM7 peptides did not affect rhodopsin dimerization. Thus, these results strongly suggest that TM4 and TM5 transmembrane domains provide a prominent rhodopsin-rhodopsin interacting surface.

The disruption of the rhodopsin dimer with TM peptides decreased protein stability. In fact, as observed previously, rhodopsin organized in dimers and higher-ordered oligomers is much more thermally stable than receptor dissociated into monomers [20, 21]. However, dimerization-disrupting TM peptides had minimal effect on the G_t activation rates *in vitro* and *in situ* in cultured cells, a situation also observed for the cholecystokinin GPCR receptor [46]. Moreover, the negligible effect of TM peptides on the G_t activation rates by Jastrzebska *et al.* [45] agrees with studies demonstrating that a single receptor incorporated into lipid nanodiscs is sufficient for full G protein activation [47]. Nonetheless, the high density of rhodopsin in disc membranes and the abundant evidence for higher-ordered organization strongly support the dimer as a primary structural unit interacting with heterotrimeric G protein.

4.1.7 Consequences of Rhodopsin Supramolecular Organization on Signaling Activation

Early studies on the lateral diffusion of rhodopsin in ROS membranes concluded that rhodopsin is a monomeric entity freely diffusing in the phospholipid bilayer [19, 33, 48, 49]. However, it is important to stress that the movement of rhodopsin is restricted essentially to a two-dimensional environment. This would reduce its dynamic freedom by orders of magnitude relative to soluble proteins. Such reduction in diffusion from three to two dimensions in biological membranes would enhance receptor clustering even in cases of low affinity interactions simply by mass action considerations. In fact, more thorough investigations of GPCR oligomerization revealed an intrinsic propensity to self-associate as a general characteristic of these membrane receptors [24, 38]. Accumulated experimental evidence indicates that the lateral mobility of membrane proteins, including GPCRs, is very much constrained by various mechanisms such as interactions with the cytoskeleton and lipid rafts. Lipid rafts recruit fatty-acyl modified signaling proteins, increasing their effective concentration and obviously affecting their function. Thus, such protein compartmentalization could be a universal mechanism for most adaptive cellular signal transduction [50]. In the case of rhodopsin, visualization of this receptor in the native ROS membranes by high-resolution AFM indicated that it is not distributed evenly, but rather localized into specific nanodomains, where it forms oligomeric arrays [16, 51]. Thus, the concept of rhodopsin

existing as freely mobile monomers had to be revised. In 2009, studies on the lateral diffusion of rhodopsin in amphibian and gecko rods using high-speed dichroic spectrophotometry confirmed an existence of a large fraction of immobile rhodopsin of different sizes in different discs [52]. These rhodopsin aggregates were not noticed in the earlier studies most likely due to not considering the formation of the Meta III photoproduct, absorption of which could easily imitate the postbleach absorbance that was attributed to rhodopsin diffusion. As proposed in these studies, rhodopsin oligomers would rather slow down and eventually stop phototransduction serving as a mechanism controlling the cascade shut-off rates. However, it is not obvious how these highly packed rhodopsin oligomers could assemble dynamically in the rates compatible with the fast responses to light activation. Molecular crowding most likely would not be ideal for efficient signaling if these processes would proceed *via* freely diffusing proteins [53]. In contrast, membrane organization into specialized rafts and nanodomains could facilitate the efficiency and sensitivity required for signaling in ROS [54, 55]. Monte Carlo simulations of stochastic encounters between photoactivated rhodopsin and G_t in the disc membranes [56] indicated that in comparison to the classical framework of freely diffusing monomeric rhodopsin, its ordered packing significantly increases diffusion coefficient of G_t by ~1.5-fold and decreases the encounter time by ~2-fold, increasing the activation rate of freely moving transducin. Thus, these studies greatly supported the concept of a GPCR dimer as the smallest functional unit, organized in higher-ordered structures that provide a kinetic benefit for rapid signaling in response to light [56, 57]. These computational analyses also supported the experimental results indicating faster rates of transducin activation by isolated rhodopsin oligomers than by the monomer [21].

4.1.8 Precoupling of G_t to Rhodopsin Oligomer and Its Effect of Signalling

To satisfy the extremely fast kinetics of the G protein-mediated signaling activation with responses occurring within milliseconds to seconds, it is highly possible that G_t precouples to rhodopsin in the dark prior to activation. Such arrangement would permit rapid light-stimulated G_t binding and signal transmission to effector proteins. Thus, the organization of rhodopsin into paracrystalline rafts would facilitate the receptor-G protein interaction in highly dense environments where diffusion is reduced. However, this binding must be relatively weak to allow proper signal amplification whereby one receptor activates about 10–100 G_t molecules. Indeed, the binding affinity of transducin to rhodopsin in dark conditions is relatively low with a dissociation constant of 60 nM–10 μM [58, 59] and very fast association/dissociation rates [60, 61] but it significantly increases to ~0.7–0.9 nM after light illumination and GDP dissociation from the G_t nucleotide-binding pocket. Light exposure stimulates massive relocation of major signaling proteins, including G_t, which translocates

between outer and inner segments (Calvert 2006). Precoupling of G_t to rhodopsin oligomers would provide a structural platform to ensure sufficient concentration of key signaling molecules in the outer segments, photoreceptor compartments where phototansduction begins, to produce immediate response to the captured photon. This interpretation agrees with particle-based reaction-diffusion simulations applied recently to investigate the consequences of rhodopsin tracks-like assemblies, identified by Cryo-ET in the native ROS, on the kinetics of signaling activation [22, 62]. These computational calculations indicated that the supramolecular organization of rhodopsin is not rate-limiting for producing active G_t and activation of G_t precomplexed with rhodopsin is kinetically advantageous resulting in quick and robust responses [52, 56, 60, 63]. Thus, it is apparent that such membrane nanoorganization of the signaling key elements would have a direct effect on signal transduction in vision.

4.1.9 Allosteric Modulation of Rhodopsin Dimer

The issue of dimer function is complicated further by the asymmetric precoupling of G_t to the rhodopsin dimer. The C-terminus of $G_{t\alpha}$ interacts only with one receptor within the dimer [60]. Conceivably, activation energy could be lost if the neighboring rhodopsin, the one not associated with $G_{t\alpha}$, would be excited by a photon. However, this loss could be prevented if asymmetric rhodopsin activation exists. In fact, ligand induced allosteric modulation between receptor protomers within the dimer has been demonstrated for other GPCRs [64, 65]. Thus, conformational changes induced by a photon in the rhodopsin monomer that does not physically interact with $G_{t\alpha}$ theoretically would induce structural evolution towards Meta II active state in the $G_{t\alpha}$-precoupled rhodopsin [66]. Such a mechanism would be critical to enhance efficiency of rhodopsin activation and potentially its desensitization. Membrane molecular dynamic (MD) simulations of rhodopsin dimers revealed that rhodopsin activation could occur in an asymmetric manner. Structural changes occurring at the dimer interface in helix TM4 of the photoactivated rhodopsin stimulated conformational rearrangements in the neighboring, un-activated rhodopsin leading to disruption of an “ionic lock”, movement of helix TM3 and the opening of the G-protein binding domain, thus triggering an evolution toward the active Meta II state of rhodopsin. In support of such asymmetric activity, cross-phosphorylation between activated and non-active receptors, wherein many of un-activated receptor molecules become phosphorylated has been reported for several GPCRs including rhodopsin expressed in mouse rod outer segment [67, 68]. The structural rearrangements at the dimer interface upon activation were also suggested for D2 dopamine receptor [28]. However, the existence of such a tandem activation mechanism and allosteric communication upon light stimulation between receptors protomers within the rhodopsin dimer has not yet been definitively tested experimentally.

4.2 Asymmetry of Rhodopsin Dimer and Its Interaction with Effector Proteins

4.2.1 Rhodopsin-G_t Complex

Evidence of functional implications of rhodopsin dimerization comes from studies of rhodopsin-transducin and rhodopsin-arrestin complexes. In the early 1980, light-dependent binding between rhodopsin and G_t has been investigated by several groups either in native, isolated disc membranes or reconstituted systems using near-infrared light scattering or surface plasmon resonance spectroscopy (SPR). These studies led to conflicting results, reporting that G_t couples to rhodopsin with 1:1, 1:2 or even 1:4 binding stoichiometry [69–72]. Later mutagenesis and crosslinking studies targeting identification of the surface between these two proteins revealed interactions which single receptors would not be able to satisfy. Ultimately, it was concluded that the rhodopsin dimer must be the smallest unit interacting with a single heterotrimeric G protein. Based on the basic knowledge provided by these early studies and later AFM images of rhodopsin dimers highly organized in oligomeric arrays, in 2004 the first structural model of the rhodopsin-transducin complex was derived and indicated that at least two rhodopsin proteins bind G_t within the complex even though the surface of G_t is large enough to couple to a rhodopsin tetramer [73]. However, to confirm this computationally derived model of the rhodopsin dimer-G_t assembly, structural evidence was necessary. Thus, we developed various strategies for purification and stabilization of this transient complex. In 2011, using single particle electron microscopy (EM) analysis the first low-resolution structure of the rhodopsin-G_t complex was resolved [74]. A 3D map of the complex was ~130 A long and 30 A thick. One end of this map was narrower and could accommodate a rhodopsin dimer, while a wider second end could accommodate G_t heterotrimer (Fig. 4.3a, b). Detailed analysis of available crystal structures of GPCRs that contain crystallographic or non-crystallographic dimers, in particular squid rhodopsin and chemokine receptor type 4 (CXCR4), and models of rhodopsin packing in the native disc membranes (pdb:1N3M) helped achieve the best arrangement of the rhodopsin dimer into the EM-map. To properly model G_t, the C-terminus of its alpha subunit was placed as observed in opsin structures with bound G_t peptide and the N-terminus was positioned in close proximity to helix 8 on the receptor, satisfying earlier light-dependent rhodopsin-G_t interactions. In such an orientation, myrystoyl and farnesyl groups attached to the G_α and G_γ, respectively could be inserted into the phospholipid bilayer. Moreover, opening of the alpha helical and ras-like domains of $G_{t\alpha}$ by about 30° was applied to achieve the 'best fitting' of G_t heterotrimer to the EM-derived molecular envelope of the complex. This is in agreement with the extreme flexibility of the $G_{t\alpha}$ in the nucleotide empty state [75]. In contrast, the high-resolution crystal structure of the complex between nanobody-modified β-adrenergic receptor-Gs complex, did not reveal dimeric organization of the receptor in this complex. However, such result could be attributed to an intrinsic characteristic of this specific GPCR and/or crystallization conditions [76].

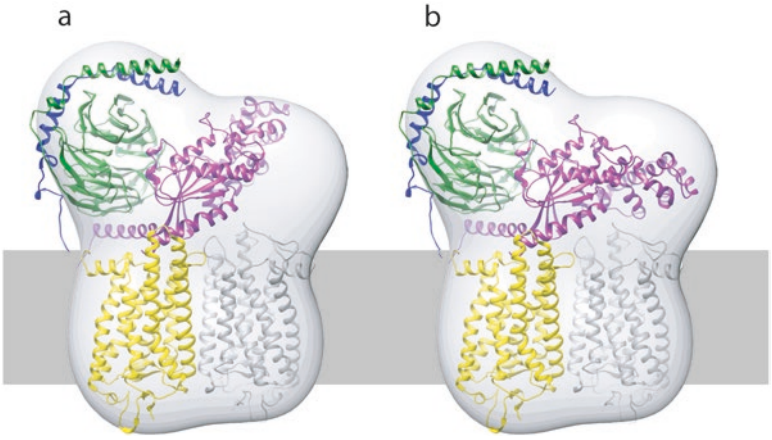

Fig. 4.3 Semi-empirical model of the rhodopsin dimer-G protein heterotrimer complex. (**a**) Fitting of rhodopsin dimer and G_t heterotrimer in the GDP bound state to EM-derived rhodopsin-G_t complex indicated large portion of unoccupied density. (**b**) Movement of the $G_{t\alpha}$ helical domain by 30° in agreement with its greater flexibility upon nucleotide release revealed better fitting of G protein into complex density map. Rhodopsin bound to C-terminal part of $G_{t\alpha}$ stabilized in its Meta II active conformation is colored *yellow*, while the second rhodopsin that eventually decays to opsin is colored *grey*. $G_{t\alpha}$ subunit is colored *pink*, β, *green* and γ *blue*

Although solving of the rhodopsin-G_t complex crystal structure will be required to reveal the molecular details of this physiological assembly, the importance of the rhodopsin dimer in the binding of G_t has been confirmed in subsequent EM studies. Labeling of rhodopsin molecules with succinylated concanavalin A unequivocally demonstrated the association of rhodopsin dimer with G_t heterotrimer within the pentameric rhodopsin-G_t complex [77]. Moreover, by tracing of each rhodopsin monomer with an isomeric variant of the chromophore, we found that rhodopsin dimer coupled to G_t heterotrimer exhibited structural and functional asymmetry [78], which could be critical to prevent loss of activation energy [66, 79]. Only one rhodopsin in the complex, most likely the one that interacts with the C-terminus of $G_{t\alpha}$, was stabilized in the active Meta II state with its chromophore trapped in the all-*trans*-retinal conformation, while the second rhodopsin molecule eventually evolved to opsin and free retinal (Fig. 4.4). Rhodopsin monomer stabilized by G_t loses its chromophore only after complex dissociation with GTPγS. All-*trans*-retinal could be also removed from the complex by the treatment with the strong nucleophile NH_2OH; however its subsequent regeneration with 11-*cis*-retinal resulted in the heteropentamer housing equal amounts of 11-*cis*-retinal and all-*trans*-retinal. This indicated that the receptor molecule coupled with G_t was stabilized in the active conformation even after chromophore depletion. Bound G_t increases receptor rigidity which most likely forces isomerization of unstable 11-*cis*-retinal to its *all*-trans configuration that can be accommodated in the binding pocket of Meta II-like rhodopsin. Tightening of the more relaxed active Meta II structure upon binding of G_t has been observed in hydrogen-deuterium exchange

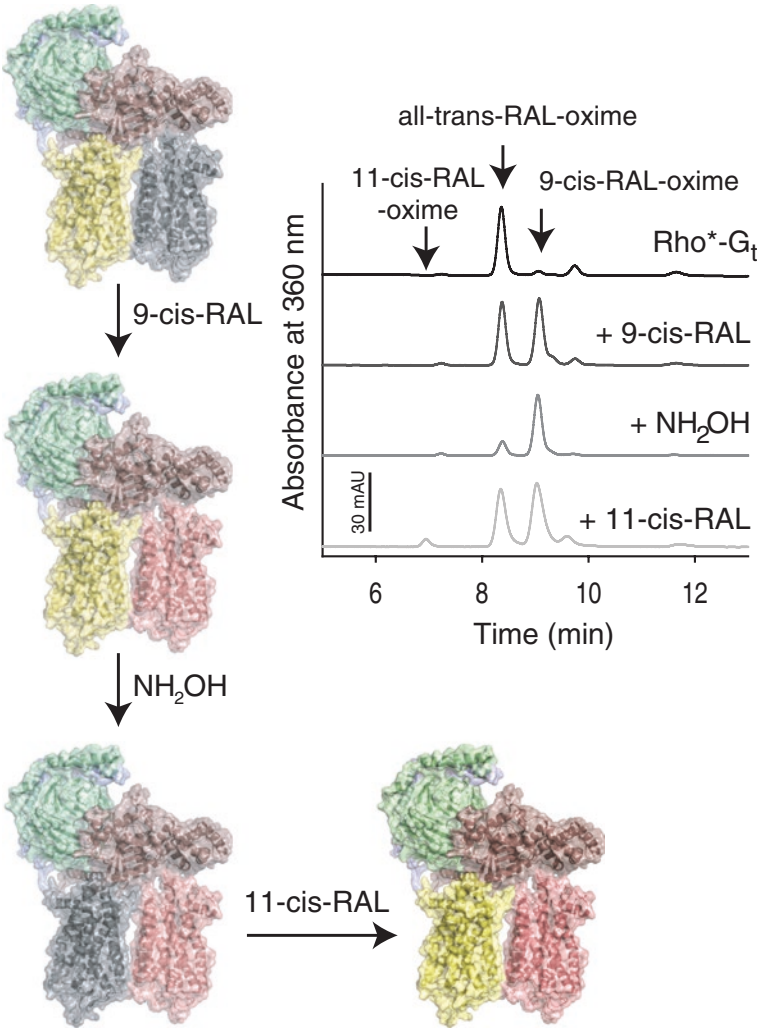

Fig. 4.4 Asymmetry of rhodopsin monomers in rhodopsin-G_t complex. The light-stimulated rhodopsin-G_t (Rho*-G_t) complex prepared on an sConA affinity resin (shown as a *yellow-grey* dimer bound to G_t) was first regenerated with 9-*cis*-retinal (shown as a *yellow-pink* dimer bound to G_t) followed by treatment with NH_2OH (modeled as a *grey-pink* dimer bound to G_t) and then regenerated with 11-*cis*-retinal (shown as a *yellow-pink* dimer bound to G_t). Isocratic analyses of retinoid oximes extracted from Rho*-G_t (*black line*), either regenerated with 9-*cis*-retinal (*dark grey line*), treated with NH_2OH (*grey line*) or regenerated with 11-*cis*-retinal (*light grey line*) are shown. Though only all-*trans*-retinal was detected in the Rho*-G_t complex, a mixture of all-*trans*-retinal and 9-*cis*-retinal with about a 1:1 stoichiometry was found in the Rho*-G_t complex incubated with 9-*cis*-retinal. Treatment of this regenerated complex with NH_2OH caused a significant reduction in all-*trans*-retinal. However, incubation of this complex with 11-*cis*-retinal resulted in formation a complex carrying a mixture of all-*trans*-retinal and 9-*cis*-retinal in about a 1:1 molar ratio. This figure originally was published in the *Journal of Structural Biology*, Jastrzebska B, Ringler P, Palczewski K, Engel A. The rhodopsin-transducin complex houses two distinct rhodopsin molecules. 2013;182(2):164–72. Copyright holder, the Elsevier

(HDX) studies of rhodopsin-transducin complex [80]. The inactive opsin molecule however could be regenerated with 11-*cis*-retinal without complex dissociation, supporting conformational similarity between inactive opsin and rhodopsin.

4.2.2 Rhodopsin-Arrestin Complexes

Rhodopsin photoactivation triggers transition of the dark state to the activated Meta II state capable of binding and activating G protein, which ultimately decays to apoprotein opsin and free retinal (Fig. 4.1a). However, before Meta II decays, its signaling is quenched by receptor phosphorylation with rhodopsin kinase and binding of arrestin-1, which blocks rebinding of G_t to the receptor. As observed by Fourier transform infrared spectroscopy (FTIR), in complex with arrestin, rhodopsin adopts its G_t-bound conformation [81, 82]. Interestingly, these studies demonstrated that during Meta II decay the stoichiometry of the rhodopsin-arrestin complex shifts from 1:1 to 2:1, where arrestin stabilizes only half of the receptor population in Meta II-like conformation, while the other half decays to inactive opsin. These results indicate that arrestin could bind either rhodopsin monomers or dimers depending on the receptor activation state, highlighting the potential functional significance of rhodopsin dimer in signaling desensitization. Moreover, arrestin bound to chromophore-depleted rhodopsin facilitated re-entry of all-*trans*-retinal, a phenomenon that does not happen in case of free opsin. However, only half of the receptor population was enabled for all-*trans*-retinal uptake. In such a scenario, it is highly likely that arrestin binds to two opsin molecules, forming a complex in which one opsin is in its active state and the other in an inactive conformation. This interpretation strongly supports functional asymmetry of rhodopsin dimer, which could be beneficial in clearance of toxic concentrations of all-*trans*-retinal released in bright light conditions, thus protecting photoreceptors from light-induced retinal degeneration. This is consistent with the observation that rod arrestin knockout mice suffer retinal degeneration [83]. Interestingly, inactive opsin molecules could be regenerated with 11-*cis*-retinal without complex dissociation, similarly as observed for the rhodopsin-G_t complex. Asymmetric arrestin-dependent increase in agonist binding to a GPCR receptor is not limited to rhodopsin. The same phenomenon has been observed for membrane-bound β-adrenergic receptor, where β-arrestin induced high affinity ligand binding in only about half of the receptor population [84]. This interpretation is supported by the arrestin structure where two clefts called the C-domain and N-domain are linked by a polar core. The rhodopsin-binding surface is exclusively located at this cleft side of the arrestin molecule. Its bimodal structure and a cross-section as large as the surface of two rhodopsin molecules [8, 85–87] suggest that a rhodopsin dimer could be accommodated by a single arrestin [88]. Conversely, the recently solved high-resolution crystal structure of the rhodopsin-arrestin complex, illustrates binding stoichiometry between these partner proteins as 1:1. This result however could be attributed to the protein construct used for these studies linking the two proteins via a 15-residue linker and the crystallization conditions [89].

4.3 Concluding Remarks

Experimental evidence accumulated over past 10+ years clearly demonstrates that rhodopsin exists in the native ROS membranes as dimers, which cluster into rows of dimers or tracks. Both computational approaches and experimental data indicate that rhodopsin molecules and several other GPCRs are capable of interacting via at least two interfaces TM4/5-TM4/5 and TM1/2/H8-TM1/2/H8. Although the functional implications of rhodopsin organization are not completely clear, a wealth of collected data strongly points towards at least two possibilities: (1) a structural role, where dense packing of rhodopsin into organized nanodomains is necessary for development and integrity of the rod outer segments of photoreceptor cells and (2) a functional role, to achieve efficient kinetics of rhodopsin signaling and sensitivity required for rod photoreceptor cells.

Despite considerable progress made on rhodopsin supramolecular membrane organization and its functional implication further studies must be conducted to delineate remaining questions.

For example, what are the specific amino acids that provide the rhodopsin dimer interface? Improper dimerization/oligomerization could be an underlying cause of some retinal degenerative diseases. More than 60 rhodopsin mutations are associated with human visual disorders called autosomal dominant and autosomal recessive retinitis pigmentosa (adRP and arRP, respectively). Interestingly, several of these mutations are located at the rhodopsin dimer contacting surface within transmembrane helical domains TM4 and TM5, suggesting that impaired rhodopsin dimerization could be implicated in this disease phenotype.

Is the rhodopsin dimer interface locked between certain helical domains or is it a dynamic feature? Does the structural helical rearrangement of the rhodopsin dimer interface occur in response to a photon capture and chromophore isomerization? The major change in the dimer interface that is formed between TM4 and TM5 in the inactive receptor state to the new dimer interface involving TM6 in the active state was demonstrated for metabotropic glutamate receptor [90]. Possibility of the existence of a less stable rhodopsin dimer interface involving TM4 and TM6 was demonstrated in CGMD simulations [26]. These predictions however must be evaluated experimentally.

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Chapter 5
Extreme Vetting of Dopamine Receptor Oligomerization

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Abstract Numerous reports have emerged over the past two decades suggesting that dopamine receptors form dimeric and/or higher-order oligomeric complexes. The existence of these complexes and their functional properties are of significant interest, as they may provide strategies for developing novel therapeutics that selectively target dopamine receptor complexes with the potential for more refined cellular ther-

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apeutics and reduced side-effects. However, there is still great debate and controversy surrounding the structural and functional aspects of dopamine receptor oligomers as well as their physiological relevance. Much of the uncertainty stems from the methodologies employed to understand these complexes, which have clear limitations and/or are not yet fully understood. Herein, we provide an overview of the literature focusing mainly on dopamine receptor homomeric complexes and selected dopamine receptor heteromeric complexes with the goal of providing a critical discussion of the methodology and the logic of the scientific inferences in this body of work.

Keywords Dopamine receptors • Dimerization • Oligomerization • Methodology • Structure • Co-immunoprecipitation • BRET • FRET • Signaling crosstalk

Abbreviations

GPCR	G protein-coupled receptor
D1	dopamine D1 receptor
D2	dopamine D2 receptor
D3	dopamine D3 receptor
D4	dopamine D4 receptor
D5	dopamine D5 receptor
mGlu5	metabotropic glutamate receptor 5
NMDA	N-methyl-D-aspartate receptors
GTP	guanosine-5′-triphosphate
A2A	adenosine A2A receptor
RET	resonance energy transfer
FRET	Förster resonance energy transfer
BRET	bioluminescence resonance energy transfer
TR-FRET	time-resolved resonance energy transfer
A:D	acceptor-to-donor ratio
BiFC	bimolecular fluorescence complementation
BiLC	bimolecular luminescence complementation
CODA-RET	complemented donor acceptor – resonance energy transfer
Rluc	*Renilla* luciferase
E_{FRET}	FRET efficiency
FCS	fluorescence correlation spectroscopy
PIE-FCCS	pulsed-interleaved excitation fluorescence cross-correlation spectroscopy
FRAP	fluorescence recovery after photobleaching
NLS	nuclear localization sequence
TM	transmembrane
IL	intracellular loop
cAMP	cyclic adenosine monophosphate

PLC	phospholipase C
CaMKIIα	calcium dependent protein kinase IIα
NAc	nucleus accumbens
PLA	proximity ligation assay

5.1 Introduction

Dopamine receptors are members of the rhodopsin-like or class A superfamily of G protein-coupled receptors (GPCRs) that mediate the physiological functions of the neurotransmitter dopamine, including modulation of neural signaling processes that play a role in motivation, reward, learning, memory, and motor control, as well as neuroendocrine function [1]. Abnormalities in dopaminergic neurotransmission are associated with various neuropsychiatric and neurodegenerative disorders such as schizophrenia, depression and anxiety, bipolar disorder, attention-deficit hyperactivity disorder, Parkinson's disease, Huntington's disease, drug addiction, and alcohol dependence [1]. Therapeutics targeting dopamine receptors have a long history of use in many of these disorders, most notably dopamine receptor antagonists and agonists, which exhibit antipsychotic and antiparkinsonian effects, respectively.

Five dopamine receptor subtypes, referred to as dopamine D1-D5 receptors (D1-D5), have been identified, and these are divided into two major subclasses based on their structural, biochemical, and pharmacological properties [1]. The D1-like family (D1 and D5) couples to the stimulatory G proteins $G\alpha_{s/olf}$ to enhance adenylate cyclase activity, while the D2-like family (D2, D3, D4) couples to the inhibitory G proteins $G\alpha_{i/o/z}$ to attenuate adenylate cyclase activity. Over the past two decades numerous reports have emerged suggesting that dopamine receptors, in addition to various other class A GPCRs, form dimeric and/or higher-order oligomeric complexes with distinctive signaling profiles and functions. Dopamine receptors of the same subtype have been shown to interact to form homomers (e.g. D2-D2), but they can also interact with different dopamine receptor subtypes (e.g. D1-D2) or different class A GPCRs (e.g. D2-adenosine receptor complexes), or even the class C metabotropic glutamate receptor 5 (e.g. D2-mGlu5) to form heteromeric complexes. While beyond the scope of this review, dopamine receptors have also been shown to interact with N-methyl-D-aspartate receptors (NMDA) from the ligand-gated ion channel family [2–4] as well as with sigma receptors [5, 6], which have been identified as ligand-regulated molecular chaperones. Table 5.1 lists the proposed dopamine receptor homomeric and heteromeric GPCR complexes reported in the literature. The existence of these complexes and their functional properties are of significant interest, as they may provide strategies for developing novel therapeutics that selectively target complexes involving the dopamine receptor, and thus provide more discriminating cellular localization and/or signaling properties, with the potential of reduced side-effects.

Table 5.1 A list of the homomeric and heteromeric dopamine receptor complexes reported in the literature

Dopamine receptor family complexes	**Complexes**	**Selected References**
Dopamine receptor homomers	D1-D1, D2-D2, D3-D3, and D4-D4	[9–23]
Dopamine receptor heteromers	D1-D2, D1-D3, D2-D5, D2-D4, D2-D3, and D1-D5	[9, 21, 24–38]
Heteromeric receptor complexes	**Complexes**	**Selected References**
Adenosine – dopamine receptor heteromers	A1-D1, A2-D2, A2-D3, A2-D4, A2-D2-mGlu5, and A2-CB1-D2	[34, 39–47]
Adrenergic – dopamine receptor heteromers	β1-D4, α1B-D4, and β2-D4	[48, 49]
Angiotensin II – dopamine receptor heteromers	AT1-D1, AT1-D2, AT1-D3, and AT1-D5	[50–53]
Cannabinoid – dopamine receptor heteromers	CB1-D2 and A2-CB1-D2	[47, 54–57]
Cholecystokinin – dopamine receptor heteromer	CCKB-D2	[58, 59]
Corticotropin-releasing hormone – dopamine receptor heteromer	CRF2-D1	[60]
Dopamine – endothelin receptor heteromer	D3-ETB	[61, 62]
Dopamine – galanin receptor heteromers	D1-GAL1 and D5-GAL1	[63]
Dopamine – ghrelin receptor heteromers	D1-GHS1 and D2-GHS1	[64–66]
Dopamine – histamine receptor heteromers	D1-H3 and D2-H3	[67–70]
Dopamine – metabotropic glutamate receptor heteromers	D2-mGlu5 and A2-D2-mGlu5	[46]
Dopamine – neurotensin receptor heteromers	D2-NTS1 and D3-NTS2	[71–73]
Dopamine – opioid receptor heteromers	D1-Mu and D4-Mu	[74, 75]
Dopamine – oxytocin receptor heteromer	D2-OXT	[76]
Dopamine – prosaposin gpr37 receptor heteromer	D2-GPR37	[77]
Dopamine – serotonin receptor heteromers	D2-5HT1A and D2-5HT2A	[78–82]
Dopamine – somatostatin receptor heteromers	D2-SST2 and D2-SST5	[83–85]
Dopamine – trace amine-associated receptor heteromer	D2-TAA1	[86]

Although the implications of dopamine receptor oligomerization have created much excitement, there is still great debate and controversy surrounding many of the structural and functional features of dopamine receptor oligomers as well as their possible physiological relevance. This discussion is part of a larger disagreement regarding oligomerization as a general property of class A GPCRs. Much of the uncertainty stems from the methodologies employed to understand these complexes, which have well-known limitations and/or are not yet fully understood [7]. Reports using similar methods have in some instances reported conflicting results, leading investigators to challenge the existence and purported functions of some dopamine receptor complexes *in vivo* [8]. Failures to replicate published studies are generally not published but are instead discussed informally, leading to further confusion and undermining confidence in the field. We find ourselves at a time when the literature is awash with reports of dimerization, yet it is difficult to conclude with confidence whether most of these complexes exist *in vivo* or play meaningful roles in physiology or pathophysiology.

In this chapter, we provide an overview of the literature focusing mainly on dopamine receptor homomers, which represent one of the most investigated groups of proposed class A GPCR complexes. In addition, we discuss illustrative dopamine receptor heteromer complexes to highlight the challenges and excitement in the field. Our primary goal is a critical discussion of the methodology and logic used in this body of work, with the hope of helping to drive the field toward consensus in the future.

5.2 Early Accounts and Ligand-Binding Studies

The proposal that dopamine receptors might form homomeric complexes was first suggested in the late 80s and early 90s based on radiation inactivation and photoaffinity labeling of solubilized D2 receptors from estrone-induced rat pituitary adenomas and transplantable tumors, which found that the apparent molecular weight of the D2 binding complex appeared to be a multiple of the receptor's monomeric molecular weight [87, 88]. In addition to these biochemical studies, radioligand binding assays revealed different estimates of D2 receptor density depending on the class of radioligand used, with antagonists in the benzamide family, such as [3H]-raclopride or [3H]-nemonapride, yielding B_{max} values significantly greater than ligands in the butyrophenone family, such as [3H]-spiperone, suggesting the existence of multiple classes of D2-like binding sites in both native- and heterologously-expressing tissue [89, 90]. Biphasic curves observed for spiperone competition of [3H]-nemonapride were interpreted as evidence of multiple independent binding sites. Only a single phase was observed when nemonapride was used to compete [3H]-spiperone binding, leading to the hypothesis that a single butyrophenone molecule preferentially bound a dimeric receptor complex whereas benzamides could bind to both protomers within a dimer [89, 91]. However, photoaffinity labeling studies revealed the presence of a higher molecular weight species bound

by the benzamide photolabel but not the butyrophenone, suggesting that benzamides labeled both monomers and dimers, while butyrophenones labeled only the monomer [11].

Other groups failed to observe the same differences in B_{max} between drug classes [92], while others revealed a critical role for assay conditions in observing these effects, especially the concentration of sodium, and proposed a model of negative cooperativity within D2 homomers such that binding of ligand to one protomer of a dimeric complex attenuates the affinity of a second ligand for the other protomer [12]. Importantly, however, this apparent ligand-binding cooperativity might also be explained by simpler models where receptors compete as monomers for a shared pool of G proteins under non-physiological assay conditions, especially conditions of GTP depletion where G protein is sequestered in complex with activated receptor [7, 93]. Nevertheless, these indirect pharmacological observations (reviewed in [94]), and early biochemical observations generated much interest in understanding how dimerization might impact the pharmacology of the dopamine receptors, leading investigators to apply more direct approaches to detect dopamine receptor homomers and heteromers in living cells and brain tissue.

5.3 Detection by Co-Immunoprecipitation and Blotting Techniques

Immunoblotting techniques were some of the earliest approaches used in an effort to detect dopamine receptor homomers and heteromers. Using these methods, investigators have detected monomers, homodimers, and higher order homo-oligomers for D1, D2 and D3 from heterologous expression systems [20, 90, 95–97]. In addition, blotting techniques also detected the presence of dopamine receptor homomers from samples prepared from mammalian brain tissue [11, 20], suggesting that homomer formation is likely not simply an artifact of anomalous or non-physiological levels of expression in heterologous systems. Co-immunoprecipitation has also been used extensively to detect several dopamine receptor heteromeric complexes, including D1-D2 [98] and A2A-D2 [41]. However, it is important to note that immunoprecipitation methods were originally designed for soluble proteins where protein interactions can be studied under their native conditions. Thus, a major concern with both immunoblotting and co-immunoprecipitation studies of GPCR interactions is the use of detergent solubilized receptors extracted from their native environment and the possibility that the detected complexes could be artifacts due to incomplete solubilization and/or aggregation in detergent of receptors that reside and function in the plasma membrane of cells. In addition, most studies fail to address the specificity of pull down by not comparing pull down of a specific receptor with that of other putatively non-interacting receptors.

5.4 Detection in Living Cells by Biophysical Methods

5.4.1 Resonance Energy Transfer Techniques

Due in part to the lack of confidence in studying receptor interactions using methods that rely on detergent solubilization, many investigations have turned to non-invasive fluorescence and luminescence assays based on resonance energy transfer (RET) techniques to detect dopamine receptor interactions in living cells [13, 18, 21, 83, 99]. In general, it has been difficult to measure a robust signal between receptors using traditional ensemble-based Förster RET (FRET) (Fig. 5.1) [100], even for receptors that show robust bioluminescence RET (BRET) and time-resolved RET (TR-FRET), although signals can be readily detected for covalent dimers using FRET in single cells (Fig. 5.1) [100]. BRET and TR-FRET are extremely sensitive and can be readily carried out in widely available plate readers, and there have been numerous reports of energy transfer between labeled receptors for both dopamine homomeric and heteromeric complexes, as well as for a vast number of Family A GPCRs. These studies present evidence that receptors in heterologous expression systems can be in close proximity to each other. However, it is important to recognize that these methods provide a time-averaged ensemble readout, and that RET can occur between receptors that pass near each other by chance as well as from receptors that are truly associated in dimeric or oligomeric complexes. Understanding and distinguishing the RET signal attributed to chance proximity (also referred to as bystander RET) from true dimerization has been a topic of major dispute in the GPCR oligomerization field for the past decade [101, 102]. Recent developments expand the experimental framework and highlight crucial controls necessary to differentiate between interacting and non-interacting receptors.

A hallmark of ensemble-based RET methods has been the notion that energy transfer from oligomerizing receptors should saturate at a maximum value when all donors are part of oligomers and acceptors are in excess. In such saturation studies, energy transfer is evaluated as a function of increasing acceptor-to-donor ratio (A:D), and a hyperbolic relationship between the two has been interpreted as evidence of specific interactions between receptor protomers. On the other hand, for non-interacting molecules, RET should increase linearly only as a function of acceptor concentration (within certain limits), as this dictates the average distance between each donor and acceptor. Therefore, bystander RET should not depend on A:D or donor concentration *per se*. However, two recent BRET studies found that titration methodology as it is most commonly applied does not reliably distinguish between interacting and non-interacting membrane proteins [103, 104]. Importantly, oligomerizing molecules can fail to reach a saturating BRET level, and also non-interacting molecules can produce a hyperbolic relationship between BRET and A:D. These problems arise when co-transfection protocols result in unintended correlations between donor and acceptor expression levels and heterogeneous cell populations within the ensemble, and when BRET is evaluated as a function of the ratio A:D instead of the acceptor density [103, 104].

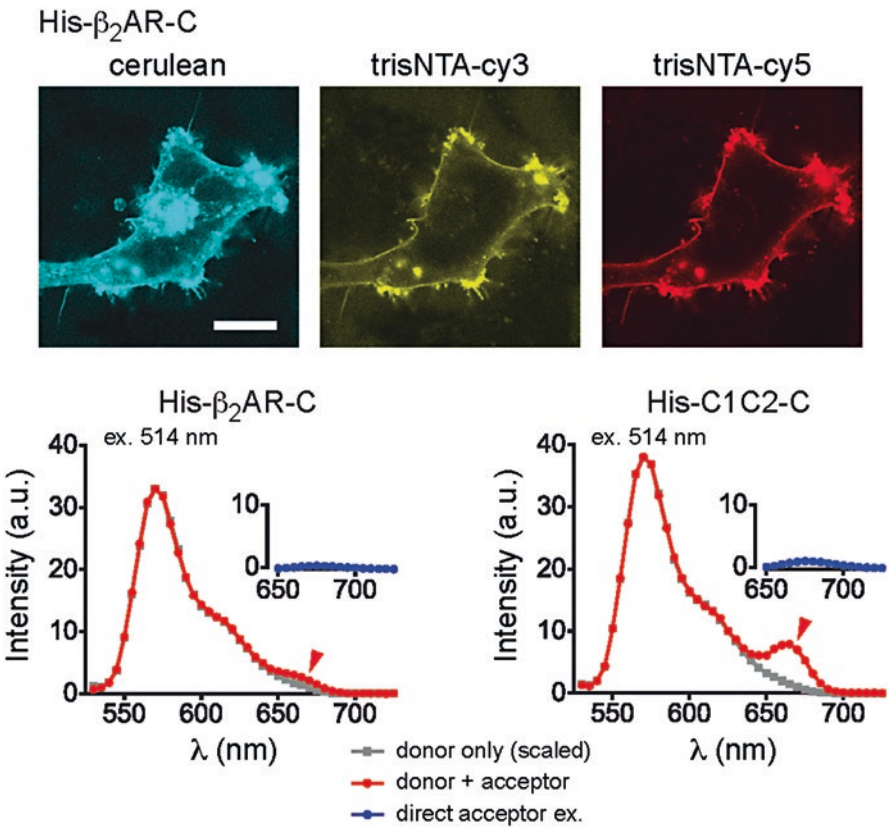

Fig. 5.1 Ensemble FRET between β_2AR protomers is relatively inefficient. *Top*, HEK 293 cells transiently transfected with β_2 adrenergic receptors tagged with 6XHis on their N-terminus and cerulean on their C-terminus (His-β_2AR-C) were labeled with a 1:1 mixture of trisNTA-cy3 and trisNTA-cy5 (a kind gift from Dr. Scott Blanchard). Individual images show cerulean, cy3 and cy5 emission. Scale bar, 20 μm. *Bottom left*, imaging spectra obtained from individual cells expressing His-β_2AR-C labeled with trisNTA-cy3 alone or a 1:1 mixture of trisNTA-cy3 and trisNTA-cy5 are superimposed and normalized to the emission peak of cy3. A small peak corresponding to cy5 emission due to direct acceptor excitation and FRET rises above the donor-only signal, as indicated by the *red arrowhead*. The *inset* shows the calculated cy5 emission component due to direct acceptor excitation with the same scaling. *Bottom right*, an experiment carried out under identical conditions with the channelrhodopsin chimera His-C1C2-cerulean, which forms covalent dimers via disulfide bonds. In this case a clear FRET component stands out above emission due to direct acceptor excitation

Typically, BRET studies of this type rely on transiently co-transfecting plasmids encoding donor- and acceptor-tagged receptors with the assumption that such a protocol produces a homogeneous cell population. For example, in saturation experiments the donor plasmid concentration is typically kept constant while the acceptor plasmid is systematically increased. Szalai et al. [103] and Lan et al. [104] established experimentally that donor expression can decrease with increasing acceptor expres-

sion, and that this can result in an artifactual hyperbolic BRET saturation curve for non-interacting receptors. As an alternative to transient co-transfection, Lan and coworkers used a cell line stably expressing the BRET acceptor under a tetracycline-inducible promoter and transiently co-transfected the donor [104]. This approach provided more uniform control of receptor expression levels, and largely mitigated unintended decreases in donor expression. The researchers also observed that, contrary to widely-accepted models, the net energy transfer generated by constitutive dimers does not saturate. The theoretical framework underlying saturation BRET is based on the assumption that net BRET will saturate if energy transfer within a dimer (intra-molecular) is efficient and energy transfer between dimers (inter-molecular) is negligible by comparison. The authors designed a series of experiments to test both intra- and inter-molecular BRET and found that for covalent constitutive dimers, both processes contribute to the overall net energy transfer. These observations highlight that the net BRET observed in heterologous expression systems is likely composed of more than just a saturation component, and that one cannot necessarily use the hyperbolic fingerprint as a tool to identify oligomerizing receptors. Instead of solely evaluating BRET as a function of A:D, the authors of both of these papers recommended a systematic evaluation of BRET as a function of acceptor concentration for at least two different donor concentrations. Both groups validated this approach with control proteins that could be switched between non-interacting and interacting states.

Another RET method frequently used to identify dopamine homomers and heteromers is TR-FRET [16, 18, 21, 24, 99, 105]. TR-FRET takes advantage of lanthanide donors with long-lived excited states that allow excitation and emission to be separated temporally. Because energy transfer is dictated by the donor lifetime, it persists long after the decay of background fluorescence, and any direct excitation of acceptor fluorophores. Recently, Lan and Lambert [106] found that the use of TR-FRET to quantify oligomerization is complicated by diffusion-mediated enhancement of FRET between lanthanide-labeled transmembrane proteins. The long-lived excited state lifetime of lanthanide donors is on a millisecond timescale, which allows for diffusion in the plasma membrane to bring originally distant donors and acceptors into proximity, thereby enhancing bystander FRET. The authors observed a density dependent increase in FRET from labeled GPCRs, but concluded based on experiments at different temperatures as well as crosslinking and fixation approaches that this effect can be attributed primarily to diffusion rather than to stable interactions.

In summary, comprehensive studies have established protocols for carefully delineating non-interacting and interacting GPCRs using RET methods. Careful characterization of effective receptor surface expression as well as both negative and positive controls for non-associating and covalently-associated molecules is essential to characterize and identify interacting receptors. Furthermore, these studies highlight that simple theoretical models can be misleading when applied to data obtained from heterogeneous cell populations and multiple causes of energy transfer. In the case of dopamine homomers and heteromers, it will be interesting to see such comprehensive protocols implemented, but it is important to note that these more robust methods have yet to be applied to any of the examples shown in Table 5.1.

5.4.2 Fluorescence and Luminescence Complementation Methods

In addition to RET-based methods, bimolecular fluorescence and luminescence complementation (BiFC and BiLC) methods have also been used to argue that D2 and D1 receptors can form homodimers [16] and BiFC and BiLC have also been combined with RET to show that four D2 protomers can interact in living cells at physiological expression levels, suggesting that receptors may form higher order homo-oligomeric structures [16]. In addition, Vidi et al. found that D2 and A2A receptors showed robust BiFC whereas the signal was weaker for D1 and A2A receptors [107]. Notably, the split constructs typically used for BiFC and BiLC form stable interactions during biosynthesis [108], making it possible to observe artifactual complementation, and again, appropriate controls are essential [109, 110].

Urizar et al. [111] devised a strategy combining protein complementation and BRET. In the complemented donor acceptor (CODA)-RET assay, receptors are fused to split versions of *Renilla* luciferase (Rluc) that exclusively operates as an energy donor when the defined receptors are in close enough proximity to complement to form a functional Rluc. The authors measured the coupling between the D1-D2 heteromer-complemented Rluc8 donor and $G\alpha_s$-venus or $G\alpha_{i1}$-venus acceptors, respectively, and demonstrated that selective agonist stimulation of either D1 or D2 resulted in a dose dependent $G\alpha_s$ or $G\alpha_{i1}$ activation, allowing interrogation of signaling from defined homodimers or heterodimers. This CODA-RET method has proven quite useful in characterizing compounds that differentially modulate mGlu homomers and heteromers [112], an example where receptors are well-known to form covalent dimers. However, the situation is more complex for class A GPCRs, since the stability and specificity of the receptor interactions are still in question. Given the irreversible nature of the complementation noted above, CODA-RET is therefore not able to confirm the existence of interactions, but is limited to investigating and differentiating the signaling properties of the defined complexes that have been stabilized using this methodology.

5.4.3 Detection by Single Cell Spectroscopy- and Microscopy-Based Methods

Single cell spectroscopy- and microscopy-based approaches circumvent issues with heterogeneous cell populations and facilitate measurements of transmembrane protein oligomerization in single cells. However, these techniques are highly labor intensive and require many cells to be investigated to achieve the statistics that support significant conclusions.

FRET microscopy imaging has been used to identify dopamine homomers and heteromers [26, 83, 113]. However, as shown in Fig. 5.1, robustly controlled single cell FRET studies of receptors widely reported to interact using multiple methodologies

show virtually undetectable FRET between receptors, whereas a control covalent dimer show a robust signal. A typical set of FRET images is based on time-averaged intensity signals from many receptors per cell, but can allow concurrent measurement of donor and acceptor densities and the efficiency of energy transfer (E_{FRET}) as a read-out of oligomerization. FRET microscopy requires careful correction of signal contamination in both donor and acceptor imaging channels to quantify the actual E_{FRET}, depending in part on the technical approach (sensitized acceptor emission, donor fluorescence recovery after acceptor photobleaching or fluorescence lifetime imaging) [114, 115]. Resolving the FRET signal from contamination produced by direct excitation of acceptor by donor laser-lines as well as cross-emission from donor in the acceptor channel is essential to discerning whether E_{FRET} can indeed be attributed to molecules in close proximity, but it is important to note that bystander FRET may also contribute to the signal. Controls quantifying the FRET levels for non-interacting and covalently-associated molecules are therefore equally important at the single cell level as in ensemble-based plate reader assays. Even more challenging are FRET experiments that use incompletely validated antibodies to label GPCRs for FRET measurements. Unfortunately, our understanding of the limitations of RET-based techniques has lagged their usage, and many reports in the literature are inadequately controlled. This does not *a priori* invalidate the conclusions, but these issues clearly plague the field, as the incentive to carefully repeat published studies and potentially invalidate them is limited.

Another single cell approach emerging to study oligomerization is fluorescence correlation spectroscopy (FCS). FCS provides measurements of receptor density, diffusional properties and particle size based on molecular brightness. FCS methods have shown that the brightness of heterologously expressed D1 receptors was approximately twice that of the purported monomer control and matched more closely to the brightness of several stable dimeric controls over a tenfold range of receptor expression levels [10]. From these results, the investigators concluded that the receptors are predominantly homodimers in the plasma membrane, but do not rule out the possibility that higher order structures may exist at greater levels of expression, for which FCS methods cannot be applied. Although FCS has been used in a number of studies of GPCR dimerization, the technique has limitations that can lead to misleading calculations and conclusions [116]. To overcome potential artifacts, more sophisticated techniques have been developed to extend FCS. One notable example is pulsed-interleaved excitation fluorescence cross-correlation spectroscopy (PIE-FCCS), which has been applied to rhodopsin interactions in the plasma membrane of living cells [117].

Fluorescence recovery after photobleaching (FRAP) is one of the most commonly used microscopy techniques to study the diffusional properties of transmembrane proteins as well as the stability of receptor complexes [118]. To directly assess the stability of D2 oligomeric complexes in the plasma membrane of living cells, Fonseca and Lambert devised an approach where a subset of D2 receptors, selectively immobilized in the membrane by antibody-crosslinking, should serve to decrease the lateral mobility of non-crosslinked receptors if they indeed reside in robust oligomeric complexes [119]. Interestingly, the authors found that the immobile

subfraction had no effect on the mobility of freely diffusing D2 receptors in the basal and agonist bound states, suggesting that the interaction between D2 protomers is at best transient and not sufficiently strong to influence lateral diffusion of each other in the membrane. In contrast, as a positive control the authors directly cross-linked the receptors, and found that immobilization of one protomer had a profound stabilizing impact on the second protomer, as expected for a stable dimer [119].

In an effort to reveal dynamic and kinetic information about dopamine receptor complex formation and dissociation, investigators have recently employed single-molecule fluorescence microscopy methods to detect dopamine receptor dimers. In contrast to ensemble methods that obscure the temporal and spatial information of individual GPCRs, single-molecule approaches might provide an unambiguous conclusion as to whether receptors are in physical contact and for how long. Using single-molecule total internal reflection fluorescence microscopy, investigators reported that approximately 30% of D2 receptors exist as transient dimers, while 70% exist as monomers in the membrane of living cells [120]. The authors used a single fluorophore labeling approach and tracked the fluorescence intensity of labeled receptors diffusing in the plasma membrane. They established the convoluted intensity distributions for a purported monomeric control and used the difference in intensity distributions between the monomer control and D2 receptors to access the overall fraction of D2 monomers and dimers. Although single-molecule methods represent an exciting new approach to detecting dopamine receptor complexes, great care must be taken in effort to validate an actual physical interaction between receptors in living cells. Intensity levels of single fluorophores are highly dependent on local chemical environment and can fluctuate greatly [121]. Furthermore, our understanding of the interactions and microdomains that impact transmembrane protein diffusion in the membrane are still quite primitive, so extensive control and comparison will be necessary. An expansion of the study mentioned above would be to implement a dual labeling approach and to co-localize receptor diffusion in the membrane, as has been done for muscarinic receptors [122]. Also, single-molecule FRET may offer a more direct proof of interaction, as it provides a resolution of 4–7 nm, in contrast to brightness and co-localization, which are subject to the diffraction barrier of ~200 nm. The strictly anti-correlated nature of a single pair of donor and acceptor fluorophores allows assessment of time-dependent changes in GPCR dimerization, and energy transfer should in principle not occur if receptors are not in physical contact [123]. Such studies are yet to be reported for any GPCR.

5.5 Trafficking and Chaperoning

In addition to detecting dimers in living cells, investigators have also explored whether dopamine receptors traffic to the cell surface as monomers or homomeric or heteromeric complexes after synthesis to study what role, if any, oligomerization plays in receptor folding and transport. For instance, co-expression of wildtype D2 with nonfunctional point mutants or truncated variants of the receptor was shown to

inhibit cell surface expression of wildtype D2 [124] and to reduce ligand binding. The authors of this study suggested that direct interactions between functional D2 and the nonfunctional variants may perturb oligomerization of wildtype receptors, which may be required for proper receptor trafficking and functioning. Other studies have observed similar effects on trafficking and/or ligand binding of D3 receptors when co-expressed with a nonfunctional truncated D3 splice variant [125, 126]. In addition, it has also been suggested that homo-oligomerization and hetero-oligomerization of the polymorphic repeat variants of D4 occur in the endoplasmic reticulum and may contribute to proper export and cell surface trafficking of the receptors [23]. In the majority of these studies, it is not clear whether the effects observed are directly associated with a physical interaction between receptors or due to effects mediated by the biological complexities of trafficking and chaperoning. Further research in this area is needed to fully understand how dopamine receptor interactions might influence trafficking and biogenesis.

Using the concept that receptors might traffic as dimers or oligomers between cellular compartments, O'Dowd and coworkers demonstrated that wildtype D1 receptors could be redirected from the cell surface to the nucleus when co-expressed with D1 receptors containing a nuclear localization sequence (D1-NLS) [9]. The authors suggested that a robust interaction between D1 and D1-NLS must exist for D1 to survive the trafficking process. Using this approach, they further demonstrated that introducing structural variations in the receptors within the complex, whether by point mutations or by inverse agonist binding, can disrupt the targeting of wildtype receptor. This observation led the authors to argue that conformational differences between the receptors may serve as a mechanism for regulating the size of the oligomeric complex [9]. It was also shown that both D1 and D2 co-localize in the nucleus when one of the receptor types contains the NLS sequence, which led to the inference that the receptors must hetero-oligomerize [9, 127]. Of note, all of these studies described above have been carried out in heterologous expression systems, and while intriguing, their relevance to native receptor interactions is not yet clear. Additionally, although the authors inferred that the interaction between receptor protomers must be robust, results of several other studies aimed at directly assessing the stability of dopamine receptor complexes as well as other class A receptors are more consistent with weakly and transiently interacting receptor complexes [119, 120, 128], and it is difficult to reconcile these two lines of inquiry. Thus, it is possible that the co-trafficking of receptors observed by the NLS method may also be related to processes associated with trafficking rather than exclusively occurring due to the affinity of one receptor for another.

5.6 Structural Organization and Stability

The structural organization of dopamine receptors in a homomeric complex has garnered much attention, with multiple studies focusing on delineating the homodimer or homo-oligomer interface(s). Initial studies used synthetic peptide sequences

derived from specific transmembrane regions of receptors to disrupt dimer formation as a probe of the interface. For instance, a peptide composed of transmembrane (TM) 6 of the β_2-adrenergic receptor was shown to block dimerization and ligand-induced activation of the same receptor [129]. Incubation of immunoprecipitated D2 with peptides containing the amino acid sequence of D2-TM6 and -TM7 was reported to disrupt the interaction between the receptors in a receptor- and TM-domain-specific manner [90]. In contrast, in a similar study a peptide sequence corresponding to D1-TM6 was unable to disrupt interactions between immunoprecipitated D1 [97], leaving open the possibility that other TM interfaces may play a role.

Using cysteine cross-linking, investigators demonstrated that TM4 can serve as a symmetrical dimer interface between D2 protomers [14], and a study using truncation variants of D2 also supported TM4 as the predominant TM-domain for homodimerization [130]. Susceptibility to crosslinking was shown to be affected by agonist and inverse agonist binding, and one crosslinking orientation of TM4 increased basal activation [15]. Taken together, these results suggest that a conformational change at the dimer interface may be involved in receptor activation. In a complementary study, it was also shown through cysteine crosslinking that residues near the extracellular end of TM1 and in intracellular helix 8 formed a second symmetrical interface for D2, which is only possible if the receptors form higher order oligomers in the plasma membrane [16], or if these interactions are transient and thus represent collisions in different configurations. While these crosslinking studies showed an apparent lack of dependence of crosslinking efficiency on receptor levels, it is nonetheless possible that receptor diffusion is fast enough and/or receptor concentration in a microdomain is sufficient to allow collisional crosslinking to confound the inferences drawn.

Consistent with the studies described above for D2, Marsango and coworkers used TR-FRET and alanine mutagenesis of residues predicted by molecular modeling to be involved in the D3 homodimer interfaces to show that the receptor protomers interact by utilizing at least two different interfaces, one composed of residues from TM1, TM2, and helix 8, and the other from TM4 and TM5 [22]. Ultimately however, the results of this study support contributions from all of the structural regions probed, including TM domains 1, 2, 4, 5, 6, and 7, as well as helix 8, suggesting multiple potential interfaces, again consistent with the possibility of collisional interactions.

Interestingly, the proposed interface(s) for dopamine receptor heteromers do not strictly involve interactions between TM regions as has been demonstrated for homomeric complexes. Instead, investigators report that heterodimer formation can be disrupted mainly by manipulating proposed electrostatic interactions between patches of negatively charged residues or phosphorylated residues on one protomer and a patch of positively charged arginine residues on the other [131]. For instance, for the D1-D2 heteromer, it has been reported that glutamic acid residues in the carboxyl tail of D1 interact with arginine residues in intracellular loop three (IL3) of D2 [132]. Likewise, it has been argued for the A2A-D2 heteromer that arginine residues in IL3 of D2 interact electrostatically with a phosphorylated serine in the carboxyl tail of A2A [133].

Overall, the results from these studies indicate that dopamine receptor homomeric and heteromeric complexes do not form disulfide linkages between protomers and are proposed to associate mainly through non-covalent intermolecular forces, as has also been suggested for many of other class A GPCR complexes [134]. The presence of multiple interfaces and contributions from nearly all of the TM bundles does not point toward a conserved or common interface between protomers of dopamine receptor complexes, which seems surprising if a stable interface is indeed formed, considering that the tertiary structure of class A GPCRs is so highly conserved. Instead, these studies seem more consistent with a model of weak and transient interactions between dopamine receptor protomers, and considering the extensive and diverse list of proposed dopamine receptor dimer complexes (see Table 5.1), imply a general model in which robust interactions are unlikely and receptor pairings may be interchangeable. Whether all or a subset of the interactions between dopamine receptor protomers are biologically relevant has yet to be elucidated, and will likely serve as a focus of new and potentially exciting research in this area. Indeed, even transient interactions have the potential to be important, and the recent estimate of the lifetime of an individual D2 receptor dimer of approximately 0.5 s [120] is certainly sufficient for such a complex to have important signaling ramifications, especially when receptor compartmentalization and membrane microdomains are considered, although demonstrating signaling from a transient dimer will be technically challenging.

5.7 Crosstalk, Signaling and Allostery

5.7.1 Signaling and Allostery from Defined Homomeric Complexes

Few studies have reported on the functional significance and characteristics of dopamine receptor homomers due to the difficulty in distinguishing one protomer from the other in a signaling unit. To overcome this challenge, Han *et. al*. developed a functional complementation assay involving D2 where the individual contributions of each receptor protomer in a dimeric unit to G protein signaling could be delineated and studied [29]. Using this approach, the authors suggested that the minimal signaling unit consists of a D2 dimer and one G protein: agonist binding to one protomer within the dimer maximally activates G protein, while agonist and inverse agonist binding to the second protomer attenuates and enhances signaling, respectively. These results suggest that the mechanism by which D2 contributes to the activation of G protein is asymmetrical, and that the two protomers in a signaling unit adopt distinct conformations in a fully activated homodimer. This conclusion is supported by a study [18] where the FRET signal observed between D2 receptors labeled with fluorescently conjugated agonists was significantly smaller than D2 receptors labeled with a mixture of fluorescently conjugated antagonists

and agonists. Again, it is important to note that while these strategies report on the population of receptors that must be together to signal or enable a FRET signal, and therefore provide important information on the function of such a complex, they do not read out on signaling of native receptor and cannot inform us as to the generalizability of the results when receptors perhaps need not interact to signal.

Based in part on the functional complementation system described above, investigators have proposed a novel mechanism for one of the first drug-like allosteric modulators of D2, SB269652 [19]. SB269652 is a bitopic ligand composed of orthosteric and allosteric pharmacophores chemically linked together, and was developed as part of a series of studies aimed at developing new subtype-selective dopamine receptor antagonists for the treatment of schizophrenia and other central nervous system disorders. The investigators showed that SB269652 binds one D2 protomer by occupying both the orthosteric site as well as a secondary binding pocket that must be engaged in order to observe the allosteric pharmacology. Thus, when SB269652 is bound to one protomer, both the orthosteric binding and secondary binding pockets are occupied, and the compound would be expected to competitively inhibit ligand in the orthosteric site. That the effect of SB269652 is in fact allosteric suggests the possibility, supported by complementation studies, that a SB269652-bound D2 receptor allosterically modulates the binding of orthosteric ligands to a second D2 protomer through the physical crosstalk in a homodimer.

While these complementation studies have begun to shed light on how engineered D2 protomers within a defined homomeric complex might interact functionally in heterologous expression systems, it is important to determine how these findings relate to native receptor interactions. Interestingly, the allosteric effects of SB269652 are preserved in native striatal membranes [19], providing one of the first proof of concept experiments that indirectly suggest that dopamine receptor homodimeric complexes might be specifically targeted to modulate receptor functionality and that such complexes represent a potential target for the development of new therapeutics. However, it is important to note that the evidence is still indirect: it is conceivable that SB269652 can bind not only in the orthosteric site, but also in an alternative pose that would not require its action at a dimer. Experiments in nanodiscs where the receptor is an isolated monomer will help to resolve these scenarios. In addition, assuming that the conformations sampled by one promoter are significantly influenced by its physical association with another, it may be possible to apply methods used to observe conformations of purified class A GPCRs in solution [135–138] to dopamine receptors in a nanodisc that can accommodate multiple receptors, or diffusing within proteoliposomes or even a cell membrane. These methods could then be used to determine how the conformations of one protomer are influenced by its interaction with another and eventually could be related to changes at the level of signaling and behavior.

5.7.2 *Studies of the D1-D2 Heteromeric Complex*

Most studies concerned with the functional significance of dopamine receptor complexes have focused on heteromeric complexes and have been reviewed recently [134]. Here, we will discuss mainly studies involving the D1-D2 heteromer, which has received much attention as well as the most scrutiny, and briefly the D2-A2A heteromer (see Sect. 5.7.3).

The dopamine D1 and D2 receptors couple to the $G\alpha_{s/olf}$ and $G\alpha_{i/o/z}$ G proteins, respectively, and their activation is known to regulate cyclic adenosine monophosphate (cAMP)-mediated second messenger signaling. Historically, reports of D1-like ligands activating phosphatidylinositol hydrolysis and mobilizing increased cytosolic calcium levels suggested that these receptors might also be involved in alternative signaling pathways and led to the search for non-canonical dopaminergic signaling mechanisms. To date, progress in the field has revealed several alternative signaling patterns through other G protein and G protein-independent pathways [1]. In this line of exploration, much effort has gone into also characterizing the biology and signaling of a putative D1-D2 heteromer [134, 139].

Lee and co-workers [98] observed a dose dependent release of intracellular calcium exclusively when D1 and D2 were co-expressed and co-activated by selective agonists (D1, SKF-81297 and D2, quinpirole) in heterologous expression systems. They further reported that the calcium signal was unique to the $G\alpha_q$ signaling pathway and involved stimulation of phospholipase C (PLC). Such a synergistic signaling pattern pointed toward the hypothesis that a D1-D2 heteromer might exist as a distinct signaling unit. In particular, the D1 partial agonist SKF-83959 has been reported to selectively activate the D1-D2 heteromer-specific calcium signal without any effect on dopaminergic cAMP signaling [36]. Injections of this drug were shown to increase the levels of calcium dependent protein kinase IIα (CaMKIIα) in the nucleus accumbens (NAc) of rats. Hasbi *et al.* [26] further linked this D1-D2 heteromer specific CaMKIIα increase to a subsequent increase in brain derived neurotropic factor and neuronal growth.

Others have questioned the pharmacological profile of SKF-83959. Lee et al. [140] failed to observe the proposed PLC activity of the compound and furthermore concluded that SKF-83959 is a partial agonist for D1-mediated cAMP synthesis [140]. Chun et al. [141] found no SKF-83959 induced calcium release in cells co-expressing D1 and D2. To the contrary, they report that SKF-83959 showed an antagonistic effect on the calcium response observed following dopamine treatment. The authors also report that a broad range of other GPCR families (serotonin, dopamine and the adrenergic receptors) are cross-activated by SKF-83959. Frederic et al. [8] utilized the CODA-RET assay (see Sect. 5.4.2) to probe the D1-D2 heteromer coupling to $G\alpha_q$ and failed to observe any dose dependent $G\alpha_q$ activation with either dopamine or a combination of quinpirole and SKF-83959. As a positive control, the authors used the $G\alpha_q$ coupled muscarinic acetylcholine M1 receptor and observed a concentration dependent activation of BRET upon agonist (acetylcholine) treatment. To test the compound *in vivo*, the researchers evaluated SKF83959-

induced behavioral effects in mice and showed that the compound induced significant locomotor and grooming responses in wildtype mice that remained intact or increased in D2 and $G\alpha_q$ knockout lines. However, these effects were eliminated in D1 knockout mice, demonstrating that D2 and $G\alpha_q$ are not essential to the SKF-83959-induced behavioral responses, and points toward SKF-83959 playing a role in classical D1 receptor signaling.

In vivo co-expression of D1 and D2 in the striatum has been the subject of much debate. Electron microscopy and *in situ* hybridization has supported segregation of the two receptors [142–144], while limited co-expression has been observed by fluorescence microscopy [37, 113, 145]. Perreault et al. [113] employed immunohistochemistry and confocal microscopy to measure the number of D1 expressing cells also expressing D2 receptors throughout the basal ganglia and found that ~20% of identified D1 neurons also expressed D2 within the striatal regions of the NAc core and shell. Furthermore, a physical interaction between D1 and D2 has been observed by combinations of FRET microscopy and immunohistochemistry in rat striatal regions [26, 113].

In contrast, Frederic et al. [8] found very limited co-expression using immunohistochemistry in the NAc shell in mice, rats and monkeys. Specifically, the authors crossed two transgenic reporter mice lines expressing fluorescently tagged D1-tdTomato and D2-eGFP respectively, and found that in the dorsal striatum core only 2% of cells co-expressed D1 and D2, whereas parts of the NAc shell displayed a maximum of 7% co-expression. Even when focusing on the cells with co-expression, no co-localization was observed using immunostaining of D1 and D2 receptors, suggesting that the receptors are segregated within the neurons [8]. Petryszyn and co-workers recently reported similar results, finding that neurons co-expressing D1 and D2 were heterogeneously scattered throughout the NAc with a representation of 14.7% and 7.2% in the core and shell respectively [146].

In a complementary approach, Frederic et al. used a proximity ligation assay (PLA) [43] to detect heteromeric complexes in wildtype, D1, and D2 knockout mice, as well as in mice with virally-mediated D1 and D2 overexpression; only following viral overexpression of both receptors was a detectable PLA signal above background observed [8]. The lack of consensus between laboratories, despite the use of related techniques, is puzzling and speaks to the complexities of the approaches and the vagaries and imperfection of the reagents, such as the anti-receptor antibodies used. Nonetheless, the studies provide a cautionary tale and point to the large number of controls necessary before concluding that a receptor heteromer exists and functions *in vivo*. Finally, the PLA approach has in fact provided evidence for observable interaction of D1-D2 receptors in neonatal mouse brain tissue [147], leaving open the possibility that such complexes may form during development and potentially in pathological conditions in adulthood.

5.7.3 Studies of the A2A-D2 Heteromeric Complex

Like D1 and D2, A2A and D2 agonism have opposing effects on adenylyl cyclase activity. While D2 signals through $G\alpha_{i/o/z}$, A2A signals through $G\alpha_{s/olf}$. At the behavioral level, A2A agonism and antagonism have opposing motor effects that can be modulated by dopamine. For example, locomotor activation produced by the non-selective A2A antagonist, caffeine, can be blocked by a D2 receptor antagonist [148, 149], while adenosine antagonism potentiates dopamine-induced locomotor activity in mice [150]. While these results may simply result from crosstalk at the level of cAMP regulation, A2A receptor agonists have also been shown to alter the receptor binding properties of some D2 ligands, specifically the adenosine agonist CGS 21680 increases the Kd of the D2 agonist propylnorapomorphine but not the D2 antagonist raclopride in rat striatal membranes [151].

In contrast to the D1 and D2 receptors, which are nearly completely expressed in different neurons, D2 and A2A receptors are co-expressed in the basal ganglia in GABAergic medium spiny neurons of the indirect striato-pallidal pathway, providing a potential opportunity for these receptors to cross-modulate signaling in distinct neuronal pathways in the striatum [152–154]. A2A agonism has been shown to inhibit D2 signaling when the receptors are co-expressed in cells [41], while D2 activity suppresses the NMDA-induced depolarization plateau potential in medium spiny neurons of the striatum. Interestingly, A2A activation was shown to inhibit this D2-mediated effect resulting in increased GABAergic firing [155]. Decreasing A2A activity might therefore promote D2-mediated suppression of NMDA-induced inhibitory firing, which could lead to increased motor function through disinhibition of thalamocortical motor drive [155]. Based on the results of these studies, along with observations of improved motor function in various animal models of Parkinson's disease [156–158], A2A antagonists are currently being tested in clinical trials to treat Parkinson's disease. While the implications of the studies above are intriguing, most of these effects could occur through downstream signaling crosstalk. Nonetheless, a large number of studies have implicated a role of direct receptor-receptor interaction in signaling crosstalk using a variety of methods including co-immunoprecipitation and RET techniques, and PLA has indeed shown that D2 and A2A receptors exist in close proximity in striatal brain slice [43]. However, these intriguing findings are subject to the same concerns reviewed above for D2 homomers, and additional studies will be required to ascertain whether signal crosstalk occurs at the level of a receptor heteromer or at the second messenger level. Experiments that show a unique signaling profile at a defined dimer, possibly by CODA-RET or functional complementation [17, 111], could begin to clarify these issues.

5.8 Concluding Remarks

The prospect of dopamine receptors interacting with themselves and with other GPCRs to form dimers and oligomers capable of unique signaling properties has generated much excitement. However, the structural properties of these complexes, their existence *in vivo*, and their relevance to receptor physiology or pathophysiology remain unclear.

One of the major challenges associated with studying dimerization and oligomerization of dopamine receptors and other class A GPCRs is related to methodological limitations as outlined throughout this chapter. Progress has been made to better understand several of the commonly used plate-reader RET methods for studying GPCR oligomerization, but these improved methods have yet to be implemented as a new standard for 're-testing' the oligomerization properties of dopamine receptors. In addition, new approaches used to study dopamine receptor complexes have emerged in recent years, including FRAP and single-molecule methods. These approaches have led to the understanding that dopamine receptor homomeric complexes in heterologous expression systems are likely not robust but rather transient in nature. It will be interesting to apply these methods to dopamine receptor heteromers as well as other GPCR complexes and membrane proteins to understand how dopamine receptor interactions compare with other protein-protein interactions.

If dopamine receptors do indeed form transient complexes, this suggests that a mix of receptor monomers and oligomers exists at any given moment, with the ratio of monomer-to-oligomer depending on the expression level of the receptor, as this must be governed by the equilibrium between the different receptor species in the membrane, as well as possibly interactions with scaffolding proteins within microdomains. Studies have shown that isolated class A receptor monomers can efficiently bind ligands, activate G protein, and recruit arrestin [159–162]. At the same time, studies highlighted throughout this chapter point toward engineered dopamine receptor homomers and heteromers also being capable of signaling in heterologous expression systems. Thus, it is conceivable that both dopamine receptor monomers and dimers may be functional. The transient nature of the complexes coupled with the possibility that both monomers and dimers may both exist in the plasma membrane and are functional present a highly heterogeneous system that will continue to be challenging to study using bulk assays alone. Single-molecule techniques hold great promise of being able to delineate monomers from dimers and may provide both the spatial and temporal resolution needed to study the functional properties of this highly heterogeneous system. However, it is important to note that much of what has been reported for dopamine receptor complexes was studied under non-physiological conditions and/or from heterologous expression systems. Whether these complexes exist *in vivo* and behave similarly to the *in vitro* setting is largely unknown. Thus, it will be important moving forward to apply methods that reveal structural and functional aspects of dopamine receptor oligomerization *in vivo.*

A major obstacle is the fact that failures to reproduce data are rarely published. Reporting on failures to observe published findings using similar methodological approaches is critical for evolving an understanding of the potential function of these putative complexes. In addition, the use of improved methods based on proximity and affinity as well as the use of new biophysical techniques to better understand dopamine receptor oligomerization may help bring the field toward a consensus in the future.

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Chapter 6
Class A GPCR: Serotonin Receptors

Ellinor Grinde and Katharine Herrick-Davis

Abstract Hundreds of studies have reported that G protein-coupled receptors self-associate to form dimers or oligomers. Yet, this topic remains controversial. This chapter reviews the current literature related to the structure and function of 5-HT receptor dimers/oligomers in recombinant cells and in native tissues. Studies designed to examine the functional significance of 5-HT receptor dimer/oligomer formation are evaluated and discussed. Emphasis is placed on the methods employed, the dimer interface, oligomer size, mechanism of G protein activation, and analysis of bivalent ligands as potential therapeutics.

Keywords Serotonin receptor • G protein-coupled receptor (GPCR) • Homodimer • Heterodimer • Biophysical methods

Abbreviations

5-HT	serotonin
AA	arachidonic acid
BiFC	bimolecular fluorescence complementation
BRET	bioluminescence resonance energy transfer
Co-IP	co-immunoprecipiation
FCS	fluorescence correlation spectroscopy
FISH	fluorescent in situ hybridization
FRET	fluorescence resonance energytransfer
GPCR	G protein-coupled receptor
IP	inositol phosphate
PLA	proximity ligation assay
SpIDA	spatial intensity distribution ananlysis
TCSPC	time correlated single photon counting

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6.1 Introduction

Serotonin (5-HT) exerts its physiological effects through interactions with 14 different 5-HT receptor subtypes expressed throughout the human body. 5-HT receptors are targets for a wide variety of currently marketed pharmaceuticals. While many of the physiological effects of 5-HT have been studied and documented, the relationship between 5-HT receptor structure and function remains less clear. In recent years, significant effort has been put forth studying the architecture of G protein-coupled receptors (GPCRs) to determine if they associate with one another to form dimeric or oligomeric complexes and to determine the influence this has on their signaling capabilities and function. In this chapter, studies designed to examine the functional significance of 5-HT receptor dimer/oligomer formation will be evaluated and discussed with an emphasis on the methods employed, the dimer interface, oligomer size, mechanism of G protein activation, and an analysis of bivalent ligands as potential therapeutics.

6.2 5-HT Receptor Homomers

In this section, studies related to the formation of 5-HT receptor homomers will be discussed for the G protein-coupled members of the 5-HT receptor super family, which includes 5-HT1, 5-HT2, 5-HT4, 5-HT5, 5-HT6, and 5-HT7 receptors. Studies examining the propensity of 5-HT receptors to form homomers are summarized in Table 6.1.

6.2.1 5-HT1 Receptors

In humans, there are five different genes encoding serotonin receptors belonging to the 5-HT1 receptor family designated 1A, 1B, 1D, 1E, and 1F. The members of this family share 40-60% overall homology to one another and all couple to Gi/o proteins to inhibit adenylate cyclase activity (reviewed in [1]). The following sections provide a detailed discussion of homodimer/oligomer studies related to the individual 5-HT1 receptor subtypes in the context of their known distribution and proposed physiological functions.

6.2.1.1 5-HT1A

The 5-HT1A receptor was first reported as a spiperone sensitive binding site labeled by ^{3}H-5-HT in rat brain [2] and was cloned by serendipity based on its high homology to beta-adrenergic receptors [3]. 5-HT1A receptors are located in the gastrointestinal tract and in brain regions implicated in mood and anxiety [4]. 5-HT1A receptors are

Table 6.1 Serotonin receptor homomers

Cells or tissue	Methods	M/D/O	Drug effect	References
5-HT1A				
HEK-293	Co-IP, WB	M & D	ND	[7]
N1E-115	FRET (FL/AP/Lux)	D	Ag↓, Cd↑	[8]
	Co-IP, WB			
HEK-293	FRET (FL/AP)	D	Ag↑, An↓	[9]
	TCSPC			
N1E-115	FRET (DQ/AS/Lux)	D	ND	[10]
N1E-115	FRET (AP/Lux)	D	ND	[11, 12]
CHO-K1	FRET (H/FL)	D & O	Ag↑, An↓, Cd↓	[13]
CHO-K1	FRET (H/TR)	D & O	NE (Ag), Cd↑	[14]
5-HT1B/1D				
HEK-293	WB	M & D	ND	[7]
Sf9	WB	M & D	ND	[24]
5-HT2A				
Cortex	RLB	D	ND	[66]
HEK-293	Co-IP, FRET (AP)	D or O?	ND	[67]
CHO	RLB, IP, AA	D	ND	[67]
HEK-293	FCS	D	ND	[68]
HEK-293	FRET (FL)	D or O?	Ag↑, An↓	[69, 70]
HEK-293	RLB, IP	D	ND	[88]
5-HT2C				
HEK-293	Co-IP, WB	D	ND	[117]
HEK-293	BRET, FRET(AP)	D or O?	ND	[117, 123]
HEK-293	BRET, FRET(AP)	D or O?	NE (An)	[118]
HEK-293	BiFC, FCS	D	NE (Ag)	[119]
Hippocampal neurons	FCS	D	ND	[119]
HEK-293T	WB, SpIDA	M, D, O	An↓	[128]
Choroid plexus epithelial cells	FCS	D	ND	[129]
HEK-293T	Cross-link, WB	D	Ag↑, An↓	[78]
5-HT4				
HEK-293/CHO	WB, Co-IP, BRET	D or O?	NE (Ag/An)	[140, 142]
COS-7	Co-IP, FRET (TR)	D	ND	[141]
	RLB, cAMP, IP			
C6 glial cells	RLB, cAMP	D	ND	[144]
5-HT7				
N1E-115	FRET (AP/Lux)	D or O?	ND	[12]
HEK-293	BRET	D or O?	ND	[170]
HEK-293	RLB, cAMP	D	ND	[170, 171]
Astrocytes	cAMP	D	ND	[172]

Summary of published literature related to 5-HT receptor homomers
Abbreviations: *AA* arachidonic acid, *Ag* agonist, *An* antagonist, *BiFC* bimolecular fluorescence complementation, *BRET* bioluminescence resonance energy transfer, *Cd* cholesterol depletion, *Co-IP* co-immunoprecipiation, *D* dimer, *D or O?* the method used does not distinguish between dimers and oligomers, *FCS* fluorescence correlation spectroscopy, *FISH* fluorescent in situ hybridization, *FRET* fluorescence resonance energy transfer, {*AP* acceptor photo bleaching, *AS* acceptor sensitization, *DQ* donor quenching, *FL* fluorescence lifetime, *H* homo, *LUX* linear unmixing TR, time resolved}, *IP* inositol phosphate, *M* monomer, *ND* not determined, *NE* no effect, *O* oligomer, *RLB* radioligand binding, *PLA* proximity ligation assay, *SpIDA* spatial intensity distribution anan-lysis, *TCSPC* time correlated single photon counting, *WB* western blot

present on serotonergic cells bodies and dendrites in the dorsal raphe where they act as auto-receptors to inhibit neuronal firing and 5-HT release (reviewed in [5]). They are also located post-synaptic to serotonergic neurons on cholinergic neurons in the septum and glutamatergic pyramidal neurons and GABAergic interneurons in the hippocampus and cortex where they cause membrane hyperpolarization and inhibition of neuronal firing. 5-HT1A receptors have been linked to anxiety, depression, schizophrenia, Parkinson's disease, pain, and suicide [5, 6].

As reported for many other biogenic amine GPCRs, Western blots of solubilized 5-HT1A receptors expressed in recombinant cell systems reveal bands the predicted size of monomers and dimers [7, 8]. In these studies, co-immunoprecipitation (Co- IP) of differentially tagged receptors provided support for the hypothesis that the higher molecular weight bands observed on Western blots represent 5-HT1A receptor homodimers. As a result, a variety of different FRET techniques have been employed to explore the possibility that 5-HT1A receptors form homodimers in intact living cells, including acceptor photobleaching with linear unmixing [8–12], fluorescence life-time [8, 9, 13], time-resolved FRET [14], and homo-FRET [13, 14]. For a detailed explanation of the different FRET methods the reader is referred to reviews by Chan and collegues [15] and Zeug and colleagues [16].

FRET studies of 5-HT1A receptors are summarized in Table 6.1. These studies used CFP- and YFP-tagged 5-HT1A receptors expressed in transfected HEK293, CHO-K1, or N1E-115 cells [8–14]. The confocal microscopy-compatible techniques used in these studies allowed the FRET measurements to be made within discrete regions of the plasma membrane of live cells. These studies acknowledged that the contribution from bystander or stochastic FRET can be significant and were careful to include appropriate controls to account for or reduce this possibility. The acceptor photobleaching studies measured FRET efficiency as a function of donor:acceptor ratio and demonstrated that the observed FRET signal was independent of receptor expression level. The need to control and account for donor:acceptor expression ratios was completely eliminated when a single fluorescent species was used, as in the homo FRET studies. In the fluorescence life-time studies the read-out was based on the decay in donor fluorescence and was independent of receptor expression level. The linear un-mixing studies employed spectral separation of CFP and YFP signals to eliminate cross-talk and bleed-through, and non-specific, background fluorescence was eliminated in studies using time-resolved FRET. Taken together, all of the published 5-HT1A receptor FRET studies have concluded that these receptors self-assemble and are present as dimers or oligomers on the plasma membrane of recombinant cells in which they are expressed.

In the studies described above, the effect of drug treatment on the observed FRET signal produced mixed results. Two studies reported that agonists (5-HT, 8-OH-DPAT) increase and antagonists (methysergide, MPPI, SCH58216) decrease the FRET signal [9, 13], while a third study reported a decrease in FRET following treatment with 5-HT [8] and a fourth study reported no effect [14]. Whether the change in FRET signal observed following drug treatment reflects a change in receptor conformation following ligand binding or an actual change in the dimer/oligmer status of the receptor is a matter of debate [8, 13].

Based on computational modeling of 5-HT1A receptor monomers and homodimers, a potential transmembrane domain (TMD) 4/5 interface has been proposed [11]. In this study, specific amino acid residues implicated by computer modeling in forming the dimer interface were mutated and subjected to lux-FRET analysis following transfection and expression in neuroblastoma cells. For wild-type receptors, the number of units participating in the complex (n value) was determined to be 2.2, indicating dimers are the predominant species. Mutation of tryptophan $175^{4.64}$ to alanine or tyrosine $198^{5.41}$ to phenylalanine reduced the n value to 1.4, suggesting that these residues form important contacts within the homodimer.

Chemical cross-linking and linear unmixing FRET analyses of differentially tagged 5-HT1A receptors expressed in recombinant cells have concluded that the homodimer is the predominant species expressed on the plasma membrane [8, 10, 11]. In contrast, Chattopadhyay and colleagues used a homo-FRET approach in which energy transfer between two identical fluorescent probes is characterized by a depolarization of the fluorescence emission and a reduction in anisotropy. Progressive photobleaching (to remove interacting partners) resulted in a progressive increase in anisotropy in a pattern consistent with a mixed population of dimers, trimers and tetramers of YFP-tagged 5-HT1A receptors expressed in CHO cells [13]. Treatment with 5-HT and actin destabilization appeared to increase the relative proportion of trimers and tetramers, while treatment with antagonist and cholesterol depletion had the opposite effect.

The membrane environment (lipid/cholesterol content) may play an important role in the assembly and/or stability of 5-HT1A homodimers/oligomers [8, 13, 14]. This hypothesis is further supported by studies using non-palmitoylated mutant receptors which are excluded from lipid rafts [8, 10]. The role of membrane lipids and cholesterol in GPCR structure and function is discussed in more detail in Chaps. 15 and 16.

Unfortunately, none of the studies described above examined the signaling properties of the 5-HT1A homodimer and none of the studies were performed using native receptors in primary culture or tissue. Therefore, elucidation of a functional role for dimer/oligomer formation in mediating the physiological effects of 5-HT1A receptors awaits future studies examining the properties of native 5-HT1A receptors in primary culture and in vivo.

6.2.1.2 5-HT1B/1D

In the central nervous system, 5-HT1B and 5-HT1D receptors are present in the dorsal raphe, locus coeruleus, basal ganglia, striatum and frontal cortex [17–19]. They are located on axon terminals of serotonergic and non-serotonergic neurons where they function as autoreceptors inhibiting neurotransmitter release. The 5-HT1B receptor is associated with impulsive and aggressive behavior in mice [20, 21] and a potential role in obsessive-compulsive behavior in humans is under investigation [22].

The 5-HT1B and 5-HT1D receptors are very similar in structure and pharmacology [23]. For example, the “triptan” drugs prescribed for migraine (eg. sumatriptan) have

high affinity for both receptor subtypes, which co-localize in the trigeminal ganglia. 5-HT1B receptors are present in cerebral arteries and in vascular smooth muscle and mediate the vasoconstrictor properties of the triptans.

5-HT1B and 5-HT1D receptors have been reported to form homodimers and to form heterodimers with each other [7, 24]. In these studies, Western blots of detergent-solublized receptors revealed the presence of bands the predicted size of monomers and dimers. Co-immunoprecipitation was used to implicate a physical association between these receptors to account for the higher molecular weight species observed on the Western blot. While the crystal structure of the 5-HT1B receptor has been reported [25, 26], it did not provide insight into potential dimer/oligomer interfaces as the receptors were aligned in a non-physiological, anti-parallel fashion within the crystal lattice.

To date, there have been no studies employing proximity-based assays or techniques with near single molecule sensitivity in primary cultures or tissues with native 5-HT1B or 5-HT1D receptors to support the existence of dimeric/oligomeric complexes. However, studies using bivalent ligands have produced interesting results. It has been reported that dimeric versions of 5-HT and 8-OH-DPAT have higher affinity for 5-HT1 receptors than their monomeric forms [27]. Similarly, the conversion of sumatriptan into a dimeric form resulted in a tenfold increase in binding affinity for 5-HT1B and 5-HT1D receptors [28]. Dimeric 5-HT1 agonists have been reported to display functional activity and increased potency in mediating contraction of the rabbit saphenous vein [29, 30] and in selectively stimulating [35S] GTPγS binding to 5-HT1B receptors in rat brain slices [28]. To date, these studies provide the strongest support favoring the presence of dimeric forms of 5-HT1B and 5-HT1D receptors in native tissues.

6.2.1.3 5-HT1E/1F

The existence of 5-HT1E receptors was first postulated based on the biphasic inhibition of ^{3}H-5-HT binding by 5-carboxamidotryptamine to human brain tissue in the presence of selective drugs to block known 5-HT receptor subtypes [31]. Subsequently, the 5-HT1E receptor was cloned from human [32, 33] and guinea pig brain [34], and was shown to have similar pharmacology and distribution in both species [35, 36]. The presence of 5-HT1E receptors in the human frontal cortex and hippocampus suggests a potential role in cognition and/or memory. However, the absence of 5-HT1E receptors in rodents and the inability to use mouse or rat preclinical models has impeded the development of selective drugs and the identification of the physiological role(s) played by this receptor.

The 5-HT1F binding site has a broad distribution throughout the brain including the cortex, striatum, thalamus, hippocampus, and amygdala [37, 38]. In addition, the 5-HT1F receptor is located within the trigeminal ganglia, making it a potential target for neuronal acting, anti-migraine drugs. Selective 5-HT1F receptor agonists have been developed [38, 39] and one, lasmitditan, has undergone preclinical [40] and phase I clinical trials for the treatment of migraine (reviewed in [41]).

Currently, studies designed to examine the potential dimeric or oligomeric nature of 5-HT1E or 5-HT1F receptors are lacking. The high structural homology of these receptors to the 5-HT1A, 5-HT1B, and 5-HT1D receptors, which have been reported to form homo- and hetero-dimers, suggests that 5-HT1E and 5-HT1F receptors are likely candidates for forming homomers and/or heteromers. However, this hypothesis awaits experimental validation.

6.2.2 5-HT2 Receptors

In humans, there are three different genes encoding serotonin receptors belonging to the 5-HT2 receptor family designated 2A, 2B, and 2C. The members of this family are highly homologous in structure and couple to Gq proteins to stimulate phospholipase C and inositol phosphate metabolism, as well as other second messenger systems (reviewed in [1]). These receptors play important roles in the cardiovascular and neuroendocrine systems, and in the regulation of cognitive function. The following sections provide a detailed discussion of homodimer/oligomer studies of 5-HT2 receptors in the context of their known distribution and proposed physiological functions.

6.2.2.1 5-HT2A

In the brain, 5-HT2A receptors are present in the cortex, hippocampus, amygdala, accumbens, striatum, hypothalamus, and brain stem [42–44]. 5-HT2A receptors are located post-synaptic to 5-HT containing neurons on glutamatergic pyramidal neurons, GABAergic interneurons, monoaminergic neurons, and glial cells [45–49]. They are found on cell bodies, processes and dendritic spines in both pre- and post-synaptic compartments [50–54]. Hallucinogens and antipsychotics display high affinity for this receptor [55–57]. 5-HT2A receptors play an important role in cognitive function and have been implicated in anxiety, depression, schizophrenia, drug addiction, and epilepsy (reviewed in [58–60]). In the periphery, 5-HT2A receptors are expressed in arterial smooth muscle and in platelets where they mediate vasoconstriction and platelet aggregation, respectively [61–63].

Radioligand binding studies provided some of the earliest evidence hinting at the presence of 5-HT2A receptor homodimers, although not reported as such at the time. Biphasic competition of antagonists for agonist-labeled 5-HT2A receptors, in the absence and presence of guanyl nucleotides, was observed in recombinant cells and human brain tissue [64–66], consistent with a negative cooperative interaction between 5-HT2A binding sites. These results have been extended to include functional assays in which several different antagonists produced both biphasic inhibition of 5-HT-stimulated arachidonic acid release and biphasic inhibition of agonist radioligand binding to 5-HT2A receptors, with potencies that were similar to their binding affinities [67]. In contrast, all antagonists tested inhibited

5-HT-stimulated inositol phosphate production in a monophasic manner, demonstrating a differential regulation of signaling by antagonists and the presence of functional 5-HT2A homodimers [67].

Co-immunoprecipitation, FRET and FCS studies of 5-HT2A receptors expressed in recombinant cells have been used to provide biochemical and biophysical evidence supporting the hypothesis that 5-HT2A receptors form homodimers [67–70]. These studies are summarized in Table 6.1. In 5-HT2A transfected HEK293 cells, treatment with 5-HT2A agonist (DOI) increased and antagonist (ketanserin) decreased FRET between CFP- and YFP-tagged receptors [69, 70]. However, the same ligands had no effect on fluorescence life-time measurements in cells expressing only CFP-tagged 5-HT2A receptors [69, 70].

Computer modeling and molecular dynamics simulations have been used to investigate the structure of the 5-HT2A ligand binding pocket and its relationship to the homodimer interface [71–73]. Different conformations of the 5-HT2A receptor were observed upon binding of agonist (5-HT), partial agonist (LSD) or inverse agonist (ketanserin) [71]. Ligand-dependent conformational differences were observed in the orientation of TMD 3 and TMD 6, along with changes in the conserved NPxxY motif at the intracellular end of TMD 7. In addition, the pattern of cholesterol association with the TMDs was ligand-dependent, as were local deformations in the lipid bilayer surrounding the receptor. Such changes were predicted to result from the tendency of the lipids to minimize the hydrophobic mismatch at exposed ends of the TMDs [71]. Hydrophobic mismatch is considered a driving force in protein-protein interactions within membranes, in an attempt to minimize the energy penalty by bringing together domains where the residual exposure energies are the greatest [74–76]. Therefore, it is has been proposed that ligand-dependent changes in receptor conformation (exposing different TMDs with varying degrees of mismatch or exposure energies) may translate into changes in dimer/oligomer formation and may even dictate which TMDs participate in the dimer/oligomer interface [74–76]. When the exposure energies surrounding the TMDs for the different ligand-bound conformations of the 5-HT2A receptor were calculated, the exposure energies were greatest at TMDs 1, 4 and 5 for ketanserin, TMDs 4 and 5 for LSD, and at TMDs 5 and 6 for 5-HT, implicating these regions as potential dimer/oligomer interfaces [71]. These results imply that the homodimer interface and shape of the 5-HT2A binding pocket are tightly linked, as reported for other biogenic amine receptors [77–79].

Molecular dynamics and cluster analysis of preferred receptor conformations of a 5-HT2A monomer predicted a binding pocket conformation favoring antagonists, which switched to agonist preferring when a predicted TMD 4/5 homodimer interface was included in the simulations [72]. In addition to transmitting anisotropic changes within the two protomers, the homodimer interface influenced the flexibility of TMD 6, a region known to be involved in the active conformation of class A GPCR [80]. Hallucinogenic and non-hallucinogenic compounds, known to bind with high affinity to 5-HT2A receptors [55], have been reported to promote changes in the second intracellular loop of the 5-HT2A receptor in a ligand-specific manner [73]. This region has been reported to play a role in G protein coupling and activation [80, 81]. These

results provide a structural basis for "functional selectivity" of hallucinogenic and non-hallucinogenic compounds, manifested as differences in G protein coupling, second messenger activation, and distinct behavioral phenotypes [82–87]. Taken together, these results are trending toward a more progressive hypothesis in which "functional selectivity" is explained by ligand-specific changes in the orientation of the TMDs which influence the composition of the dimer/oligomer interface and shape the G protein binding cavity at the intracellular surface of the homodimer, thereby regulating G protein coupling specificity and efficiency.

Additional pharmacological evidence supporting asymmetry and functional cross-talk between protomers within a 5-HT2A homodimer was provided by Teitler and colleagues using drug-induced inactivation followed by reactivation [88]. In the inactivation phase, recombinant cells expressing 5-HT2A receptors were pretreated in the presence of an antagonist (ritanserin) with very slow dissociation kinetics that binds to the receptor in a pseudo-irreversible or wash-resistant manner. When the cells were treated with ritanserin and then thoroughly washed, 5-HT-stimulated IP production was abolished. In the reactivation phase, the ritanserin pretreated and inactivated cells were washed and then exposed to a competitive antagonist (spiperone). Following a second drug wash-out step (to remove the competitive antagonist), 5-HT-stimulated IP production was restored. The reactivation of inactivated receptors is best explained by a 5-HT2A homodimer model with functionally interacting protomers. Ritanserin, bound pseudo-irreversibly to one protomer, is released upon spiperone binding to the second (unoccupied) protomer. Following drug wash out, both protomers are now unoccupied and receptor signaling (5-HT-stimulated IP production) is restored. This phenomenon can be modeled based on the magnitude of the right-ward shift in the inactivator (ritanserin) dose-response curve produced by a saturating concentration of reactivator (spiperone), and thus can be used to predict the number of participating protomers within the receptor complex [88]. When this experimental design was applied to investigate 5-HT2A receptor function, spiperone produced a tenfold right-ward shift in risperidone's inactivation dose-response curve and predicted an oligomer number of 2.2 [88]. These results are consistent with FCS studies in which the homodimer was determined to be the predominant species for plasma membrane 5-HT2A receptors expressed in HEK293 cells [68].

Attempts to develop bivalent ligands targeting 5-HT2A homodimers have not provided any leads in terms of enhancing the pharmacological profile of the ligand. In contrast to studies with the 5-HT1B/1D receptors, where a homodimeric ligand showed increased affinity and potency [27–30], homodimeric forms of the 5-HT2A partial agonists, pergolide and terguride, displayed reduced affinity and lost their agonist activity [89]. In a subsequent study, the selective 5-HT2A antagonist, M-100907, was used as a starting template for bivalent ligand development [90]. Homodimeric forms of M-100907 with linkers in the range of 12 to 18 atoms retained their antagonist properties and were the most potent inhibitors of 5-HT-stimulated intracellular calcium release in 5-HT2A expressing CHO cells. However, bivalency did not improve affinity or potency.

6.2.2.2 5-HT2B

The 5-HT2B receptor, originally named 5-HT2F, was first identified as a 5-HT-sensitive receptor stimulating smooth muscle contraction in the rat fundus [91, 92]. This receptor is expressed in the gut, heart, lung, kidney, liver, smooth muscle, and brain [93]. 5-HT2B receptors mediate vasorelaxation in pulmonary arteries and muscle contraction in the small intestine, and may play a role in pulmonary hypertension and ventricular hypertrophy [94, 95]. The importance of this receptor in mammalian physiology is demonstrated by the lethality of 5-HT2B receptor knock-out [96]. 5-HT2B receptors are present in various brain regions including the cerebellum, hypothalamus, amygdala and septum, but are less abundant in brain than 5-HT2A and 5-HT2C receptors [97]. Stimulation of 5-HT2B receptors in the amygdala has been reported to be anxiolytic in rats [98]. More recently, 5-HT2B receptors on astrocytes have been suggested to play a role in the therapeutic efficacy of serotonin reuptake inhibitors in the treatment of major depression [99–101].

While there are many studies reporting the homodimeric nature of 5-HT2A and 5-HT2C receptors, studies examining potential homodimer formation of 5-HT2B receptors are lacking. Recently, the crystal structure of the 5-HT2B receptor was solved in the agonist bound conformation [25, 26]. The crystal structure predicts an active state involving rotation of transmembrane domains (TMDs) 3, 5 and 6 accompanied by rearrangement of TMDs 5, 6 and 7 at their intracellular interface to facilitate G protein coupling and activation. However, the receptors were aligned in anti-parallel fashion within the crystal lattice, yielding no physiologically relevant information regarding potential dimer/oligomer interfaces.

6.2.2.3 5-HT2C

Early autoradiography studies reported a predominant localization of this receptor in choroid plexus tissue [102, 103]. However, subsequent in situ hybridization studies revealed a wide spread distribution throughout the basal ganglia, limbic system and prefrontal cortex [64, 104–106]. In fact, 5-HT2C receptor mRNA has been reported to be more abundant and wide spread throughout the CNS than mRNA of the closely related 5-HT2A receptor [107, 108]. Thus the 5-HT2C receptor is well positioned throughout the CNS to mediate many of the central actions of serotonin including regulation of cognitive function, mood, movement, appetite, and pain modulation. 5-HT_{2C} receptors are located postsynaptic to serotonergic neurons on GABAergic, glutamatergic, dopaminergic, neuropeptidergic, and cholinergic neurons [109–113]. The 5-HT2C receptor is a therapeutic target of great interest as it has been implicated in anxiety, depression, schizophrenia, addiction, obesity and epilepsy (reviewed in [114–116]).

5-HT2C receptor homodimerization has been investigated using Western blot, chemical cross-linking, Co-IP, BRET, FRET, BiFC, FCS, and SpIDA (Table 6.1). These studies have been performed in transfected HEK293 cells, in cultured hippocampal neurons and choroid plexus epithelial cells. In the initial study, Western

blots revealed the presence of detergent-sensitive 5-HT2C homodimers, and positive BRET signals were observed following co-expression of differentially-tagged 5-HT2C receptors in HEK293 cells [117]. The specificity of the BRET signal observed for 5-HT2C receptors was demonstrated using non-interacting receptor pairs (eg. 5-HT2C/M4-muscarinic and 5-HT2C/beta2-adrenergic). Positive FRET signals were observed on the plasma membrane of living cells co-expressing CFP- and YFP-tagged 5-HT2C receptors [117]. The specificity of the signal was confirmed by demonstrating the dependence of FRET efficiency on the donor/acceptor ratio and independence of receptor expression level. Treatment with 5-HT or clozapine had no effect on FRET efficiency, indicating that these compounds did not alter the homodimeric structure of the 5-HT2C receptor [118]. Time-lapse fluorescence confocal microscopy provided direct visualization of beta-arrestin2 recruitment to the plasma membrane following 5-HT binding to the homodimer [118, 119].

Homodimers can form between the different isoforms of the 5-HT2C receptor that are generated by RNA editing. Homodimerization was observed for the unedited (INI) and the fully edited (VSV and VGV) isoforms [118]. In transfected HEK293 cells, positive BRET was observed between the INI/VSV, INI/VGV and VSV/VGV isoform pairs [120]. Since these isoforms of the 5-HT2C receptor differ in their signaling capabilities [111, 122] it is possible that dimers of different isoform pairs may have unique signaling properties. However, their signaling properties and their presence in vivo have not been investigated.

Acceptor photobleaching FRET was used to monitor 5-HT2C receptor homodimer formation in the endoplasmic reticulum (ER) of transfected HEK293 cells [123]. In this study, time-lapse confocal microscopy was used to track the synthesis and trafficking of 5-H2C receptors in the presence of specific markers to label the ER and Golgi compartments. At early time points post-transfection, positive FRET signals between CFP- and YFP-tagged 5-HT2C receptors were observed in the ER and Golgi compartments and were present once the receptors reached the plasma membrane [123]. These results are consistent with the hypothesis that homodimer biogenesis begins in the ER, as has been reported for other biogenic amine receptors (reviewed in [124]).

Fluorescence correlation spectroscopy (FCS) is another confocal microscopy-based technique that can be used to examine the dimer/oligomer status of interacting proteins (reviewed in [125, 126]). FCS uses photon counting detectors to monitor the diffusion of fluorescence-tagged proteins and their molecular brightness. Since the molecular brightness of a protein cluster is directly proportional to the number of fluorescent proteins within the cluster, this method can be used to determine the oligomer status of mobile proteins freely diffusing within the plasma membrane of living cells [119, 127]. FCS studies of fluorescence-tagged biogenic amine receptors in HEK293 cells, including 5-HT2A and 5-HT2C receptors, identified the homodimer as the predominant species in the mobile fraction [68, 119]. The same result was obtained when fluorescence-tagged 5-HT2C receptors were expressed in primary hippocampal neurons [119]. The FCS results are supported by fluorescence complementation (BiFC) between 5-HT2C receptor pairs expressing non-fluorescent halves of GFP, which was observed in the ER and on the plasma membrane [119].

In the FCS studies, homodimers were the predominant mobile species when receptor expression levels ranged from 10 to 100 receptors/μm^2 of plasma membrane [68]. These results were extended using a different fluorescence intensity-based technique called spatial intensity distribution analysis, SpIDA (reviewed in Chap. 2 and [128]). Consistent with FCS studies, SpIDA reported homodimers as the predominant species at expression levels < 100 receptors/μm^2. However, higher order oligomers were prominent at expression levels above 100 receptors/μm^2 [128]. Antagonist binding was accompanied by a decrease in fluorescence intensity, interpreted as an increase in the monomeric fraction induced by a change in the overall quaternary structure of the receptor following ligand binding [128].

To date, only one study has examined 5-HT receptors endogenously expressed in their native cellular environment. Native 5-HT2C receptors, endogenous to the choroid plexus, have been reported to be expressed as homodimers on the apical surface of the epithelial cells at a density of 32 receptors/μm^2 [129]. While this is similar to a reported density of 20 receptors/μm^2 for native beta2-adrenergic receptors in alveolar epithelial cells [130], expression levels in neurons are even lower at 4.5 receptors/μm^2 [130]. When expressed within normal physiological levels, homodimers appear to be the predominant mobile species for 5-HT2C receptors in HEK293 cells [119, 128, 129], hippocampal neurons [119] and choroid plexus epithelial cells [129]. The signaling properties of the 5-HT2C homodimer were investigated using agonists that bind in a wash-resistant manner to one or both protomers of the 5-HT2C homodimer [129]. Agonist binding to one protomer stimulated a half-maximal inositol phosphate response, while binding to both protomers was required to produce a maximal response. These experiments provide pharmacological evidence supporting the hypothesis that for 5-HT2C receptors homodimers are the basic signaling unit.

Hendrickson and colleagues used a cysteine cross-linking approach in an attempt to elucidate potential dimer interfaces responsible for the formation of 5-HT2C homodimers [78]. In these studies, homology modeling based on the crystalline structure of rhodopsin provided a 5-HT2C receptor model that was used to identify residues with appropriate surface exposure for cysteine cross-linking. Candidate residues were mutated to cysteine and the formation of disulfide-linked dimers was evaluated by Western blot. These experiments identified potential dimer interfaces at the extracellular end of TMD1 and also at TMD4/5 [78]. However, higher-order oligomers of 5-HT2C receptors were not observed following cysteine cross-linking in cells co-expressing TMD1 and TMD4/5 cysteine mutant receptors. Based on these results and on experiments using receptor-Gα fusion proteins, it was concluded that 5-HT2C receptor homodimers are quasisymmetrical at the TMD4/5 interface and asymmetrical with respect to G protein coupling [78].

6.2.3 5-HT4 Receptors

5-HT4 receptors were first identified in guinea-pig hippocampal membranes and mouse colliculi neurons as a novel 5-HT receptor subtype that stimulates adenylate cyclase activity [131]. In the periphery, 5-HT4 receptors are found in the

gastrointestinal tract, heart, bladder and adrenal gland. In the brain they are found in the basal ganglia, accumbens, hippocampus and substantia nigra (reviewed in [132, 133]). 5-HT4 receptors are heteroreceptors with both somatodendritic and axon terminal locations on GABAergic neurons in the accumbens and striatum [134]. They are also present on cholinergic and glutamatergic neurons in the hippocampus [135, 136] and prefrontal cortex [137].

Ten different splice variants of the 5-HT4 receptor have been identified with truncations of the C-tail, which increases the constitutive activity of this receptor [138]. The splice variants have different distributions in the brain and periphery, but their physiological relevance remains unknown. 5-HT4 receptor agonists have been marketed for the treatment of gastro-oesophageal reflux and irritable bowel syndrome, and more recently are being investigated as potential therapeutics for Alzheimer's disease (reviewed in [139]).

As with other class A GPCR, the first evidence for 5-HT4 receptor homodimers was provided by Co-IP and the appearance of higher molecular weight bands on a Western blot [140]. These results, obtained in transfected CHO and HEK293 cells, were confirmed using BRET. The specificity of the BRET signal between Rluc- and YFP-tagged 5-HT4 receptors was verified by co-expression of 5-HT4/Rluc with M1-muscrainic/YFP, which reduced the BRET signal by two-thirds [140]. Treatment with agonists and antagonists did not modify the BRET signal [140]. Positive BRET signals were observed between splice variants of the 5-HT4 receptor with different length C-tails and different levels of constitutive activity [140].

Consistent with the BRET results, the 5-HT4 receptor was solubilized as a homodimer in complex with a single G protein [141]. Radioligand binding, GTPγS binding, and FRET experiments using various combinations of ligand binding selective (RASSL) and signaling defective mutant receptors were used to demonstrate that a single Gs protein associates with the 5-HT4 homodimer and that the C-terminus of the Gs subunit always orients itself toward the ligand occupied protomer within the homodimer [141]. GTPγS binding was most efficiently stimulated when both protomers of the homodimer were occupied by agonist. Although 5-HT4 receptors have been reported to form constitutive homodimers in recombinant cell lines, and the signaling properties have been characterized, these observations have not been verified for native 5-HT4 receptors in native cells or tissues.

When computer simulations of the 5-HT4 receptor were generated, based on the crystal structure of bovine rhodopsin, molecular modeling and protein docking identified a potential TMD3/TMD4 homodimer interface stabilized by disulfide linkage between Cys112 and Cys145 [142]. In addition, a potential hydrophobic dimer interface motif (A, X_3, G) was identified in TMD4. While treatment with reducing agent (DTT) produced a modest decrease (30%) in the observed BRET signal, Western blots revealed a much larger increase in the relative proportion of bands the predicted size of monomers [142]. These results were interpreted as evidence for both disulfide and hydrophobic interactions within a TMD4 dimer interface.

Bivalent ligands have been synthesized and tested for activity at 5-HT4 receptors. ML10302, a 5-HT4 partial agonist, lost its agonist properties upon conversion into its bivalent form [143]. In this study, a BRET assay was used to confirm that the bivalent form of the ligand was in fact binding to both protomers of the 5-HT4 receptor homodi-

mer. In contrast to the 12–18 atom linker length found to be optimal for the 5-HT2A bivalent ligand M-100907 [90], spacer arms of 20–24 atoms were found to be optimal for a series 5-HT4 receptor bivalent ligands [144]. While these studies have identified some of the basic characteristics for designing novel 5-HT4 bivalent ligands, functional bivalent ligands with improved affinity and potency have yet to be developed.

6.2.4 5-HT5 Receptors

5-HT5 receptors are the least well characterized of the 5-HT receptor family. Two different genes, designated 5-HT5a and 5-HT5b, have been cloned (reviewed in [1]), but the 5-HT5b does not encode a functional protein. While the mRNA encoding these receptors has been identified in the cortex, hippocampus and cerebellum [145], functional binding sites have not been identified in native cells or tissues and their physiological function remains unknown. It is tempting to speculate that perhaps 5-HT5 receptors require the assistance of another GPCR to achieve functional competency. For example, the alpha1D-adrenergic receptor requires heterodimerization with other alpha1-adrenergic receptor sub-types to reach the plasma membrane and become functional [146]. The most well characterized example of this phenomenon is the class C GABAbR1/R2 heterodimer that requires co-expression of both receptor sub-types for full function [147–150]. At the present time, a similar hypothesis for 5-HT5 receptor function remains purely speculative and there have been no published reports of the homomeric or heteromeric status of recombinant 5-HT5 receptors.

6.2.5 5-HT6 Receptors

The 5-HT6 receptor was first characterized in the striatum as a novel 5-HT receptor subtype positively coupled to adenylate cyclase [151]. In addition to the striatum, mRNA for this receptor is found in the amygdala, accumbens, hippocampus and cortex [152, 153]. 5-HT6 receptors are believed to have a post-synaptic localization in the brain, and there is little evidence to suggest their presence in the periphery. The 5-HT6 receptor is thought to play a role in learning and memory and has been targeted for the development novel Alzheimer's disease therapeutics (reviewed in [154]). As with the 5-HT5 receptor, there have been no published reports of studies related to the monomeric/oligomeric status of 5-HT6 receptors.

6.2.6 5-HT7 Receptors

The 5-HT7 receptor was cloned in 1993 by six different groups and is the most recently discovered 5-HT receptor subtype coupled to Gs and adenylate cyclase (for an excellent review of 5-HT7 receptor structure and function see [155]). In the

periphery, this receptor is present in the vascular system [156] and gastrointestinal tract, where it mediates smooth muscle relaxation [157]. In the brain this receptor is expressed on neurons and glial cells of the hippocampus, hypothalamus, thalamus, prefrontal cortex, amygdala, and dorsal raphe [158–162].

The 5-HT7 receptor has been proposed to play a role in circadian rhythms [163] and thermoregulation [164, 165]. In the prefrontal cortex and hippocampus 5-HT7 receptors regulate the activity of GABAergic and glutamatergic pyramidal neurons, providing an anatomical basis for their proposed role in learning, memory and cognition (reviewed in [166]). A variety of different antidepressants and antipsychotics have been demonstrated to have high affinity for this receptor [167]. Recently, two non-selective 5-HT7 antagonists (lurasidone and vortioxetine) have undergone clinical trials and have received marketing approval for the treatment of schizophrenia and depression [168, 169].

BRET and FRET studies have reported that 5-HT7 receptors form homodimers when expressed in recombinant cells [12, 170]. In addition to the traditional biophysical techniques, Teitler and colleagues have used a novel approach called inactivation/reactivation to monitor 5-HT7 homodimer formation. In these studies, the pseudo-irreversible binding of risperidone to the orthosteric binding site of the 5-HT7 receptor was used as a tool to evaluate the functional consequences of receptor dimerization [170, 171]. In the inactivation phase, treatment with saturating concentrations of risperidone, followed by repeated drug wash-out, completely eliminated 5-HT-stimulated cAMP production. Radioligand binding studies performed in parallel revealed that risperidone was producing its insurmountable, inactivating effect by occupying only 50% of the available orthosteric binding sites [170, 171]. In the reactivation phase, the risperidone pretreated and inactivated cells were washed and then exposed to a competitive antagonist (clozapine). Following a second drug wash-out step to remove the competitive antagonist, 5-HT-stimulated cAMP production was completely restored. These results indicate that risperidone was binding in a wash-resistant manner to only one protomer of the homodimer and that it was released when competitive antagonist occupied the second protomer. This was demonstrated using radioligand binding to show that both inactivator and reactivator drugs were operating at the orthosteric binding site [170]. In this study, six different competitive antagonists reactivated the risperidone inactivated receptors in a dose-dependent manner with potencies that closely matched their binding affinities for the orthosteric binding site. These results nicely demonstrate the homodimeric nature of the 5-HT7 receptor when expressed in recombinant cells. However, more importantly similar results with the inactivation/reactivation approach were obtained when experiments were performed on native 5-HT7 receptors endogenously expressed in astrocytes [172], providing some of the most convincing evidence to date for the presence of native 5-HT receptor homodimers. The inactivation/reactivation method can be applied to determine the oligomer number of native GPCR in primary cultures and in vivo [88].

6.3 Convincing Evidence or Experimental Artifact?

Currently, there are hundreds of reports in the published literature describing the dimeric or oligomeric nature of GPCR expressed in recombinant cell systems. Dimer/oligomer formation has been reported to regulate all aspects of GPCR function including synthesis, ligand binding, G protein coupling, and trafficking (reviewed in [124, 173]. Even so, the presence and functional relevance of GPCR dimerization in vivo is widely debated (reviewed in [174]). Concerns have been raised about the functional relevance of GPCR dimerization as monomeric receptors have been reported to activate G proteins in reconstituted systems [175, 176] and the crystal structure of the agonist occupied beta2-adrenergic receptor revealed a monomeric receptor in complex with a single G protein [81]. On the other hand, structural studies of native rhodopsin receptors have revealed their dimeric organization in rod outer segments [177] and their association with G transducin [178]. Additionally, leukotriene, dopamine, and serotonin receptors have been solubilized as homodimers in complex with a single G protein [141, 179, 180].

The presence of GPCR dimers or oligomers in intact native tissues and in vivo is inferred from studies employing indirect biophysical methods, immunofluorescence in fixed tissue sections, and functional studies in transgenic animals. For example, ligand-based FRET studies identified oxytocin homodimers in rat mammary gland tissue [181], and heterodimers of D1 and D2 dopamine receptors in striatal neurons are inferred from antibody-based FRET in fixed brain slices [182]. Supporting evidence is provided by studies using heterodimer selective antibodies [183], transgenic mice expressing mutant receptors that restore [184] or inhibit [185] normal receptor function, and pharmacological studies using heterodimer selective agonists [186, 187].

The majority of studies performed to date on 5-HT receptors have used recombinant cell expression systems. 5-HT receptor homomers have been investigated using a wide variety of techniques including Co-IP, radioligand binding, second messenger activation, BRET, FRET, BiFC, FCS, and SpIDA. It is recognized that each method has its own associated caveats and potential for artifact. However, the use of proper controls can eliminate many of these concerns. For example, proper controls in Co-IP experiments can be used to rule out the possibility of non-specific protein association or aggregation during solubilization and denaturation prior to Western blot. Therefore, a properly designed Co-IP study can provide evidence for the dimeric/oligomeric organization of GPCR in native tissues.

One of the more common criticisms of receptor oligomerization studies is the potential for non-specific interactions due to protein over-crowding in the plasma membrane. With resonance energy transfer (RET) studies, the difficulty lies in discriminating between a specific RET signal and non-specific or bystander RET, as the contribution from bystander RET can be significant. However, the specificity of the signal can be confirmed by demonstrating the dependence of RET efficiency on the donor/acceptor ratio and independence of receptor expression level. In the case of 5-HT receptors, studies have been performed using a wide variety of different BRET and FRET techniques (AP, FL, Homo, Lux, TR, summarized in Table 6.1) each with their own advantages in terms of eliminating potential artifacts. The

observation of positive and specific BRET and FRET signals obtained with each of these methods adds validity to the results and strongly supports the hypothesis that 5-HT receptors form homodimers. In addition, the potential for non-specific receptor-receptor interactions or aggregation due to receptor over-expression is eliminated in FCS studies, as applied to 5-HT2C receptors, since this technique requires very low receptor expression levels in order to be able to track changes in fluorescence intensity of individual fluorescent proteins [125, 126].

It should be noted that RET studies designed to examine the effect of ligand on dimer/oligomer status must be interpreted with caution. It is tempting to speculate that an increase or decrease in RET results from an increase or decrease in dimer/oligomer formation, as has been done in the literature. However, changes in RET following ligand binding can just as easily be attributed to a change in receptor conformation and need not reflect changes in dimer/oligomer status.

The majority of studies to date agree that 5-HT receptors are predominantly expressed as homodimers on the plasma membrane (Table 6.1). There are a few differences in reports regarding the overall size of 5-HT receptor complexes, in terms of dimers versus tetramers, but there have been no reports that 5-HT receptors do not form dimers/oligomers. Studies using native 5-HT receptors expressed in primary cultures of astrocytes [172] and choroid plexus epithelial cells [129] indicate that native 5-HT receptors do form and function as homodimers. These studies employed both biophysical and pharmacological methods and provide the most convincing evidence supporting the physiological relevance of 5-HT receptor homodimers.

6.4 Functional Significance for G Protein Activation

The functional significance of homodimerization, in terms of G protein activation, has been examined for 5-HT2A, 5-HT2C, 5-HT4 and 5-HT7 receptors. In an elegant series of experiments designed to examine the functional significance of 5-HT2A homodimer signaling, antagonists were observed to display biphasic inhibition of radioligand binding (negative cooperativity) and differential regulation of inositol phosphate production versus arachodonic acid release in a monophasic or biphasic manner, respectively [67]. For the antagonists examined in this study, the negative cooperativity observed for the inhibition of second messenger production mirrored the negative cooperativity observed for radioligand binding. These studies are among the first to provide evidence that antagonists can display functional selectivity at homodimeric GPCR, and may have important implications for future drug development as many GPCR-targeted therapeutics are antagonists.

In the case of 5-HT2C receptors, a mutant (S138R) receptor was created that was incapable of binding 5-HT and lost its basal activity and ability to activate G proteins [188]. Co-expression of wild-type (WT) receptors with the S138R mutant receptor resulted in the formation of heterodimers comprised of one active and one inactive protomer, with respect to G protein signaling. It was observed that the WT/S138R heterodimer was incapable of activating G proteins, did not stimulate inositol phosphate production, and did not internalize in response to 5-HT stimulation

[188]. Similar results were reported for the 5-HT7 receptor using the antagonist risperidone which displays very slow dissociation kinetics for binding to one protomer of the homodimer [170, 172]. These experiments revealed that the pseudo-irreversible, wash-resistant binding of risperidone to one protomer did not interfere with 5-HT binding to the second protomer, but rendered the dimer inactive with respect to G protein activation. Taken together, these studies provide evidence that the 5-HT homodimer interacts with a single G protein, that both protomers participate in signaling, and that both protomers must be functional in order for signaling to occur.

The 5-HT4 receptor has been solubilized as a homodimer in complex with a single G protein [141]. Elegant studies using mutant receptors with different ligand binding properties (5-HT4-RASSL) demonstrated that agonist binding to one protomer is capable of G protein activation, although G protein coupling efficiency was two-times greater when both protomers of the dimer were activated by agonist binding [141]. Similar results were reported for 5-HT2C receptors expressed in HEK293 cells [129]. In this study, agonists that bind in a wash-resistant manner to one (cabergoline) or both protomers (ergotamine) within the homodimer produced half-maximal and maximal G protein activation, respectively. These results suggest that each protomer of the homodimer contributes equally toward the signaling process. However, studies with other GPCR have reported allosteric interactions between protomers with one protomer contributing a greater response than the other (see Chap. 17 and [174]). Studies with glycoprotein hormone receptors have established a link between cooperativity and constitutive activity in which receptors displaying the greatest basal activity lost nearly all of their cooperative allosteric regulation [189]. This may explain the more balanced signaling observed between protomers of 5-HT receptor homodimers that display high constitutive activity, such as 5-HT2C [121] and 5-HT4 [190].

Taken together, these studies indicate that the 5-HT receptor homodimer interacts with a single G protein and that both protomers of the homodimer must make functional contacts with the G protein in order for signaling to occur. For receptors displaying constutive activity, both protomers of the homodimer may contribute equally to the signaling process. For 5-HT receptors with low levels of constitutive activity, it remains to be determined whether both protomers will contribute equally to the signaling process or if the signaling will be more asymmetrical in nature.

Studies examining the effect of ligand on GPCR dimer/oligomer status have produced differing results ranging from no effect, to dissociation or association of dimers into oligomers. BRET and FRET studies that examined the effect of ligand on 5-HT receptor oligomer status concluded that ligand binding did not promote the dissociation of dimers into monomers or association into higher order oligomers of 5-HT1A [8, 14], 5-HT2C [118] and 5-HT4 receptors [140, 142]. In a similar fashion, FCS studies of 5-HT2C receptors [119], along with FRAP (fluorescence recovery after photobleaching) studies of β1- and β2- adrenergic receptors [191] and FRET studies of M1 and M2 muscarinic receptors [192], report no effect of agonist on GPCR homodimer/oligomer status in intact cells. On the other hand, agonists have been reported to increase and antagonists to decrease FRET for 5-HT1A [9, 13],

5-HT2A [69, 70] and 5-HT2C receptors [128]. In addition, studies of 5-HT1A [13], 5-HT2C [128], β2-adrenergic [79], M3 muscarinic [193], and D1 and D2 dopamine receptors [194, 195] suggest that these receptors form tetramers or higher order oligomers that are differentially regulated by treatment with various ligands.

At the present time, the great diversity in the results reported for biogenic amine dimer/oligomer status in the absence and following ligand treatment are best interpreted as being method, ligand and receptor dependent. Additional studies, using techniques with single molecule sensitivity and pharmacological methods that can be performed on native receptors in primary cultures and in vivo will be required to determine whether all biogenic amine receptors behave in a similar fashion with respect to their oligomer number and/or whether different ligands are able to induce different dimeric/oligomeric states within a given receptor family. Consistent with this hypothesis, computer simulations of the 5-HT2A receptor indicate a link between the topology of the binding pocket and the dimer interface [71–73]. Different ligand bound conformations of the receptor demonstrate different dimer interface arrangements [71–73], which ultimately could influence the shape of the G protein binding pocket along with G protein coupling specificity and efficiency.

6.5 5-HT Receptor Heteromers

The potential formation of heteromers between different members of the GPCR family greatly expands their potential signaling repertoire and opens up new avenues for the development of novel therapeutics with enhanced selectivity. Indeed, there is a rich literature implicating GPCR heteromers as novel signaling complexes and their potential involvement in neurological and psychiatric disorders (see Chaps. 19 and 20). To facilitate the classification of heteromers, criteria were established by the International Union of Basic and Clinical Pharmacology (IUPHAR) [196]. The specific IUPHAR criteria that must be satisfied in order for a heteromer to be considered functionally relevant are listed in Table 6.2.

Multiple studies have revealed interacting GPCR partners for the 5-HT1A, 5-HT2A and 5-HT2C receptors that satisfy all three of the IUPHAR criteria [203] for classification as functional heteromers, and they are listed in part A of Table 6.2. Additional interacting partners for 5-HT1A and 5-HT2C receptors that satisfy one or two of the criteria are listed in part B of Table 6.2. It should be noted that two of these interacting pairs, 5-HT1A/GalR1-2 and 5-HT2C/MT2, satisfy two of the IUPHAR criteria considered to be sufficient for categorization as functional heteromers [196]. There is preliminary evidence in recombinant cells suggesting the potential for heteromeric associations between 5-HT1A and μ-opioid or adensoine2A, 5-HT2C and MT1, and 5-HT4 with β2-adrenergic receptors. These studies are summarized in part C of Table 6.2. However, additional studies are required to determine if such associations exist in vivo and if so, to determine their potential physiological relevance.

Table 6.2 Serotonin receptor heteromers

Summary of receptor subtypes reported to form heteromers with 5-HT receptors. The receptors were categorized using the IUPHAR criteria for functional heteromers [203]. The table is divided into three parts: A) Meets all IUPHAR criteria; B) Partial IUPHAR criteria; and C) Preliminary studies
IUPHAR Criteria for Functional Heteromers:
1. Demonstrated physical association of cognate receptors:
(a). Co-localization of receptors in primary culture or native tissue (insufficient by itself) and
(b) Co-IP or proximity assay (considered sufficient by itself, without co- localization, when performed in primary culture or native tissue)
2. Pharmacological or functional properties different from the cognate homomers (positive/negative allosteric interactions or novel signaling properties)
3. Use of knock-out animals or siRNA knock-down
Abbreviations: *A2A* adenosine A2A receptor, *AC* adenylate cyclase, *Ag* agonist, *An* antagonist, *BiFC* bimolecular fluorescence complementation, *B2-AR* beta2-adrenergic receptor, *BiFC* biomolecular functional complementation, *BRET* bioluminescence resonance energy transfer, *Ca* calcium, *CB1* cannabinoid receptor1, *Co-IP* co-immunoprecipitation, *D2* dopamine D2 receptor, *ERK* extracellular regulated kinase, *FGFR1* Fibroblast growth factor receptor 1, *FISH* fluorescent in situ hybridization, *FRET* fluorescence resonance energy transfer {*3-FRET* with three fluorescent proteins, *AP* acceptor photo-bleaching, *FCM* flow cytometry, *FL* fluorescence lifetime, *HTRF* homogenous time resolved, *Lux* linear un-mixing}, *GalR1* Galanin Receptor 1, *GHS-R1a* Ghrelin receptor, *GIRK* G protein-gated inwardly rectifying potassium channel, *ICV* intracerebralventricular injection, *IP* inositol phosphate, *KO* knock-out, *mGlu2* metabotropic glutamate receptor2, *MAPK* mitogen activated protien kinase, *mPFC* median prefrontal cortex, *MT2* Melatonin receptor 2, *NA* not applicable, *PLA* proximity ligation assay, *PLC* phospholipase C, *RLB* radioligand binding, *RT-PCR* real-time polymerase chain reaction, *siRNA* silencing RNA, *TIRF* total internal reflective fluorescence microscopy, *WT* wild-type

A). Meets all three IUPHAR criteria

5-HT1A/5-HT7	**Method**	**Measured effect**	**Criteria**	**References**
Mouse brain	Co-IP	Proximity	1b	[12]
N1E-115 cells	FRET(AP/Lux)	Proximity	–	[12]
Hippocampal neuron	RT-PCR	5-HT1A:5-HT7*	–	[12]
N1E-115 cells	GTPγS	↓Gi	2+	[12]
N1E-115 cells	Phosphorylation	↑Erk	2+	[12]
N1E-115 cells	TIRF	Internalization	2+	[12]
Hippocampal neuron & Xenopous Oocyte	Electrophysiology recording	↓GIRK	2^	[12]
Hippocampal neuron	siRNA	↑GIRK	3#	[12]

* Relative expression ratios of 5-HT1A to 5-HT7 change over tenfold during development from 3:1 at P2 to 35:1 at p90.

+ Co-expression of 5-HT7 with 5-HT1A decreased 5-HT1A signaling through Gi, had no affect on 5-HT7 stimulation of Gs, and increased 5-HT1A-mediated Erk phosphorylation. 5-HT1A receptor internalization only occurred in cells co-transfected with 5-HT7 receptors, and 5-HT1A internalization was blocked by 5-HT7 antagonist (SB269970) but not by 5-HT1A antagonist (WAY100635).

^ Co-expression of 5-HT7 decreases 5-HT1A-mediated activation of GIRK.

siRNA knock-down of 5-HT7 receptors restored 5-HT1A-mediated activation of GIRK.

5-HT1A/FGFR1	**Method**	**Measured effect**	**Criteria**	**References**
Rat midbrain raphe	Immunostaining	Proximity	1a	[197]
Rat midbrain raphe	In situ PLA	Proximity	1b	[197]
Raphe RN33B cells	PLA	Proximity	1b*	[197]

(continued)

Table 6.2 (continued)

HEK293T cells	BRET, FRET(AP)	Proximity	–	[197, 198]
Rat midbrain raphe	Phosphorylation	↑Erk1/2	2+	[199]
Raphe RN33B cells	Phosphorylation	↑Erk1/2	2+	[199]
Raphe RN33B cells	TMDV peptide	↓Heteromer^	–	[199]
Raphe RN33B cells	siRNA	↓Erk1/2	3^	[197]
* 5-HT1A/FGFR1 co-activation (8-OH-DPAT/FGF-2) increased heteromer formation.				
+5-HT1A agonist stimulates both ERK1/2 and FGFR1 phosphorylation. Synergistic effects on ERK1/2signalling following co-activation were observed in RN33B cells of the rat midbrain raphe. Co-activation increased cell differentiation and growth in RN33B cells.				
^ PLA, synergistic signaling and differentiation were reduced by a 5-HT1A TMDV peptide (but not by TMDII peptide) and following siRNA knock-down of either 5-HT1A or FGFR1.				
5-HT2A/5-HT2C	**Method**	**Measured effect**	**Criteria**	**References**
Rat prefrontal cortex	Immunostaining	Co-localization	1a	[54]
Rat prefrontal cortex	Co-IP	Proximity	1b	[200]
High impulsivity in outbred rats	Ratio of 2A:2C receptor expression	Motor impulsivity, impulsive behavior	2*	[200]
Rat prefrontal cortex	siRNA	Motor impulsivity, impulsive behavior	2, 3+	[200]
* The ratio of 5-HT2A to 5-HT2C receptor levels in the mPFC was positively correlated with motor impulsivity in individual outbred rats. High phenotypic motor impulsivity was associated with reduced heterodimer formation in the mPFC, measured by Co-IP.				
+5-HT2C siRNA knock-down in the mPFC increased motor impulsivity; increased 5-HT2A receptor expression; and produced a leftward shift in M100907 potency (5-HT2A antagonist) in suppressing impulsive behavior.				
5-HT2A/CB1	**Method**	**Measured effect**	**Criteria**	**References**
Hippocampus, cortex, striatum	In situ PLA	Proximity	1b*	[202]
HEK293T cells	BRET, BiFC, PLA	Proximity	–	[202]
HEK293T cells	cAMP, Ca	↓Gq/11; ↑Gi/o	2+	[202]
WT mice	TMDV/VI peptide	↓Heteromer	3^	[202]
5-HT2A KO mice	Behavioral assay	Memory, Anxiety	3#	[202]
* In situ PLA was performed in WT, 5-HT2A KO and CB1 KO mice.				
+ Co-expression with CB1 switched 5-HT2A coupling from Gq to Gi. CB1 agonist (WIN552122). and 5-HT2A agonist (DOI) signaling were inhibited by pertussis toxin but not by Gq inhibition (YM 254890). Selective antagonism of the 5-HT2A protomer blocked CB1 signaling and selective antagonism of CB1 blocked 5-HT2A signaling.				
^ CB1R-derived TMDV/VI peptides blocked heteromer formation and abolished THC memory impairment in vivo.				
# The amnesic, anxiolytic and increased social interaction effects of THC observed in WT mice were eliminated in the 5-HT2A knock-out mice, with no effect on nociception or locomotion.				
5-HT2A/D2	**Method**	**Measured effect**	**Criteria**	**References**
Rat cortex, striatum, accumbens, s. nigra	Immunostaining	Co-localization	1a	[69]
Rat striatum	In situ PLA	Proximity	1b	[203]
HEK293 cells	FRET (FL/AP)	Proximity	–	[69]
	BRET	(Ag↓, An↑)		[204]

(continued)

Table 6.2 (continued)

HEK293 cells	Co-IP	Proximity	–	[205]
HEK293 cells	PLC, AC, Ca	↑Gq/11; ↑↓Gi/o	2*	[203, 204]
Mouse striatum	RLB	↑binding affinity	2+	[205]
HEK293 cells	IP	↑ efficacy	2^	[205]
5-HT2A KO mice	Behavioral assay	Locomotion	3#	[205]
*5-HT2A-mediated Gq/11 signaling was synergistically enhanced by activation of the D2 protomer with quinpirole. D2-mediated Gi/o signaling was synergistically enhanced by activation of the 5-HT2A protomer with LSD or DOI but was inhibited by 5-HT.				
+ Occupancy of the D2 protomer with agonist (quinpirole) increased DOI binding affinity for 5-HT2A. Occupancy of the 5-HT2A protomer with LSD or DOI, but not 5-HT, increased the agonist high affinity binding of dopamine for the D2 protomer.				
^ Co-expression with D2 increased DOI efficacy for 5-HT2A-mediated activation of Gq/PLC signaling, but had no effect on 5-HT-mediated signaling.				
# 5-HT2A receptor expression is required for haloperidol inhibition of MK-801-stimulated locomotor activity.				
5-HT2A/mGluR2	**Method**	**Measured effect**	**Criteria**	**References**
Mouse cortex	Immunostaining	Co-localization	1a	[206, 208]
	FISH			
Human/mouse brain	Co-IP	Proximity	1b	[206, 209]
HEK293 cells	Co-IP, BRET	Proximity[X]	–	[206, 208]
	BiFC, FRET (HTRF/AP/FCM/3)			[210, 211]
Mouse cortex	RLB	Binding affinity	2*	[206, 207]
HEK293 cells	Ca, GTPγS	mGluR2/3 Ag activates Gq/11	2+	[210]
Xenopus oocyte	Voltage recording	↓Gq/11; ↑Gi/o	2^	[209]
Mouse cortex & cortical neurons	GTPγS	↓Gq/11; ↑Gi/o	2^	[206, 209]
5-HT2A-KO mice	Behavioral assay	Head twitch, Locomotion	3#	[206–209]
mGluR2-KO mice				
5-HT2A-KO mice	GTPγS	Gq/11; Gi/o	3~	[210]
mGluR2-KO mice	Co-IP			
[X] Heteromer formation involves a TMD4/5 interface between 5-HT2A and mGluR2 homodimers.				
* mGluR2/3 agonist LY379 increased the affinity of hallucinogens for 5-HT2A. 5-HT2A agonist (DOI) decreased the affinity of mGluR agonists for mGluR.				
+ mGluR2/3 agonists increased intracellular calcium only in cells co-expressing 5-HT2A and mGluR2. This effect was blocked by selective 5-HT2A antagonist (M100,907) and inactivating mutations, and required a functional mGluR2 homodimer complex with Gi and functional 5-HT2A homodimer complex with Gq.				

(continued)

Table 6.2 (continued)

^ Co-expression with mGluR2 decreased 5-HT2A-mediated activation of Gq by 5-HT. This effect was enhanced by mGluR2/3 agonist and blocked by antagonist LY341495. Co-expression with 5-HT2A enhanced mGluR-mediated activation of Gi by glutamate. 5-HT2A neutral antagonist (methysergide) had no effect while agonist (DOI) decreased and inverse agonist (clozapine) increased glutamate-mediated Gi signaling.

Occupancy of the 5-HT2A receptor in the 5-HT2A/mGluR2 heteromer with mGluR2/3 agonist (LY379268) reduced head twitch induced by 5-HT2A agonists (DOI, LSD) [206]. Head twitch and erg-2 induction by 5-HT2A agonists (LSD, DOI) were abolished in mGluR2-KO mice [207]. Substitution of Ala-677$^{4.40}$, Ala-681$^{4.44}$ and Ala-685$^{4.48}$ in mGluR2 abolished head twitch response induced by DOI [208]. Locomotor-induced behavior by MK801 in WT mice was reduced with mGluR2 agonist LY379268, but not in 5-HT2A-KO mice [209]. MK801 stimulated activity was reduced by 5-HT2A inverse agonist (clozapine) in WT but not in mGluR2-KO mice [209].

~ mGluR2/3 agonist L379268 increased Gi and Gq activity in WT mice, while the mGluR2/3 antagonist LY341495 reduced their activity. LY379268 did not activate Gi and Gq in mGluR2-KO mice. LY379268 stimulated Gi activity in 5-HT2A-KO mice and the LY379268-dependent activation of Gq was reduced.

B). Partial IUPHAR criteria

5-HT1A/D2	**Method**	**Measured effect**	**Criteria**	**References**
Mouse cortex	Immunostaining	Co-localization	1a	[214]
HEK293 cells	FRET (FL/HTRF)	Proximity (An↑)	–	[214]
HEK293 cells	cAMP, IP	↓Gi, ↑Gq/11	2*	[214]
HEK293 cells	Phosphorylation	↑ERK	2+	[214]

* Simultaneous application of clozapine and 8-OH-DPAT to cells co-expressing 5-HT1A and D2 receptors decreased cAMP production and activated a novel signaling pathway, Gq activation of IP production. The opposite effect was observed when the drugs were added separately, or when added simultaneously to cells individually expressing either 5-HT1A or D2.

+ Clozapine and 8-OH-DPAT enhanced ERK phosphorylation only in cells co-expressing 5-HT1A and D2 receptors.

5-HT1A/GalR1-2	**Method**	**Measured effect**	**Criteria**	**References**
Rat hippocampus & dorsal raphe	PLA	Proximity	1b	[215]
HEK-293 cells	Co-IP, FRET (AP)	Proximity	–	[221, 222]
Purified receptors	Surface plasmon resonance	Heteromer formation*	–	[223]
Rat hippocampus & dorsal raphe	RLB	↑GalR affinity	2+	[215–220]
		↓5-HT1A affinity		
HEK-293 cells	Luciferase assay	↓AC, ↓MAPK	2^	[221]
Rat – ICV injection	Behavioral assay	Passive avoidance	2#	[224]

* Purified 5-HT1A and GalR1 formed heteromers that were disrupted by pre-exposure to Zinc.

+5-HT1A receptor activation increased the affinity of GalRs in rat brain homogenates and sections. Galanin peptide (GAL1–15) decreased 5-HT1A agonist binding affinity in brain homogenates, an effect blocked by GalR1-2 antagonist (M35).

^ Transinhibition of AC and MAPK signaling in cells co-expressing 5-HT1A and GalR1.

ICV galanin attenuated 5-HT1A-mediated (8-OH-DPAT) passive avoidance retention deficit.

5-HT2C/GHS-R1a	**Method**	**Measured effect**	**Criteria**	**References**

(continued)

Table 6.2 (continued)

Primary neurons (hippocamp/hypothal)	Immunostaining	Co-localization	1a	[225]
HEK293A cells	FRET (FC)	Proximity	–	[225]
HEK293A cells	Fluorescence	↓ calcium	2*	[226]
C57BL/6 mice	Food consumption	↑, ↓	2+	[225]
* Co-expression with 5-HT2C attenuated GHS-R1a-mediated intracellular calcium release, an effect that was blocked by 5-HT2C antagonist.				
+ Ghrelin's orexigenic effect was potentiated or attenuated following intraperitoneal administration of selective 5-HT2C antagonist (SB242084) or agonist (lorcaserin), respectively.				
5-HT2C/MT2	**Method**	**Measured effect**	**Criteria**	**References**
Cortex, choroid & hippocampus	Co-IP	Proximity	1b*	[228]
HEK293 cells	BRET	Proximity (Ag↑)	–	[228]
HEK293 cells	IP	↑Gq/11	2+	[228]
HEK293 cells	Western blot	Receptor level^	–	[228]
* Co-IP was performed in human brain.				
+ Co-expression with MT2 enhanced 5-HT2C activation of Gq/PLC. Melatonin binding to MT2 produced a unidirectional transactivation of the 5-HT2C protomer. Agomelatine displayed biased signaling.				
^ Co-transfection with MT2 doubled the cell surface expression of 5-HT2C receptors, increasing 5-HT-stimulated IP production.				
C). Preliminary studies				
5-HT1A/μ-opioid	**Method**	**Measured effect**	**Criteria**	**References**
Cos-7/CHO-K1 cells	Co-IP	Proximity	–	[229]
HEK293 cells	BRET	Proximity	–	[229]
CHO-K1 cells	Phosphorylation	↓ERK1/2	2	[229]
CHO-K1 cells	[35S]GTPγs	Transactivation	2	[229]
5-HT1A/A2A	**Method**	**Measured effect**	**Criteria**	**References**
HEK-293 cells	FRET (FL/AP)	Proximity	-	[9]
		(Ag↑, An↓)		
5-HT2C/MT1	**Method**	**Measured effect**	**Criteria**	**References**
HEK293 cells	Co-IP	Proximity	–	[228]
HEK293 cells	BRET	Proximity	–	[228]
5-HT4/β2-AR				
CHO-K1 cells	BRET	Proximity	–	[140]

6.5.1 5-HT Receptor Heteromers Satisfying the IUPHAR Criteria for Functional Heteromers

6.5.1.1 5-HT1A/5-HT7

5-HT1A receptors have been reported to form heterodimers with 5-HT7 receptors. 5-HT1A/5-HT7 heteromers have been reported in mouse hippocampal neurons where they regulate GIRK (G protein-gated inwardly rectifying potassium channel) activity [12]. Heterodimerization was reported to decrease 5-HT1A activation of

Gαi and GIRK activity with no effect on 5-HT7 activation of Gαs, indicating an inhibitory role of the 5-HT7 protomer on 5-HT1A receptor function [12]. In this study, a Lux-FRET method was used to demonstrate the preferential formation of 5-HT7 homodimers over 5-HT1A/5-HT7 heterodimers and 5-HT1A homodimers. Interestingly, 5-HT7 receptor production in the hippocampus decreased during postnatal development, while 5-HT1A expression remained constant. The overall relative proportion of 5-HT1A/5-HT7 heterodimers decreased from 9% at postnatal stage 2 (P2) to 2% at P90, indicating that the concentration of heterodimers and their functional significance would change over time during development [12].

6.5.1.2 5-HT1A/FGFR1

Co-localization, close proximity, and synergistic signaling between the 5-HT1A receptor and the fibroblast growth factor (FGFR1) tyrosine kinase receptor have been reported in rat midbrain raphe neurons [197–199]. The in situ proximity ligation (PLA), positive BRET, and the synergistic effects on second messenger activation were reduced by a peptide corresponding to TMDV of the 5-HT1A receptor, implicating a requirement of functional 5-HT1A homodimers in mediating the observed effects. Knock-down (siRNA) of either 5-HT1A or FGFR1 reduced the observed synergistic effects on ERK (extracellular regulated kinase) signaling. The 5-HT1A/FGFR1 signaling complex was reported to have neurotrophic effects (increase neurite density, protrusions and growth cone development) in hippocampal neurons [197, 199]. Based on these results, the 5-HT1A/FGFR1 heterocomplex has been proposed to play a role in hippocampal plasticity and may represent a novel target for reversing depression-induced atrophy of hippocampal neurons [197, 199].

6.5.1.3 5-HT2A/5-HT2C

Given the overall similarity in structure between 5-HT2A and 5-HT2C receptors, the 5-HT2C receptor would be the most likely member of the 5-HT receptor family for forming heteromers with the 5-HT2A receptor. Immunohistochemical analyses indicate that 5-HT2A and 5-HT2C co-localize in the same GABAergic neurons as well as in a population of pyramidal projection neurons in the rat medial prefrontal cortex (mPFC) [54]. Co-IP studies suggest that both receptors are found in the same protein complex in the rat mPFC [200]. The ratio of 5-HT2A to 5-HT2C receptor protein levels in the mPFC was positively correlated with motor impulsivity in individual outbred rats [200]. In this study, high phenotypic motor impulsivity was associated with reduced heterodimer formation in the mPFC. 5-HT2C siRNA knock-down in the mPFC increased motor impulsivity, increased 5-HT2A receptor expression, and produced a leftward shift in M100907 (5-HT2A antagonist) potency in suppressing impulsive behavior. The combination of low doses of the selective 5-HT2A antagonist M100907 *plus* the preferential 5-HT2C agonist MK212 evoked

modest effects, but resulted in an approximately additive suppression of cocaine-evoked hyperlocomotion and c-Fos expression in the caudate putamen [201]. In a second study, the combination of subthreshold doses of M100907 *plus* the selective 5-HT2C agonist WAY163909 synergistically suppressed inherent and cocaine-evoked motor impulsivity as well as cocaine-induced hyperactivity and cocaine-seeking behavior [60]. Thus, the 5-HT2A/5-HT2C system provides a rich avenue for further studies related to the mechanisms and treatment of drug addicition.

6.5.1.4 5-HT2A/CB1

Compelling evidence for the close proximity of 5-HT2A and cannabinoid CB1 receptors is provided by in situ PLA in mouse brain, an observation that was abolished in either 5-HT2A or CB1 knock-out (KO) mice [202]. When the signaling properties of the proposed heteromer were investigated in HEK293 cells, it was observed that co-expression of the CB1 receptor resulted in a switching of 5-HT2A receptor coupling from Gq to Gi, and bidirectional cross antagonism was observed following selective blockade of 5-HT2A or CB1 receptors [202]. Interestingly, the amnesic, anxiolytic and social interaction effects of THC observed in WT mice were abolished in 5-HT2A KO mice, while the analgesic properties of THC were unaltered. These results suggest the 5-HT2A/CB1 heteromer as a potential target for the dissociation of the memory impairing effects of THC from its therapeutically beneficial analgesic properties [202].

6.5.1.5 5-HT2A/D2

Heteromers of 5-HT2A and D2 dopamine receptors have been postulated based on co-localization, proximity, synergistic and transinhibition of binding affinity and signaling, and behavioral studies in knock-out mice [69, 70, 203–205]. Co-immunostaining and in situ PLA provide evidence favoring the close proximity of 5-HT2A and D2 receptors in the rat cortex, striatum, accumbens, and substantia nigra [69, 203], and positive FRET/BRET signals between 5-HT2A and D2 receptors have been reported in recombinant cells [69, 70, 204]. In these studies, FRET efficiency was differentially regulated by agonists and antagonists, and was reduced by mutations in the C-terminus of the 5-HT2A receptor (including the polymorphism H452Y) and mutations in the third intracellular loop of the D2 receptor [69, 70]. 5-HT2A agonists with hallucinogenic properties (LSD, DOI), but not 5-HT, increased the density of ^{3}H-raclopride binding sites in the striatum and increased dopamine binding affinity, effects that were blocked by the 5-HT2A antagonist, ketaniserin [203]. In a similar fashion, D2 activation with quinpirole increased the binding affinity of DOI at 5-HT2A receptors [205]. Co-activation of 5-HT2A and D2 receptors with 5-HT and quinpirole synergistically enhanced 5-HT2A signaling through Gq/PLC, while decreasing D2-mediated Gi signaling [203–205]. In contrast to its effect on 5-HT

signaling, quinpirole decreased DOI-mediated activation of Gq/PLC signaling through 5-HT2A receptors [205]. Studies using knock-out mice demonstrated a role for 5-HT2A receptors in haloperidol-mediated inhibition of MK-801-stimulated locomoter activity [203]. Since 5-HT2A and D2 receptors are expressed in brain regions predicted to play a role in psychosis, and are targets for currently marketed anti-psychotics, the identification of 5-HT2A/D2 heteromers in the brain could have important implications for the pathology and treatment of schizophrenia.

6.5.1.6 5-HT2A/mGluR2

5-HT2A receptors have been reported to form heteromers with glutamate mGluR2 receptors and the heterocomplex has been suggested to play a role in psychosis [206–210]. Their existence in vivo is postulated based on co-localization and Co-IP from mouse and human cortical tissue, studies using knock-out mice and studies in post-mortem tissue from schizophrenic subjects [206–210]. In HEK293 cells co-expressing 5-HT2A and mGluR2 receptors, but not in cells expressing either receptor alone, activation of mGluR2 stimulated 5-HT2A-receptor mediated intracellular calcium release [210]. When both 5-HT2A and mGluR2 were present, 5-HT-mediated Gq signaling was reduced and glutamate-mediated Gi signaling was enhanced [209]. The 5-HT2A/mGluR2 heterocomplex is hypothesized to regulate the balance between Gq and Gi signaling and is suggested to predict the anti-psychotic and pro-psychotic activity of drugs that target 5-HT2A or mGluR2 receptors [209]. An elegant series of experiments (using ligand binding and signaling defective mutant receptors) demonstrated that the observed cross-talk required the presence of functional homodimers of both 5-HT2A and mGluR2, each in complex with their own G protein, joined together at TMD4/5 to form a heterotetramer [210]. In the heterocomplex, activation of the mGluR2 protomer was reported to inhibit signaling and behavioral responses to hallucinogens [206–210]. Knock-out of the mGluR2 receptor eliminated the well known head-twitch behavioral response to hallucinogens mediated through 5-HT2A receptors, implicating a role for the heterocomplex in mediating behavioral responses to hallucinogens [206–208, 210]. The potential differential regulation of 5-HT2A and mGluR2 receptor expression levels and signaling properties in post-mortem brain tissue from schizophrenic subjects has been suggested to provide a role for the heterocomplex in the etiology of schizophrenia [206, 210]. Several studies examining allosteric signaling properties have implicated the heterocomplex as a potential target for the development of novel antipsychotic drugs [208–210]. However, the biological relevance of this heterocomplex has been questioned based on the lack of differential regulation of signaling by serotonergic and glutamatergic agonists in HEK293 cells co-expressing 5-HT2A and mGluR2 receptors and questions have been raised about their neuronal co-localization [211, 212]. The apparent discrepancy in results obtained by the two different groups awaits clarification.

6.5.2 5-HT Receptor Heteromers with Partial IUPHAR Criteria

6.5.2.1 5-HT1A/D2

It is well established that 5-HT1A and D2 dopamine receptors are expressed in brain regions implicated in schizophrenia, and both receptors have been cited as playing a role in the efficacy of antipsychotic drugs [213]. Immunohistochemical labeling studies have reported the co-localization of 5-HT1A and D2 receptors in mouse cortical neurons, and antipsychotic drugs have been reported to produce differential effects on signaling through cAMP, IP and ERK in HEK293 cells co-expressing 5-HT1A and D2 receptors [214]. Positive FRET signals in co-transfected HEK293 cells are suggestive of heteromer formation that is regulated by antipsychotic drugs [214]. These studies provide strong rationale for future in vivo studies aimed at elucidating the potential role of this heteromer in the pathology and treatment of schizophrenia.

6.5.2.2 5-HT1A/GalR1-2

5-HT1A/galanin GalR1-2 heteromers have been reported to be expressed in rat hippocampus and dorsal raphe, and have been suggested to represent a novel target for the treatment of depression [215]. Differential regulation of signaling through this heterocomplex was reported as a decrease in 5-HT1A agonist radioligand binding affinity following GalR activation and an increase in GalR affinity following activation of 5-HT1A receptors in rat brain homogenates [216–220]. Co-immunoprecipiation, positive FRET and trans-inhibition of adenylate cyclase and MAPK signaling have been reported in HEK293 co-expressing 5-HT1A and GalR1-2 [221, 222]. Purified receptors were evaluated using surface plasmon resonance spectroscopy to determine the kinetics of heteromer formation, which was disrupted following treatment with zinc [223]. Intracerebroventricular administration of galanin attenuated 5-HT1A-mediated passive avoidance retention deficit in a rats [224]. These studies provide support for the design of 5-HT1A/GalR1-2 specific compounds to be tested in future studies aimed at understanding the role of this heterocomplex in the physiology and pharmacotherapy of depression.

6.5.2.3 5-HT2C/GHS-R1a and 5-HT2C/MT2

Two different heterocomplexes, both involving the 5-HT2C receptor, have been suggested to play a role in appetite regulation and obesity. The 5-HT2C receptor has been reported to co-localize with the ghrelin GHS-R1a receptor in cultured primary hypothalamic and hippocampal neurons from the rat and to form heterodimers with GHS-R1a when co-expressed in HEK293 cells [225, 226]. In these studies,

pharmacological activation and blockade of 5-HT2C receptors attenuated and potentiated, respectively, the orexigenic effects of ghrelin in mice. Based on these results, a role for the 5-HT2C/GHS-R1a heteromer in appetite regulation has been postulated (reviewed in [227]). In addition, 5-HT2C and melatonin MT2 receptors have been are present in human cortex and hippocampus and are suggested to play a role in obesity [228]. Interestingly, the novel antidepressant agomelatine displayed biased signaling as a 5-HT2C antagonist and MT2 agonist, suggesting the heterodimer as a potential target for the development of a novel class of therapeutics for the treatment of psychiatric disorders as well as eating disorders and obesity [228].

6.6 Implications for the Development of Novel Therapeutics

A largely unanswered question is how the discovery of GPCR dimers/oligomers will impact future drug development: will bivalent ligands targeting 5-HT receptors have enhanced therapeutic potential? Currently, we do not have the answer to this question. Many of the published studies related to 5-HT receptors have explored the binding properties of homodimeric ligands. Some studies have reported increased potency or efficacy for homodimeric agonists [27–30], while other studies have reported no improvement of a homodimeric antagonist over the monomeric version of the ligand [90]. In some cases, the agonist character of the monomeric parent compound was lost in the homodimeric form [89]. The mixed results observed were both ligand and 5-HT receptor sub-type dependent.

Studies with 5-HT2C and 5-HT7 receptors indicate that when one protomer of the homodimer is held in an inactive conformation, either by mutation or by occupancy with antagonist, the binding of full agonist to the second, unoccupied protomer does not stimulate G protein activation or signaling [170, 172, 188]. Consistent with these results, studies involving 5-HT1A/5-HT7 [12] and 5-HT2A/CB1 [202] heteromers have reported that binding of a selective antagonist to one protomer blocks the signaling of a selective agonist bound to the other protomer. These results suggest that perhaps antagonists are just as effective at blocking signaling in their monovalent form and that synthesis of bivalent antagonists may not provide additional benefit for blockade of signaling. This hypothesis could easily be tested for a given receptor subtype by applying the inactivation/reactivation method using an antagonist with slow dissociation kinetics [88]. If antagonist occupancy of a single protomer within the dimer complex is sufficient to block signaling, then pretreatment with a saturating concentration of antagonist (one that binds with very slow dissociation kinetics in a wash-resistant manner to one protomer of the dimer) followed by drug wash-out would result in a 50% loss of agonist radioligand binding and complete loss of agonist-mediated signaling.

Given that computer modeling, radioligand binding, and pharmacological studies suggest asymmetry and in some cases cooperativity between the protomers within the homodimer (reviewed in Chap. 17), perhaps bivalent agonists incorporating two different pharmacophores may prove to be of greater utility than

homodimeric ligands. In this case, computer modeling studies with a specified pharmacophore bound to one protomer may be useful in predicting the conformation of the binding pocket of the second protomer and may aid in the design of an appropriate compound targeting the second protomer. This approach may also be advantageous for designing heteromer selective ligands. At the present time, it is unclear whether bivalent ligands will have improved clinical efficacy over their monomeric counterparts. Clearly, additional studies are needed to fully explore the potential of bivalent ligands as novel 5-HT receptor therapeutics.

6.7 Summary

During the past two decades, there have been more than 100 published reports related to 5-HT receptor dimer/oligomerization. These studies have used a wide variety of biochemical, biophysical and pharmacological methods to demonstrate that 5-HT receptors are not isolated entities, but rather that they self-associate to form functional complexes. This self-association process leading to the formation of homodimers appears to begin during receptor synthesis in the ER as a natural step in receptor maturation and processing [123] and has been proposed as a general mechanism necessary for trafficking of class A GPCR to the plasma membrane (reviewed in [124]).

The majority of studies have reported the homodimer as the preferred signaling unit for 5-HT1, 5-HT2, 5-HT4 and 5-HT7 receptors [8, 11, 67, 68, 78, 119, 129, 141, 172]. However, FRET and SpIDA studies indicate that 5-HT1A and 5-HT2C receptors can form higher order oligomers at higher receptor expression levels [13, 128]. Functional studies indicate that the 5-HT receptor homodimer interacts with a single G protein [67, 141, 172, 188]. In addition to pharmacological evidence, this hypothesis is supported by biochemical studies in which 5-HT4 receptors were purified as a pentameric assembly of two receptors and a single heterotrimeric G protein [141]. The same 2:1 receptor:G protein stoichiometry was observed for purified leukotriene [179], dopamine [180], and rhodopsin receptors [230]. All of these studies support the hypothesis that the homodimer is the minimal functional signaling unit for 5-HT receptors, as well as other biogenic amine receptors.

The strongest evidence supporting the hypothesis that 5-HT receptors form and function as homodimers comes from studies of native 5-HT2C receptors in choroid plexus epithelial cells [129] and native 5-HT7 receptors in astrocytes [172]. In these studies, native receptors endogenously expressed in their natural cellular environment were identified as homodimers on the plasma membrane and demonstrated signaling properties consistent with a homodimeric structure. To date, these studies provide the most compelling evidence demonstrating the functionality and physiological relevance of native 5-HT receptor homodimers.

Studies designed to explore potential dimer interface(s) of biogenic amine GPCR suggest a TMD1/2-H8 homodimer interface and a TMD4/5 interface potentially responsible for the association of dimers into higher order oligomers (see Chap. 15 and [231–234]). This appears to be the case for the 5-HT2A/mGluR heteromer

[210]. However, studies with 5-HT receptor homodimers seem to implicate a TMD4/5 homodimer interface [11, 72, 78, 142, 208].

Pharmacological studies indicate that both protomers of 5-HT2, 5-HT4 and 5-HT7 homodimers participate in signaling through G proteins. Activation of one protomer produces a sub-maximal response and both protomers are required for maximal G protein stimulation [141, 172, 188]. For the 5-HT2A receptor, guanyl nucleotide-insensitive biphasic radioligand binding curves were observed for a series of antagonists, suggestive of negative cooperativity in a homodimeric complex [67]. Asymmetry in G protein coupling and G protein activation has been reported for other class A GPCR. For constitutively active receptors, such as 5-HT2C and 5-HT4, the signaling appears to be more balanced with both protomers contributing to the signaling process in an equal manner [129, 189].

Heterodimerization opens up a new avenue for differential regulation of signaling, either by enhancement or inhibition of the original pathways activated by the cognate protomers or by activation of new pathways. For example, heteromers of 5-HT1A/5-HT7 [12] and 5-HT2A/mGluR2 receptors [206–210] have been shown to display functional characteristics different than their respective homodimer counterparts. Changes in expression level of one partner of a heteromer either during development, aging, chronic drug treatment or disease progression, would have profound effects on the relative concentration and thus physiological consequence of heteromer signaling. This has important implications for the pharmacological intervention of physiological processes regulated by GPCR heteromers and for diseases caused by altered GPCR expression and or function. Heteromers involving 5-HT receptors have been suggested to play physiological roles in schizophrenia (5-HT2A/D2; 5-HT2A/mGluR2; 5-HT1A/D2), depression (5-HT1A/FGFR1; 5-HT1A/GalR1-2), drug addiction (5-HT2A/5-HT2C), appetite and obesity (5-HT2C/GHS-R1a; 5-HT2C/MT2), memory and anxiety (5-HT2A/CB1), and neuronal development (5-HT1A/5-HT7).

Studies with bivalent ligands targeting 5-HT receptors have been in progress for over 20 years. So far, the results of the studies described herein indicate that simultaneous occupancy of both protomers of a 5-HT homodimer with a bivalent agonist ligand may enhance ligand selectivity, potency or efficacy, or may even change the pharmacological profile of the ligand from agonist to antagonist. To date, studies with bivalent antagonists targeting 5-HT receptors have not yielded compounds with improved binding or signaling properties. 5-HT receptor heteromer selective drugs have yet to be synthesized and tested. In conclusion, much more work is needed in this area to realize the full potential of this approach for designing better therapeutics targeting 5-HT-related disorders.

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Chapter 7
Class A GPCRs: Cannabinoid and Opioid Receptor Heteromers

Salvador Sierra, Ivone Gomes, and Lakshmi A. Devi

Abstract Cannabinoid and opioid receptors mediate a variety of physiological processes including pain and drug reward. Both receptors couple to $G_{\alpha i/o}$ proteins and their activation leads to inhibition of adenylyl cyclase and potassium channel activity ultimately leading to inhibition of synaptic transmission. Over the last decade increasing anatomical, biochemical and pharmacological evidence demonstrated interactions between the opioid and cannabinoid receptor systems that could modulate not only the physiological but also the pathophysiological roles of these receptors. The present review aims to summarize the literature surrounding the properties of cannabinoid and opioid receptors and the unique signaling following their interaction as well as the novel interventions in the management of disorders ranging from pain to addiction.

Keywords Opioid receptor • Cannabinoid receptor • Heteromers • Addiction • Pain

7.1 Introduction

Cannabinoid and opioid receptors are members of the family A of G-protein coupled receptors (GPCRs) that share a number of features: (i) both receptors couple to $G_{\alpha i/o}$ proteins; thus activation of either receptor leads to the inhibition of adenylyl cyclase activity, membrane depolarization and neurotransmitter release [56, 114]; (ii) the activation of either receptor leads to similar physiological responses such as pain attenuation, euphoria and decreased gastrointestinal motility [56, 114]; (iii) both cannabinoid and opioid receptors and their endogenous ligands exhibit overlapping distributions in sensory and reward circuits [158, 166]; and (iv) both receptors are targets for drugs of abuse like heroin for the μ opioid receptor (μOR) and marijuana

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for the CB_1 cannabinoid receptor (CB_1R). Together these common features make cannabinoid and opioid receptors very attractive targets for the development of therapeutics for the treatment of pain and addiction [159].

For a number of years it was classically accepted that a functional GPCR comprised of a single polypeptide receptor (i.e. a receptor protomer or monomer) associated with a single heterotrimeric G-protein that was capable of turning an input signal into a cellular response. In addition, the receptor protomer could be trafficked from the cell surface to intracellular compartments and vice-versa, and its expression levels could be regulated [69]. However, several pharmacological and biochemical studies carried out in the early 80s provided indirect evidence suggesting that the functional receptor comprised of two or more polypeptides (reviewed in [208]). The availability of differentially epitope tagged receptors in the 90s facilitated the use of co-immunoprecipitation studies that showed that GPCRs could exist as dimers or higher order structures called oligomers. Receptor dimers can be of two types: homomers (association between identical protomers) or heteromers (association between protomers of different subtypes of a receptor or between two different receptors). Today using a variety of techniques and approaches it is clear that a number of GPCRs including opioid and cannabinoid receptors can form heteromers (reviewed in [91]) and that receptor heteromerization provides a mechanism for introducing versatility, diversity and fine-tuning of biological systems.

In this review we will briefly describe the distribution, regulation and pharmacology of cannabinoid and opioid receptors. We will also describe evidence for interactions between these two receptor systems. In addition, we will describe evidence for formation of cannabinoid-opioid receptor heteromers with a focus on the recently proposed criteria for receptor heteromerization (reviewed in [91]). Finally we will describe the relevance of these heteromeric complexes in disease and in drug discovery.

7.2 The Cannabinoid System

The cannabinoid system, also known as the endocannabinoid (eCB) system, comprises the cannabinoid receptor 1 (CB_1R) [167], considered to be the main GPCR along the central nervous system (CNS) [108], the cannabinoid receptor 2 (also named CB_2R) [178], also known as the "immune cannabinoid receptor" along with their lipid based endogenous ligands, 2-arachidonoylglycerol (2-AG), and anandamide (AEA). More recently, endogenous peptidic ligands to CB_1R, named hemopressins, have been identified [92, 105]. Studies show that lipidic cannabinoid ligands can also target peroxisome proliferator activated receptors (PPARs) [188], transient receptor potential channels (i.e. TRPV1) [268], or GPR55 [106]. However, since most studies involving the eCB system have examined CB_1R and CB_2R, these two receptors will be the focus of this review.

7.2.1 Cannabinoid Receptors

7.2.1.1 Cannabinoid 1 Receptor (CB_1R)

Human CB_1R is encoded by the CNR1 gene at the 6q15 locus [190]. This receptor was first cloned in 1990 from a rat cerebral cortex cDNA library [167]. CB_1R is present both in the periphery and CNS, although with greater expression in the CNS. Within the CNS the receptor is highly expressed in the somatosensory cortex (layers II, III, V and VI), hippocampus (CA3 layer and dentate gyrus) and cerebellum (molecular layer); the receptor exhibits moderate to low expression in the motor cortex (layer V), thalamus, nucleus accumbens (NAc), ventral pallidum, subthalamic nucleus, ventral tegmental area (VTA), substantia nigra *pars compacta* (SNpc) and spinal cord [107, 154]. With regards to its subcellular location, CB_1R is mainly present at the cell membrane of preterminal axon shafts and within somatodendritic endosomes and lysosomes, and to a lesser extent at synaptic terminals [237]. Interestingly, the inhibition of CB_1R basal activity leads to decreases, whereas its activation leads to an increase in endosomal location [237]. In addition, studies have detected the presence of CB_1R in mitochondria suggesting a role for the receptor in the control of cellular respiration and energy production [7].

CB_1R couples to $G_{\alpha i/o}$ proteins and its activation leads to inhibition of adenylyl cyclase activity and of N- and P/Q-type calcium currents and to activation of mitogen-activated protein kinase, and of A-type and inwardly rectifying potassium currents [20, 150]. Several studies have shown that in addition to $G_{\alpha i/o}$ proteins CB_1R can also signal through other G proteins ($G_{\alpha s,}$ $G_{\alpha q}$ or $G_{\alpha z}$) depending on ligand treatment or the cell type being investigated [80, 88, 156, 180] (see Fig. 7.1). Ultimately, CB_1R activation leads to the inhibition of the release of a number of neurotransmitters such as glutamate, GABA, glycine, acetylcholine, norepinephrine, dopamine, serotonin and cholecystokinin [124].

The physiological role of CB_1R has been revealed through the use of mice where receptor levels have been knocked-down or knocked-out. Studies with these animals revealed a role for CB_1R in antinociception [61], reward [73, 165], object recognition [232], memory storage and retrieval [204], motor learning [133] and movement disorders [226].

7.2.1.2 Cannabinoid 2 Receptor (CB_2R)

Human CB_2R is encoded by the CNR2 gene at the 1p36.11 locus [190]. This receptor was cloned in 1993 from a human promyelocytic leukemic line HL60 cDNA library [178]. In contrast to CB_1R, CB_2R is highly expressed in primary and secondary lymphoid organs (for example, spleen), and within the CNS in cells of immune lineage such as microglia [161, 262] particularly under pathological conditions, where it modulates cytokine release [174]. Although the presence of CB_2R in neurons has been somewhat controversial, several studies have detected its presence in

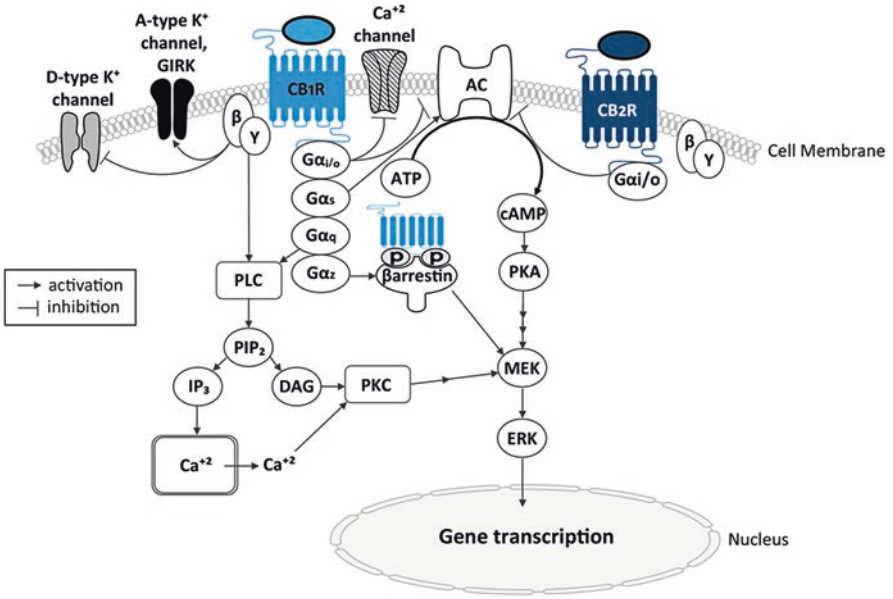

Fig. 7.1 Cannabinoid receptor-mediated signaling
The stimulation of CB_1R or CB_2R by an agonist leads to the dissociation of the $G_{\alpha i/o}$ subunit from the $G_{\beta\gamma}$ (under certain conditions CB_1R can couple to $G_{\alpha s}$, $G_{\alpha q}$ or $G_{\alpha z}$). This leads to $G_{\alpha i/o}$-mediated inhibition of cAMP production and of calcium channels and to $G_{\beta\gamma}$-mediated modulation of potassium channels, and ultimately to inhibition of neurotransmitter release. The activation of $G_{\alpha s}$, $G_{\alpha q}$, or $G_{\alpha z}$ leads to different effects from the activation of $G_{\alpha i}$ proteins, either increasing cAMP production (for $G_{\alpha s}$), stimulating PLC activity and calcium release (for $G_{\alpha q}$), or recruiting β-arrestin (for $G_{\alpha z}$).
Abbreviations: *AC* adenylyl cyclase, *PLC* phospholipase C, *PIP2* phosphatidylinositol diphosphate, *IP3* inositol triphosphate, *DAG* diacyl glycerol, *PKA* protein kinase A, *P* phosphate group

neurons from the cortex, cerebellum, amygdala, striatum, globus pallidus and substantia nigra *pars reticulata* (SN*pr)* although at far lower levels than in spleen [95]. With regards to its subcellular location, CB_2R is found mainly at somatodendritic sites, and in intracellular membranous structures such as mitochondria [54, 225].

CB_2R like CB_1R couples to $G_{\alpha i/o}$ proteins and its activation leads to inhibition of adenylyl cyclase activity and activation of mitogen-activated protein kinase [22](see Fig. 7.1). In addition, CB_2R activation leads to a rapid, and transient increase in intracellular free Ca^{+2} in neuronal and immune cells [233]. However, the signaling pathways mediated by CB_2R in neurons have not yet been elucidated. Nevertheless, a few reports have suggested an involvement of CB_2R in modulating glutamatergic, dopaminergic and GABAergic synaptic transmission [78, 132, 261]. With regards to the physiological role of CB_2R, studies using knock-out animals suggested an involvement of the receptor in antinociception [117], reward [199], memory [143] and movement disorders [192, 226].

7.2.2 Cannabinoid Ligands

7.2.2.1 2-arachidonoylglycerol (2-AG)

2-AG is the most abundant eCB in the brain with highest levels being present in the brainstem, hippocampus, striatum and medulla and lowest levels in the diencephalon, cerebellum and cortex [16]. Under physiological conditions, 2-AG is synthesized and released by the postsynaptic neuron [98] although microglial cells could also contribute to its synthesis during inflammation [30]. 2-AG is produced by the sequential hydrolysis of 1-stearoyl-2-arachidonoyl-sn-glycerol by phospholipase Cβ (PLCβ) followed by hydrolysis of the resulting diacylglycerol (DAG) by DAG lipase (DAGL) [179]. Even though 2-AG can be oxidized or hydrolyzed by cyclooxygenase-2 (COX-2) and fatty acid amide hydrolase (FAAH) respectively, it is primarily degraded by three hydrolytic enzymes, monoacylglycerol lipase (MAGL), α/β domain-containing hydrolase 6 (ABHD6) and α/β domain containing hydrolase 12 (ABHD12) [19]. Compared to classic neurotransmitters eCBs are not stored in vesicles, they are secreted "on demand" into the extracellular space in response to elevations of intracellular calcium levels [151]. Studies have shown an involvement of pre- and postsynaptic neurons as well as astrocytes in regulating 2-AG levels in the brain [247].

2-AG behaves as a partial CB_1R/CB_2R agonist with higher affinity and intrinsic activity for CB_1R [233, 234]. Ligand bias analysis in CHO cells expressing human CB_1R indicate that 2-AG exhibits very little bias towards decreases in cAMP levels or increases in phosphorylated ERK 1/2 levels [128]. That 2-AG could be an endogenous CB_1R agonist is supported by DAGLα knock-out mice (lacking the enzyme needed for 2-AG synthesis) that exhibit a phenotype similar to mice lacking CB_1R [77]. Interestingly, studies with MAGL knock-out mice indicate that sustained increases in 2-AG levels result in increased hippocampal long term potentiation (LTP) as well as cognitive and spatial learning [194].

7.2.2.2 Anandamide (AEA)

Anandamide (AEA) is the second most abundant eCB in the CNS [16] with highest levels of expression in brainstem (mesencephalon), hippocampus, striatum and medulla and lowest levels in diencephalon, cerebellum and cortex [17]. AEA can be generated by multiple pathways depending on the availability of precursors, the brain region involved and the particular physiologic or pathologic process [151, 177]. Nevertheless, the major pathway of synthesis of AEA involves hydrolysis of N-arachidonoyl phosphatidylethanol (NAPE) by NAPE-phospholipase D (NAPE-PLD) [57]. Fatty acid amide hydrolase (FAAH) is the main enzyme responsible for AEA degradation [45] although a second pathway through oxidation by cyclooxygenase-2 (COX-2) has been described [257].

AEA acts as a partial agonist of CB_1R and CB_2R with higher affinity and intrinsic activity for CB_1R [233, 234] and the vanilloid receptor TRPV1 [176]. Ligand bias analysis in CHO cells expressing human CB_1R indicate that AEA exhibits bias towards decreases in cAMP levels compared to increases in phosphorylated ERK 1/2 levels [128]. In contrast to 2-AG, increased levels of AEA lead to impairments in CB_1R-mediated LTP and consequently of learning and memory [6]. Attempts to generate knock-out mice that cannot synthesize AEA in order to explore its physiological role have not been successful largely because of the presence of alternative pathways that can compensate for the inactivated one or to increases in the levels of other eCB such as 2-AG [142, 177]. However, mice with a genetic deletion of the enzyme that degrades FAAH have been successfully generated. These animals exhibit higher levels of AEA in the brain [45] and CB_1R-mediated behaviors including hypoalgesia [145], emotional phenotype [31], antinociception, anxiolytic and antidepressant effects [5]. Exogenous administration of AEA to FAAH knock-out mice leads to more robust antinociception, catelepsy, hypothermia and disruption of working memory than in wild-type mice [244, 255].

7.2.2.3 Hemopressins

Recently peptidic ligands for CB_1R, named hemopressins have been identified. A nine amino acid peptide named hemopressin (PVNFKFLSH) was isolated from hot acid extracts of rat brain using an enzyme capture assay. This peptide is derived from the α1 chain of hemoglobin [49, 206] and was shown to function as an inverse agonist of CB_1R [105]. Hemopressin administration induces hypotension [18, 206], antinociception in a model of inflammatory pain [50], attenuation of carrageenan-induced hyperalgesia [50, 105] and decrease in food intake [58]. Questions arose about whether hemopressin was a naturally occurring peptidic cannabinoid ligand given that cleavage of the aspartic acid-proline bond in hemoglobin required to generate this peptide can occur under the acidic conditions used to extract peptides from rat brain. Peptidomic analysis of endogenous peptides extracted from mouse brain under conditions that did not involve use of acid extraction detected the presence of longer N-terminally extended peptides of hemopressin named RVD-Hpα (RVDPVNFKFLSH) and VD-Hpα (VDPVNFKFLSH) [92]. In addition, a peptide derived from the β chain of hemoglobin that exhibited sequence similarity to hemopressin was identified and named VD-Hpβ (VDPENFRLLCNM)[92]. Studies show that RVD-Hpα and VD-Hpα exhibit agonistic activity at CB_1R and VD-Hpβ at CB_1R and CB_2R [92]. Very little is known about the physiological roles of these longer hemopressin peptides except that RVD-Hpα inhibits bombesin-mediated increase in catecholamine levels [235], and administration of VD-Hpα leads to antinociception, hypothermia and hypoactivity [265]. Not much is known about the generation of hemopressin peptides from hemoglobin and about its degradation. Interestingly, although the α and β chains of hemoglobin are classically known to comprise the main constituents of red blood cells, the mRNA for these polypeptide chains and/or protein has been detected in other cell types including macrophages,

crystalline lens, lung cells, and neuronal and glial cells in the brain [13, 14, 51, 85, 149, 184, 205, 258]. More recently a study used monoclonal antibodies to RVD-Hpα and detected the presence of this peptide in the brain in noradrenergic neurons, peptidergic axons throughout the brain and in chromaffin cells in the adrenal medulla [109]. Together, this indicates site-specific generation of peptidic endocannabinoids although not much is known about what regulates their synthesis and secretion.

7.3 The Opioid System

The opioid system consist of the three "classical" opioid receptors, mu (MOR)[38], delta (DOR) [65, 129] and kappa (KOR) [173], and their endogenous ligands, endorphins, enkephalins and dynorphins [231]. Opioid receptors are members of the family A of GPCRs and the three subtypes exhibit ~60% sequence homology with a high degree of sequence conservation in the transmembrane domains and intracellular loops [138]. Although additional receptor types such as sigma, epsilon and orphanin have been considered to belong to the opioid receptor system [231], their characterization as belonging to the opioid system is still under investigation and as such they will not be described in this chapter.

7.3.1 Opioid Receptors

7.3.1.1 Mu Opioid Receptor (MOR)

Human MOR is encoded by the OPRM1 gene at the 6q25.2 locus [190]. This receptor was first cloned in 1993 from a rat brain cDNA library [38]. Anatomical studies detect highest expression of MOR in the caudate nucleus, cerebellum and NAc with lower expression in the neocortex, putamen, thalamus, hippocampus, amygdala, dorsal horn of the spinal cord, periaqueductal gray, raphe nuclei and substantia nigra [12, 195]. With regards to its subcellular localization MOR is present at the cell membrane of dopaminergic, cholinergic, GABAergic, glutamatergic and serotonergic neurons [37, 99, 112, 198, 202, 243]. In addition to neurons, the presence of MOR has been detected in microglial cells [175] and in astrocytes [64].

In general MOR couples to $G_{\alpha i/o}$ proteins, although studies have shown that it can also couple to $G_{\alpha z}$ proteins, leading to the inhibition of adenylyl cyclase activity and thereby cAMP production [33] (see Fig. 7.2). MOR activation has been shown to lead to suppression of Ca^{2+} influx, promoting the opening of G-protein-coupled inwardly rectifying K^+ (GIRK) channels, and inhibiting the activity of several channels as well as excitatory postsynaptic currents (EPSC) evoked by glutamate; this results in attenuation of neuronal excitability, reduction of neurotransmitter release ultimately leading to decreased neurotransmission [28, 89, 90, 118, 187, 229].

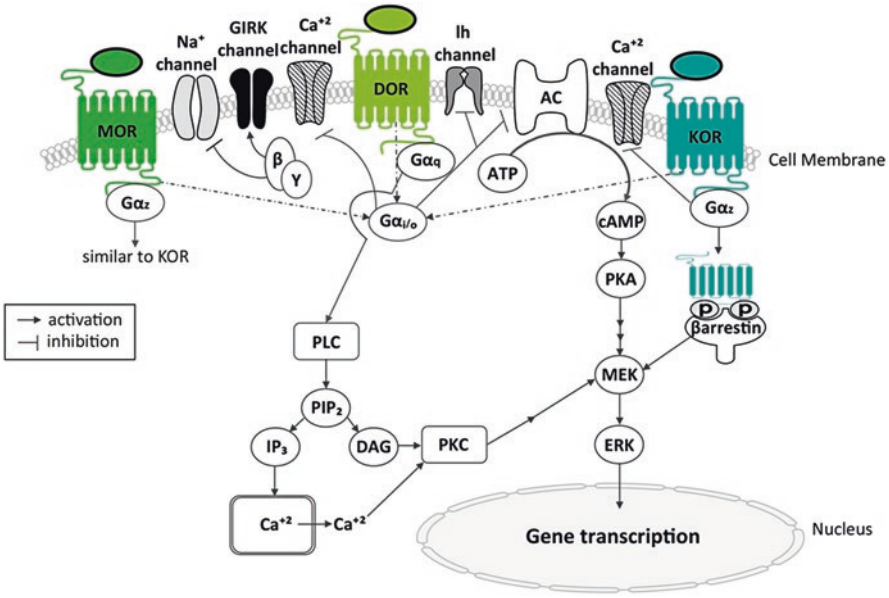

Fig. 7.2 Opioid receptor-mediated signaling
The stimulation of all three opioid receptors by agonist ligands leads to the activation of the $G_{\alpha i/o}$ protein (MOR and KOR coupling to $G_{\alpha z}$ and DOR coupling to $G_{\alpha q}$ has been reported). This leads to inhibition of cAMP production by adenylyl cyclase, of calcium and of Ih channels (in sensory neurons TPRV1 and ASIC channels are inhibited). Coupling of MOR and KOR to $G_{\alpha z}$ leads to β-arrestin recruitment and MEK signaling while coupling of $G_{\alpha q}$ to DOR leads to stimulation of PLC and release of intracellular calcium.
Abbreviations: *AC* adenylyl cyclase, *PLC* phospholipase C, *PIP2* phosphatidylinositol diphosphate, *IP3* inositol triphosphate, *DAG* diacyl glycerol, *PKA* protein kinase A, *P* phosphate group, *Ih* hyperpolarization-activated cation current channel, *GIRK* G protein-coupled inwardly-rectifying potassium

Mice lacking MOR have been generated and behavioral studies with these animals demonstrate a role for the receptor in antinociception, reward, mood disorders, locomotor activity and immune responses [82, 130, 168, 260].

7.3.1.2 Delta Opioid Receptor (DOR)

Human DOR is encoded by the OPRD1 gene at the 1p35.3 locus [190]. The receptor was first cloned by two independent groups in 1992 [65, 129]. DOR is highly expressed in the neocortex, caudate-putamen and NAc with moderate to low expression levels in thalamus, hypothalamus, brainstem, cerebellum and spinal cord [157, 195]. DOR is primarily found in neurons and astrocytes [59, 63, 64]. Subcellular localization studies detect the presence of DOR mainly within the cytoplasm in large dense-core vesicles (LDCV) [264] in cholinergic [10], dopaminergic [76], GABAergic [148], glutamatergic [15] and serotonergic [4] neurons.

DOR generally couples to $G_{\alpha i/o}$ proteins leading to inhibition of adenylyl cyclase activity and of excitatory postsynaptic currents (EPSC) evoked by glutamate [89]. Studies have shown that DOR can also couple to $G_{\alpha q}$ proteins leading to the activation of phospholipase C [139] (see Fig. 7.2). Mice lacking DOR have been generated and studies with these animals indicate a role for DOR in antinociception [83, 201, 266], reward [35, 185, 209], mood disorders [72], locomotor activity [72], and epilepsy [121].

7.3.1.3 Kappa Opioid Receptor (KOR)

Human KOR is encoded by the OPRK1 gene at the 8q11.23 locus [190]. The receptor was cloned in 1993 from a mouse brain cDNA library [259]. The expression of KOR in the brain is comparably lower than MOR and DOR, with highest levels in the NAc, claustrum, dorsal endopiriform and interpeduncular nuclei and low levels in the cerebellum and spinal cord [186, 195]. KOR can be detected in neurons, astrocytes and microglia [63, 102, 175]. Subcellular localization studies detect the presence of KOR at the cell membrane [102] in cholinergic [68], dopaminergic [62], GABAergic [123], and glutamatergic [162] neurons.

In general KOR couples to $G_{\alpha i/o}$ proteins, although studies have shown that it can also couple to $G_{\alpha z}$ proteins, leading to the inhibition of adenylyl cyclase activity and thereby cAMP production [137] (see Fig. 7.2). Animals lacking KOR have been generated and studies with these animals indicate a role for KOR in antinociception [3], reward [135], mood disorders [66], epilepsy [122] and immune responses [84].

7.3.2 Opioid Ligands

7.3.2.1 Endorphins

Endorphins are generated by proteolytic processing of the precursor protein, pro-opiomelanocortin (POMC) [197]. The human POMC gene is present at the 2p23.3 locus [190]. POMC is proteolytically cleaved by prohormone convertases 1/3 and by prohormone convertase 2 to yield a variety of bioactive peptides including endorphins [200].

Of the endorphins generated from POMC processing, β-endorphin(1-31) (also known as β-endorphin) is the major analgesic peptide. The primary sites for generation of β-endorphin in the brain are the hypothalamus and the pituitary gland [60, 230]. POMC derived peptides are sorted into large dense core vesicles prior to secretion through the regulated secretory pathway [200]. A number of enzymes have been implicated in the degradation of β-endorphin. These include enkephalinase (also termed as neprylisin), angiotensin-converting-enzyme (ACE), aminopeptidase M (APM) and insulin degrading enzyme (IDE) [75, 97, 203]. These enzymes are widely distributed throughout the CNS and are present in pre- and post- synaptic sites in neurons, astroglia and microglia cells [46, 96].

Endorphins play a major role in regulating the hypothalamic-pituitary-gonadal axis, in stress-induced analgesia and they have mood-enhancing and anxiolytic effects [104]. β-endorphin can bind with similar affinities at MOR and DOR and with much lower affinity at KOR [119]. Studies with mice lacking β-endorphin indicate a role for this peptide in incentive-motivation and reward associated with food intake [103], in stress-induced analgesia [217], and in the rewarding actions of cocaine [183].

7.3.2.2 Enkephalins

Enkephalins are primarily derived from the proteolytic processing of the precursor peptide proenkephalin (PENK) [197]. Human PENK is encoded by the PENK gene at the 8q12.1 locus [190]. PENK is processed by prohormone convertases and carboxypeptidase E to yield a variety of peptides including one copy of Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu), four copies of Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and two copies of extended Met-enkephalin peptides [197]. In addition, Leu-enkephalin can be generated by proteolytic processing of another opioid peptide precursor, prodynorphin [131]. Enkephalinergic neurons are spread through the CNS particularly in regions involved in processing of painful stimuli [100]. Besides neurons, enkephalins can also be released from astrocytes [256]. Enkephalins are rapidly degraded by the same enzymes that degrade endorphins [152]. The major catabolic step involves cleavage of the Tyr-Gly bond by membrane associated aminopeptidases [152].

Met- and Leu-enkephalin show high affinities for DOR and tenfold lower affinity for MOR and negligible affinity for KOR [119]. Studies with mice lacking PENK have demonstrated the importance of PENK-derived peptides in feeding [171], reward [103, 241], antinociception [9] and anxiety disorders [170].

7.3.2.3 Dynorphins

Dynorphins are derived from the proteolytic processing of the precursor peptide prodynorphin (PDYN). This precursor in humans is encoded by the PDYN gene at the 20p13 locus [190]. Dynorphinergic neurons are widely spread in the brain especially in the supraoptic and paraventricular nucleus of the hypothalamus and striatum (D_1R positive neurons), brainstem, spinal cord (B5-I interneurons) and dorsal root ganglia [21, 125, 250, 252]. Besides neurons dynorphins can also be released from astrocytes [251]. The proteolytic processing of prodynorphin by prohormone convertases and carboxypeptidase E yields a number of dynorphin peptides including dynorphin A and dynorphin B [236]. Dynorphins are degraded by the same enzymes that degrade endorphins and enkephalins [94].

Dynorphin A and dynorphin B can bind to all three opioid receptors but show a greater affinity toward KOR [94]. Mice lacking the PDYN gene have been generated. Studies with these mice demonstrate an involvement of dynorphins in reward [224], antinociception [267], anxiety disorders [169] and locomotion [34].

7.4 Interactions Between Cannabinoid and Opioid Systems

As described in Sects. 7.2 and 7.3 several studies have demonstrated the involvement of the cannabinoid and opioid systems in a number of physiological processes including antinociception and addiction. Moreover, studies have provided evidence for functional interactions between these two receptor systems. Cross-talk between the cannabinoid and the opioid system has been described in eating behavior [44], alcohol intake [160], antinociception, development of dependence, tolerance and withdrawal [210, 221], reward and reinforcement behavior [210], learning, memory and emotional responses [210]. A number of mechanisms could account for the reported interactions between cannabinoid and opioid receptor systems. These include (i) both opioid and cannabinoid receptors share a pool of second messengers taking part in convergent signal transduction [220]; (ii) the ligand of one receptor modulates the release of the endogenous ligand for the other receptor [253]; or (iii) opioid and cannabinoid receptors directly associate (heteromerize) to generate an entity with novels properties compared to individual receptor protomers. Below we describe studies showing interactions between cannabinoid and opioid receptors in pain, development of tolerance and addiction. In Sects. 7.5 and 7.6 we will describe evidence for cannabinoid-opioid heteromers and the relevance of these heteromers in disease states such as pain and addiction.

7.4.1 Biochemical Evidence for Cannabinoid-Opioid Interactions

A number of studies have suggested interactions between the cannabinoid and opioid systems. These studies show that cannabinoid agonists modulate the release of endogenous opioid peptides and vice-versa. This includes studies showing that cannabinoid agonists increase extracellular levels of β-endorphin in the periphery, of POMC in the hypothalamus, and enkephalins and dynorphin in the spinal cord, and of enkephalins in the NAc [41, 42, 113, 116] and that the opioid agonist morphine can modulate levels of AEA and 2-AG in the brain [249]. At the receptor level studies show that activation of one receptor can lead to changes in the levels of the other receptor. Thus studies show that (i) chronic intrathecal administration of morphine increases expression of CB_1R and CB_2R in the spinal cord [147]; (ii) chronic treatment with a cannabinoid agonist leads to a small increase in MOR in supraspinal regions involved in regulation of painful stimuli [43, 67, 248]; (iii) THC increases the dissociation of ligands specific for MOR and DOR [126, 245]; (iv) treatment with a CB_1R agonist or a DOR agonist induces asymmetric cross-desensitization in N18TG2 cells [223]; (v) cortical and substantia nigra membranes from DOR knock-out mice exhibit increases in CB_1R binding and signaling [8, 214]; and (vi) membranes from CB_1R knock-out mice exhibit an increase of DOR $G_{\alpha i}$-mediated activity [193].

7.4.2 *Cannabinoid-Opioid Interactions in Pain*

Among the different receptors, neurotransmitters and circuits involved in the control of pain, endogenous opioid and cannabinoid systems modulate several pathways regulating pain sensation [71]. Activation of the opioid and cannabinoid systems in the periphery (i.e. via nociceptive primary afferent neurons) and centrally (i.e. via spinal cord and brain) contribute to alleviate transient and acute pain [127, 222]. Anatomical data indicate that cannabinoid and opioid receptors colocalize in multiple regions involved in pain control. MOR, DOR and KOR (in order of frequency) and CB_1R are present at presynaptic (end of primary afferent axon terminals) and postsynaptic sites (second order neuron, and possibly interneurons) in the spinal dorsal horn [1, 11, 39, 120, 219] where they inhibit substance P release and modulate postsynaptic signaling (see Fig. 7.3).

Studies show that chronic pain leads to the upregulation of opioid [27, 120] and cannabinoid receptors [146, 262] through the peripheral and central nervous systems, although some discrepancies have been reported depending on the region and

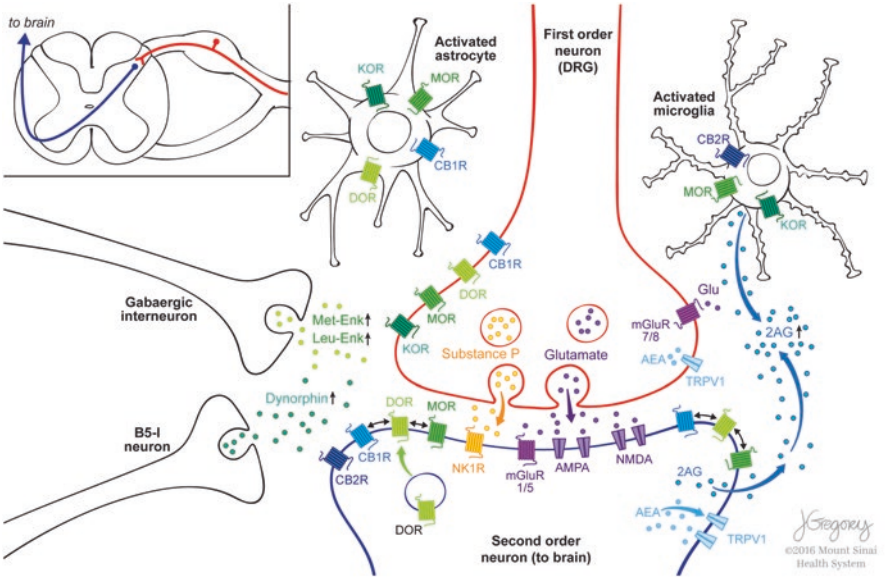

Fig. 7.3 Cannabinoid and opioid receptors within the spinal cord in chronic pain
Chronic pain leads to the release of enkephalins and dynorphin by interneurons and endocannabinoids by both first and second order neurons and activated microglia. Along with this there is an upregulation of cannabinoid and opioid receptors in both pre and postsynaptic locations and eventually a physical and functional interaction among CB_1R, MOR and DOR in the second order neuron. Ultimately, these adaptive changes fail to counteract the proinflammatory state that causes central sensitization and prolongation of pathological pain.
Abbreviations: *mGluR* metabotropic glutamate receptor, *AMPA* α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, *NMDA N-methyl-D-aspartate* receptor, *NK1R* neurokinin 1 receptor, *B5-I* B5 interneuron

the animal model used [26]. Furthermore, upregulation of the endocannabinoids, AEA and 2-AG, and the main endogenous opioids endowed with antinociceptive properties, Met-, Leu-enkephalin and dynorphins in the spinal cord during pain [155, 213], favors the role of both receptor systems in analgesia. Whereas acute pain is effectively alleviated by opioids [81], in chronic pain where cannabinoid receptors are permanently stimulated, the effectiveness of cannabinoid agonist drugs alone is limited, particularly in humans [136].

Previous research showed that interactions between cannabinoid and opioid systems during antinociception can either be bidirectional or synergistic. Bidirectional studies include those (i) showing that morphine-mediated antinociception is blocked by the CB_1R antagonist AM251, while cannabinoid agonist-mediated antinociception is blocked by the opioid antagonist, naloxone or by a KOR antagonist, norbinaltorphimine [47, 159]; (ii) indicating that opioid-mediated analgesia can be enhanced by increasing endogenous anandamide levels and antinociception mediated by AEA can be blocked by a KOR antagonist [101, 191]; (iii) showing that acute administration of cannabinoid agonists leads to the release of opioid peptides while chronic administration of THC induces the expression of opioid peptide precursors (reviewed in [26]). Synergistic studies show that low doses of cannabinoid agonists can potentiate the analgesia mediated by low doses of opioid agonists [40, 153, 254]. More recent studies report that the co-administration of low, but not high-dose CB_2R agonists and morphine increased analgesia and attenuated development of tolerance [2, 263].

Studies using subtype selective antagonists to opioid receptors or antisense oligonucleotides support the involvement of MOR and KOR but not DOR in the antinociceptive effects of THC [159]. In addition studies show that intrathecal administration of a KOR antagonist reversed analgesia mediated by a CB_1R/CB_2R agonist, but not by CB_1R selective agonists [189], and that CB_1R antagonists block antinoception mediated by salvinorin (a KOR agonist) in a model of visceral pain [70]. However, studies using knock-out animals to evaluate cannabinoid-opioid interactions during analgesia do not provide clear-cut data. These studies report that a lack of MOR, DOR or KOR or of both MOR and DOR has no effect on THC-mediated antinociception in the tail immersion and hot plate tests [32, 87]. However, decreased THC-induced antinociception was reported in mice lacking either the pre-proenkephalin or the pro-dynorphin gene in the tail immersion test [241, 267]. In the case of mice lacking pro-dynorphin, another study did not observe any change in THC-mediated antinociception in the tail immersion test [79]. Studies with mice lacking CB_1R show no effect on opioid-mediated antinociception in the tail immersion and hot plate tests while opioid-mediated stress-induced analgesia was attenuated [240]. In the formalin test, a tonic pain model characterized by an acute phase involving activation of sensory receptors and a second phase involving an inflammatory response and development of CNS sensitization, studies report a decrease in morphine (administered locally or spinally) antinociception during the acute phase only in mice lacking CB_1R but not CB_2R and during the inflammatory phase in mice lacking either CB_1R or CB_2R; these changes were not observed when morphine was systemically administered [55].

7.4.3 Cannabinoid-Opioid Interactions in Development of Tolerance

It is well established that prolonged administration of opioids or cannabinoids leads to the development of antinociceptive tolerance where increasing doses have to be administered in order to maintain the same antinociceptive effect. Studies have shown that tolerance to morphine leads to reduced THC antinociception and tolerance to THC leads to attenuation of morphine antinociception in mice [238]. However, in rats tolerance to morphine was shown to lead to increased THC antinociception [216]. Interestingly, administration of subanalgesic doses of a synthetic cannabinoid, CP55,940, induced significant antinociception in morphine tolerant rats while subanalgesic doses of morphine had no antinociceptive activity in animals tolerant to CP55,940 [248]. Studies using knock-out animals indicate that the development of antinociceptive tolerance to THC is attenuated in mice lacking preproenkephalin while development of tolerance to opioids is not affected in mice lacking CB_1R [141, 241].

7.4.4 Cannabinoid-Opioid Interactions in Addiction

Addiction is a pathological reward system characterized by impaired self-control over drug-taking despite negative consequences [115]. The reward system is a phylogenetically ancient circuit intended to satisfy primary necessities (food, sex, water) by producing pleasure feelings ("liking") that elicit motivation ("wanting") and prioritize behavioral options (goal-pursuit) to ensure self-preservation and the survival of the species. Dopaminergic, cholinergic, glutamatergic and GABAergic projections are involved in this complex circuit [134], the so-called mesocorticolimbic pathway. Normally, a pleasant experience produces hedonic effects by increasing β-endorphin levels in the VTA that suppress tonic GABAergic inputs into dopaminergic projections thereby increasing the release of dopamine in nuclei targeted by ascending ventral tegmental area (VTA) fibers. These nuclei include the NAc, medial prefrontal cortex (mPFC), basolateral amygdala (BLA) and hippocampus, and they encode stimuli value, regulate executive control, form associative fear and reward-related memories and establish memories of rewarding experiences, respectively [218].

As with natural rewards, addictive drugs are reinforcing and exhibit many commonalities. However, two major differences explain why recruitment and hijacking of the reward system (i.e. addiction) by these drugs is particularly harmful. First, drug rewards became overvalued contributing to compulsion and prioritizing drug consumption over other life goals. Second, drugs do not serve any homeostatic purpose or reproduction but prove detrimental to health and functioning [115]. These clinical phenomena are tied to specific neural mechanisms, which will be briefly described. The rewarding drug (eg. morphine or THC) produces an exaggerated

β-endorphin response (thereby inhibiting GABAergic VTA interneurons) and therefore induces higher dopamine release in the NAc, which results in euphoria and promotes its repetitive use leading to positive reinforcement (see Fig. 7.4). Eventually, addictive drugs dysregulate the reward circuit leading to dopamine and β-endorphin hypoactivity [86, 48] and the absence of drug produces negative reinforcement in terms of dysphoria, which manifests as withdrawal (symptoms upon drug discontinuation), craving (urge for drug consumption) and hedonic dysregulation (negative emotional state when the access to the drug is prevented). Chronic consumption alternates euphoria and dysphoria states thereby closing the vicious cycle of addiction [48]. Moreover, it has been proposed that release of cytokines induced by activated microglia during addiction could, *per se*, facilitate addiction development [211].

Studies have detected the presence of cannabinoid and opioid systems in areas of the brain involved in reward [210, 239]. Moreover, intake of cannabinoids or opioids has rewarding effects that often lead to their abuse. A number of studies have suggested involvement of the opioid system in addiction to cannabinoids and of the cannabinoid system in addiction to opioids. These include studies showing that (i) the opioid receptor antagonist, naloxone induces withdrawal in mice that exhibit dependence to the cannabinoid agonist, Hu-210 while the CB_1R antagonist SR141716A induces withdrawal in morphine dependent animals [182]; (ii) the endogenous cannabinoid anandamide or a inhibitor of its uptake attenuates naloxone-precipitated withdrawal signs in morphine dependent animals [53, 246]; (iii) prolonged treatment with CB_1R agonists or antagonists attenuates naloxone withdrawal in morphine dependent animals [215, 242]; (iv) co-administration of a CB_1R antagonist and morphine for 5 days decreases symptoms of morphine withdrawal [164]; (v) THC-mediated dopamine efflux from the NAc is blocked by naloxone [36]; (vi) a CB_1R antagonist attenuates opioid self-administration and conditioned-place preference (CPP) in rodents [52, 181, 227]; (vii) the MOR antagonist, naltrexone, decreases cannabinoid mediated CPP and self-administration [23, 24].

Studies with knock-out animals also suggested interactions between the cannabinoid and opioid systems during different aspects of addiction. Thus mice lacking pre-proenkephalin exhibit attenuated withdrawal to cannabinoids [241], and mice lacking prodynorphin do not exhibit aversion to high doses of cannabinoids but exhibit facilitation to the reinforcing effects of a cannabinoid agonist [172, 267]. Studies with mice lacking opioid receptors indicate that a lack of KOR leads to loss of aversion to high doses of cannabinoids [87]; a lack of MOR leads to either no change or to attenuation of withdrawal to cannabinoids [87, 144], to a lack of THC-mediated CPP , and to attenuated aversion to high doses of cannabinoids [87]; while a lack of both MOR and DOR leads to attenuated withdrawal to cannabinoids [32]. Studies with mice lacking CB_1R demonstrate attenuation of morphine withdrawal symptoms, and a lack of morphine CPP, self-administration and sensitization to chronic morphine administration [141, 163]. Together these studies indicate either unidirectional or bidirectional cross-talk between opioids and cannabinoids during addiction.

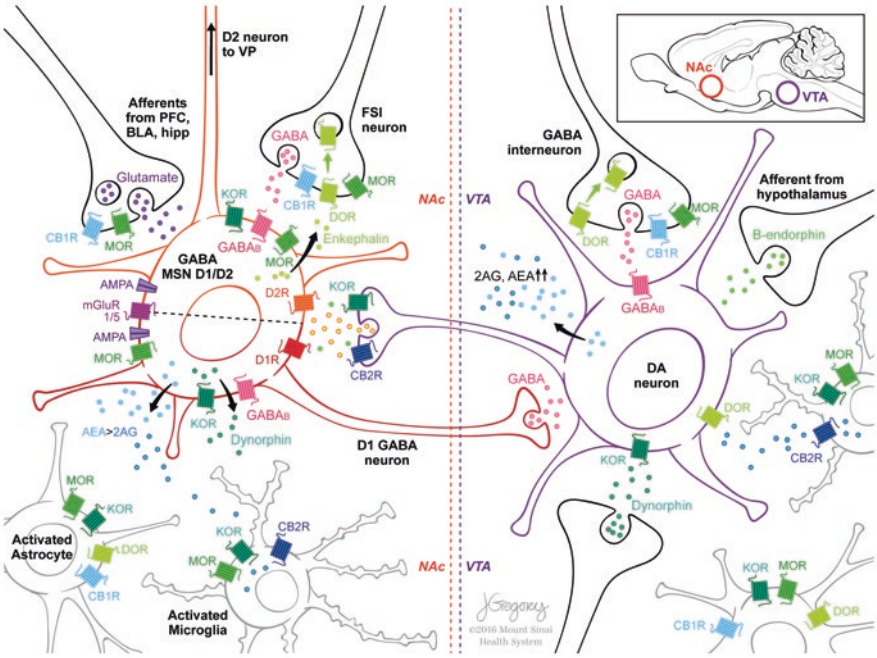

Fig. 7.4 Cannabinoid and opioid receptors within the VTA and NAc in addiction
Addictive drugs induce the release of β-endorphin (by hypothalamic efferents) and eCBs, AEA and 2-AG, by neurons and microglia in the VTA thereby inhibiting the release of GABA from interneurons. As a result dopaminergic neurons release dopamine into the NAc, increasing D_1R activation (which is necessary for conditioning) and triggering neuroadaptations in basal ganglia circuits. Cannabinoid and opioid systems in the NAc modulate fast spiking GABAergic interneurons (FSI) and glutamatergic afferents from PFC, BLA and hippocampus ultimately leading to an unbalanced D_1R and D_2R signaling. Microglia activation leads to the release of eCBs thereby facilitating the addictive state. To date, no dimers have been characterized in these nuclei.
Abbreviations: *eCBs* endocannabinoids, *NAc* nucleus accumbens, *VTA* ventral tegmental area, *PFC* prefrontal cortex, *BLA* basolateral amygdala, *hipp* hippocampus, *FSI* fast-spiking interneuron, *mGluR* metabotropic glutamate receptor, *AMPA* α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, *$GABA_B$* metabotropic gamma-aminobutyric acid receptor, *DA* dopaminergic

7.5 Evidence for Cannabinoid-Opioid Receptor Heteromers

In order to demonstrate receptor heteromerization the following three criteria need to be fulfilled: (i) heteromer components should colocalize and physically interact, (ii) heteromers should exhibit properties distinct from those of the protomers and (iii) heteromer disruption should lead to a loss of heteromer-specific properties (reviewed in [91]). Most studies examining GPCR heteromerization have addressed criteria (i) using a combination of co-localization studies, co-immunoprecipitation

studies and proximity based assays. This is because although co-localization studies can demonstrate that the two receptor protomers are in the same subcellular compartment they do not have sufficient resolution to demonstrate close proximity for direct interactions (reviewed in [91]). Similarly, although co-immunoprecipitation studies indicate that the two receptors form interacting complexes they do not rule out the presence of additional proteins between the two interacting receptors (reviewed in [91]). Close proximity between two receptors for direct interactions has been addressed through the use of proximity based biophysical techniques such as bioluminescence resonance energy transfer (BRET) or fluorescence resonance energy transfer (FRET) (reviewed in [91]). These approaches are amenable for use in heterologous cells expressing tagged receptors and recent modifications of the FRET-based approach allow it to be used to detect heteromers in endogenous tissues (reviewed in [91]). More recently, a proximity ligation assay developed to demonstrate protein-protein interactions [74] and in eventually identifying receptor and signaling associated protein complexes [228] has also been used to demonstrate the presence of GPCR heteromers in endogenous tissue (reviewed in [93].

Fulfillment of criteria (ii) for receptor heteromerization requires demonstrating that the heteromer exhibits properties (i.e. binding, signaling, trafficking) that are different from the individual receptor homomers i.e. a unique "biochemical fingerprint" (reviewed in [91]). It is easier to carry out these studies in vitro using cells expressing recombinant receptors since these cell lines can be easily generated and in addition cell lines expressing individual receptors can be used as controls that help assign the unique fingerprint to the heteromer. Such studies are more challenging when using endogenous tissue, since within a tissue we may have different cell types some co-expressing both receptors and some expressing individual receptors; this could make data interpretation challenging. Thus the suggested approach is to establish the heteromer fingerprint in vitro and then try to recapitulate it in endogenous systems using tissues from wild-type animals that co-express both receptors and the same tissues from animals lacking individual receptors as controls (reviewed in [91]).

Fulfilment of criteria (iii) for receptor heteromerization requires demonstration that heteromer disruption leads to a loss/attenuation of the heteromer's biochemical fingerprint. Progress with identification of reagents that can alter the heteromer biochemical fingerprint has been slow because of the need to demonstrate that these reagents do not affect individual receptor homomers and poor knowledge about the heteromeric interfaces. Some such reagents have been generated including membrane-permeable peptides that disrupt heteromeric interfaces, transgenic animals that express mutant protomers that cannot form heteromers and heteromer-selective agents like antibodies and ligands (reviewed in [91]). More effort has been put towards the generation of reagents that selectively recognize and/or activate heteromers in endogenous tissues such as antibodies, bivalent ligands, bifunctional/multivalent ligands and small molecule ligands [91]). In the following sections we describe whether evidence for cannabinoid-opioid heteromerization in vitro and in endogenous tissue fulfill the criteria proposed for receptor heteromerization with a focus of CB_1R-MOR and CB_1R-DOR heteromers.

7.5.1 Evidence for CB_1R-MOR Heteromerization

In heterologous cells expressing differentially epitope tagged CB_1R and MOR, colocalization studies show that both receptors can be detected in the same subcellular compartment, co-immunoprecipitation studies show that they form interacting complexes and proximity based assays show that they are in close proximity for direct interactions [111, 207]. Thus in in vitro systems CB_1R and MOR fulfill criteria (i) for receptor heteromerization. In endogenous systems immunostaining studies detect the presence of CB_1R and MOR in common subcellular compartments in the locus coeruleus, NAc putamen and dorsal horn of the spinal cord [110, 196, 212, 219, 220]. Given that the resolution of this technique is not sufficient to demonstrate close proximity for direct interaction together with a lack of co-immunoprecipitation and proximity based studies in endogenous systems (cells/tissues) criteria (i) for CB_1R-MOR heteromers in endogenous tissue is yet to be fulfilled. Signaling studies show that in heterologous cells co-expressing CB_1R and MOR the constitutive activity of CB_1R negatively modulates MOR function [29] while the simultaneous addition of a CB_1R and a MOR agonist leads to a significant decrease of the response compared to that seen upon activation of individual receptors [207]. Moreover in Neuro 2A cells endogenously expressing CB_1R and stably expressing epitope tagged MOR, treatment with individual receptor agonists promoted neurite outgrowth while a combination of a CB_1R and a MOR agonist led to attenuation of neurite outgrowth [207]. These studies indicate that CB_1R-MOR heteromers exhibit a unique biochemical fingerprint in heterologous cells. Given that attenuation of signaling observed with a combination of a CB_1R and a MOR agonist was also detected in SKNSH cells and with striatal membranes (both endogenously express CB_1R and MOR) [207] would indicate that the biochemical fingerprint detected in heterologous cells is maintained in endogenous systems which would fulfill criteria (ii) for CB_1R-MOR heteromerization. Fulfilment of criteria (iii) for receptor heteromerization would require demonstrating that heteromer disruption would lead to a loss of the unique biochemical fingerprint; such studies have not yet been carried out. However, unique reagents targeting CB_1R-MOR heteromers have been generated. These comprise bivalent ligands where a MOR selective agonist is connected by spacers of different lengths to a CB_1R antagonist/inverse agonist [140]. Preliminary studies indicate that a 20-atom spacer is needed to bridge both the receptor protomers and administration of this compound leads to potent antinociception without development of tolerance [140].

7.5.2 Evidence for CB_1R-DOR Heteromerization

In Neuro 2A cells endogenously expressing CB_1R and stably expressing epitope tagged DOR co-localization studies demonstrate that both receptors are present in the same subcellular compartment and co-immunoprecipitation studies show

that they form interacting complexes while proximity based assays in HEK-293 cells expressing differentially epitope CB_1R and DOR show that both receptors are in close proximity for direct interactions [207, 214]. Thus in in vitro systems CB_1R and DOR fulfill criteria (i) for receptor heteromerization. In endogenous systems colocalization of CB_1R and DOR has been demonstrated in primary cortical neuronal cultures and forebrain [26] and more recently by a proximity based ligation assay (PLA) in mice spinal cord (unpublished results). Although co-immunoprecipitation studies demonstrating formation of CB_1R-DOR interacting complexes in endogenous tissues are lacking, the demonstration of colocalization and of close proximity between the two receptors in an endogenous system would indicate that these receptors satisfy the criteria for heteromer formation in endogenous tissue. With regards to demonstrating that CB_1R-DOR heteromers exhibit a unique biochemical fingerprint (criteria (ii) for heteromerization) studies show that in Neuro 2A cells endogenously expressing CB_1R the receptor is associated with the adapter protein AP-3 and present mainly in an intracellular compartment but upon stable expression of epitope tagged DOR it associates with the adapter protein AP-2 and is present at the cell surface [214]. Signaling studies show that the presence of DOR decreases the potency of a CB_1R agonist, increases phospholipase C-mediated arrestin 3 recruiment and leads to activation of novel signaling pathways that enhance cell survival [214]. Some of these observations could also be made in endogenous systems. For example deletion of DOR in F11 cells that endogenously express CB_1R and DOR changes the subcellular localization of CB_1R [214]. In addition, in cortical membranes the presence of DOR leads to attenuation of cannabinoid mediated signaling [214]. Moreover, in cortical membranes expressing CB_1R and DOR but not in membranes expressing only CB_1R (i.e. from DOR knock-out mice) treatment with the CB_1R antagonist decreases neuronal survival, supporting that activation of the CB_1R-DOR heteromer leads to cell survival [214]. Together, these studies indicate that the CB_1R-DOR heteromer exhibits a unique biochemical fingerprint thereby satisfying criteria (ii) for receptor heteromerization.

To fulfill criteria (iii) for CB_1R-DOR heteromerization would require demonstrating that heteromer disruption would lead to a loss of the unique biochemical fingerprint; agents that directly disrupt CB_1R-DOR heteromer formation are currently unavailable. However, studies using lentiviral mediated knockdown of DOR in F11 cells show that this leads to changes in the localization of CB_1R from the cell surface to an intracellular compartment [214]. In addition, signaling studies using membranes from DOR knockout animals show increased cannabinoid-mediated signaling compared to wild-type controls indicating that lack of DOR disrupts heteromer-mediated signaling [214]. Moreover, a CB_1R-DOR heteromer selective antibody has been generated; this antibody detects increased heteromer levels in certain brain regions during neuropathic pain and blocks the enhancement of DOR activity observed upon co-administration of non-signaling doses of CB_1R ligands in these animals [25].

7.6 Therapeutic Potential of Cannabinoid-Opioid Receptor Heteromers

Cannabinoid-opioid heteromers could be potential targets for the development of therapeutics with reduced side-effects to treat pain. One reason for reduced side-effects is that the heteromers would have restricted tissue distribution, since heteromer formation requires the co-expression of both receptors in the same subcellular compartment. Another reason is that heteromerization provides a means to allosterically modulate the function of an individual receptor which could lead to a reduction of side-effects.

In the case of CB_1R-MOR heteromers not much is known about changes in its levels during pathology. However, this heteromer could be a good target for the development of therapeutics to treat pain since studies with bivalent ligands targeting this heteromer show that it induces potent antinociception without development of tolerance [140]. In the case of CB_1R-DOR heteromers, antibodies that selectively recognize this heteromer have been generated [26]. Studies using these antibodies detected increased heteromer levels in the cortex, hypothalamus and striatum of animals with neuropathic pain due to peripheral nerve lesion [26]. Furthermore, in these animals low non-signaling doses of CB_1R ligands significantly enhance DOR signaling in cortical membranes and this could be blocked by the heteromer-selective antibody [26]. These results suggest that DOR function in the cortex is altered during neuropathic pain and that targeting this heteromer could lead to the identification of novel therapeutics to treat neuropathic pain that is often refractory to commonly used opioids and cannabinoids.

7.7 Conclusions and Perspectives

Over the last decade increasing evidence indicates that GPCRs can form heteromers. In this review we describe evidence for cannabinoid-opioid heteromerization under the criteria proposed for demonstration of receptor heteromerization (reviewed in [91]). Studies indicate that CB_1R-MOR and CB_1R-DOR fulfill some of the proposed criteria. Currently, there is a need in the field of GPCR heteromerization, particularly cannabinoid-opioid heteromerization, for reagents that specifically disrupt a given heteromer pair in endogenous systems; studies with these reagents would help elucidate the physiological role of the heteromer. Generation of such reagents would require identification of heteromeric interfaces; these would be unique for a given heteromer. Knowledge of these heteromeric interfaces could facilitate the generation of heteromer-deficient animals; the use of such animals would help evaluate the physiological role of cannabinoid-opioid heteromers and differentiate the contribution of the heteromer from individual receptor homomers for a particular physiological response or during pathology. In addition to reagents that disrupt the heteromer, identification of heteromer selective agonists and antagonists would also help assess

their physiological role as well as their potential as targets for the development of drugs to treat specific pathologies with reduced side-effects.

Other potential areas of research on cannabinoid-opioid heteromers involve mapping of the tissue distribution of these heteromers. This could be achieved using heteromer-selective antibodies. In addition, research on how cannabinoid-opioid heteromers are formed, on what regulates the formation of a given heteromer pair for eg. CB_1R-MOR versus CB_1R-DOR as well as their expression at the cell surface, on the mechanisms regulating their levels during pathology could lead to the identification of novel therapeutic targets to treat pathologies involving the cannabinoid and opioid systems.

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Chapter 8
Class A GPCR: Di/Oligomerization of Glycoprotein Hormone Receptors

Aylin C. Hanyaloglu, F. Fanelli, and K.C. Jonas

Abstract G protein-coupled receptor (GPCR) dimerization and oligomerization was first described over 2 decades ago, contributing to the recent paradigm shift in GPCR signaling of a simplistic, archetypal view involving single receptors activating specific heterotrimeric G proteins at the cell surface, to one of an increasing complex receptor signaling system. However, our understanding of how dimerization and oligomerization, particularly homomerization, generates functional diversity in GPCR signaling is poorly understood. For the Class A/rhodopsin subfamily of glycoprotein hormone receptors (GpHRs), di/oligomerization has been demonstrated to play a significant role in regulating its signal activity at a cellular and physiological level and even pathophysiologically. Here we will describe and discuss the developments in our understanding of GPCR oligomerization, primarily the role of homomeric receptor complexes, in both health and disease, from the study of this unique and complex subfamily of GPCRs.

Keywords Glycoprotein hormone receptor • Dimer • Oligomer • Super-resolution imaging • Modelling • Signaling • Luteinzing hormone • Chorionic gonadotropin • Thyrotropin-stimulating hormone • Follicle-stimulating hormone

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Abbreviations

αGSU	common α subunit
BRET	bioluminescence resonance energy transfer
CG	chorionic gonadotropin
ECD	ectodomain
FCS	fluorescence correlation spectroscopy
FSHR	follicle-stimulating hormone receptor
FRET	fluorescence resonance energy transfer
GnRH	gonadotropin releasing hormone
GPCR	G protein-coupled receptor
GpH	glycoprotein hormone
GpHR	glycoprotein hormone receptor
H	helix
HPG	hypothalamic-pituitary-gonadal
HPT	hypothalamic-pituitary-thyroid
KO	knockout
LHR	luteinizing hormone receptor
LHR^B	luteinizing hormone receptor binding-deficient mutant
LHR^S	luteinizing hormone receptor signal-deficient mutant
PALM	photoactivatable-localization microscopy
PD	photoactivatable dyes
TM	transmembrane
TRH	thyrotropin releasing hormone
TSHR	thyroid stimulating hormone receptor
WT	wildtype

8.1 Introduction

The family of glycoprotein hormone receptors (GpHRs) consists of receptors for luteinizing hormone (LH) and chorionic gonadotropin (CG) (LHR), follicle-stimulating hormone (FSHR), and thyroid-stimulating hormone (TSHR). These members of the G protein-coupled receptor (GPCR) superfamily comprise a unique subgroup within the Family A/Rhodopsin GPCRs due to their leucine-rich repeat-containing N-termini with a large glycoprotein extracellular ectodomain (ECD). In turn the high glycosylation status of the ligands make them the most complex of protein hormones. These hormone receptors play critical roles in the endocrine axis of the hypothalamic-pituitary gonadal (LHR, FSHR) and hypothalamic-pituitary-thyroid pathways, identified via numerous studies in both animal models and disease causing mutations in humans (reviewed by [1–3]).

The current evolved model of GPCR signaling incorporates ever-increasing complexity in its signal pathways and mechanisms of regulation, by which GPCR

homo/hetero-merization has made a significant contribution to. Such complexity in receptor regulation provides key mechanism/s for the multiple and dynamic roles these receptors play *in vivo*. All three GpHRs have been demonstrated to exist as monomers, dimers and higher order oligomers, primarily as homomers, but for LHR/FSHR also as heteromers. Although the earliest biochemical observations were made over 20 years ago, only studies in the last 6 years have unveiled the complexities in the molecular mechanisms and roles of GpHr di/oligomers. Therefore, in this chapter we will discuss the recent developments in our understanding of GPCR dimerization and oligomerization via the study of GpHRs. We will first outline the key physiological roles and structural/activity features that have been exploited to answer key questions on the roles of GPCR di/oligomerization. Specific focus will then be how distinct approaches have been used to study GpHR oligomers from *in vitro* single molecule studies of individual protomers and structural modeling, to the impact of oligomerization on cellular signaling, physiology and human disease. Therefore, despite their unique structural features, GpHRs have proven to be useful receptor models to answer key questions and thus, enhance our understanding of the significance of di/oligomerization to GPCR biology and its implications in molecular medicine.

8.2 Glycoprotein Hormones and Their Receptors

8.2.1 Glycoprotein Hormones

The glycoprotein hormone (GpH) family is comprised of four peptide hormones: luteinizing hormone (LH), follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH) and chorionic gonadotropin (CG). Belonging to the cysteine knot superfamily [4], they are secreted from the anterior pituitary (with the notable exception of CG) in response to endocrine stimuli. The GpHs are heterodimeric proteins comprised of two subunits; a shared common α subunit (αGSU) and a hormone specific β subunit that confers functional specificity and biological activity [5]). The αGSU is synthesized in excess, while formation of functional heterodimeric GpHs is limited by synthesis of the hormone-specific beta subunit. Structurally, the αGSU and beta subunits are linked via non-covalent association and are subjected to post-translational modifications via glycosylation (reviewed by [6]). Glycosylation is essential in dictating hormone activity, as such, differential glycosylation of the GpHs have been previously reported [7–10] to potentially produce naturally occurring biased ligands for differential regulation of signal pathways and gene transcription [11].

The control of GpH synthesis, packaging and secretion is governed by feedback from the hypothalamic-pituitary-thyroid (HPT) and hypothalamic-pituitary-gonadal (HPG) axes, as well as additional neuronal, endocrine and physiological inputs (Fig. 8.1). With the exception of CG, the synthesis and secretion of the GpHs share

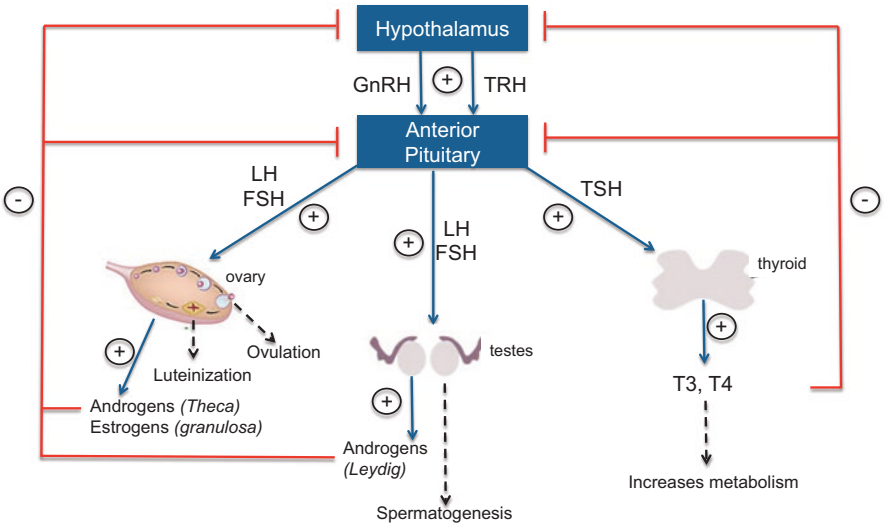

Fig. 8.1 The Hypothalamic-Pituitary-Thyroid (HPT) and Hypothalamic-Pituitary-Gonadal (HPG) axes. The hypothalamus secretes the hormones GnRH and TRH into the hypophyseal portal system and activates their GPCRs in the anterior pituitary gland. The GnRH and TRH receptors are expressed in the gonadotropes and thyrotrope cells respectively. Activation of these receptors leads to secretion of the gonadotropins, LH and FSH, and TSH. Activation of TSHR by TSH on follicular thyroid cells leads to production of T3 and T4 that has essential roles in metabolism (see text). In male reproduction LH activation of LHR in testicular Leydig cells results in production of the androgen testosterone, that plays key roles in primary and secondary sexual functions, while FSH activates its receptor on Sertoli cells to regulate spermatogenesis. In females, LHR is expressed in theca cells of the follicle, mural granulosa cells and luteal cells. LH activation of LHR mediates androgen production, ovulation and progesterone production from the corpus luteum. FSH activates FSHR in granulosa cells and is important in follicular development and estrodial production. Estrodial, progesterone, and T3 and T4 negatively feedback to the hypothalamus and pituitary to control GnRH/LH/FSH and TRH/TSH levels

similar regulatory mechanisms, with synthesis of TSH, LH and FSH positively regulated by the hypothalamic pulsatile secretion of thryotropin-releasing hormone (TRH) [12, 13] and gonadotropin-releasing hormone (GnRH) [14], respectively (Fig. 8.1). On activation of their GPCRs located on the cell surface of the thyrotrope and gonadotrope cells of the anterior pituitary, TRH and GnRH stimulate the synthesis and secretion of TSH and FSH/LH (for a more detailed coverage on this subject matter, readers are referred to [15] for TSH/HPT axis, and [16, 17]for FSH/LH and HPG axis). Following the secretion of TSH and FSH/LH into the portal circulation, TSH and FSH/LH in turn bind to their cognate GpHRs located in cellular compartments of the thyroid and gonads, respectively (Fig. 8.1). The gonadotropin CG is a specific hormone produced in humans and certain primates [18]. It must be noted that while horses independently evolved to produce CG that activates its LHRs, this equineCG has both FSHR and LHR activities in other species. The expression of CG is localized to and secreted from the pre-implantation conceptus,

primarily secreted by the syncytiotrophoblast, and also the extravillous trophoblast (reviewed by [7]). The control of CG synthesis and secretion is via paracrine and autocrine signals from both the maternal hormonal secretion and the developing embryo (for further, more detailed coverage, see [19]). Compared to LH, CG is considered to be a 'super-agonist' of the LHR due to its longer biological half-life and known differences in binding affinities [20]. More recent studies suggest that LH and CG may induce even further distinct activities via LHR [21, 22].

Functionally, these hormones mediate diverse endocrine and paracrine roles, knowledge of which has been refined by both transgenic mouse studies of the later 1990s and early 2000s, and identification of disease causing mutations in humans (we refer the reader to the following excellent reviews for further details [1, 23]). TSH is essential for thyroid function, which acts to regulate cellular metabolism via the production of the thyroid hormones triiodothyronine (T3) and tetraiodothyronine, (also known as thyroxine (T4)). Although T4 is weakly biologically active, T3 is considered the fully biologically active thyroid hormone, and as such, T4 is converted to T3 via deiodination. The HPT axis provides the fundamental regulation of basal metabolic rate, controlling a plethora of physiological functions including heart rate, bone turnover and thermogenesis [24]. TSH production is subject to negative feedback via direct inhibition of thyroid hormones at the anterior pituitary, and via indirect inhibition by T3 and T4 control of hypothalamic TRH synthesis [25] (Fig. 8.1). The gonadotropin hormones and their receptors collectively control and regulate reproductive functions in both males and females. In males, pulsatile release of GnRH remains tonic, ensuring continuous pulsatile release of FSH and LH to regulate spermatogenesis and control androgen production, respectively. In females, however, the differential regulation of LH and FSH by GnRH pulse frequency forms the basis of the endocrine control of the ovarian cycle, with fast pulses favoring LH and slow pulses favoring FSH (for further mechanistic detail refer to [16]). In females, FSH is essential for antral follicle growth during the follicular phase of the ovarian cycle, controlling estradiol production. LH is essential for androgen production (for conversion to estradiol) in the developing follicle, ovulation, coordinating the remodeling of the follicle remnant into the corpus luteum and resulting progesterone production (Fig. 8.1). In the event of a pregnancy, the gonadotropin CG produced by the embryo acts on and prevents regression of the corpus luteum. This continued progesterone production maintains the uterine lining and supports receptivity to embryo implantation and placental development. These critical roles of CG in early pregnancy have also been attributed to act via direct paracrine action within the uterus [26].

8.2.2 Glycoprotein Hormone Receptors (GpHRs)

The TSHR is expressed in the cells of the spherical follicles (or thyrocytes) of the thyroid gland. LHR is expressed specifically within Leydig cells of the testes, while in the ovary, LHR is found in three distinct cell types; the theca cells of the early

antral follicle, the mural granulosa cells of the peri-ovulatory Graafian follicle, induced by FSH/FSHR, and luteal cells of the corpus luteum. FSHR expression in the testes is restricted to the Sertoli cells where it acts to regulate spermatogenesis. In females, the FSHR is expressed in granulosa cells of the developing ovarian follicle and in the corpus luteum. These distinct expression patterns of LHR/FSHR in males and females are pertinent to studies of both receptor homo and heteromerization that will be discussed below. For all three GpHRs, several studies have reported extra-gonadal/thyroid expression across many tissue types, however, definitive functional roles are yet to be fully determined.

GpHrs are multi-exon genes; 11 for LHR and 10 for FSHR and TSHR. The multiple exons primarily encode the complex large extracellular leucine-rich repeat hormone binding domain that makes these receptors distinct from other members of the Rhodopsin-like/Class A family (reviewed by [6, 27]). Consequently numerous splice variants have been reported for each receptor with distinct expression patterns, and are primarily thought to negatively impact on receptor function, either as they are non-functional and/or exhibit dominant negative effects on full-length receptors [28–30]. Interestingly, the TSHR undergoes a unique posttranslational proteolytic event amongst the GpHRs that results in cleavage of the TSHR into an α and β subunit. This is due to the presence of a unique ~50 amino acid sequence (termed the C-peptide) in the ECD, although the precise boundaries of this region and the cleavage sites are still uncertain [31, 32]. Likewise, the enzyme/s that mediate this cleavage remains to be determined, though studies indicate likely involvement of a membrane-associated protease e.g. ADAM (a disintegrin and metalloproteinase domain-containing proteins). Cysteine residues on the α and β subunits enable linkage via disulfide-bonds. Although this is the predominant form of TSHR in thyrotropes, it has been suggested the single polypeptide, 'uncleaved' form of the receptor may also be a mature cell surface receptor that can activate signaling [32]. These α-β disulfide links are broken, which leads to shedding of the α subunit and has pathophysiological significance as it is thought that certain individuals are more susceptible to thyroid stimulating autoantibodies and autoimmune hyperthyroidism (Graves Disease). These TSHR autoantibodies compete with TSH for receptor occupancy and activate TSHR signaling. In contrast, those that compete but antagonize the receptor have been linked to hypothyroidism, although interestingly both patient groups can exhibit both types of antibodies and even 'switching' of antibody content and consequently thyroid state has been reported [33, 34].

Despite the explosion of crystal structures reported within the GPCR family in the last decade, there is, as yet, no crystal structure of the GpHRs with its transmembrane (TM) domains. However, crystal structures of the FSHR ECD bound to FSH [35], and TSHR 1-220 ECD amino acids bound to M22 antibody [36] found remarkable similarities between the two resolved structures. Both structures revealed that FSHR and TSHR exhibit clasped ligands at 90 degrees relative to the receptor axis. A second, more complete crystal structure of FSHR, containing the entire extracellular region of FSHR has also suggested that FSH binds to FSHR in a 'hand-clasp' mode, with FSH orientating to interact with the extracellular loops and juxtamembrane

regions of the transmembrane domain [37]. This study also suggested FSHR to reside as a trimer, however this will be discussed in further detail in following sections.

The dominant cognate heterotrimeric G protein pathway for all three GpHRs is the Gαs pathway, leading to activation of adenylate cyclase, increases in intracellular cAMP and activation of cAMP effector proteins such as protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac). However, as for many GPCRs these receptors can couple to multiple G protein-dependent and G protein-independent pathways, the ability of which may be cell type-dependent, but in turn has significance for studying GPCR di/oligomerization. For LHR, coupling to Gαq/11 occurs under high receptor and high hormone levels; physiological relevant conditions that occur in the mural granulosa cells of the ovulatory follicle and the LH surge prior to ovulation [38]. TSHR is also able to activate Gαq/11 signaling, which via activation of phospholipase C, leads to formation of inositol phosphates and increases in intracellular calcium. This pathway may underlie TSH-mediated expression of metallothioneins, proteins that protect cells from reactive oxygen species and toxic metal ions and are upregulated in thyroid cancer and Graves disease [39]. Furthermore, in follicular carcinoma cells the ability of TSHR to signal via Gα12/13 activates MAPK signaling via EGFR transactivation, leading to a proliferative and de-differentiation signal [40]. FSHR has been reported to dually couple to both Gαs and Gαi/o family in Sertoli cells [41], and both LHR and FSHR can activate G protein-independent signaling via the GPCR adaptor proteins, the arrestins [42, 43]. Interestingly, 'neutral' antibodies to the TSHR bind allosterically (as opposed to stimulating and blocking antibodies that compete for the orthosteric TSH site) and in turn may act as biased ligands for MAPK over cAMP signaling [44].

8.3 Evidence of Glycoprotein Hormone Receptor Di/Oligomerization in Cells and Impact on Receptor Activity

The organization of GPCRs, not only as single monomeric entities, but also as dimers and higher order oligomeric complexes, impacts biosynthetic trafficking, ligand specificity and efficacy, signaling and endocytosis. The earliest biochemical reports that GPCRs may not just exist and function as monomeric entities challenged the paradigms of ligand binding and signal transduction that were based on this hypothesis. These studies included reports that the GpHrs exhibit molecular weights, via electrophoresis, equivalent to two or more receptors that were both SDS resistant and occurred under reducing conditions, not only in transfected cells overexpressing these receptors but also in testes (FSHR) and native thryocytes [45, 46], suggesting involvement of TM domains in these interactions. Subsequent studies have employed primarily biophysical approaches, namely fluorescence, or bioluminescence, resonance energy transfer (FRET and BRET respectively) to measure real-time

receptor-receptor interactions in intact cells that confirmed these complexes were preformed and likely to occur during biosynthesis [47–49]. These approaches to study these homomeric complexes have also been employed to identify potential interfaces and the ability of di/oligomers be modulated by ligand. For LHR and FSHR, a complex dimer interface was proposed (also supported by more recent single-molecule approaches and molecular modeling-see below) involving both TM and ECD interfaces [47, 48], while for TSHR the beta subunit was sufficient for interaction, suggesting this interface may be exclusively TM-mediated (see Sect. 8.5). How, and if, receptor activation modulates these complexes is still poorly understood. The approach used to address this question must also be considered. All three receptors appear to form preformed complexes during biosynthesis, possibly indicating requirements for oligomerization for its trafficking and expression to the surface. Modulation of these complexes following ligand activation via FRET/BRET approaches has detected no alteration in the level LHR or FSHR homomers. Interestingly, for TSHR addition of TSH resulted in a loss of oligomer formation [49]. FRET and BRET are highly distance dependent between donor and acceptor molecules; therefore, while changes in energy transfer following TSH could reflect conformational alterations at the protomer level, resulting in a larger distance between donor and acceptor without altering di/oligomer formation. Despite this, co-immunoprecipitation studies have also confirmed TSH-dependent decrease in oligomers [49]. However, it raises the question of the functional role of TSHR oligomerization in receptor signaling and is also inconsistent with other studies employing resonance energy transfer approaches and assessment of binding co-operativity [50]. By combining a variety of experimental approaches this study demonstrated that ligand binding did not alter dimer formation. Although, the interface was primarily TM-mediated, the ECD may modulate receptor-receptor interactions [50]. Indeed a subsequent study has demonstrated that a single tyrosine residue in the TSHR ECD can stabilize constitutive or preformed oligomers [51]. Interestingly, it is likely that for all three GpHR homodimers only one of the protomers will be hormone bound due to strong negative co-operativity. This has been demonstrated to occur via transmission of conformational changes upon GpH binding from the hormone-bound ECD of one protomer to the other protomer, via the TM bundles, as opposed to direct communication of the ECDs [50, 52]. For FSHR, this negative cooperatively may also be present in trimers depending on the glycosylation status of FSH [27]. Endocrine systems such as the HPT and HPG axes, may benefit from systems such as negative co-operativity since it provides a means to respond to a wider range of ligand concentrations, e.g. as in the cyclical changes in LH levels, with maximal sensitivity in the lower concentration range.

Unpicking the functional roles of GpHR di/oligomers, and indeed GPCR homomers in general, has been more challenging than the study of heteromers, particularly those where the heteromers have very distinct pharmacological and signaling properties from the homomer. The application of functional complementation approaches, however, has enabled researchers to infer the impact of these interactions on receptor activity and initiated wider interest in GPCR oligomerization. Functional complementation, also termed transactivation or intermolecular

co-operation, exploits the ability of direct receptor-receptor interactions to rescue functional activity of mutant receptors. As first shown by Maggio and co-workers, pairs of GPCR chimeras or truncated fragments, which were inactive when individually expressed, regained binding and signaling activity if coexpressed in the same membrane [53, 54]. Similarly, for all three GpHRs various mutations or receptor deletions have been generated that create distinct ligand binding-deficient and G protein-signal-deficient mutant (yet retain ligand-binding functions) receptors, that are non-functional when expressed on their own yet co-expression in cells enabled rescue of Gαs-cAMP signaling [55]. In general, this supports the work described above that a single hormone is bound per dimer, and that this is sufficient for G protein activation, at least for Gαs signaling, although the degree of 'functional rescue' reported varies depending on the mutations used, expression levels and ratios between the two mutant receptors [56–60]. An interesting finding in functional complementation studies is that the ability of receptor protomers to transactivate neighboring protomers may be sufficient for Gαs-cAMP signaling but not necessarily sufficient for other G protein pathways that the GpHRs are known to couple to, e.g. Gαq/11. For both TSHR and LHR activation of this pathway by TSH and LH requires cis-activation across the same protomer, rather than trans-activation to an interacting receptor, observed with cAMP signaling. For TSHR it was concluded that both protomers in a dimer must bind TSH to activate Gαq/11 signaling to explain the differences in potencies between these two pathways [61]. We have observed similar findings for LHR, however, it was concluded that the weaker activation of LH-induced Gαq/11 coupling and signaling in cells expressing ligand and binding-deficient mutants is a requirement for LH to transmit activation across the same protomer that binds ligand [60]. Interestingly for LHR, activation of Gαq/11 by its second endogenous ligand CG, via functional complementation, is sufficient for normal inositol phosphate/calcium signaling [60]. This suggest that LH and CG exhibit distinct abilities to activate LHR in terms of directing conformational changes from extracellular ligand binding domains across the TM region to intracellular G protein-coupling. In the context of the WT receptor it cannot be excluded that LH-mediated cis-activation of LHR, leading to Gαq/11 signaling, either may be via individual receptor protomers within a complex or via receptor monomers. Why these two hormones differ in their activation of receptor complexes remains to be determined, but one could speculate that there may be physiological advantages for LHR to exhibit altered sensitivity to Gαq/11 signaling between these ligands at different concentrations. In other words, LH-mediated Gαq/11 signaling could be specifically 'tuned' for the high concentrations during the LH surge leading to ovulation, while activation of this pathway by CG can occur at lower concentrations in very early pregnancy.

Functional complementation has also been employed as a tool to decipher the dimer interface domains required for functional activation of GpHRs. Tethering the ECD of LHR to a GPI-anchor protein or its own TM1 (signal-deficient mutant) have reported conflicting requirements of the involvement of the ECD and TM domains in LHR functional complementation/transactivation and inferred potential interface [56, 57]. However, given the ability of these receptors to also form oligomers, the

isolated ECDs could be associating with receptor oligomers rather than a single protomer as part of a dimer. Although it must be highlighted these studies indicate minimal requirements for functional complementation and not necessarily the di/oligomer interfaces.

8.3.1 Functional Impact of GpHR Heteromerization

The focus of study for these receptors, in the context of GPCR oligomerization, has primarily been on the homomeric forms. Within any cell across different systems, many different kinds of GPCRs will be expressed and thus these receptors have the potential to undergo cross talk with distinct receptors. Despite this, to date, only the FSHR and LHR have been demonstrated to form heteromers with a functional impact on LH/FSH activity [62]. These receptors are not co-expressed in the testes, but in females at a specific maturation stage of ovarian granulosa cells in preovulatory follicles will express both LHR and FSHR. Earlier data may have provided evidence for a possible requirement of heteromerization for ovulation, as it was observed that this was only possible when both LHR and FSHR are coexpressed. Specifically it has been shown that ovulation can be triggered in hypophysectomised rodents by treatment with high doses of FSH [63]. However, LHR knockout (KO) mice were insensitive to FSH-mediated induction of ovulation despite the expression of FSHR in their granulosa cells [64]. One possible explanation for this insensitivity to FSH is that functional LHR, even in the absence of ligand (as after hypophysectomy), is necessary for the granulosa cells to respond to FSH in the preovulatory phase possibly by formation of functional heteromers. FSHR and LHR have been shown to form complexes in cell lines using BRET and functional studies indicate an interesting bidirectional negative modulation on cAMP signalling, such that FSHR attenuates LH-induced signaling and LHR attenuates FSH-mediated signaling via FSHR and increases the dissociation of FSH [62]. This study is consistent with prior work in rodent granulosa cells overexpressing recombinant human LHR resulting in an attenuation of FSH-stimulated cAMP production in these cells [65], however, it remains unclear if these two receptors do form functional complexes *in vivo*.

8.3.2 Single-Molecule Fluorescence Studies of GpHR Monomers, Dimers and Oligomers

A significant technological development in the study of GPCR homo- and heteromerization in the past 6 years has been the application of single molecule and super-resolution imaging approaches, enabling researchers to directly visualize receptor monomers, dimers, and oligomers, and unveil unprecedented detail in kinetics,

spatial organization and even functional roles of specific complexes. These developments in microscopy to studying GPCRs and GPCR oligomerization have been recently reviewed [66–68]. Herein, focus will be on the application of these techniques to GpHRs.

So far there are two distinct studies that have used high- and super-resolution fluorescent and imaging techniques to understand GpHR oligomerization at the nano-scale. Recently, Mazurkiewicz and colleagues [69] have used fluorescence correlation spectroscopy (FCS) (which measures fluorescence intensity fluctuations due to protein diffusion or aggregation within a small volume), with post acquisition photon counting histogram analysis, to measure LHR and FSHR complexes. This technique assesses the stoichiometry of receptor complexes formed, and identified that LHR and FSHR are homodimers and can also form heteromers [69]. Although such techniques have high temporal resolution, the discrepancies of FCS methodologies with other studies may be due to the lower receptor expression levels employed and/or the fact that oligomers, compared to dimers, may be slower diffusing and thus detected inefficiently by the limited spatial volume that is imaged via FCS.

We have employed super-resolution imaging via photoactivatable-localization microscopy (PALM), to map single LHR molecules to <10 nm resolution, compared to conventional light microscopy that achieves ~200 nm maximal resolution, enabling quantitation of LHR complexes at the cell surface [60]. PALM is dependent upon the use of photo-activated fluorophores, which remain in the dark state until unmasked or activated by UV light, emitting fluorescence in a fluorophore-defined wavelength range and subsequently photo-bleached. This activation occurs in a stochastic manner, allowing for spatially separate detection of the activated molecules, and single molecule detection. Repetition of cycles until all fluorophores are activated and bleached into the dark state ensures accurate and defined coordinate specific spatial detection of proteins [66]. For labeling of proteins two methods are employed; either tagging of proteins with photo-switchable proteins or photo-activatable dyes (PD). In this study, PD-PALM was employed to visualize monomers, dimers and oligomers of LHR at the cell surface [60]. The very high spatial resolution via PD-PALM is advantageous compared to prior single molecule imaging studies of GPCR oligomers as it enables localization of labelled receptors expressed at higher densities (~200 molecules/μm^2) than single molecule tracking techniques (usually 1–3 molecules/μm^2). Thus, imaging of LHR di/oligomers was resolved and quantified at receptor densities of 2000–8000 receptors/cell, in line with previously reported *in vivo* expression levels of LHR in ovarian and testicular compartments of 4000–20,000 receptors/cell [70, 71].

To determine the role of protomers within an individual oligomer we employed previously characterized LHR mutants that undergo functional complementation (as discussed above in Sect. 8.3) as these receptors have pre-defined roles; either to bind ligand or activate G protein signaling, thus creating oligomers with defined functional asymmetry. We found that WT LHR, and signal- and binding-deficient mutant receptors form monomers, dimers (consistent with FCS studies (69)) and lower and higher order oligomers. Additionally, that the formation of lower order oligomers (<5 receptors within a complex) was independent of receptor density. Interestingly, 'heteromeric'

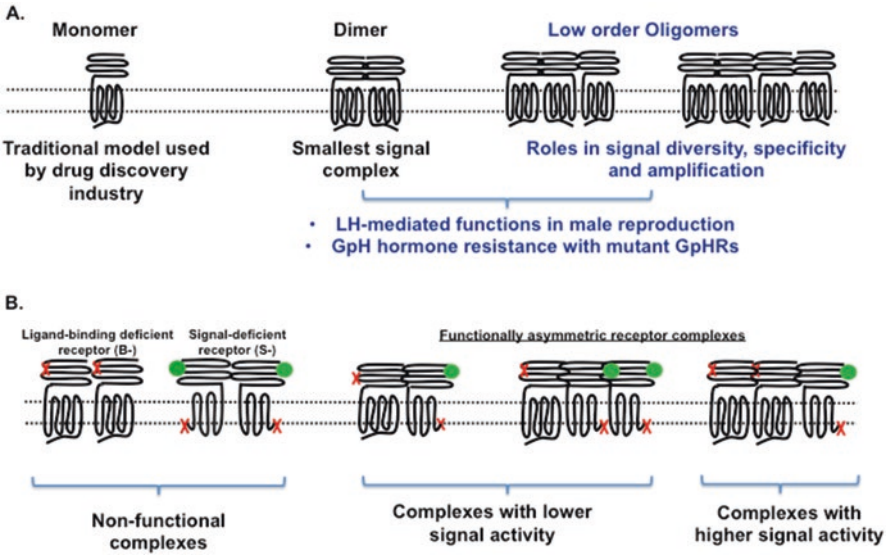

Fig. 8.2 The role of oligomerization on GpHR function. (**a**) Proposed models of the functional roles of monomer, dimer and oligomer to the GPCR family highlighting the complexes with identified roles *in vivo***.** (**b**) Two distinct non-functional mutant receptors, ligand binding deficient (B-) and a signal-deficient (S-) can associate to form functional complexes in vivo. For LHR it has been identified that functionally asymmetric oligomers with more B- receptors have enhanced signal activity over dimers or complexes with more S- receptors (see text and [60]). *Green circle* = Ligand; *red cross* = mutation site

complexes of binding- and signal-deficient mutants preferentially formed oligomeric complexes. A key finding of this study was that altering the functional role of an individual protomer in a complex oligomer could dramatically modulate receptor activity. Specifically, oligomers observed by PD-PALM to have a higher ratio of binding-deficient mutant receptor over signal-deficient mutant receptor exhibited dramatically enhanced signal strength (Fig. 8.2), suggesting that signaling can be 'fine-tuned' by altering the functions of individual protomers within an oligomer, although ligand-dependent alterations in complexes were not observed when observing overall levels of dimers, lower and higher order oligomeric forms [60]. This study provides evidence towards current conceptual models where GPCR oligomerization is viewed as a platform for diversifying/amplifying signal responses (Fig. 8.2).

8.4 Physiological and Pathophysiological Roles of GpHR Di/Oligomerization

A challenging, but essential question in the field has been to understand the role of each individual receptor form, i.e., monomers, dimers and oligomers, in their native system *in vivo.* Furthermore, understanding how perturbations in the organization

of GPCR complexes may also be altered in disease and would provide further impetus for therapeutic targeting of not only heteromers, but also homomeric complexes. The first *in vivo* evidence that formation of homomeric di/oligomers is a physiological relevant form of GPCR signaling for the well-studied Class A/rhodopsin family of GPCR was by using the mouse LHR [72]. Again functional complementation was exploited as a system where 'forced' dimerization/oligomerization must occur to regain ligand-induced signaling. As described above, co-expression of two non-functional mutant LHRs, one that cannot bind ligand with a mutant LHR lacking the ability to generate intracellular signals, formed heteromers and activated cAMP signaling *in vitro*. In order to address whether this form of signaling was also functional *in vivo*, it was necessary to have a system that did not express the WT LHR, thus the LHR knockout (LuRKO) animals were employed [73]. Male LuRKO mice display a phenotype expected in the absence of LH action, exhibiting normal prenatal development of the reproductive tract, but disrupted postnatal reproductive function, presenting with cryptorchidism, testicular hypoplasia, underdeveloped seminiferous tubules and Leydig cell (LHR expressing cells) hypoplasia. Spermatogenesis was also blocked at the round spermatid stage and consequently these animals are infertile [73]. The LHR mutants were created in bacterial artificial chromosome constructs containing the entire LHR gene with the aim to create transgenic animals of each mutant receptor that are expressed with the correct spatio-temporal regulation as for the endogenous LHR. These transgenic animals were crossed to the LuRKO mice so each mutant was expressed in the absence of endogenous WT LHR and the two transgenic lines were also bred to create the double transgenic line expressing both signal and binding-deficient mutant LHRs. Through this approach it was clearly demonstrated that while mice expressing either mutant were still infertile, transgenic mice co-expressing the binding-deficient and signaling-deficient forms of LHR could re-establish normal LH actions through intermolecular functional complementation of the mutant receptors, in the absence of functional wild type receptors [72] (Fig. 8.2). These animals exhibited a reversal in the phenotype of the LuRKO mice, with full restoration in spermatogenesis and testis size. Although testosterone levels were slightly lower in the double transgenic mice than WT animals, they had normal fertility, siring an equivalent number of pups as WT male mice [72].

Whilst animal models are excellent tools to study the role of GpHR complexes *in vivo*, identification of links to human diseases have also highlighted the importance of receptor-receptor interactions physiologically and pathophysiologically. Reports to date are primarily involving GPCR heteromers, where distinct GPCRs may be inappropriately expressed in disease (e.g. preeclampsia [74]), where undesirable side-effects of certain drugs have been attributed to cross-talk or GPCR heteromerization (e.g. A2A-adenosine and dopamine D2 receptor in Parkinson's disease [75]) or, where targeting heteromers may be beneficial (e.g. serotonin 2A receptor-mGluR2 in psychosis [76]). There have been few reports directly linking GPCR homo-oligomerization to human disease but an excellent example is that identified for the TSHR [77]. Patients with TSH resistance is one cause of congenital hypothyroidism with the thyroid gland *in situ* and can exhibit a broad degree of

resistance from elevated levels of TSH, with normal thyroid levels (partial TSH resistance) to complete hypothyroidism with complete TSH resistance. Families have been identified with a history of hypothyroidism with a dominant mode of inheritance of partial TSH resistance, due to heterozygous inactivating mutations in the TSHR gene. Three distinct inactivating mutations in TSHR were assessed and all had a dominant negative effect on WT TSHR function, via intracellular retention during biosynthesis, as a result of WT/mutant TSHR oligomerization [77]. This work has potential implications to other endocrine disorders including known disease-causing mutations of LHR and FSHR. For LHR, there are known inactivating mutations resulting in LH resistance due to a deletion of two amino acids within the 7th TM domain of the receptor [78] that are not solely trafficking impaired, but also signal impaired. Further assessment of how similar LHR mutants, which are signal-impaired only, on WT receptor function, demonstrated that the mutant and WT LHRs form heteromers and that the signal-impaired mutant had negative allosteric effects on WT receptor activity [79]. Such observations may provide molecular explanations underlying the broad phenotypic spectrum in males with LHR-inactivating mutations [80].

The clinical significance of GPCR oligomerization also lies in drug design that can either selectively target homo- or hetero- GPCR dimers/oligomers, e.g. bivalent ligands. Intermolecular cooperation of GPCR dimers has the potential for selective signal activation as has been shown with FSHR and LHR [58, 60]. Bivalent allosteric GpHR ligands using small molecule compounds have been generated with the premise of increasing receptor specificity of these allosteric small molecules between LHR and FSHR that target receptors complexed within homomers and heteromers. Interestingly, these studies suggest that allosteric targeting of LHR dimers or oligomers, via bivalent ligands, are potentially beneficial by improving the potency and specificity of these small molecule ligands [81, 82], and is consistent with our study demonstrating that LHR oligomers regulate signal strength [60].

8.5 Structural Complexity of GpHR Oligomers

Despite extensive experimental evidence for GPCR dimerization/oligomerization, there is very little insight into the architecture of the supramolecular receptor assemblies. X-ray structures have provided some information on the likely architecture of selected receptor dimers. Rhodopsin crystals show dimeric architectures characterized by TM or helix (H) H1–H1 or H8–H8 contacts all compatible with atomic force microscopy data [83]. A similar architecture was also found for the constitutively active opsin apoprotein [84]. With respect to the β_2-adrenergic receptor (β_2-AR), crystal packing in the presence of cholesterol shows a significant involvement of the hydrophobic molecule in the inter-monomer interface [85]. In this framework, protein-protein contacts are minimal and include a pair of salt bridges between H1 and H8 [85]. The crystal structure of the CXCR4 chemokine receptor in complex with the IT1t antagonist shows a dimer interface essentially contributed by amino acids from

the extracellular halves of H5 and H6, with emphasis on the former [86]. Additional contacts involving the cytosolic ends of H3, H4, H5, and intracellular loop 2 (I2) also contribute to the interface [86]. This dimer architecture characterizes the highest resolved complex with IT1t, encoded as 3ODU, as well as the lower resolution complexes encoded as 3OE8 and 3OE9 [86]. The 3OE8 structure is a trimeric assembly characterized by an additional interface involving H5 and H6 from one protomer and H1 from the other protomer. The structure of μ-opioid receptor exhibits receptor molecules intimately associated into pairs along the crystallographic twofold axis through two different interfaces [87]. The first interface is a more limited parallel association mediated by H1, H2, and H8, whereas the second and more prominent interface involves H5 and H6 [87]. Finally, in the crystallographic dimer of the κ-OR bound to the JDTic antagonist, the extracellular half of the interface relies on H1–H1 and H1–H2 contacts from both monomers, whereas the cytosolic end is made by H8-H8 contacts [88]. Although the involvement of the intracellular regions cannot be excluded [89], consensus emerges on H1, H4, and H8 being involved in GPCR dimerization/oligomerization (reviewed also in [90]). However, it should be also considered the possibility that different dimeric architectures may exist in a dynamic equilibrium, as recently inferred from a study on M3 muscarinic receptor dimerization/oligomerization [91]. Furthermore, different receptor portions are expected to be simultaneously involved in the formation of higher order oligomers.

As discussed above for GpHRs it is likely that both ECD and TM domains are important in mediating and regulating complex formation. The role of the ECD in oligomerization was highlighted by the crystal structure of the FSHR ECD in complex with FSH as a trimer, where one molecule of glycosylated FSH is present. However this model is almost incompatible with inter-monomer contacts mediated by the TM helices [35], regions shown to be involved in the interface for all three GpHRs. However, this trimeric model has been demonstrated to be the primary functional form of the FSHR and recent molecular modeling of ‘full length’ FSHR trimer seems to suggest that multiple configurations (and hence multiple TM interfaces) of oligomers may be possible to accommodate the large ECDs that participate in both cis- and trans-activation of its protomers [27], as has been demonstrated for both LHR (see below) and TSHR. For identifying TSHR dimer interfaces the computational method of Brownian Dynamics has been recently applied, focusing on the TM domains (or the TSHR β subunit) by modeling on the rhodopsin crystal structure. This study identified H1 and H5 as the dominant interfaces, with additional involvement of H2 and H4 [92]. As modeling was characterized in the absence of the ECD, putative interfaces were investigated by mutating or deleting these sites in the full length receptor, confirming a role for H1 and H2, particularly H1. Interestingly, substitution of H1 of TSHR with H1 of LHR (that retains some conserved residues) exhibited reduced capacity to oligomerize. However, co-expression of the chimeric receptor with WT TSHR rescued this decrease, and the authors suggest that dimer interface is not a specific set of TM residues, but rather there are ‘regions of attraction’ in TM domains [92]. Such a model exemplifies the complex, potentially multi-di/oligomeric interface of the GpHRs, a phenomena further demonstrated with LHR by combining high-resolution modeling with super-resolution imaging data.

8.5.1 The FiPD-Based Computational Strategy to Quaternary Structure Predictions of Transmembrane Proteins

So far, only very few predictions of the architecture of GPCRs dimers/oligomers have utilized docking sampling and a significant part of such predictions were obtained by a computational protocol developed in our laboratory, i.e. hereafter defined as FiPD-based approach [93–100]. Indeed, such a protocol is based on the combination of protein-protein docking by the ZDOCK software [101] and solution filtering by the FiPD software, which aids minimizing false positives (visit "Software" at http://www.csbl.unimore.it) [102]. The approach consists of rigid-body docking using a version of the ZDOCK program devoid of desolvation as a component of the docking score [101]. It does not employ symmetry constraints either for improving sampling or in the filtering step. Furthermore, there are no size-limitations for the systems under study, which are not limited to the TM regions but include the loop regions as well. In the case of homo-dimerization, two identical copies of the structural protomer model are docked together, i.e. one protomer is used as a fixed protein (target) and the other as a mobile protein (probe). For predicting heterodimers, the structural model of protomer A is taken as a target, whereas the structural model of protomer B is taken as a probe and/or vice versa. For each run the best 4000 solutions are retained and ranked according to the ZDOCK score. These solutions are then filtered using the "membrane topology" filter implemented in the FiPD software [102], which discards all the solutions that violate the membrane topology requirements. Such a filter discards more than 94% of the solutions selected according to the docking score. The filtered solutions are clustered using a Cα-RMSD (Root Mean Square Deviation of the Cα-atoms) threshold generally equal to 3.0 Å. The best scored docking solution/s from the most populated and reliable cluster/s is/are finally chosen to build the dimer/oligomer. Docking, filtering, and clustering are iteratively repeated several times to predict higher order oligomers, by using the intermediate dimer/oligomer as a target and the protomer as a probe.

Benchmarks of the approach have been carried out on: (a) the tetrameric potassium channel (Kch, 384 amino acids); (b) the pentameric large conductance mechanosensitive channels, MscL (540 amino acids) and eptameric MscS (1771 amino acids) and (c) the trimeric bacteriorhodopsin (698 amino acids). These led to native-like quaternary structures, i.e., with the Cα-RMSD lower than 2.5 Å from the native oligomer [102]. The availability of crystal structures for the CXCR4 chemokine receptor and the κ-opioid receptor dimers enabled validation of this approach on GPCRs as well [103]. The FiPD-based protein-protein docking protocol has been employed for quaternary structure predictions of a number of GPCRs, including members of the amine [97], GpH [60, 95], peptide [96], prostanoid [99], and purine [97, 98] subfamilies. Collectively, quaternary structure predictions by the FiPD-based approach on Class A GPCRs emphasized the role of H1 and H4 in mediating dimerization, consistent with evidence from structure determinations and *in vitro* experiments [100, 103].

8.5.2 *Combination of FiPD-Based Approach and Super-Resolution Imaging to Predict Likely Architectures of LHR Oligomers*

The FiPD-based approach has been more recently combined with super-resolution imaging (PD-PALM) resolving single GPCR molecules into functional asymmetric dimers and oligomers to gain insight into the molecular complexity, as well as spatial and structural signatures involved in GPCR di/oligomerization as described above [60].

PD-PALM identified that trimers were primarily organized in triangular formations, whilst tetramers exhibited a higher variety of distributions with at least one triangular sub-arrangement [60] (Fig. 8.3). As the localization of molecules identified

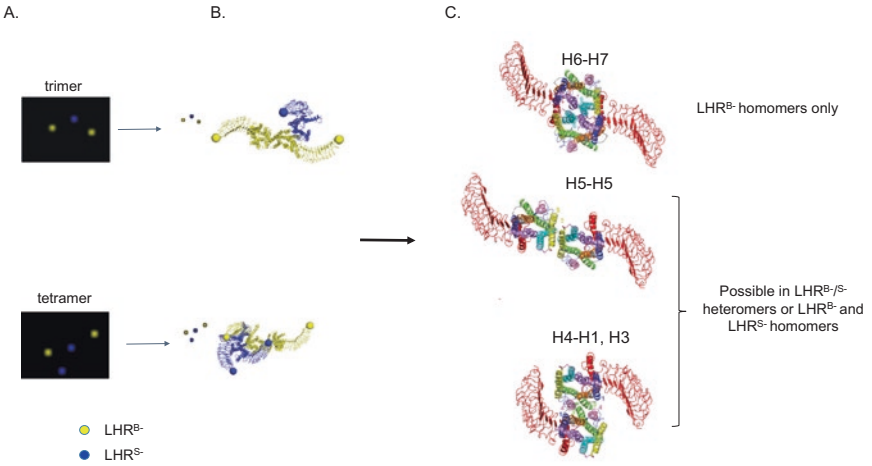

Fig. 8.3 Combining PD-PALM and FiPD structural modeling of LHR trimers and tetramers to identify protomer interfaces. (**a**) Example of LHR binding-deficient (LHR^{B-}) and signal-deficient (LHR^{S-}) trimer and tetramer visualized by PD-PALM and comparison of spatial arrangements with structural data that closely aligned with PD-PALM resolved images. In **b** structures are imaged from the extracellular side perpendicular to the membrane surface. *Yellow* and *blue* colors indicate LHR^{B-} and LHR^{S-} forms respectively. Spheres are centered on the Cα-atom of the first amino acid. (**c**) Examples of FiPD modeling demonstrating potential homo and heteromer interfaces that occurred in both dimers and oligomers. The interfaces are shown from the intracellular side, perpendicular to the membrane plane. The receptor is divided into different colors, ECD- *red*, TM helices: 1- *blue*, 2- *orange*, 3- *green*, 4- *pink*, 5- *yellow*, 6- *cyan*, and 7-*purple* with helix 8 also represented in purple. Intracellular and extracellular loops (IL and EL, respectively) are depicted as, 1- *slate*, 2- *gray* and 3- *magenta*. The helices participating in the interface are labeled by the corresponding helix number (Adapted from [60]. This research was originally published in The Journal Of Biological Chemistry. Jonas, K.C, Fanelli, F, Huhtaniemi, I and Hanyaloglu, A.C. Single molecule analysis of functionally asymmetric G protein-coupled receptor (GPCR) oligomers reveals diverse spatial and structural assemblies. *The Journal of Biological Chemistry*. 2015; 290:3875–3892. © the American Society for Biochemistry and Molecular Biology)

by PD-PALM represents the dye position of the antibody-bound to the epitope tag on the N-terminus of the ECD, the technique provides unprecedented information on the geometric arrangements of trimers and tetramers in intact cells, in their native membrane environment, but not highly detailed structural information on inter-protomer interfaces mediating associations in these complexes. Therefore, docking simulations on surrogates of binding- and signaling-deficient LHR forms (LHR^{B-} and LHR^{S-}, respectively) were used to predict spatial organization and interacting interfaces of protomers within an oligomer, and how these aligned with the super-resolution imaging data [60]. Of note to these studies, in order to create a completely signal-deficient form of LHR, H6-7 were deleted. Despite the removal of these TMs, this mutant retained its ability to traffick to the cell surface [60, 72].

The structural models were achieved by comparative modelling using the two available crystal structures of the FSHR ECD [35, 37] and crystal structures of constitutively active opsin [84] as templates. Unexpectedly, the predicted spatial organization based on the first amino acid of the N-terminus (yellow or blue sphere centred on the Cα-atom) closely matched PD-PALM resolved geometries of trimers and tetramers (Fig. 8.3), with >90% of PD-PALM imaged complexes aligning with structural predictions. Spatial organization of these LHR complexes were dictated by contacts between helices and the distinct shape of the large N-terminus and revealed that the quaternary structure of the helix bundles of each protomer in trimers and tetramers were primarily linear (Fig. 8.3 and [60]). Therefore, the spatial organization of molecules inferred from PD-PALM and structural modelling are due to the rigid and defined shape of the ECD, in line with the crystallographic complex of the FSHR ECD trimer [37].

This study also identified potential multiple, and hence complex di/oligomer interfaces. Some helix contact signatures were observed in all three possible pairings of the two mutant LHRs (self-associating LHR^{B-}, LHR^{S-} dimers, and LHR^{B-}/LHR^{S-} dimers), whilst others were only feasible for either LHR^{B-} or LHR^{S-}. H4-H1, H3 or H5 contacts were observed in all three possible dimeric pairings (Fig. 8.3). As H6-7 was absent in LHR^{S-}, H6–H7 contacts were unique to the LHR^{B-} homomer. The H5-H5 interface, possible in both self-associating and intermolecular cooperating LHR^{B-}/LHR^{S-} complexes (Fig. 8.3), and the H6–H7 interface unique to LHR^{B-} homomers permits the largest separation between the two N-termini. Interestingly, despite deletion of H6 and H7 in LHR^{S-} limiting the number of possible interfaces in the self-associating LHR^{S-} homodimer, this deletion increased the number of possible interacting interfaces in the LHR^{B-}/LHR^{S-} complexes, providing an opportunity for larger interfaces within an intermolecular cooperating LHR^{B-}/LHR^{S-} complex [60]. Collectively, the predicted and PD-PALM visualized LHR^{B-}/LHR^{S-} complexes are comprised of diverse and complex combinations of helix interfaces, with multiple possible inter-protomer interactions consistent with prior studies of both FSHR and TSHR. These combined functional and modeling approaches have also predicted how many heterotrimeric G proteins could associate with lower order oligomers. For FSHR, a combination of the bacteriorhodopsin, which assembles as a trimer in crystals and in solution, with the crystal structure of the β2-adrenegric receptor-Gαs complex were used as templates. In this study it was predicted that only one G protein would fit in to the tightly

associated FSHR trimer that was modeled [27]. However, enrichment of LHR^{B-} within LHR^{B-}/LHR^{S-} oligomers, which exhibit enhanced signaling, permits the association of two heterotrimeric G proteins via the H5 or H4-H1, H3 interfaces [60]. In contrast, cells favoring the formation of tetramers with an excess of LHR^{S-}exhibit the lowest ligand-induced signal responses and are possibly due to the ability of such oligomers to accommodate only one G protein, indicating that the broad spectrum of receptor complexes observed could regulate LHR activity. Overall the complexity in di/oligomer receptor interfaces may reflect the unique structural features of the GpHrs and/or an advantageous feature of combining cellular and computational approaches that study GPCRs at the protomer level.

8.6 Future Perspectives and Conclusions

The GpHRs and their hormones represent a unique subfamily within the GPCR super-family, and much of the molecular complexity from its ligands, to the monomeric receptor structure and signal regulation may underlie the subsequent intricacy in the molecular make-up of GpHR dimers and oligomers. There are obvious advantages physiologically to such complexity as it potentially provides a highly tuned system to respond and rapidly reprogram to a cyclical and dynamic hormonal environment. But in turn, studies with these receptors have provided key information on the significance of these complexes at the individual oligomer, cellular, physiological, and pathophysiological level pertinent across the GPCR superfamily.

While significant technical developments have been made in the study of GPCR oligomers, a significant advance would be the crystal structure of a full length GpHR that could provide key molecular pieces of the puzzle and what, if any, oligomers could be captured by such an approach. It is worth noting that LHR, and likely for the other GpHRs, a significant portion are monomeric (~60% via PD-PALM [60]). Therefore there are outstanding questions related to our understanding of how both monomeric receptors and oligomeric complexes each contribute to (or perhaps even inter-communicate), to regulate cellular signaling. Furthermore, if, and how, these complexes may be altered under pathological conditions outside of disease-causing receptor mutations. For example, LHR and FSHR have been found in distinct non-gonadal cancers [104, 105] where studying receptor oligomerization and activity in these cells may unveil novel therapeutic targets, especially given the development of low molecular weight allosteric compounds to these receptors. Although significant headway in demonstrating physiological relevance of GpHR oligomers has been made, a likely next step is to ‘see’ these complexes in native tissue at the individual protomer lever, and potentially identify novel heteromeric associations of the GpHRs with other GPCRs outside of this subfamily. Identification of such novel GPCR heteromers may underlie observations, which as yet, have no molecular clarification, for example the inability of CG, but not prostaglandin E2, to active Gαs-adenylate cyclase-cAMP signaling in human endometrium, despite

the presence and known downstream activity of LHR in this tissue [106]. Overall, our advances, both technically and at the molecular level, in the study of GpHR oligomers have made a significant contribution to our more general understanding of GPCR di/oligomers, an area that has had a history of controversy. The integration of disciplines and methodologies will truly facilitate the progression in our understanding of these complex signaling molecules, and the ability to pharmacologically target these complexes with high precision to create efficacious and specific therapeutics of the future.

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Chapter 9
Chemokine Receptor Oligomerization to Tweak Chemotactic Responses

Henry F. Vischer

Abstract Chemokine receptors guide cell migration by responding to local chemokine gradients during immune surveillance and inflammation. Similar to other G protein-coupled receptors, chemokine receptors can form oligomeric complexes that might have distinct pharmacological and biochemical properties as compared to their individual constituents. The majority of evidence for chemokine receptor oligomers came from transfected cells using tagged receptors to monitor their close proximity or physical association. However, translation of these observations to (patho)-physiological consequences is puzzling for the majority of chemokine receptor oligomers due to experimental limitations and challenges to distinguish oligomer- from downstream signaling-mediated crosstalk. Recent methodological advances allow in situ validation of chemokine receptor oligomers in native cells, disruption of oligomers, and detection of oligomer-mediated signaling. Chemokine receptor oligomerization modulates cell migration in (patho)-physiology and consequently offers novel therapeutic targets.

Keywords Chemokine • Chemokine receptor • Receptor dimerization • Negative binding cooperavity • Chemotaxis

Abbreviation

7TM	seven transmembrane
α_{1A}-AR	α_{1A}-adrenergic receptor
α_{1B}-AR	α_{1B}-adrenergic receptor
α_{1D}-AR	α_{1D}-adrenergic receptor
α_{2A}-AR	α_{2A}-adrenergic receptor
α_{2B}-AR	α_{2B}-adrenergic receptor

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α_{2C}-AR	α_{2C}-adrenergic receptor
ACKR	atypical chemokine receptor
AIDS	acquired immune deficiency syndrome
AngII	angiotensin II
APC	antigen-presenting cell
AT_1R	angiotensin II receptor type 1
β_1-AR	β_1-adrenergic receptor
β_2-AR	β_2-adrenergic receptor
BRET	bioluminescence resonance energy transfer
BSMC	bronchial smooth muscle cell
CB2	cannabinoid receptor 2
CLL	chronic lymphocytic leukemia
CODA-RET	complemented donor-acceptor resonance energy transfer
CoIP	co-immunoprecipitation
COPD	chronic obstructive pulmonary disease
CRS1	chemokine recognition site 1
CRS2	chemokine recognition site 2
D2R	dopamine receptor 2
DOR	δ-opioid receptor
EBI2	Epstein-Barr virus-induced receptor 2
EBV	Epstein-Barr virus
ER	endoplasmatic reticulum
FC	functional complementation
FRET	fluorescence resonance energy transfer
GDP	guanosine diphosphate
GPCR	G protein-coupled receptor
GPCR-HIT	GPCR Heteromer Identification Technology
GRK	G protein-coupled receptor kinase
GTP	guanosine triphosphate
HHV	human herpesvirus
HIV	human immunodeficiency virus
IBD	inflammatory bowel disease
IGF-1R	insulin-like growth factor-1 receptor
IL	intracellular loop
IS	immunological synapse
IUPHAR	International Union of Basic and Clinical Pharmacology
LE	lymphatic endothelial cell
LN	lymph nodes
MHC	major histocompatibility complex
MoDC	monocyte-derived dendritic cell
MOR	μ-opioid receptor
NAM	negative allosteric modulator
NBC	negative binding cooperativity
NHERF1	NA^+/H^+ exchanger regulatory factor-1

PFC	protein fragment complementation
PGE2	prostaglandin 2
PLA	proximity ligation assay
PLC	phospholipase C
PTX	pertussis toxin
RET	resonance energy transfer
Rluc	Renilla luciferase
RTK	receptor tyrosine kinase
S1PR1	sphingosine-1-phosphate receptor 1
SNP	single-nucleotide polymorphism
TM	transmembrane
trFRET	time-resolved fluorescence resonance energy transfer
VE	vascular endothelial cell
VSMC	vascular smooth muscle cell

9.1 Introduction

Chemokines regulate localization, migration, activation, development and differentiation of immune cells in the body [1]. Approximately 45 endogenous chemokine subtypes have so far been identified in human. These chemokines are subdivided into four groups (C, CC, CXC, and CX3C) based on their conserved cysteine signature [2]. Some chemokine subtypes are secreted in lymphoid organs and involved in immune cell homing during homeostasis, whereas other chemokines are secreted upon trauma to attract immune cells to inflammatory sites [1]. Chemokine receptor-expressing immune cells are guided towards the source of chemokine secretion by a local chemotactic gradient of bound chemokine to glycosaminoglycans that are tethered to the surface of endothelial cells and extracellular matrix [3]. In addition, chemokine receptors are expressed on various other cell types (e.g. endothelial, epithelial, smooth muscle, and neural cells) and involved in tissue development and remodeling [2].

Chemokine receptors belong to the structural family of class A G protein-coupled receptors (GPCRs) and 22 different subtypes have been identified in human [2]. Eighteen of these chemokine receptors are actual GPCRs (XCR1, CCR1-10, CXCR1-6, and CX3CR1) and activate heterotrimeric G_i proteins upon chemokine binding, whereas four so-called atypical chemokine receptors (ACKR1-4) bind and internalize chemokines without signaling through heterotrimeric G proteins [2].

The majority of chemokine receptors bind multiple chemokine subtypes (and vice versa), whereas others are highly specific (Fig. 9.1). Distinct spatiotemporal expression of chemokine receptor subtypes allows selective subsets of immune cells to sense and migrate towards gradients of corresponding chemokines in lymphoid organs and/or inflammatory sites (Fig. 9.2) [1]. Dysregulated chemokine and/or chemokine receptor expression results in chronic inflammation, autoimmune

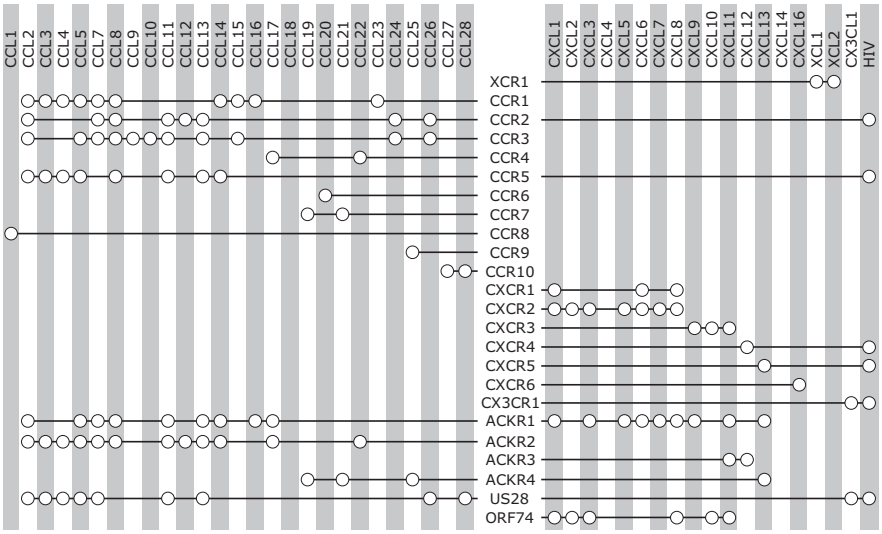

Fig. 9.1 Chemokines (*horizontal*) and chemokine receptors (*vertical*) interactions are indicated by *open circles*. The atypical chemokine receptors (ACKR) were recently renamed: DARC = ACKR1; D6 = ACKR2; CXCR7 = ACKR3; CCX-CKR = ACKR4 [153] (Figure is modified with permission from Ref. [154])

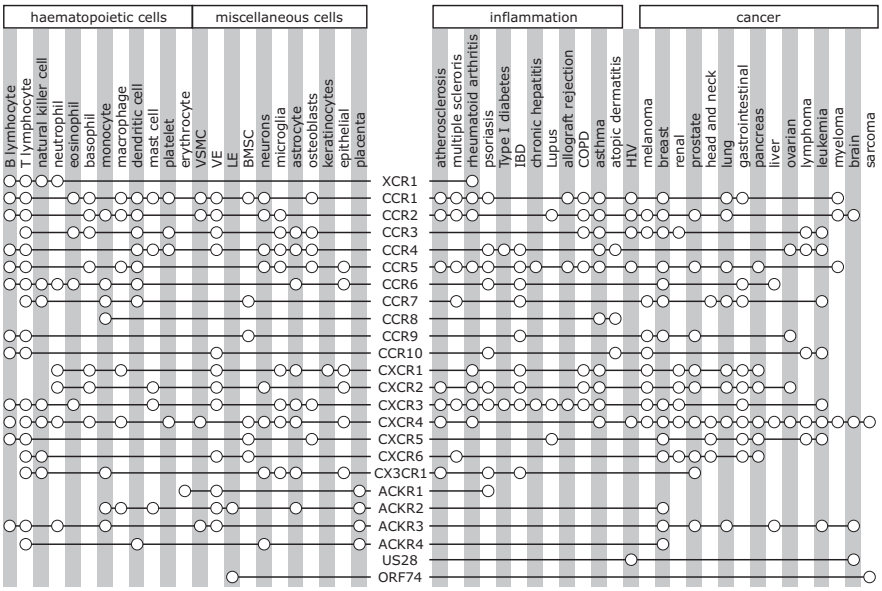

Fig. 9.2 Chemokine receptor expression (*left-hand side*) and association with pathologies (*right-hand side*) are indicated by *open circles*. Abbreviations: *VSMC* vascular smooth muscle cell; *VE* vascular endothelial cell; *LE* lymphatic endothelial cell; *BSMC* bronchial smooth muscle cell; *IBD* inflammatory bowel disease; *COPD* chronic obstructive pulmonary disease (Figure is modified with permission from Ref. [154])

diseases, tumorigenesis and/or metastasis [4, 5]. Moreover, infection with human herpesviruses (HHV) such as cytomegalovirus and Kaposi's sarcoma-associated herpesvirus, may result in ectopic expression of viral chemokine receptors on infected cells (Fig. 9.2). These viral chemokine receptors are constitutively active and/or modulated by human chemokines to facilitate viral dissemination but also inflammatory and proliferative diseases [6].

Finally, CCR5 and CXCR4 are co-receptors for the entry of R5- and X4-tropic human immunodeficiency virus (HIV) strains, respectively, in $CD4^+$ macrophages and T cells [7].

9.2 Chemokine and Chemokine Receptor Stoichiometry

Chemokines can form homo- and/or hetero-oligomers in solution or upon binding to glycosaminoglycans, and this multimerization is essential to orchestrate immune cells migration in vivo. In contrast, monomeric (mutant) chemokines efficiently induce chemokine receptor-mediated signaling and chemotaxis in vitro, indicating that chemokines activate their cognate receptors as a monomer [8].

Chemokines bind with their globular core to the extracellular N-terminus and loops of chemokines receptor (i.e. chemokine recognition site 1; CRS1), whereas their flexible N-tail subsequently enters the pocket formed by the seven-transmembrane (7TM) domain (chemokine recognition site 2; CRS2) to induce receptor activation [9]. Similar to other GPCRs, chemokine receptors can form dimers (see below), which would allow one chemokine to simultaneously bind to CRS1 of one receptor and CRS2 of the other receptor within the dimer (i.e. 1:2 stoichiometry) [10]. However, co-expression of chemokine binding-deficient (CRS1) with signaling-deficient (CRS2) CXCR4 mutants did not rescue intracellular Ca^{2+} mobilization in response to CXCL12 stimulation, even though all mutants had the same propensity as wild type CXCR4 to form dimers [11]. Hence, the lack of functional complementation (FC) indicates that CXCL12 binds to CXCR4 in a 1:1 stoichiometry. Indeed, recent high resolution crystal structures of CXCR4 in complex with viral chemokine antagonist vMIP-II, and viral chemokine receptor US28 in complex with human CX3CL1, confirmed that one chemokine binds to CRS1 and CRS2 of one receptor [12, 13].

9.3 Chemokine Receptor Dimerization

Class C GPCRs such as $GABA_B$ receptor, taste 1 receptors 1–3, and metabotropic glutamate 2 receptors function as obligate dimers (see Chaps. 17 and 18) [14, 15]. In contrast, most class A/B GPCRs can signal as monomers through heterotrimeric G proteins, and/or recruit G protein-coupled receptor kinases (GRK), and β-arrestins upon agonist stimulation [16–24]. Yet, heteromerization of class A/B GPCRs may

unmask novel biochemical properties as a result of allosteric interactions within the macromolecular receptor complex that are different from those of the individual GPCR subtypes [25–27]. However, it is technically challenging to distinguish altered biochemical signaling by physically interacting GPCRs from heterologous regulation and crosstalk at downstream signaling hubs [25–27].

Evidence for di- and oligomeric GPCR complexes, including chemokine receptors (see Table 9.1), came the last two decades predominantly from recombinant expression in cell lines using various biochemical and resonance-energy transfer-based methodologies (see Chaps. 2, 3, 4, 5 and 6). However, many of these identified GPCR complexes still awaits in situ validation in native tissues, which is often limited by the availability of GPCR subtype-selective antibodies [25, 26]. Coimmunoprecipitation (CoIP) of dimerized chemokine receptors from solubilized native cells has been reported for CCR2, CCR5, CCR7, CXCR2, CXCR4, ACKR3, and ACKR4 homo- and/or heteromers (Fig. 9.3a; see Table 9.1). More recently, proximity ligation assay (PLA) was developed to detect native GPCR heteromers in situ by labeling them first with subtype-selective primary antibodies, subsequently followed by oligonucleotide-conjugated secondary antibodies (Fig. 9.3b). If the distance between these secondary antibodies is less than 16 nm, which corresponds to an approximate distance of less than 40 nm between their epitopes (i.e. GPCRs), the conjugated oligonucleotide can be enzymatically ligated, amplified, and hybridized with fluorescent complementary probe allowing microscopy analysis [28]. Native CXCR4/ACKR3 and CXCR4/$\alpha_{1A/B}$-adrenergic receptors ($\alpha_{1A/B}$-AR) heteromers were detected on the cell surface of aortic vascular smooth muscle cells (VSMC) by PLA, whereas CXCR4 was not in close proxity with α_{1D}-AR, α_{2A}-AR, α_{2B}-AR, and α_{2C}-AR [29, 30]. Peptide analogs of CXCR4 TM2 or TM4 specifically reduced PLA between CXCR4 and α_{1A}-AR or ACKR3, respectively, without affecting receptor levels at the surface of these cells. Interestingly, PLA signal between endogenous CXCR4 and cannabinoid receptor 2 (CB2) on the surface of human prostate and breast cancer cells was only detectable upon co-stimulation with CXCL12 and CB2 agonist AM1241 [31]. CCR7 homodimers were detected by PLA upon stimulation of native human monocyte-derived dendritic cells (MoDCs) with prostaglandin E2 (PGE2 (see below) [32].

Time-resolved fluorescence resonance energy transfer (trFRET) between lanthanide- and acceptor fluophore-conjugated ligands upon receptor binding revealed presence of GPCR oligomers in native tissues [33, 34], and might be applied as well to detect chemokine receptors using fluorescently labeled chemokines and/or monovalent llama-derived nanobodies (Fig. 9.3c) [35–37]. However, possible negative binding cooperativity between chemokine receptor binding sites may hamper this approach and should be taken into account in particular for labeled agonists (see below) [33].

Table 9.1 Chemokine receptor oligomer detection and biochemical consequences

R1	R2	Native cells	Heterologous cells	CoIP	BRET	FRET	PFC	trFRET	PLA	BirA PB	GPCR-HIT	CODA-RET	FC	Co-internalization	Dimer disruption	Mutagenesis	Bivalent ligand	Biochemical consequences	References
CCR2	CCR2	X	X	I	–	–	–	–	–	–	–	–	–	–	–	–	–	Bivalent antibody-induced dimerization and signaling	[62, 63, 66]
CCR2	CCR2	X	X	–	–	–	–	–	–	–	–	–	–	–	X	X	–	Interface mutation and TM peptide inhibit signaling and monocyte migration	[40, 42]
CCR2	CCR2	–	X	–	M	–	–	–	–	–	–	–	–	–	X	–	–	TM peptide affected chemokine-induced BRET changes	[44]
CCR2	CCR2	–	X	–	M	–	C	–	–	–	–	X	–	–	–	–	–	Proximal signaling	[47]
CCR2	CCR5	–	X	I	–	–	–	–	–	–	–	–	–	N	–	–	–	Chemokine co-stimulation switched signaling from G_i to $G_{q/11}$	[66]
CCR2	CCR5	–	X	I	–	M	–	–	–	–	–	–	–	–	–	X	–	Bivalent antibody-induced dimerization blocked HIV-1 replication	[40, 43, 63]
CCR2	CCR5	–	X	–	–	–	–	–	–	–	X	–	–	–	–	–	–	Proximal signaling	[94]
CCR2	CCR5	X	X	C	C	–	–	–	–	–	–	–	–	N	–	–	–	NBC agonists and antagonists; functional cross-antagonism	[67, 80]
CCR2	CCR5/ CXCR4	X	X	–	M	–	M	–	–	–	–	M	–	–	–	–	–	NBC agonists and antagonists; functional cross-antagonism	[49]
CCR2	CXCR4	X	X	–	M	–	–	–	–	–	–	–	–	–	–	–	–	NBC agonists and antagonists; functional cross-antagonism	[59]

(continued)

Table 9.1 (continued)

R1	R2	Native cells	Heterologous cells	CoIP	BRET	FRET	PFC	trFRET	PLA	BirA PB	GPCR-HIT	CODA-RET	FC	Co-internalization	Dimer disruption	Mutagenesis	Bivalent ligand	Biochemical consequences	References
CCR2	CXCR4	–	X	I	–	–	–	–	–	–	–	–	–	–	–	X	–	Bivalent antibody-induced dimerization blocked HIV-1 replication	[40, 43]
CCR2	CXCR4	–	X	–	M	–	–	–	–	–	–	–	–	–	X	–	–	TM peptide affected chemokine-induced BRET changes	[44]
CCR2	CXCR4	–	X	–	M	–	C	–	–	–	X	X	–	–	–	–	–	Proximal signaling	[47, 94]
CCR2	CXCR4	X	X	C	–	–	C	–	–	C	–	–	–	–	–	–	–	Differential anterograde trafficking	[68, 155]
CCR2	AT1R	–	X	–	–	–	–	–	–	–	X	–	–	–	–	–	–	Heteromer β-arrestin2 recruitment impaired G_i coupling	[96]
CCR5	CCR5	–	X	I	M	–	–	–	–	–	–	–	–	Y	–	–	–	Bivalent antibody-induced dimerization blocked HIV-1 replication	[64, 66, 156]
CCR5	CCR5	–	X	M	M	M	–	–	–	–	–	–	–	–	X	X	–	Interface mutation and TM peptide disrupted homomerization	[40, 57]
CCR5	CCR5	–	X	C	C	–	C	–	–	–	–	–	X	–	–	–	–	Homodimer signaling and internalization	[41, 52, 105]
CCR5	CCR5	–	X	–	–	–	C	–	–	–	–	–	–	–	–	–	–	Rab1/11-mediated anterograde trafficking	[102]
CCR5	CCR5/ CD4	–	X	–	–	–	C	–	–	–	–	–	–	–	–	–	–	Rab1/8-mediated anterograde trafficking	[102]

CCR5	CCR5-Δ32	–	X	C	–	–	–	–	–	–	–	–	–	–	–	–	Reduced RSE and HIV-1 infection	[97, 99]
CCR5	CXCR4	–	X	–	M	M	–	–	–	–	–	–	–	–	–	X	–	–	[45, 57]
CCR5	CXCR4	–	X	C	C	–	–	–	–	–	–	–	–	–	–	–	–	Co-recruited to immunological synaps; cross-inhibited by ligands	[90]
CCR5	CXCR4	–	X	–	C	–	–	–	–	C	–	–	–	–	–	–	–	–	[155, 157]
CCR5	CXCR4	–	X	–	–	–	C	–	–	–	–	–	–	–	–	–	–	Rab1/2/11-mediated anterograde trafficking; impaired NHERF1 interaction	[102, 105]
CCR5	CXCR4/ CD4	–	X	–	–	–	C	–	–	–	–	–	–	–	–	–	–	Rab1-mediated anterograde trafficking; increased RSE; reduced HIV entry	[69, 102, 104]
CCR5	ACKR1	–	X	C	C	–	–	–	–	–	–	–	–	N	–	–	–	Reduced CCR5-mediated chemotaxis and Ca^{2+} mobilization	[109]
CCR5	C5aR	–	X	C	C	–	–	–	–	–	–	–	–	Y	–	–	–	Co-internalization	[41]
CCR5	DOR	X	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	Chemokine-induced CCR5 downregulation impaired by morphine	[158]
CCR5	KOR	X	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	Chemokine-induced CCR5 downregulation impaired by morphine	[158]
CCR5	MOR	X	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	Chemokine-induced CCR5 downregulation impaired by morphine	[158]
CCR5	MOR	–	X	C	–	–	–	–	–	–	–	–	–	N	–	–	–	Cross-phosphorylation and desensitization	[89]
CCR5	MOR	X	–	–	–	–	–	–	–	–	–	–	–	–	–	–	X	Reduced HIV infection in presence of morphine	[143–145]
CCR5	MOR	X	–	–	–	–	–	–	–	–	–	–	–	–	–	–	X	Increased antinociception without tolerance	[141]

(continued)

Table 9.1 (continued)

R1	R2	Native cells	Heterologous cells	CoIP	BRET	FRET	PFC	trFRET	PLA	BirA PB	GPCR-HIT	CODA-RET	FC	Co-internalization	Dimer disruption	Mutagenesis	Bivalent ligand	Biochemical consequences	References
CCR5	**UL33**	**–**	**X**	**C**	**C**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**Increased RSE; decreased signaling and HIV infection**	**[126]**
CCR5	UL78	–	X	C	C	–	–	–	–	–	–	–	–	–	–	–	–	Increased RSE; modulated signaling; decreased HIV infection	[126]
CCR5	CD4	X	X	C	C	–	–	–	–	–	–	–	–	–	–	–	–	Increased RSE	[103]
CCR6	BILF1	–	X	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	[116]
CCR7	CCR7	X	X	–	–	M	M	–	M	–	–	–	–	–	X	X	–	Increased Src/SHP2-mediated chemotaxis into LN	[32]
CCR7	ChemR23	X	X	–	C	–	–	C	–	–	–	–	–	N	–	–	–	NBC agonists	[83]
CCR7	β_2-AR	X	X	C	–	–	–	–	–	–	–	–	–	–	–	–	–	Increased B cell retention in LN	[135]
CCR7	BILF1	–	X	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	[116]
CCR9	BILF1	–	X	C	C	–	–	C	–	–	–	–	–	–	–	–	–	–	[116]
CCR10	BILF1	–	X	C	C	–	–	C	–	–	–	–	–	–	–	–	–	–	[116]
CXCR1	CXCR1	X	X	C	C	M	–	–	–	–	–	–	–	–	–	X	–	Anterograde trafficking?	[51, 71, 159]
CXCR1	CXCR2	X	X	C	C	M	–	–	–	–	–	–	–	–	–	X	–	Anterograde trafficking?	[51, 71, 159]
CXCR1	CXCR4	–	X	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	[159]
CXCR1	CXCR5	–	X	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	[159]
CXCR1	CXCR6	–	X	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	[159]
CXCR1	ACK3	–	X	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	[159]

CXCR2	CXCR2	–	X	C	C	M	–	–	–	–	–	–	–	–	–	–	–	–	[51, 71, 157, 159]
CXCR2	CXCR2	X	X	–	–	–	–	–	–	–	–	–	–	–	–	–	X	–	[149]
CXCR2	CXCR2	X	X	C	–	–	–	–	–	–	–	–	–	–	–	X	–	TM3 required for homomerization	[160]
CXCR2	CXCR4	–	X	–	C	C	–	–	–	–	–	–	–	–	–	–	–	–	[157, 159]
CXCR2	CXCR5	–	X	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	[159]
CXCR2	CXCR6	–	X	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	[159]
CXCR2	ACKR3	–	X	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	[159]
CXCR2	DOR	–	X	C	C	C	–	C	–	–	–	–	X	–	–	–	–	CXCR2 antagonist enhanced DOR signaling	[91]
CXCR2	α_1-AR	–	X	–	C	–	–	–	–	–	X	–	–	Y	–	–	–	Increased β-arrestin2 recruitment and α1-AR internalization	[95]
CXCR2	GluR1	X	X	C	–	–	–	–	–	–	–	–	–	–	–	–	–	Reduced CXCR2 homomerization and chemotaxis	[131, 160]
CXCR3	CXCR3	–	X	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	[81, 157, 159]
CXCR3	CXCR4	–	X	–	C	C	–	–	–	–	–	–	–	–	–	–	–	–	[157, 159]
CXCR3	CXCR4	–	X	C	C	C	–	C	–	–	X	–	–	–	–	–	–	NBC agonists; heteromer β-arrestin2 recruitment	[81]
CXCR3	CXCR6	–	X	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	[159]
CXCR3	ACKR3	–	X	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	[159]
CXCR3	ACKR4	X	X	–	–	C	–	–	–	–	–	–	–	–	–	–	–	NBC agonists; decreased CXCR3-mediated chemotaxis	[82]
CXCR3	BILF1	–	X	C	C	–	–	C	–	–	–	–	–	–	–	–	–	–	[116]
CXCR4	CXCR4	X	–	I	–	–	–	–	–	–	–	–	–	–	–	–	–	JAK/STAT activation	[65]
CXCR4	CXCR4	–	X	–	C	–	C	–	–	C	–	C	–	–	–	–	–	–	[52, 105, 155, 157, 159]
CXCR4	CXCR4	–	X	–	–	C	–	–	–	–	–	–	–	–	X	–	–	TM peptide decreased homomerization and chemotaxis	[46]

(continued)

Table 9.1 (continued)

R1	R2	Native cells	Heterologous cells	CoIP	BRET	FRET	PFC	trFRET	PLA	BirA PB	GPCR-HIT	CODA-RET	FC	Co-internalization	Dimer disruption	Mutagenesis	Bivalent ligand	Biochemical consequences	References
CXCR4	CXCR4	–	X	–	–	–	C	–	–	–	–	–	–	–	–	–	–	Rab2/6/8-mediated anterograde trafficking	[102]
CXCR4	CXCR4	–	X	C	C	–	–	–	–	–	–	–	X	–	–	–	–	CXCL12 binding to one protomer within dimer	[11]
CXCR4	CXCR4	–	X	C	M	M	M	–	–	–	–	–	–	–	X	–	–	TM peptide affected chemokine-induced BRET changes	[44, 57, 58, 60, 61]
CXCR4	CXCR4	–	X	–	M	–	C	–	–	–	X	X	–	–	–	–	–	Proximal signaling	[47]
CXCR4	CXCR4	X	X	–	–	–	–	–	–	–	–	–	–	–	–	–	X	–	[146–148, 150, 161]
CXCR4	CXCR4-WHIM	–	X	–	C	–	–	–	–	–	–	–	–	–	–	–	–	Reduced internalization and enhanced signaling	[162]
CXCR4	CCR5-Δ32	–	X	C	–	–	–	–	–	–	–	–	–	–	–	–	–	Reduced RSE and HIV-1 infection	[99]
CXCR4	CXCR5	–	X	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	[159]
CXCR4	CXCR6	–	X	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	[159]
CXCR4	ACKR3	X	X	–	–	–	C	C	C	–	–	–	–	–	–	–	–	–	[29, 60, 159]
CXCR4	ACKR3	X	X	C	–	C	–	–	–	–	–	–	–	–	–	–	–	Increased CXCR4-mediated Ca^{2+} mobilization; only delayed pERK1/2	[111]
CXCR4	ACKR3	–	X	C	M	–	–	–	–	–	–	–	–	–	–	–	–	Decreased CXCR4-mediated G_i signaling; increased β-arrestin2 signaling	[61, 112]

CXCR4	α_{1A}-AR	X	–	C	–	–	–	–	C	–	–	–	–	–	–	–	–	Increased vasoconstriction	[29]
CXCR4	α_{1B}-AR	X	–	C	–	–	–	–	C	–	–	–	–	–	–	–	–	Increased vasoconstriction	[29]
CXCR4	β_2-AR	X	X	M	C	–	–	–	–	–	–	–	–	–	–	–	–	Activated CXCR4 impaired β_2-AR signaling	[138]
CXCR4	β_2-AR	–	X	C	–	–	–	–	–	C	–	–	–	–	–	–	–	Increased T cell retention in LN	[135, 155]
CXCR4	CB2	X	–	M	–	–	–	–	M	–	–	–	–	–	–	–	–	Co-stimulation increased oligomerization and decreased CXCR4 signaling	[31]
CXCR4	ChemR23	X	X	–	C	–	–	C	–	–	–	–	–	N	–	–	–	NBC agonists	[83]
CXCR4	DOR	X	X	M	–	M	–	–	–	–	–	–	–	N	–	–	–	Co-stimulation stabilized oligomerization and impaired signaling	[72]
CXCR4	MOR	–	X	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	[58]
CXCR4	UL33	–	X	C	C	–	–	–	–	–	–	–	–	Y	–	–	–	Decreased RSE, CXCL12 signaling, and HIV infection	[126]
CXCR4	UL78	–	X	C	C	–	–	–	–	–	–	–	–	Y	–	–	–	Decreased RSE, CXCL12 signaling, and HIV infection	[126]
CXCR4	BILF1	–	X	C	C	–	C	C	–	–	–	C	–	–	–	–	–	Decreased CXCL12 binding to CXCR4 due to G_i scavenging	[48, 116]
CXCR4	IGF-1R	X	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	Decreased CXCR4-mediated chemotaxis	[127]
CXCR4	CD4	–	X	C	–	C	–	–	–	–	–	–	–	–	–	–	–	–	[58, 103]
CXCR5	CXCR5	–	X	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	[133, 159]
CXCR5	CXCR6	–	X	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	[159]
CXCR5	ACKR3	–	X	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	[159]
CXCR5	BILF1	–	X	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	[116]
CXCR5	EBI2	–	X	–	–	C	–	–	–	–	–	–	–	N	–	–	–	Reduced CXCL13 binding and CXCR5-mediated signaling	[133]
CXCR6	CXCR6	–	X	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	[159]

(continued)

Table 9.1 (continued)

R1	R2	Native cells	Heterologous cells	CoIP	BRET	FRET	PFC	trFRET	PLA	BirA PB	GPCR-HIT	CODA-RET	FC	Co-internalization	Dimer disruption	Mutagenesis	Bivalent ligand	Biochemical consequences	References
CXCR6	ACKR3	–	X	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	[159]
ACKR3	ACKR3	–	X	–	M	C	M	–	–	–	–	–	–	–	–	–	–	–	[60, 61, 159]
ACKR3	ACKR3	X	X	–	–	–	–	–	–	–	–	–	–	–	–	–	X	–	[151]
ACKR3	BILF1	–	X	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	[116]
ACKR3	EGF-R	X	–	–	–	–	–	–	M	–	–	–	–	–	–	–	–	Increased proliferation	[128]
ORF74	IGF-1R	–	X	–	–	–	–	–	C	–	–	–	–	–	–	–	–	Constitutive activity ORF74 unmasked IGF-1R-mediated signaling to PLC	[130]
US28	UL33	–	X	C	C	–	–	–	–	–	–	–	–	Y	–	–	–	Decreased NF-κB activity	[124]
US28	UL78	–	X	C	C	–	C	–	–	–	–	–	–	Y	–	–	–	Decreased NF-κB activity	[124, 125]
US28	US27	–	X	C	C	–	–	–	–	–	–	–	–	Y	–	–	–	–	[124]

Used techniques to identify chemokine receptor dimers are indicated by "X", whereas "C" is constitutive; "I" is ligand- or antibody-induced; "M" is ligand- or antibody-modulated; "Y" is yes; "N" is no; FC is functional complementation; NBC is negative binding cooperativity; RSE is receptor surface expression; LN is lymph node

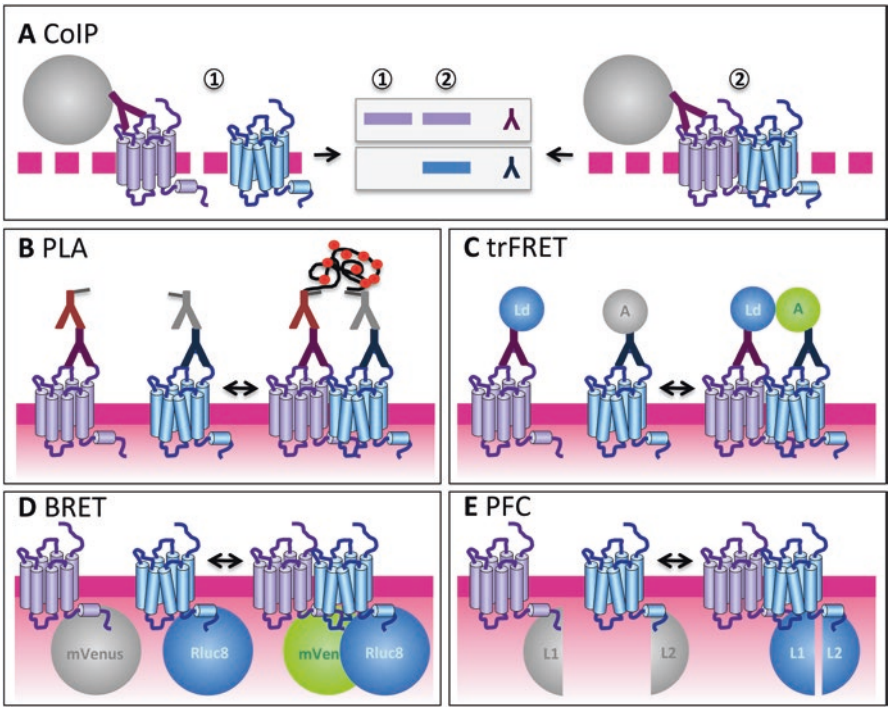

Fig. 9.3 GPCR dimer detection methods. (**a**) Co-immunoprecipitation (CoIP) assay. Immobilized antibody is used to isolate specific (epitope-tagged) GPCR subtype (*purple*) from solubilized cells. The "blue" GPCR is only co-immunoprecipitated and detected on Western blot (*middle panel*) if physically associated with the "purple" GPCR (*right panel*). (**b**) Proximity ligation assay (PLA). Close proximity between primary antibody-bound GPCRs (<16–40 nm) is detected using proximity ligation and DNA amplification of oligonucleotide-conjugated secondary antibodies. (**c**) Time-resolved fluorescence resonance energy transfer (trFRET). Close proximity (<10 nm) between antibody-bound GPCRs is detected by trFRET between lanthanide donor and fluorescent acceptor that are conjugated to the antibodies. (**d**) Bioluminescence resonance energy transfer (BRET). Resonance energy transfer between bioluminescent donor molecule (e.g. modified Renilla luciferase; Rluc8) and fluorescent acceptor molecule (e.g. modified yellow fluorescent protein; mVenus) that are genetically fused to GPCRs occurs when brought in close proximity (<10 nm) by interacting GPCRs. If both donor and acceptor molecules are compatible fluorescent proteins, fluorescence resonance energy transfer (FRET) will occur. (**e**) Protein-fragment complementation (PFC). Non-functional biosensor protein fragments (e.g. bioluminescent or fluorescent proteins) are genetically fused to GPCRs and reconstitute into functional biosensors when brought in close proximity (<2–5 nm) by interacting GPCRs

9.4 Chemokine Receptor Dimerization Interface(s)?

Several GPCRs are packed as homomers in high-resolution crystal structures providing some insight in possible dimerization interfaces. However, experimental validation by dimerization-disrupting agents or mutations is essential as irrelevant dimerization might result from crystal packing, such as for example antiparallel

dimerization of bovine rhodopsin [38]. Crystallized CXCR4 homodimerizes with transmembrane (TM)5/TM6 interface when bound to various antagonist classes [10, 12], whereas maraviroc-bound CCR5 homodimerizes via TM1/TM4 and TM1/TM7/helix 8 interactions at the extracellular and intracellular site, respectively [39]. Mutation of $I52^{1.54}$ and $V150^{4.47}$ residues in TM1 and TM4, respectively, disrupted CCR5 homodimerization and heterodimerization with CCR2 [40]. Moreover, treatment of CCR5 expressing cells with synthetic peptides that correspond to TM1 (MLVILIL) or TM4 (VTSVITW) impaired CCR5 homomerization in fluorescence resonance energy transfer (FRET) experiments (Fig. 9.3d), whereas peptides that harbor $I52^{1.54}$V and $V150^{4.47}$A mutations were ineffective [40]. In contrast, these TM1 and TM4 peptides did not affect CCR5 homodimerization as measured by bioluminescence resonance energy transfer (BRET) [41]. Mutagenesis of corresponding residues in CCR2 ($V64^{1.54}$A and $V162^{4.47}$A) or treatment with CCR2-TM1 peptide (MLVVLIL) impaired homodimer-mediated signaling, suggesting that CCR2 and CCR5 share a similar dimerization interface [40, 42]. The natural occurring CCR2-$V64^{1.54}$I mutation (10–25% of population) is associated with delayed AIDS development in HIV-1 infected individuals and initially found to stabilize heteromerization with CCR5 and CXCR4 in CoIP experiments [43]. In contrast, CCR2 and CCR2-$V64^{1.54}$I had equal propensity to heteromerize with CXCR4 in BRET experiments [44]. Truncation of the C-tail of CCR5 or alanine-substitution of serine and threonine residues in the C-tail reduced heteromerization with CXCR4 [45]. Synthetic peptides of CXCR4 TM2, TM4, TM6, and TM7 did not affect basal BRET between CXCR4/CXCR4 or CCR2/CXCR4 dimers, but modulated ligand-induced BRET changes, suggesting that these peptides impair conformational rearrangements within dimers rather than distort receptor dimerization [44]. However, a comparable CXCR4 TM4 peptide reduced basal FRET between CXCR4-eCFP and CXCR4-eYFP, and consequently chemotaxis of immune and cancer cells towards CXCL12 [46]. Interestingly, synthetic CXCR4 TM2 and TM4 peptides specifically reduced PLA signal between CXCR4/$\alpha_{1A/B}$-AR and CXCR4/ACKR3 heteromers, respectively, without affecting receptor levels at the surface of native VSMC [29, 30]. Based on selective disruption of CXCR4 heteromers by these TM2 and TM4 peptides and the homodimeric CXCR4 crystal structure, a heterohexameric receptor configuration was proposed in which CXCR4 homodimerizes via TM5/6 and each CXCR4 protomer heteromerizes with $\alpha_{1A/B}$-AR and ACKR3 via TM2 and TM4, respectively [30]. Higher order CXCR4 oligomers were previously detected using complemented donor-acceptor resonance energy transfer (CODA-RET; Fig. 9.3e), and indeed implies that these receptors have multiple interaction interfaces [47–49].

Chemical crosslinking of introduced cysteines in CCR7 homodimers identified interactions between the cytoplasmic and extracellular ends of TM3/TM7 and TM1/TM2/TM3/TM7, respectively [32]. Synthetic peptides mimics of TM1, TM2, and TM4 indeed disrupted CCR7 homodimerization. In addition, random site-directed mutagenesis of intracellular loop (IL)2 and TM7/helix8 identified CCR7-$A315^{7.42}$G and CCR7-$L325^{7.52}$S as mutants with decreased homodimerization. Double mutant CCR7-$A315^{7.42}$G/$L325^{7.52}$S was even less able to form homodimerize due to a

reduced hydrophic TM7/helix8 interface. Reversely, enlarging the hydrophobic interface by A315$^{7.42}$V or single-nucleotide polymorphism (SNP) variant V317$^{7.44}$I increased CCR7 homodimerization [32].

Multiple GPCR dimerization interfaces allow formation of hetero-oligomeric chemokine receptor complexes, and agents that disrupt selective interfaces may therefore be used to target specific receptor pairs.

9.5 Constitutive or Ligand-Induced Chemokine Receptor Dimerization?

Class C GPCR $GABA_B$ receptor requires heteromerization of $GABA_{B1}$ and $GABA_{B2}$ in the endoplasmatic reticulum (ER) in order to mask an ER-retention motif and be consequently targeted to the cell surface [50]. Fusion of an ER retention motif to the intracellular C-tail of HA-tagged CXCR1 prevented cell surface targeting of this receptor. Moreover, co-expressed FLAG-tagged CXCR1 or CXCR2 were also retained intracellular, suggesting that CXCR1 homo- and heteromers are readily formed in the endoplasmatic reticulum during protein synthesis [51]. In line, CCR5 dimers were both detected in the endoplasmatic reticulum and cell surface by BRET measurements after cell fractionation [52]. Co-expression of a chemokine binding-deficient CCR5 mutant with a phosphorylation/internalization-deficient CCR5 mutant rescued GRK-mediated receptor phosphorylation, β-arrestin translocation and receptor internalization upon stimulation with CCL5 [41]. These data suggest that chemokine receptors form constitutive oligomeric complexes during their entire life-cycle from biosynthesis and cell surface targeting all the way to agonist-induced co-internalization, which is supported by the many studies that report ligand-independent chemokine receptor oligomerization using CoIP, resonance energy transfer (RET) and protein fragment complementation (PFC)-based techniques (Fig. 9.3). However, other chemokine receptor heteromers did not co-internalize upon agonist stimulation (see Table 9.1). Indeed, real-time single-molecule imaging techniques and affinity-based forced seggregation of differentially tagged GPCRs into microdomains, revealed that some GPCRs form only transient oligomers that dissociate in a ligand-independent manner within seconds, whereas other GPCRs are engaged in more stable oligomers [53–56]. Interestingly, PGE2 induces CCR7 dimerization in native human monocyte-derived dendritic cells (MoDCs) by lowering cellular cholesterol levels [32]. Treatment of transfected HEK293 cells with methyl-β-cyclodextrin to sequester cholesterol confirmed the increase in CCR7 oligomerization. On contrary, methyl-β-cyclodextrin slightly decreased FRET within CXCR4 homodimers [46].

Observed changes in constitutive RET and/or PFC in response to chemokine stimulation have been interpreted as conformational changes within existing chemokine receptor complexes, rather than formation or dissociation of chemokine receptor oligomers [32, 40, 44–47, 49, 52, 57–61]. The presence of cognate chemokines or specific antibodies was strictly required for the immunoprecipitation of

cross-linked CCR2, CCR5, and CXCR4 homo- and heterodimers, under denaturation conditions that did not allow detection of constitutive chemokine receptor dimers [40, 62–66]. However, CoIP of these chemokine receptors was observed in the absence of chemokines under less stringent conditions [52, 57, 67–69]. Binding of chemokines to (existing) chemokine receptor dimers might indeed change existing chemokine receptor dimer conformations so that the efficiency of disuccinmidyl suberate-mediated cross-linking is significantly increased so that dimers are detected under more stringent denaturation [40, 70]. Simultaneous stimulation of native PC3 human prostate cancer cells with CXCL12 and AM1241 increased CoIP of CXCR4/CB2 heteromers, and was required to detect these heteromers in situ using PLA [31]. The common chemokine CXCL8 stabilizes CXCR1 and CXCR2 homomers while disturbing heteromers of these receptors, resulting in distinct internalization kinetics [71]. Interestingly, stimulation with CXCL12 or [D-Pen2, D-Pen5]-enkephalin resulted in dissociation of CXCR4 and δ-opioid receptor (DOR) heteromers and subsequent G_i-mediated signaling, whereas co-stimulation with both agonists stabilizes CXCR4-DOR heteromers that are unable to signal or internalize [72]. Hence, both evidence for ligand-independent and –dependent chemokine receptor oligomerization has been reported and apparent discrepancies for specific receptor pairs might be the consequence of methodological limitations. Ongoing refinements in high-resolution imaging methods that can monitor single molecules in real time may unambiguously chemokine receptor complexes, their origin and fate, and effect of their cognate ligands on these processes [26, 73].

9.6 Functional Consequences of Chemokine Receptor Dimerization

Chemokine receptor oligomers and in particular heteromers are only physiological relevant if their biochemical properties such as ligand pharmacology, signaling, localization or trafficking, are different as compared to those of the individual receptors as monomers [25, 26, 74]. However, to unambiguously proof that these changed properties are the consequence of GPCR oligomerization rather than crosstalk between activated signaling pathways of GPCR monomers, is experimentally challenging, and have let to quite some controversy in literature [25, 27, 73, 75].

9.7 Negative Binding Cooperativity Within Chemokine Receptor Heteromers?

Heteromerized GPCRs can affect each other's conformation [76, 77]. Consequently, agonist binding to one GPCR can allosterically change binding affinities of the associated GPCR for its specific ligands [78, 79]. Indeed, negative binding cooperativity (NBC) has been reported within chemokine receptor heteromers (Table 9.1)

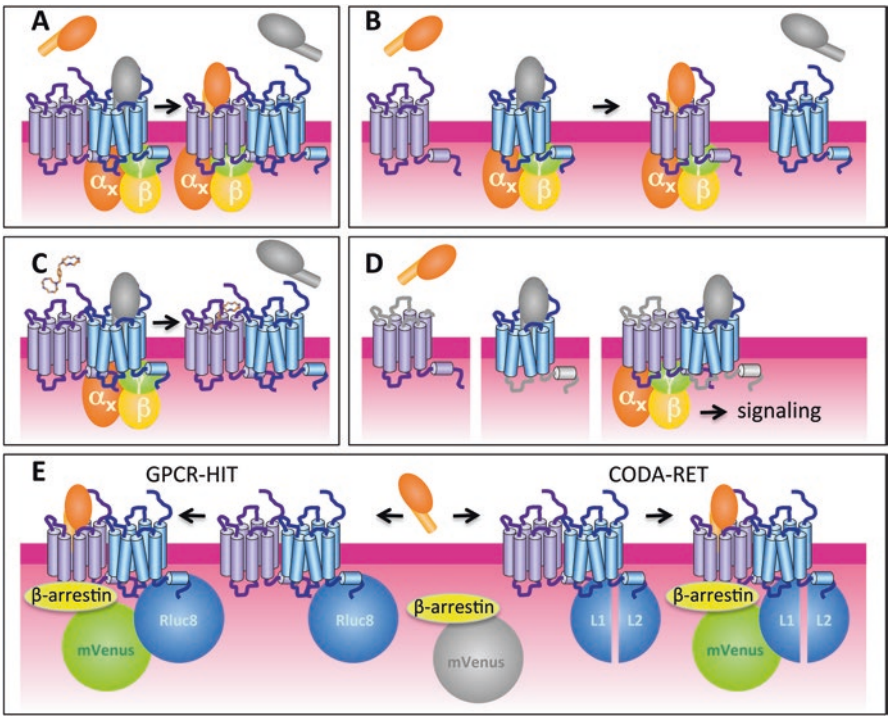

Fig. 9.4 Binding cooperativity and signaling by GPCR dimers. (**a**) Negative binding cooperativity between high-affinity chemokine binding sites involves transduction of conformational changes between heteromerized chemokine receptors and switched G protein coupling. (**b**) Apparent negative binding cooperativity might be observed if high-affinity agonist binding is G protein-dependent and a shared pool of G proteins is depleted by one GPCR subtype at the expense of other GPCR subtypes. GPCRs do not necessarily physically interact. (**c**) Cross-antagonism within chemokine receptor heteromers involves transduction of conformational changes between heteromerized chemokine receptors without switching G protein coupling. (**d**) Functional complementation (FC) experiment. Mutation of purple GPCR impairs chemokine binding, whereas mutation of blue GPCR impairs receptor-mediated signaling. Co-expression of purple and blue GPCR mutants restores chemokine-induced signaling by receptor heteromer. (**e**) GPCR heteromerization identification technology (GPCR-HIT; *left-hand side*) and complemented donor-acceptor resonance energy transfer (CODA-RET; *right-hand side*). BRET between GPCR subtype (*blue*) and signaling molecule (e.g. β-arrestin) that are genetically fused to BRET donor and acceptor molecules upon agonist binding to untagged GPCR (*purple*) indicates that two GPCR subtypes are in close proximity. CODA-RET detects GPCR dimerization using PFC and subsequent recruitment of signaling molecule (e.g. β-arrestin) to GPCR dimer by BRET upon agonist binding

[49, 59, 67, 80–83]. However, apparent negative binding cooperativity between agonists of co-expressed GPCRs might not necessarily be the consequence of conformational transmission by physically interacting GPCRs. High-affinity agonist binding to its GPCR appears to be often G protein dependent, and depletion of shared G proteins by agonist-bound GPCRs might subsequently decrease the binding of another agonist to co-expressed GPCRs, which do not necessarily form oligomers (Fig. 9.4a, b) [22, 48, 78, 80, 84]. Particularly the routinely used equilibrium

binding assays on membrane homogenates might be prone to G protein scavenging, as G protein coupling to agonist-bound receptors is nearly irreversible in the absence of free guanosine triphosphate (GTP) to replace released guanosine diphosphates (GDP) [84]. On the other hand, equilibrium agonist binding to intact cells might be less sensitive to G protein scavenging by another agonist-bound GPCR as GTP is available in the cytoplasm. Chemokines that selectively interact with CCR2 (i.e. CCL2 and CCL7), CCR5 (i.e. CCL4), or CXCR4 (i.e. CXCL12) inhibited each other's binding to heteromers of their receptors in equilibrium-binding experiments on both membrane preparations and native intact cells [49, 59, 67, 80]. Moreover, these chemokines accelerated each other's dissociation kinetics from membranes that co-express their corresponding receptor subtypes, which confirms their allosteric mode-of-action as this induced dissociation of pre-bound radiolabeled chemokine cannot be explained by G protein scavenging to receptors that bind unlabeled chemokines [49, 59, 80]. Negative binding cooperativity was also observed in both equilibrium and dissociation binding assay formats between chemokines CXCL10 and CXCL12 that are selective for CXCR3 and CXCR4, respectively, on membrane preparations that co-express their corresponding chemokine receptor subtypes [81]. However, these chemokines did not affect each other's binding to intact cells expressing CXCR3/CXCR4 heteromers. On the other hand, CXCL10 binding to intact cells expressing CXCR3/ACKR4 heteromers was cross-inhibited by ACKR4-binding chemokines CCL19, CCL21, and CCL25 [82]. Interestingly, CXCL11 binding to CXCR3 on these intact cells was only cross-inhibited by CCL19 and vice versa, but not by CCL21 and CCL25. This apparent discrepancy might be related to the probe-dependent nature of allosterism [85, 86], and/or binding of CXCL10 and CXCL11 to distinct conformational populations of CXCR3 [87, 88]. Agonists of G_i-coupled DOR, μ-opioid receptor (MOR), and CB2 did not affect chemokine binding to intact cells expressing CXCR4/DOR, CCR5/MOR, and CXCR4/CB2 heteromers, respectively, suggesting indeed that not all agonist-stabilized receptor conformations can allosterically affect the ligand-binding pocket of the associated receptor within heteromers [31, 72, 89].

The allosteric small CXCR3 agonist VUF10661 attenuated binding of CXCL12 to membranes co-expressing CXCR3 and CXCR4, whereas allosteric CXCR3 antagonists VUF10085 and TAK-779 did not affect CXCL12 binding [81]. Likewise, allosteric CXCR4 antagonist AMD3100 did not affect CXCL10 and chemerin binding to CXCR3/CXCR4-expressing membranes and CXCR4/ChemR23-expressing cells, respectively [81, 83]. These findings suggest that only agonists and not antagonists can transduce a conformational change from one receptor to the other within heteromers, which is in line with the observed change in intramolecular FRET in norepinephrine-bound α_{2A}-adrenergic receptor upon agonist binding to heteromerized μ-opioid receptor (MOR), but not in response to MOR antagonist binding [76]. However, CCR2 and CCR5 antagonist TAK-779 cross-inhibited CXCL12 binding to CXCR4 and consequently recruitment of CCR2/CCR5/CXCR4-expressing native immune cells in vitro and in vivo, whereas CXCR4 antagonist AMD3100 cross-inhibited CCL2 and CCL4 binding and chemotaxis to these cells (Fig. 9.4c) [49, 59]. In contrast to these findings, however, pretreatment of transfected Jurkat T cells co-expressing CCR5 and CXCR4 with TAK-779 or

AMD3100 did not affect chemotaxis towards CXCL12 or CCL5, respectively [90], and CXCR4 inverse agonist TC14012 was unable to cross-inhibit β-arrestin2 recruitment to CCR2/CXCR4 heteromers in response to CCL2 [47]. No clear mechanistic explanation can be given why small molecules (e.g. AMD3100 and TAK-779) display negative cooperativity on chemokine binding to CCR2/CCR5/CXCR4 heteromers, but are ineffective on chemokine and chemerin binding to CXCR3/CXCR4 and CXCR4/ChemR23 heteromers, respectively. Therefore, possible allosteric cross-inhibition of chemokine receptors by small molecule antagonists that selectively interact with their partner receptor within heteromers should be kept in drug discovery campaigns. Moreover, evaluation should not only be limited to binding studies but also extended to heteromer-mediated signaling as small CXCR2 antagonist SB225002, but not CXCR2 chemokine CXCL8, enhanced G protein activation by CXCR2/DOR heteromers upon stimulation with opioid agonists [91].

Negative binding cooperativity within chemokine receptor heteromers would allow cells to bias their responsiveness towards the chemokine with the highest concentration in multiple chemotactic gradients by allosterically reducing the affinity of associated chemokine receptors towards their cognate chemokine subtype(s). Pre-treatment of peripheral blood mononuclear cells from chronic lymphocytic leukemia (CLL) patients with CXCR3 chemokines (CXCL9, CXCL10, or CXCL11) or small agonist VUF11418 inhibited CXCR4-mediated cell migration towards CXCL12, without affecting CXCR4 cell surface levels [92]. Co-stimulation with CXCL9 also attenuated chemotaxis of Th1 memory cells towards CXCL12, which however was accompanied by a reduction in CXCR4 levels at the surface [93]. CXCL12 did not affect migration of these cells towards CXCL9, which might be related to lower surface expression of CXCR4 as compared to CXCR3. Inhibition of Rho GTPase Rac abolished the inhibitory effect of CXCL9 pretreatment on migration towards CXCL12, indicating that CXCR3/CXCR4 crosstalk is at the level of intracellular signaling pathways [93]. In addition, CXCR3 agonists did not affect CXCR4 signaling to Akt and ERK in CLL cells upon co-stimulation with CXCL12, suggesting the absence of negative binding cooperativity within CXCR3/CXCR4 heteromers [92]. CXCL11 increased BRET between β-arrestin2-Venus and CXCR4-Renilla luciferase (Rluc) in transfected HEK293F cells only if CXCR3 is co-expressed, suggesting that CXCR3 and CXCR4-Rluc heteromerize [81]. Interestingly, co-stimulation with equimolar concentrations CXCL11 and CXCL12 increased BRET in this GPCR Heteromer Identification Technology (GPCR-HIT) approach as compared to stimulation with CXCL12 alone (Fig. 9.4e), which seems difficult to reconcile with negative binding cooperativity between the agonist binding pockets of CXCR3 and CXCR4 [81]. This GPCR-HIT method confirmed heteromerization between CCR2/CCR5 and CCR2/CXCR4 [94]. Similarly, co-stimulation with equimolar concentrations CCL2 and CCL4 or CXCL12 resulted in a more than additive increase in BRET between β-arrestin2-Venus and CCR5-Rluc or CXCR4-Rluc, respectively, in cells co-expressing CCR2. In contrast, co-stimulation with CXCL12 and CCL2 resulted in a similar β-arrestin2 recruitment to CXCR4/CCR2 heteromers as compared to stimulation with CCL2 alone in a CODA-RET assay (Fig. 9.4e) [47].

The functional consequences of negative binding cooperativity on chemokine receptor heteromer signaling are difficult to separate from downstream crosstalk and/or heterologous desensitization. Linking experimental separation of chemokine receptor heteromers to the loss of chemokine binding cooperativity and effect on multigradient chemotaxis might be helpful to distinguish allosteric modulation within heteromers from intracellular cross-regulation.

9.8 Signaling by Chemokine Receptor Oligomers

GPCR-HIT and CODA-RET have been used to evaluate chemokine receptor oligomer interactions with G proteins and/or β-arrestin (Fig. 9.4e). In addition, supportive evidence for chemokine receptor oligomer-mediated signaling came from functional complementation (FC) mutants and changed signaling upon perturbation of dimerization interfaces.

GPCR-hit revealed β-arrestin2 recruitment to heteromers consisting of CCR2/CCR5, CCR2/CXCR4 [94], CXCR2/α_{1A}-adrenergic receptor (α_{1A}-AR) [95], CXCR3/CXCR4 [81], CCR2/angiotensin II receptor type 1 (AT_1R) [96].

CXCL12 increased BRET between CXCR4 homodimers and Gαi_1-Rluc, as measured by CODA-RET between reconstituted fluorescent protein Venus (CXCR4-V1/CXCR4-V2), and Gαi_1-Rluc [47]. Moreover, activation of co-expressed CCR2 by CCL2 stimulation increased BRET between CXCR4-V1/CXCR4-V2 and Gαi_1-Rluc, but did not induce enhanced BRET between the G protein-uncoupled mutant CXCR4-N119$^{3.35}$K-vYFP and Gαi_1-Rluc [47]. Hence, functional protomers seem required for G protein coupling and indeed suggest receptor transactivation within CCR2/CXCR4 heteromers, which is in line with the CCL2-induced conformational change in CXCR4 homomers as measured by BRET in cells co-expressing CCR2 [47]. Interestingly, CXCR4-N119$^{3.35}$K did not recruit β-arrestin2 in response to CXCL12 but showed increased recruitment in cells co-expressing CCR2 upon stimulation with CCL2. This suggests that β-arrestin2 to CCR2 protomer is not hampered by allosteric interactions with the mutated CXCR4, and might even be facilitated by the absence of G protein coupling to CXCR4 protomers. Importantly, activation of the G_i-coupled dopamine receptor 2 (D_2R) by quinpirole did not induce G protein or β-arrestin2 recruitment to CXCR4, which matches the fact that D_2R and CXCR4 did not form heteromers [47].

CXCR2/DOR heteromer-mediated signaling in response to agonist stimulation was shown by functional complementation (FC) approach (Fig. 9.4d). Fusion of DOR to pertussis toxin (PTX)-insensitive Gα_{i2}-C^{352}I with an additional mutation G^{204}A to prevent guanine-nucleotide resulted in a construct that was not able to induce $GTP_\gamma S$ binding to HEK293 membranes in which endogenous G$\alpha_{i/o}$ proteins were inhibited by PTX pre-treatment upon stimulation with DOR agonist DADLE [91]. Likewise, the signaling incompetent mutant CXCR2-I148$^{3.54}$E fused to Gα_{i2}-C^{352}I was unable to incorporate $GTP_\gamma S$ in response to CXCL8. However, co-expression of these two functionally inactive fusion constructs resulted in

DADLE-induced G protein activation, which was allosterically enhanced by co-treatment with CXCR2 antagonist SB225002 [91].

CCR7 oligomerization is required for efficient Src-dependent phosphorylation of CCR7 on $Y155^{3.51}$ in the DRY motif and subsequent recruitment of SHP2 upon stimulation with CCL19 and CCL21 [32]. The interaction with Src was reduced for oligomerization-impaired CCR7 mutants $A315^{7.42}G$, $L325^{7.52}S$, and $A315^{7.42}G/L325^{7.52}S$, whereas oligomerization-promoting mutations $A315^{7.42}V$ and $V317^{7.44}I$ increased Src recruitment. Src inhibitor PP2 decreased CCR7-mediated chemotaxis of primary T cells towards CCL19 or CCL21, whereas inhibition of SHP2 using NSC-87877 significantly reduced migration towards CCL21 but not towards CCL19. Interestingly, $G\alpha_i$ protein inhibitor pertussis toxin impaired CCR7-mediated T cell migration, but was less effective in activated T cells with increased CCR7 oligomerization. Pertussis toxin did not affect Src-dependent CCR7 and SHP2 phosphorylation, even though Src was found to form a complex with CCR7 oligomer and $G\alpha_i$, $G\beta_2$, and $G\gamma_2$ [32].

9.9 Differential Trafficking by Chemokine Receptor Oligomers

The CCR5Δ32 mutation is a deletion of 32 base pairs resulting in a truncated protein consisting of 4 TM domains that is not expressed on the cell surface [2]. Co-expression and heteromerization of CCR5Δ32 and CCR5 retained both receptors in the endoplasmic reticulum, which might explain the delayed onset of AIDS in heterozygous individuals [97–99]. However, this sequestration of CCR5 by CCR5Δ32 in the endoplasmic reticulum was not confirmed in epithelial and T cells in another study [100].

CCR2 and CXCR4 expression are expressed at low levels on prostate cells but are highly upregulated on localized and metastatic prostate cancer, and enhance proliferation, invasiveness, metastasis and adhesion of prostate cancer cells to bone marrow and endothelial cells [68]. Rab GTPases regulate transport of GPCRs from the endoplasmic reticulum to the cell surface [101]. Cell surface targeting of CCR2/CXCR4 heteromers in PC3 prostate cancer cells is dependent on Rab1 and Rab8. Transport of CCR2 to the plasma membrane of these cells requires Rab1, Rab6, and Rab8, whereas CXCR4 requires Rab2, Rab6, and Rab8 [68]. Similarly, anterograde targeting of CXCR4 homodimer in Jurkat T cells is dependent on Rab2, Rab6, and Rab8, and was not affected by CD4 [102], even though CD4 was found to CoIP and BRET with CXCR4 in transfected CHO-K1 and HEK293T cells, respectively [103, 104]. In contrast, surface expression of CCR5 homodimers requires Rab1 and Rab11 in the absence of CD4, but Rab1 and Rab8 in the presence of CD4. Downregulation of CD4 by RNA interference in native THP-1 and primary T cells decreased CCR5 expression on the cell surface [103]. CoIP and BRET revealed CCR5/CD4 interaction in the ER of transfected cells [103]. CCR5/CXCR4 heterodimer trafficking to the cell surface relies on Rab1, Rab2, and Rab11 in the

absence of CD4, and only Rab1 when CD4 is present [102]. Hence, chemokine receptor heteromers anterograde traffic differently as compared to the individual monomers or homodimers and may be affected by associated membrane proteins such as CD4. CoIP, CODA-RET and sequential BRET-FRET suggested indeed heterotrimerization of CCR5, CXCR4, and CD4 [69, 104]. Importantly, CCR5 co-expression inhibited HIV-1 envelope protein gp120IIIB binding to CD4/CXCR4 heteromers and consequently attenuated X4 HIV-1 infection of Jurkat and primary human CD4+ T cells, which could be inhibited by CCL5-induced internalization of CCR5 [104]. These findings corroborated the observation that CCR5 decreased X4 HIV-1 entry in NIH3T3 cells co-expressing CD4 and CXCR4, as compared cells that do not co-express CCR5 [69]. Similarly, CXCR4 reduced CD4/CCR5 heteromer-mediated entry of R5 HIV-1 in Jurkat cells transfected with CCR5, which was reduced by CXCR4 internalization upon CXCL12 binding [104]. The fact that chemokine-induced CCR5 or CXCR4 internalization reduced the inhibitory effect on HIV-1 entry via the opposite chemokine receptor suggests that these receptors do not co-internalize as heteromers. However, CCL5 modulates CXCR4 cell surface levels with similar internalization and recycling kinetics as CCR5 if both receptors are co-expressed in Jurkat T cells [90]. Similar internalization and recycling was observed for CCR5 upon stimulation with CXCL12, which was dependent on CXCR4 co-expression. In contrast, CXCR4 is targeted for degradation in these cells upon CXCL12 stimulation, suggesting segregation of CXCL12-bound heteromers in the endosomes [90]. Interestingly, NA^+/H^+ exchanger regulatory factor-1 (NHERF1) interacts constitutively via its PDZ2 domain with CCR5 homodimers but not with CCR5/CXCR4 heteromers or CXCR4 homodimers [105]. This CCR5-NHERF1 interaction increased CCL5-induced β-arrestin2 recruitment, internalization and β-arrestin2-independent ERK1/2 [105].

CXCR4 is expressed in both resting and activated T cells, whereas CCR5 is only expressed in the latter T cell population [90]. T cell activation by chemokines secreted from antigen-presenting cells (APC) results in the co-recruitment and accumulation of CCR5/CXCR4 heteromers into the immunological synapse (IS) between these cells, which can be inhibited by CCR5- or CXCR4-selective antagonists TAK779 or AMD3100, respectively [90]. In the IS, CCR5 and CXCR4 switches from G_i-induced chemotaxis to $G_{q/11}$-mediated cell adhesiveness in response to chemokine co-stimulation [106]. Consequently, CCR5/CXCR4 heteromers in the IS reduced migratory T cell responsiveness while increasing T cell-APC interaction, T cell proliferation and interferon-γ production [90, 106]. Similar switch from G_i- to $G_{q/11}$-mediated signaling has been observed for CCR2/CCR5 heteromers upon co-stimulation with CCL2 and CCL5 [66].

Norepinephrine does not induced β-arrestin2 recruitment or internalization of its cognate α_{1A}-AR in HEK293FT cells. However, co-expression of CXCR2 resulted in α_{1A}-AR-mediated β-arrestin2 recruitment in GPCR-HIT assay and subsequent receptor internalization into punctuated vesicles upon norepinephrine stimulation, which could be blocked by α_{1A}-AR antagonist Terazosin but also CXCR2 inverse agonist SB265610 suggesting CXCR2/α_{1A}-AR heteromerization [95]. Close proximity of CXCR2 and α_{1A}-AR was confirmed by saturation BRET between both

receptors. CXCR2 and α_{1A}-AR are co-expressed in stromal smooth muscle layer of prostate and α_{1A}-AR interaction with β-arrestin2 was indeed observed in prostate stroma [107]. Interestingly, the nonselective β-adrenergic receptor (β-AR) antagonist Labetalol was identified as CXCR2/α_{1A}-AR heteromer-biased agonist by partially activating α_{1A}-AR-mediated inositol-1-phospate accumulation but acting as full agonist on β-arrestin2 recruitment [95].

9.10 Chemokine Receptor Heteromerization with Atypical Chemokine Receptors

The subfamily of ACKRs consists so far of four members (i.e. ACKR1-4) with similar 7TM folding and chemokine binding properties as conventional chemokine receptors, but characteristically inability to conventionally activate G protein-mediated signaling [2]. These ACKRs regulate chemokine availability by scavenging and/or transcytosis. In addition, ACKRs heteromerize with conventional chemokine receptors and allosterically modulate their G protein and/or β-arrestin-mediated responses.

Atypical chemokine receptor 1 (ACKR1) is expressed on venular endothelial cells and involved in CC and CXC chemokine transcytosis from basolateral to apical membrane of these cells, resulting in increased leukocyte extravasation [108]. Both ACKR1 and CCR5 are upregulated on these cells in response to treatment with the proinflammatory cytokine tumor necrosis factor-α [109]. ACKR1/CCR5 heteromers are constitutively formed and not affected by chemokine stimulation. ACKR1 inhibites CCR5-mediated Ca^{2+} signaling and chemotaxis in transfected L1.2 pre-B cells upon CCL5 stimulation without affecting subsequent CCR5 internalization [109].

Atypical chemokine receptor 3 (ACKR3) is present on endothelial and various immune cells, and highly upregulated during inflammation and hypoxia [110, 111]. Indeed, multiple tumors showed ACKR3 upregulation, in addition to increased expression of CXCR4 and their common ligand CXCL12 [2]. ACKR3 constitutively heteromerize with CXCR4 [60, 61, 111, 112]. Modulation of maximal BRET signal between CXCR4 and CXCR7 by CXCL12 stimulation, but not reconstitution of firefly luciferase in PFC assay, suggested conformational change within existing heteromers upon chemokine binding [60, 61]. ACKR3 decreased both basal and CXCL12-induced BRET between CXCR4-YFP and $G\alpha_{i1}$-Rluc in HEK293T cells, which might be due to allosteric interactions within heteromers and/or CXCL12 scavenging [61]. Consequently, CXCR4-mediated $GTP_{\gamma}S$ binding, intracellular Ca^{2+} mobilization, and decreased adenylyl cyclase activity in response to CXCL12 stimulation was attenuated upon ACKR3 co-expression (61,112). In contrast, an earlier study reported an increase in CXCR4-mediated Ca^{2+} signaling in HEK293 cells co-expressing ACKR3 [111]. Similar discrepancy was observed for β-arrestin2 recruitment in cells co-expressing CXCR4/ACKR3 heteromers as compared to cells that expressed CXCR4 or ACKR3 individually. CXCR4/ACKR3 expressing cells

showed decreased magnitude and duration of CXCL12-induced β-arrestin2 recruitment towards CXCR4, whereas recruitment to CXCR7 was unaffected as measured by dual color luciferase complementation assay [113]. On contrary, basal and CXCL12-induced CoIP of β-arrestin2 with CXCR7 was increased in the presence of CXCR4, resulting in increased activation of β-arrestin2-dependent signaling to ERK1/2, p38 MAPK and SAPK/JNK [112]. In addition, co-expression of CXCR4 and ACKR3 on MDA-MB-231 breast cancer cells and U87 glioblastoma cells displayed increased chemotaxis towards 1–100 nM CXCL12 as compared to cells expressing only CXCR4. This increase in chemotaxis was β-arrestin2 dependent and reversed by co-expression of dominant negative β-arrestin or treatment with β-arrestin2 siRNA, and at least in part mediated by ACKR3 as revealed by chemotaxis towards ACKR chemokine CXCL11 [112]. On the other hand, downregulation of ACKR3 by RNA interference also increased CXCR4-mediated chemotaxis of T lymphocytes towards 0.3 nM CXCL12, whereas no difference was observed for 3–30 nM CXCL12 [61]. These discrepancies might be related to differences in cellular background. In contrast to CCR5 (see above), ACKR3 did not affect CD4/CXCR4-mediated entry of X4-HIV into HEK293T cells [61].

Atypical chemokine receptor 4 (ACKR4) form heteromers with CXCR3 and allosterically reduced the number of chemokine binding sites without affecting receptor expression levels [82]. Consequently, ACKR4 inhibits CXCR3-mediated chemotaxis towards CXCL9 and CXCL10. Moreover, negative binding cooperativity was observed between some of the ACKR4 and CXCR3 chemokines (see above). Activation of microglia cells or T lymphocytes by respectively bacterial lipopolysaccharide or successively phytohemagglutinin and interleukin-2, resulted in a downregulation of ACKR4 and upregulation of CXCR3, and consequently increased migration towards CXCR3 chemokines that are secreted at inflammatory sites [82]. Hence, ACKRs seem to inhibit G protein-mediated signaling by heteromerized chemokine receptors.

9.11 Chemokine Receptor Heteromerization with Virally Encoded GPCRs

Human herpesviruses are widespread pathogens that establish lifelong latent infections in humans. Most HHVs encode one or more viral GPCRs that are expressed by infected host cells and hijack cellular signaling constitutively and/or in response to human chemokines to optimize their own survival and dissemination [6]. In addition, some viral GPCRs have been associated with inflammatory and proliferative diseases.

The Epstein-Barr virus (EBV) resides latently in long-living memory B lymphocytes but can cause infectious monocytosis and various carcinomas and lymphomas. The EBV-encoded GPCR BILF1 is an orphan receptor that is expressed during viral reactivation in antibody-secreting plasma B lymphocytes [6]. BILF1

constitutively activates G_i-mediated pathways and inhibits protein kinase R-mediated repression of translational machinery to facilitate viral replication [114]. BILF1 downregulates major histocompatibility complex (MHC) class I molecules resulting in a decreased recognition and elimination of infected cells by cytotoxic T lymphocytes [115]. In addition, BILF1 can heteromerize with human chemokine receptors that are expressed by B cells (Table 9.1) [116]. BILF1 constitutively scavenges G_i protein and consequently impairs G_i-dependent high affinity CXCL12 binding to CXCR4, whereas the constitutively inactive BILF1-K122$^{3.50}$A mutant did not affect CXCL12-induced CXCR4 signaling [48]. Overexpression of additional $G\alpha_{i1}$ proteins rescued CXCL12-induced signaling in cells co-expressing BILF1 and CXCR4, suggesting that crosstalk between BILF1 and heteromerized CXCR4 is at the level G proteins and may not necessarily involve allosteric interactions within heteromers [48].

Human cytomegalovirus (HCMV) has been associated with vascular diseases and considered to act as oncomodulator in various tumors [117, 118]. HCMV-encoded chemokine receptor US28 is expressed in glioblastoma and shown to constitutively activate proliferative and angiogenic signaling [119, 120]. In addition, US28 mediates VSMC migration towards CC chemokines and may contribute to vascular pathologies [121]. The three other HCMV-encoded GPCRs US27, UL33, and UL78 are still orphan GPCRs. UL33 is constitutively active, whereas US27 can enhance HEK293 cell proliferation [122, 123]. US28 can heteromerize with the other three HCMV-encoded GPCRs in transfected HEK293 cells [124]. UL33/US28 and UL78/US28 heteromers showed decreased NF-κB activation as compared to US28 on its own, which was hypothesized to be important to establish viral latency or reactivation. In contrast, US28-mediated phospholipase C (PLC) activation was not affected by UL33 and UL78, suggesting that these receptors do not allosterically affect the active US28 conformation [124, 125]. In addition, UL33 and UL78 heteromerize with CCR5 and CXCR4, resulting in increased CCR5 expression on the cell surface but translocation of CXCR4 to intracellular sites [126]. UL33 attenuated CCR5- and CXCR4-mediated migration of transfected monocytic cells towards CCL5 and CXCL12, respectively, without affecting chemokine binding. In contrast, UL78 reduced only CCR5-mediated chemotaxis. Interestingly, both UL33 and UL78 impaired CCR5 and CXCR4 coreceptor activity and consequently blocked infection of monocytic cells by R5- and X4-topic HIV strains [126].

9.12 Chemokine Receptor Heteromerization with Non-GPCRs Membrane Receptors

Chemokine receptors can also heteromerize with non-GPCR membrane receptors to modulate trafficking, ligand binding, and/or signaling.

The glycoprotein CD4 is involved in development of T lymphocytes and antigen recognition. CD4 is the primary receptor for HIV entry and forms heteromeric

complexes with CCR5 and CXCR4, which function as co-receptors for infection with R5 HIV and X4 HIV, respectively (see above) [69, 103, 104].

CXCR4 and the receptor tyrosine kinase (RTK) insulin-like growth factor-1 receptor (IGF-1R) physically associates with heterotrimeric $G\alpha_{i2}$ proteins in both non-metastatic (MCF-7) and metastatic (MDA-MB-231) breast cancer cell lines [127]. CXCR4 expression is high in both non-metastatic and metastatic cell lines, whereas IGF-1R expression is high in non-metastatic but low in metastatic breast cancer cells. Interestingly, CXCL12-induced migration of metastatic cells but not non-metastatic cells. Migration of non-metastatic cells towards IGF-1 is PTX-insensitive, whereas the chemotactic response of metastatic cells to IGF-1 is pertussis toxin-sensitive and required co-expression of CXCR4. Hence, IGF-1R-induced migration in metastatic cells might involve transactivation of CXCR4, which results in the release of $G\alpha_{i2}$ proteins from the complex in MDA-MB-231 but not in MCF-7 cells [127].

Expression of ACKR3 is higher in estrogen receptor positive (ER^+) breast cancer cells as compared to normal breast tissue [128]. Downregulation of ACKR3 by RNA interference decreased cell proliferation and EGF-induced activation of epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase (ERK)1/2. PLA suggests that ACKR3 and EGFR heteromerize on MCF-7 cells, which is increased upon stimulation with EGF and involving β-arrestin2 as scaffold [128]. Hence, ACKR3/EGFR/β-arrestin2 complex might be promising target to inhibit cancer cell proliferation.

The constitutively active KSHV-encoded chemokine receptor ORF74 is involved in the development of Kaposi's sarcoma, whereas IGF-1R plays a role in growth and survival of this tumor [6, 129]. Expression of ORF74 in HEK293T unmasks signaling of endogenous IGF-1R to phospholipase C in response to IGF-1 [130]. This transactivation required constitutive signaling by ORF74 and could be attenuated by CXCL10 that acts as inverse agonist on ORF74. ORF74 might form heteromers with IGF-1R on the cell surface as shown by PLA, however, this interaction could not be confirmed by CoIP.

Heteromerization of CXCR2 with the ligand-gated ion channel AMPA receptor in native cerebellar granule neurons impairs chemotaxis towards CXCL1 and CXCL8 [131]. Expression of AMPA receptor is developmentally upregulated and highest at the end of CGN migration from the external to inner granule layer of the cerebellum.

9.13 Physiological Consequences of Chemokine Receptor Dimerization

Vasoconstriction CXCR4 heteromerizes with $\alpha_{1A/B}$-ARs on the surface of native VSMCs [29, 30]. CXCL12 and extracellular ubiquitin enhanced the potency but not efficacy of phenylephrine to increase blood pressure in rats by increasing

vasoconstriction without affecting myocardial function, which could be blocked by CXCR4 antagonist AMD3100. Disruption of CXCR4/$\alpha_{1A/B}$-AR heteromerization using a synthetic CXCR4 TM2 peptide impaired intracellular Ca^{2+} mobilization, myosin light chain 2 phosphorylation, and contraction of mesenteric artery smooth muscle cells in response to phenylephrine [29, 30]. Interestingly, the atypical chemokine receptor ACKR3 is also expressed on native VSMCs and reduced phenylephrine-stimulated vasoconstriction in response to selective ACKR3 agonists [132].

CCR7 Homomers: DC Homing to Lymph Nodes (LN) Dendritic cells are involved in adaptive immune responses by presenting antigen to T lymphocytes in lymphoid organs. Infectious or inflammatory signals activate immature dendritic cells (DCs) that reside in skin or mucosal tissue resulting in an upregulation of CCR7 expression [1]. PGE_2-induced CCR7 oligomerization increased migration efficiency of mature DCs towards CCL21-producing lymphatic endothelial cells and consequently enhanced homing to T cell zones in lymph nodes [32]. Disruption of CCR7 oligomers using synthetic peptides that mimick the oligomerization interface reduced migration of primary T cells towards CCL19 and CCL21, confirming that CCR7 oligomerization is essential for efficient immune cell chemotaxis.

CXCR5/EBI2 Heteromers: B Cell Homing into Lymph Nodes CXCR5 regulates B cell homing towards CXCL13 from follicular DCs and marginal reticular cells in lymph nodes, whereas Epstein-Barr virus-induced receptor 2 (EBI2) mediates their positioning within these lymph nodes to generate an appropriate antibody response [1]. EBI2 is upregulated in activated B cells and attenuates CXCL13-induced CXCR5 signaling (Ca^{2+} and ERK1/2) and chemotaxis in primary activated B cells independent of its agonist 7α,25-dihydroxycholesterol, as compared to primary activated B cells obtained from EBI2 knockout mice [133]. EBI2 reduced binding affinity of CXCL13 for CXCR5, suggesting allosteric interactions within CXCR5/EBI2 heteromers. Indeed, fluorescence resonance energy transfer confirmed close proximity between CXCR5 and EBI2, which might control the localization of activated B cell in lymph nodes upon upregulation of EBI2.

CCR7/β_2-AR and CXCR4/β_2-AR Heteromers: Neural Control of Lymphocyte Egress from Lymph Nodes Lymphocytes express β_2-adrenergic receptors (β_2-ARs) and are consequently sensitive to norepinephrine secreted by the sympathetic nervous system [134]. Single dose or continuous administration of β_2-adrenergic receptor (β_2-AR) agonists to mimic adrenergic innervation inhibited egress of lymphocytes from lymph nodes in mice [135]. β_2-AR activation did not induce lymphocyte migration but enhanced CCR7- and CXCR4-mediated chemotactic responses, whereas CXCR5- and egress-promoting sphingosine-1-phosphate receptor 1 (S1PR1)-dependent chemotaxis was unaffected. CoIP revealed that β_2-AR physically interacts with CCR7 and CXCR4, but not with CXCR5 and S1P1R1, suggesting that heteromerization facilitates synergized signaling. Hence, adrenergic nerves control lymphocyte trafficking by reducing their recruitment into the peripheral tissues

(lymhopenia) and consequently suppressed T-cell mediated inflammation in autoimmune encephalomyelitis and delayed-type hypersensitivity mouse models [135].

CXCR4/β_2-AR Heteromers: Heart Failure β-adrenergic receptors (β-AR) are key regulators of cardiac contractility in response to catecholamine. In heart failure, the ventricular myocardium shifts from β_1-adrenergic receptors (β_1-AR) towards β_2-adrenergic receptors (β_2-AR) predominated responses, due to downregulation of β_1-AR expression on cardiomyocytes [136]. In addition, CXCR4 and CXCL12 are upregulated due to hypoxic and inflammatory conditions in the myocardium [137]. Interestingly, CXCR4/β_2-AR heteromerization on native ventricular myocytes was increased upon co-stimulation with CXCL12 and β-AR agonist isoproterenol as compared to stimulation with either agonist alone [138]. Treatment with CXCL12 prevented β_2-AR from adopting an active conformation in response to isoproterenol as determined by conformational selective antibodies. Consequently, CXCL12-induced CXCR4 activity negatively modulates β_2-AR signaling through G_s proteins and limits diastolic Ca^{2+} accumulation and contractility in ventricular myocytes.

CCR2/AT1R Heteromers: Chronic Kidney Disease CCR2 and angiotensin II receptor type 1 (AT_1R) overexpression in glomerular podocytes is associated with chronic kidney disease, and their cognate agonists CCL2 and angiotensin II (AngII), respectively, induce podocyte apoptosis and consequently progressive proteinuria [96]. In addition, CCL2 activates kidney interstitium and induces macrophage infiltration. CCL2 did not affect AngII-induced inositol-1-phosphate formation in cells co-expressing AT_1R and CCR2. However, co-stimulation of these receptors reduced CCL2-induced $G\alpha_{i1}$ coupling, while synergistically increasing β-arrestin2 recruitment to CCR2/AT_1R heteromers in GPCR-HIT assays [96]. Combined pre-treatment with selective antagonists for CCR2 and AT_1R (i.e. RS504393 and Irbesartan, respectively) impaired β-arrestin2 recruitment in response to CCL2 or AngII, as well as, co-stimulation with both agonists. Moreover, combined treatment of subtotal nephrectomized rats with CCR2- and AT_1R-selective inhibitors synergistically decreased proteinuria, podocyte depletion, and macrophage infiltration.

CXCR4/CB2 Heteromers: Cancer Metastasis Cannabinoids reduce metastasis and invasiveness of cancer cells by reducing CXCR4-mediated chemotaxis towards CXCL12. Co-stimulation of prostate and breast cancer cells with CXCL12 and CB2 agonist AM1241 induced heteromerization of CXCR4 with CB2, resulting in allosteric silencing of CXCR4-mediated signaling [31].

9.14 Bivalent Ligands to Target Chemokine Receptor Heteromers

Chemokine receptor heteromers might represent promising therapeutic targets as their co-expression profiles are likely more cell type- or disease state-specific as compared to individual receptor subtypes. Bivalent ligands have been generated by

fusing GPCR-selective pharmacophores using a spacer of 18–25 atoms to bridge two GPCR binding pockets within dimers [139]. Chemokine and opioid receptors co-localizes in neurons and glia cells in peripheral and central pain processing areas, and inflammatory chemokines impair the efficacy of morphine in chronic pain treatment, which ultimately results in hyperalgesia [140]. A bivalent ligand (MCC22) consisting of MOR agonist oxymorphone fused to CCR5 negative allosteric modulator (NAM) TAK220 with a 22-atom linker was much more anti-nociceptive in mice as compared to a mix of both ligands or morphine, without showing tolerance [141].

Opioid drug abuse and addiction increase CCR5 and CXCR4 expression on immune and glia cells by activating MOR, and consequently enhance HIV entry and replication [142]. Moreover, morphine impaired the antiretroviral effect of the CCR5 NAM Maraviroc [143]. Fusion of MOR antagonist naltrexone to Maraviroc using a 21-atom linker resulted in a bivalent ligand with decreased affinity but increased effectiveness to inhibited HIV-1 entry in human astrocytes as compared to a mix of Maraviroc and naltraxone, both in the absence and presence of morphine [143–145]. However, this bivalent ligand was less effective than Maraviroc in inhibiting viral entry in microglia in the presence of morphine, which might be related to the much higher CCR5 levels at the cell surface as compared to MOR [143].

Various bivalent CXCR4 ligands have been reported that all displayed increased affinity for cells expressing CXCR4 as compared to their monovalent counterparts. Bivalent ligands consisting of two molecules of the CXCR4 antagonist cyclopentapeptide FC131 with a rigid 6 nm poly(L-proline) linker displayed higher affinity as compared to other spacer lengths or monovalent FC131 [146]. The trivalent variant of FC131 with a three-branched spacer interacted with CXCR4 dimers but not trimers [147]. Linking two peptides consisting of 21 amino acids of the N-terminus of antagonistic chemokine vMIP-II by a disulfide bridge increased binding affinity and anti-HIV activity as compared to monovalent peptides [148]. Likewise, fusion of two monovalent CXCR2, CXCR4 and CXCR7 nanobodies increased the binding affinity for their respective receptor [149–151]. Two classes of monovalent CXCR2 nanobodies were identified with one class binding to N-terminus and the other to extracellular loops (EL)1-3 in a conformation-sensitive manner. Fusion of these two nanobody classes resulted in biparatopic CXCR2 nanobody with reduced binding affinity for cells expressing either a N-terminal or extracellular loop CXCR2 mutant. However, its binding affinity was restored upon co-expression of both CXCR2 mutants, confirming that this biparatopic nanobody specifically interacts CXCR2 dimers [149]. Biparatopic nanobodies consisting of monovalent domains that target different GPCR subtypes will be valuable tools to selectively detect native GPCR heteromers.

9.15 Conclusion

Chemokine receptor oligomers have been first reported nearly two decades ago. Yet, in situ validation of native oligomers and their unique functional significance are only available for some receptor combinations often due to technical limitations.

Moreover, only few studies attempted to disrupt GPCR oligomers using for example interfering TM peptides to impair oligomer-specific functions. With the ongoing development of (novel) techniques these three requisites for GPCR heteromers acceptance as physiologically relevant units by the International Union of Basic and Clinical Pharmacology (IUPHAR) might eventually be met for increasing number of identified chemokine receptor oligomers [25, 74]. In addition, increasing evidence revealed that GPCR oligomers are dynamic complexes that can be promoted, stabilized, or destabilized by individual ligands and/or ligand combinations [31, 72, 152]. Chemokine receptor heteromerization further increases the complexity of the chemokine system, but might also offer therapeutic opportunities in the future to target chemotactic responses of those cells that co-express the heteromeric receptor subtypes more selectively using for instance bivalent ligands.

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Chapter 10
Secretin Receptor Dimerization. Prototypic of Class B GPCR Behavior

Kaleeckal G. Harikumar and Laurence J. Miller

Abstract The secretin receptor is a class B1 GPCR that has been demonstrated to associate with itself, with structurally-related receptors, and even with select receptors from another class of GPCRs. The predominant complex formed with itself represents a symmetrical homo-dimer, along the lipid face of transmembrane segment four. This exists constitutively, arriving at the plasma membrane in this form after biosynthesis and being unaffected by agonist binding. There is evidence to suggest that this state of the receptor is functionally important, contributing to its high affinity binding of natural agonist and the high potency of responses to secretin. It also explains the negative cooperativity responsible for the reduced affinity binding and biological activity of agonist occupation of the second protomer comprising this complex. These themes appear to be consistent among other family members, and most members of the class B GPCR family can not only form homodimers, but also associate with each other to form hetero-complexes. Such complexes can explain agonist-induced cross-internalization of the associated receptors, providing an important mechanism for receptor regulation. It will be important to explore the physiologic implications of these processes, and to examine whether they are important *in vivo*.

Keywords Secretin • Secretin receptor • Class B GPCR • Dimerization • Negative cooperativity

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Abbreviations

BiFC	bimolecular fluorescence complementation
BRET	bioluminescence resonance energy transfer
FRET	fluorescence resonance energy transfer
GPCR	G protein-coupled receptor
PACAP	pituitary adenylate cyclase-activating peptide
PAC1	receptor recognizing predominantly PACAP
Rlu	*Renilla* luciferase
SCTR	secretin receptor
TM	transmembrane segment of a GPCR
VIP	vasoactive intestinal polypeptide
VPAC	receptor recognizing both VIP and PACAP
YFP	yellow fluorescent protein

Protein-protein interactions are a fundamental molecular mechanism for the exchange of information in signaling pathways. In addition to propagation of a signal through a cell, protein-protein interactions occur at the level of the plasma membrane where circulating hormones and transmitters initiate the activation of these pathways by binding to cell surface receptors. Proteins can directly interact with those cell surface receptors to regulate their activity or to mediate subsequent signaling events. Additionally, cell surface receptors can associate with each other to achieve similar results. The classical example is the group of single transmembrane receptor tyrosine kinases that must interact with each other to become activated by cross-phosphorylation [1].

It is now well accepted that seven-transmembrane guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs) can also associate with each other on the cell surface [2]. However, the details of these processes and the functional implications for different receptors in this superfamily can be quite distinct. It is important to keep in mind that GPCRs have been demonstrated to be capable of functioning as isolated units that are able to bind agonist ligand and to associate with its cognate heterotrimeric G protein [3]. In spite of this set of observations, GPCRs are capable of associating with each other to form dimeric structures and even higher-order oligomeric structures. Various members of this superfamily can have their biosynthesis and intracellular trafficking affected by association with other receptors [4]. They can have their ligand binding specificity and affinity affected by association with other receptors [5]. They can have their biological responses qualitatively or quantitatively affected by association with other receptors [6].

The best current organizing principle to understand this follows the major families of GPCRs. At one extreme, GPCR association to form dimers is required for class C GPCRs, that can even have these dimeric structures stabilized by covalent disulfide bonds [7]. The nature of ligand recognition is dependent on the composition of the

class C dimers and the molecular basis for G protein association and initiation of signaling has been well worked out for this group of GPCRs [8]. At the other extreme is the class A GPCRs, where association events can be transient and kinetically unstable, often utilizing multiple different interfaces, and having a broad variety of functional effects. This can include the spectrum listed above, as well as having no effect at all. The tendency of class B GPCRs to associate to form complexes is intermediate between that of class C and class A GPCRs. The secretin receptor studies that we will focus on in the current chapter seem to be representative of the class B group of GPCRs, which form relatively stable and structurally-defined dimeric structures that appear to be functionally important, based on *in vitro* studies. We now know that the helical bundle domains that seem to be major determinants for GPCR association are quite distinct from family to family [9–11], in spite of all three families sharing the heptahelical conformation and G protein association events.

The concept of GPCR oligomerization has been somewhat controversial over the years [12, 13]. This reflects the hydrophobic nature of seven-transmembrane (TM) structures that tend to associate non-specifically when expressed at high density and that can be extracted from the lipid bilayer within a large micelle based on the conditions utilized. The methodology utilized is, therefore, very important to be certain that an association event is potentially meaningful. This includes the use of a variety of complementary and confirmatory experimental conditions and approaches, as well as use of a number of critical controls.

10.1 Class B GPCR Ligands

The natural ligands for the class B1 GPCRs are all moderate length peptides, ranging from 27 to 99 residues in length, and being linear as well as some containing an intrachain disulfide bond near its amino terminus [14]. Alanine scanning mutagenesis and peptide truncation studies have supported the presence of a pharmacophoric domain spanning almost the entire length of most of these peptides [15–18]. Structure-activity studies have established that determinants for binding affinity span the entire length of the peptides, while the biological activity resides predominantly in the peptide amino terminus [19]. Structural determinations of these peptide ligands in solution have demonstrated helical segments in the mid-regions and carboxyl-terminal regions of many of these [20]. The helical content of these peptides has often been increased by using solvents mimicking a membrane environment as well.

10.2 Class B GPCRs

The class B GPCRs are similar to all the members of the GPCR superfamily, having seven predicted transmembrane helical segments that associate with each other to form a helical bundle [21]. The nature of this bundle in class B GPCRs, however, is

quite distinct, not sharing the typical signature sequences in those regions typical of the other major families of GPCRs [21]. Like the other members of the superfamily, the class B GPCRs also couple with heterotrimeric G proteins through their cytosolic face. This is actually quite highly conserved across the superfamily and the structural themes for this portion of GPCRs are shared.

In the evolution of each group of GPCRs, there has been the opportunity and need to develop specializations to accommodate vast differences in the structures of natural ligand agonists. As noted above, for the class B GPCRs, the natural ligands share the properties of being moderately long peptides with helical segments and with biologically active amino-terminal ends. This group of receptors has developed a characteristic moderately long extracellular amino-terminal tail domain (100–200 residues) that can bind the helical portion of these ligands within a cleft that is formed above its Sushi motif [22]. This is formed by the presence of six highly conserve cysteine residues that form three disulfide bonds by linking the first to the third, the second to the fifth, and the fourth to the sixth cysteines. Additionally, there are two pairs of antiparallel beta sheets, loop regions, and an amino-terminal alpha helical segment. This domain is highly characteristic of this family of receptors and appears to play an analogous role for all of them. Kinetic studies have shown that the first event in docking natural ligands is the siting of this portion of the peptide ligand into the receptor amino terminus. Then, the siting of the peptide amino terminus into the helical bundle proceeds. How these two major domains are oriented relative to each other are not yet clear, in the absence of a holoreceptor structure. A number of lines of evidence suggest substantial mobility between these domains, but it is not yet clear how much the conformation of each domain might change and in what way.

Overlaid on these uncertainties are possible roles of the receptor dimeric complexes in affecting orientation and movements. This has not been studied for any member of this family.

10.3 Secretin and Secretin Receptor and Its Physiology

To date, only a single molecular form of secretin and only a single secretin receptor have been isolated. Both of these are typical of other ligands and receptors in this family. Secretin is a linear 27-residue peptide with residues spread along its entire length that contribute to binding affinity and with the first four residues at the amino terminus contributing its biological activity. The other peptides within this family most closely related include vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP) [23]. The secretin receptor can bind and respond with a cAMP response to VIP, although with very low affinity and potency (loss of approximately three orders of magnitude) [24].

The secretin receptor is also prototypic of this family and most structurally related to the VPAC1, VPAC2, and PACAP (PAC1) receptors [23, 24]. These receptors can also recognize secretin, binding it with lower affinity and potency to stimulate cAMP responses than their natural agonist ligands (potency of secretin at VPAC1 approxi-

mately 300-fold lower than VIP; potency of secretin at VPAC2 more than 1000-fold lower than VIP; potency of secretin to stimulate PAC1 more than 1000-fold lower than PACAP) [25]. Like other receptors in this family, the secretin receptor best couples with Gs to activate adenylate cyclase and to yield cAMP, and it also can couple with Gq to yield an intracellular calcium response, although the latter requires very high concentrations of agonist [26]. Agonist stimulation of the secretin receptor can also stimulate translocation of arrestins to the plasma membrane [27].

Secretin is synthesized and secreted from enteroendocrine S cells in the proximal small intestine in response to luminal acid and amino acids. The receptors for this hormone are present in the pancreas on duct cells, with lower concentrations in islets and vascular structures. They are present in the liver exclusively on duct cells. They are present on gut smooth muscle and neurons, as well as on vagal afferent neurons, and in various areas of the central nervous system. Highest levels are in cerebellum, with lower levels in the cortex, thalamus, hypothalamus, hippocampus, striatum, and midbrain. They are also present in epididymis, kidney, heart and lung.

Consistent with this broad tissue and cellular distribution, secretin has many biological effects. Most prominent among these is its stimulation of alkaline secretions from pancreatic and biliary ductular systems to neutralize the acid being emptied from the stomach into the duodenum, where pH is critical for optimal digestive processes and for ultimate nutrient absorption. Consistent with this goal, secretin also reduces gastric acid secretion and slows gastric emptying. Its effects on fluids and electrolytes are important also. It has effects on cardiac muscle, vascular perfusion, and renal perfusion, as well as on appetite and glucose homeostasis. All this provides many opportunities to target this receptor in various types of therapy.

10.4 Evidence for Secretin Receptor-Secretin Receptor Association

Evidence for secretin receptor associating with itself to form receptor dimers and possibly higher order receptor oligomers come from a variety of techniques. The most crude of these is co-immunoprecipitation of detergent-solubilized membranes. This approach can be misleading due to the large size of the micelles formed by some detergents incorporating neighboring proteins, as well as by potential artefacts of overexpression of receptor, with high levels of expression driving non-specific interactions.

Resonance transfer techniques have become a mainstay of this field. These include both bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET). The latter can be utilized quantitatively for determination of distances between donor and acceptor, based on photochemical considerations described by Forster [28]. The former is more generally utilized due to fewer constraints, such as relative orientation of donor to acceptor and donor bleaching [29]. Both methods are highly dependent on distances, with signals being reduced by a power of six relative to the separation of donor and acceptor.

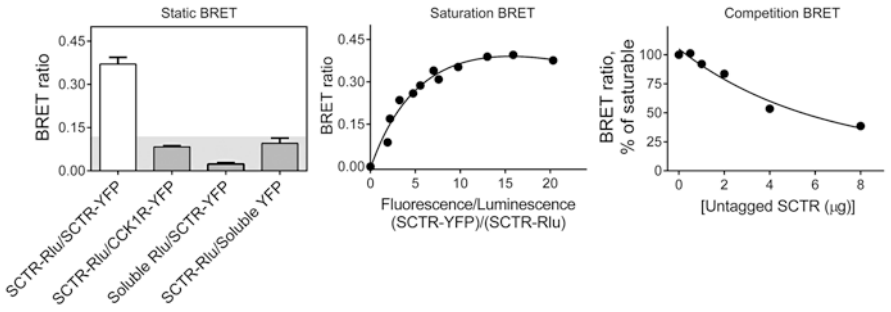

Fig. 10.1 *Receptor BRET.* Shown are data from typical experiments performed to demonstrate the tendency of the secretin receptor to associate with itself. The *first panel* shows a significant receptor BRET signal, above the controls representing the resonance transfer signal with paired soluble complementary donor or acceptor and with a complementary tagged structurally-distinct GPCR in another family. The *second panel* shows data from a typical saturation BRET experiment, in which increasing the amounts of acceptor relative to donor yields saturation of the receptor BRET signal. The *third panel* shows data from a typical competition BRET experiment in which unlabeled receptor reduces the saturable receptor BRET signal

Secretin receptor association was demonstrated using receptor BRET (Fig. 10.1), with constructs tagged at their carboxyl terminus with Rlu and YFP [30]. Background signal was determined using the complementary tag as a soluble construct to be expressed in the cytosol and/or preferentially as a non-associating protein expressed on the plasma membrane in similar concentration to the receptor of interest. The challenge is to be certain that the control truly does not associate in a biologically relevant manner. This typically begins by using a structurally unrelated receptor, but that does not always work out, since structurally unrelated GPCRs have been reported to associate with the secretin receptor. Another key aspect to these studies is the level of receptor expression, realizing that over-expression can lead to crowding in the plasma membrane and to non-specific interactions. Indeed, this was the major basis for the early criticism by James [13]. For that reason, it is also important to study resonance transfer using levels of donor and/or acceptor similar to that present physiologically. It is also important to do saturation BRET studies. Both of these have been performed for the secretin receptor and are fully consistent with self-association at physiologic levels of expression [31, 32]. This complex is not disrupted or amplified by agonist occupation [30].

Another approach to establish receptor association involves the use of tagged forms of the receptor of interest using non-fluorescent halves of a fluorescent indicator that requires complementation for induction of a signal [33]. This has been termed bimolecular fluorescence complementation (BiFC) [33]. It has the advantage of being able to see what cellular compartment the complexes reside in using morphologic techniques. It has the disadvantage that energy is necessary to disrupt an intact fluorophore, thereby having the possibility that such complexes last longer than they might normally. Here, too, this technique has been applied to the secretin receptor [31], and used to show that secretin receptor complexes form during bio-

synthesis and traverse the biosynthetic machinery to be delivered to the cell surface as intact complexes [34]. As described below, this approach also lends itself to determination of the presence of higher order complexes as well.

Covalent cross-linking can also be utilized to demonstrate the presence of receptor complexes. This has been applied to the secretin receptor with cross-linkable cysteine residues used at the interface to stabilize the homo-dimeric receptor complex after it was demonstrated to exist (see below) [35].

10.5 Determinants for Complex Formation

Once it is clear that receptors associate to form complexes, it becomes important to understand the determinants for complex formation. One approach to gain such insight involves the truncation of receptor domains, determining whether the residual portion of the receptor can still associate with itself. For the secretin receptor, we were able to truncate the extracellular amino-terminal tail and still have a receptor BRET signal [34]. We were also able to truncate the intracellular carboxyl-terminal tail without disrupting this signal [34]. This suggested that key determinants were present within the receptor core. A good way to start exploring parts of the core is with the competitive overexpression of transmembrane segments. This was first applied to the beta-2 adrenergic receptor [36]. For the secretin receptor, only TM4 was able to reduce and eliminate the secretin receptor BRET signal [32]. As an important control, the faces of the TM4 segment were then modified by alanine scanning [32]. Only the lipid-exposed face disrupted the secretin receptor BRET signal [32]. This then needed to be further confirmed by building in mutations of the lipid–exposed face of TM4 in an intact secretin receptor construct [32]. This required mutation of more than one residue at the interface, but two was adequate. Here, residues Gly^{243} and Ile^{247} were mutated to alanines to eliminate the receptor BRET signal (Fig. 10.2). Again, this disrupted the previously described BRET signal and this was confirmed with saturation BRET studies.

10.6 Receptor Dimers Versus Higher-Order Oligomers

Once it became clear that there was a single dominant interface for the secretin receptor to associate with itself, it seemed likely that these complexes might be limited to dimers and not form higher order complexes. This was tested using the BiFC approach described above [31]. The homo-dimeric BiFC complex was used as acceptor (fluorescent YFP), and another secretin receptor construct tagged with Rlu was used as donor. While the BiFC interaction worked well to yield fluorescent intact YFP, demonstrating formation of a secretin receptor homo-dimeric structure,

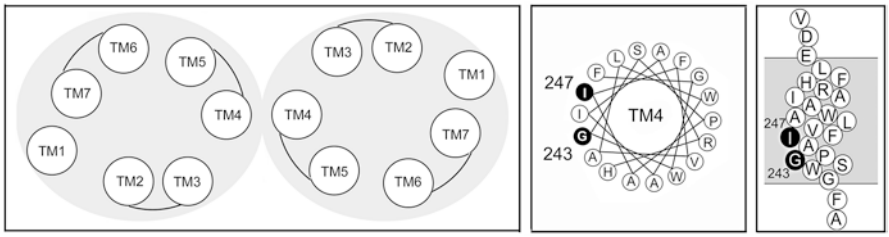

Fig. 10.2 *Secretin receptor dimer interface.* Shown is an illustration of the proposed secretin receptor homo-dimer interface at the lipid-exposed face of transmembrane segment 4 (TM4) bringing together two intact helical bundles, with the view from the extracellular side of the membrane. The key residues that can be mutated to disrupt this structure are Gly^{243} and Ile^{247}, shown to reside at the lipid-exposed face of TM4, viewed laterally as well as in helical wheel format

there was no BRET signal generated between secretin receptor-Rlu and the receptor dimer. Controls were shown to be capable of generating a BRET signal from these complementary tags, although there is still the possibility that higher order secretin receptor complexes might have donor and acceptor situated too far from each other to elicit a significant BRET signal. While this provides soft evidence against such a higher order complex, it is difficult to understand how more than two secretin receptors could utilize the same lipid-face of TM4 as an interface for such a complex.

In another important series of studies, intrinsic photoaffinity labeling was performed utilizing photolabile analogues of secretin to covalently label the receptor [37]. By incorporating various pairs of photolabile residues in positions known to label distinct regions of the secretin receptor, we were able to definitively demonstrate that only one receptor protomer within the homo-dimeric complex was involved in docking one secretin ligand, and that peptide does not span the two receptor protomers comprising this homo-dimer.

10.7 Hetero-Receptor Complexes Within Class B GPCRs

There are a series of reports documenting the ability of different class B GPCRs to associate with each other to form hetero-receptor complexes [38–40]. However, since all of these receptors follow the same signaling paradigm, with high potency cAMP responses and low potency intracellular calcium responses, it is perhaps not surprising that no clear functional effect of such complexes has yet been recognized. Further, if all of these receptors follow the theme set by the secretin receptor, they might be expected to associate through the lipid face of their TM4 segment. This has not yet been directly demonstrated. If this turns out to be true, such hetero-receptor complexes might be competitive with the homo-dimeric receptor complexes described above. It will be quite interesting to understand the relative propensity of a given receptor to form homo-dimeric complexes versus hetero-dimeric complexes on the same cell. Here, too, it might be quite difficult to determine functional

implications of this, since co-expression of two class B GPCRs on the same cell would be expected to yield monomers and dimers of each receptor, as well as the possibility of hetero-dimers. Relative stability of each type of complex would determine the relative frequency of those forms.

10.8 Cross-Class Hetero-Receptor Complexes Involving the Secretin Receptor

The secretin receptor is unique within class B GPCRs in being described to also associate with non-class B GPCRs [41]. Physiologic insights described above resulted in examination for possible interaction between the class B secretin receptor and the class A angiotensin 1a receptor [41]. Indeed, receptor BRET studies supported the possibility of a direct interaction between these receptors and there was even evidence provided for a functional effect of such a complex. Based on the ability of competitive disruption of this complex with transmembrane segment peptides, the suggestion was made for association of an inactive angiotensin receptor to reduce the cAMP response at the secretin receptor, while a similar association event with an activated angiotensin receptor increased the cAMP response to secretin [41]. The molecular nature of this complex and these events need to be more fully characterized.

The suggestion of functional interactions between secretin and vasopressin in the kidney has also been made [42]. This, too, will need to be carefully studied to understand whether it might be explained at the level of a direct receptor-receptor interaction.

In addition to possible effects on ligand binding and biological activity, hetero-receptor complexes are likely important for receptor regulation. Occupation of either the secretin receptor or the associated angiotensin receptor with its agonist ligand stimulated not only the internalization of the occupied receptor, but also the associated receptor [41]. This might also play a role in the hetero-receptor complexes within the class B GPCRs.

10.9 Functional Significance of Secretin Receptor Complexes

Just as it has been challenging to establish the existence of biologically relevant complexes involving the secretin receptor, it has been quite challenging to understand the functional significance of such complexes. This relates to the variety of forms of receptor on a given cell and, particularly, to applying methods to explore function in a natural cell with its natural variety of expressed receptors. This is further complicated by levels of receptors, proximal mediators, and other potentially-interacting elements in the expression systems utilized. Other variables, such as

stage in cell cycle, and degree of confluence of cultured cells used in these studies can also affect the results. Given all of these variables, *in vitro* data from cultured cells is still a good place to start the exploration of possible functional effects, but whatever is observed, will need to ultimately be studied and validated in natural cells and *in vivo* if possible.

The best current approach toward understanding possible function of a receptor complex is to disrupt it (without directly interfering with function, such as hormone binding), and to examine function. As noted above, there is the ability to mutate the lipid face of TM4 of the secretin receptor without modifying the ability of secretin to bind or to yield a cAMP response [32]. This modification has been reported to shift the competition-binding curve to the right, reducing high affinity binding, and to shift the cAMP concentration-response curve to the right as well [35]. These data have been interpreted as suggesting that the homo-dimeric secretin receptor complex facilitates high affinity secretin binding and potent secretin-induced biological activity [35]. This was shown to be sensitive to non-hydrolyzable forms of GTP (GppNHp) and was believed to stabilize the complex between receptors and G proteins [35]. The stoichiometry of such a complex has not yet been definitively established for a class B GPCR. It is quite possible that one heterotrimeric G protein associates with the homo-dimeric secretin receptor complex, but it is also not yet clear whether such a G protein associates with the cis- or trans-protomer, based on hormone occupation. This, too, will have to be carefully examined in future studies.

Roed et al. [39] provided a clear discussion of the link between GPCR association (oligomerization) and binding cooperativity. The high affinity binding and potent response to natural agonists are also associated with a shift to low affinity binding and low potency, presumably at the second protomer of the class B GPCR homo-dimeric complexes. This analysis might also suggest that the G protein associates with the symmetrical homo-dimeric receptor complex in an asymmetric manner that would support a stoichiometry of one G protein to two receptor molecules.

In a particularly interesting series of studies, cysteine residues were incorporated along the lipid face of TM4 of the secretin receptor and disulfide bonds were induced to form using cuprous phenanthroline. This yielded two distinct conformations of covalently-stabilized secretin receptor homo-dimers. Of interest, only one conformation was consistent with the high affinity state of this complex. Cross-linking through Gly^{243}, Ile^{247}, and Ala^{250} resulted in a GTP-sensitive state of the receptor, while cross-linking through Ala^{246} and Phe^{240} resulted in a GTP-insensitive lower affinity state of the receptor.

10.10 Oligomerization of Other Class B GPCRs

The themes developed above for the secretin receptor are quite typical of events described for other members of this family. Table 10.1 summarizes reports in the literature for other members of this family.

Table 10.1 Receptor interactions involving class B G protein-coupled receptors

Receptor self-association (homo-receptor complexes)			
Receptors	**Functional impact of complexes**	**Methods employed**	**References**
Secretin receptor (SCTR)	Signaling, ligand binding	BRET, FRET, BiFC	[32, 35, 44]
Calcitonin receptor (CALCR)	Signaling	BRET, FRET, Co-IP	[45, 46]
Corticotropin releasing factor receptor (CRF1R)	No effect	FLIM-FRET, FCS, FRET	[47–50]
Corticotropin releasing factor receptor (CRF2R)	No effect	FRET	[48]
Calcitonin receptor like receptor (CALCRL)	ND	BRET, BiFC	[51]
GHRH receptor (GHRHR)	ND	Co-IP	[52]
Glucagon receptor (GCGR)	No effect	BRET, FRET	[40, 53]
Glucagon-like peptide-1 receptor (GLP-1R)	Signaling	BRET, BiFC	[43]
Gastric-inhibitory polypeptide receptor (GIPR)	No effect	BRET, FRET	[40, 53]
PAC1 receptor (PAC1R)	Signaling	BRET, BiFC, Co-IP	[54, 55]
Parathyroid hormone receptor (PTH1R)	No effect	BRET	[56]
Vasoactive intestinal peptide 1 receptor (VPAC1R)	No effect	BRET, FRET, Co-IP	[30, 57]
Vasoactive intestinal peptide 2 receptor, (VPAC2R)	No effect	BRET, FRET, Co-IP	[30, 57]

Receptor association with different receptors (hetero-receptor complexes)				
Receptors	**Associated Receptor**	**Functional impact of complexes**	**Methods employed**	**References**
SCTR	VPAC1R	ND	BRET	[30, 38]
	VPAC2R	ND	BRET	[30, 38]
	PTH1R	ND	BRET	[38]
	PTH2R	ND	BRET	[38]
	GLP-1R	ND	BRET	[38]
	GLP-2R	ND	BRET	[38]
	GHRHR	ND	BRET	[38]
	CALCRL	ND	BRET	[38]
	Atr1a (class A)	Signaling	BRET, FRET	[41]
GCGR	GLP-1R	Signaling	BRET, FRET	[40, 53]
	GIPR	No effect	BRET, FRET	[40, 53]
GLP-1R	GIPR	Signaling	BRET, FRET	[40, 53, 58]
VPAC1R	VPAC2R	No effect	BRET, Co-IP	[30, 57]
CRHR1	VT_2R (class A)	Signaling	BRET	[59]

Atr1a type 1a angiotensin receptor, *Co-IP* co-immunoprecipitation, *FCS* fluorescence correlation spectroscopy, *FLIM* fluorescence lifetime imaging, *ND* not determined, *VT_2R* vasotocin receptor

For some of these receptors, observations have extended beyond what we can currently achieve with the secretin receptor. A prominent example of this is the ability to examine how small molecule agonists might be affected differently from natural peptide agonists. Since both of these exist for the glucagon-like peptide-1 (GLP-1) receptor, it was possible to disrupt the homo-dimeric receptor complexes and examine biological effects [43]. Disruption of such complexes resulted in less than tenfold reduction in cAMP responses to GLP-1, but a marked loss in the intracellular calcium responses to the same peptide agonist. In contrast, there was nearly complete elimination of the cAMP response to a small molecule agonist, while ERK phosphorylation remained intact. Thus, the ability of small molecule ligands to allosterically regulate signaling at this receptor was markedly affected by the dimeric complex.

10.11 Summary

The secretin receptor is prototypic of the class B GPCR family, having a tendency to form relatively stable symmetrical homo-dimeric receptor complexes along the lipid-exposed face of TM4. Such a complex contributes to high affinity natural ligand binding and potent biological activity, while also explaining negative cooperativity for ligand occupation and activation of the second receptor protomer. Similar hetero-dimeric receptor complexes form with most members of this family of receptors as well. There were also examples of cross-class hetero-receptor complexes, such as that involving the secretin receptor and the angiotensin 1a receptor.

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Chapter 11
Class B GPCR: Receptors and RAMPs

Joseph J. Gingell, Christopher S. Walker, and Debbie L. Hay

Abstract Receptor activity-modifying proteins (RAMPs) are a family of three single transmembrane spanning proteins. They were first identified based on their ability to facilitate the cell surface expression of a G protein-coupled receptor (GPCR), named the calcitonin receptor-like receptor (CLR). They also determine whether CLR can be activated by calcitonin-gene related peptide (CGRP) or adrenomedullin (AM). We now know that RAMPs can interact with a number of GPCRs, including the calcitonin receptor. The interaction between a GPCR and RAMP can influence cell surface expression and trafficking, determine hormone specificity and regulate intracellular signaling and/or G protein-coupling in a receptor-specific manner. This chapter will review recent advances in the RAMP field, paying particular attention to receptors formed by the co-expression of CLR and RAMPs. We will detail the known GPCR partners for RAMPs and describe how these interactions with RAMP can influence GPCR function.

Keywords Amylin • Adrenomedullin • Calcitonin • CGRP • Calcitonin receptor • GPCR • Receptor activity-modifying protein • RAMP

11.1 Introduction

Historically, identifying receptors for the peptide hormones amylin, calcitonin gene-related peptide (CGRP) and adrenomedullin (AM) proved difficult. However, the calcitonin receptor (CTR), a class B GPCR, had been identified as the cognate receptor for the closely related hormone calcitonin [1]. Based on amino acid sequence identity with CTR, the calcitonin receptor-like receptor (CLR) was proposed as a possible candidate. Unfortunately, CLR did not display affinities for amylin, CGRP or AM [2–4]. In 1998 a group led by Steven Foord used a molecular cloning strategy, discovering that two proteins were required to form CGRP and AM

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receptors [5]. This heterodimer was formed by CLR, and a single transmembrane spanning receptor activity modifying protein (RAMP). Three members of RAMP family have been identified, RAMP1, RAMP2 and RAMP3 [5]. The three RAMPs display a similar structure, consisting of a large N-terminal extracellular domain (ECD) (~150 amino acids) and a short (~10 amino acid) intracellular C-terminus linked by a single transmembrane domain [5]. Co-expression of CLR with RAMP1 forms the CGRP receptor, whereas co-expression with RAMP2 or RAMP3 results in one of two distinct AM receptors, AM_1 and AM_2 respectively [5]. This work was followed quickly by the observation that the co-expression of CTR with RAMP 1, 2 or 3 forms three amylin receptor subtypes, AMY_1, AMY_2 and AMY_3 respectively [6–8]. Given the widespread expression of RAMPs throughout the body, it was suggested that RAMPs may interact with other GPCRs [9, 10]. It is now clear that RAMPs interact with a wide range of GPCRs and can affect their associated GPCRs in a number of different ways (Fig. 11.1 and Table 11.1). This chapter reviews the current known GPCR partners for RAMPs and describes how these interactions with RAMP can influence GPCR function. We describe how RAMPs control cell surface expression, regulate ligand binding, modulate G protein-coupling and intracellular signaling and modulate receptor internalization and trafficking for their associated GPCR.

11.2 RAMP Influence on Ligand Binding

RAMPs alter the pharmacology of the CLR and CTR to produce receptors with distinct pharmacological profiles. The large ECD of class B GPCRs is important for ligand binding [11]. It is through this region of the receptor that RAMPs appear to have the biggest influence on receptor pharmacology. Early studies utilizing chimeras between RAMP1 and RAMP2 [12, 13], revealed that the RAMP ECD is a critical determinant of receptor pharmacology and that the ECD of RAMP1 alone is capable of forming a heterodimer with the CLR (albeit with a large reduction in affinity for CGRP) [14]. This is supported by the fact that the isolated ECDs of the AM_1 and CGRP receptors exhibit the same pharmacological profile as the full length receptors [15, 16], underlining the importance of this region of the RAMP as a critical determinant of the ligand binding properties of the receptor.

11.3 Structural Basis of RAMP Interactions with the Receptor ECD

Crystal structures of ECDs of RAMP1 and RAMP2 have been determined alone and also in complex with the CLR ECD [16–19]. The structures of the RAMP1 and RAMP2 ECD are very similar, comprising a three helix bundle, stabilized by three and two disulfide bonds respectively. There are no significant differences in the

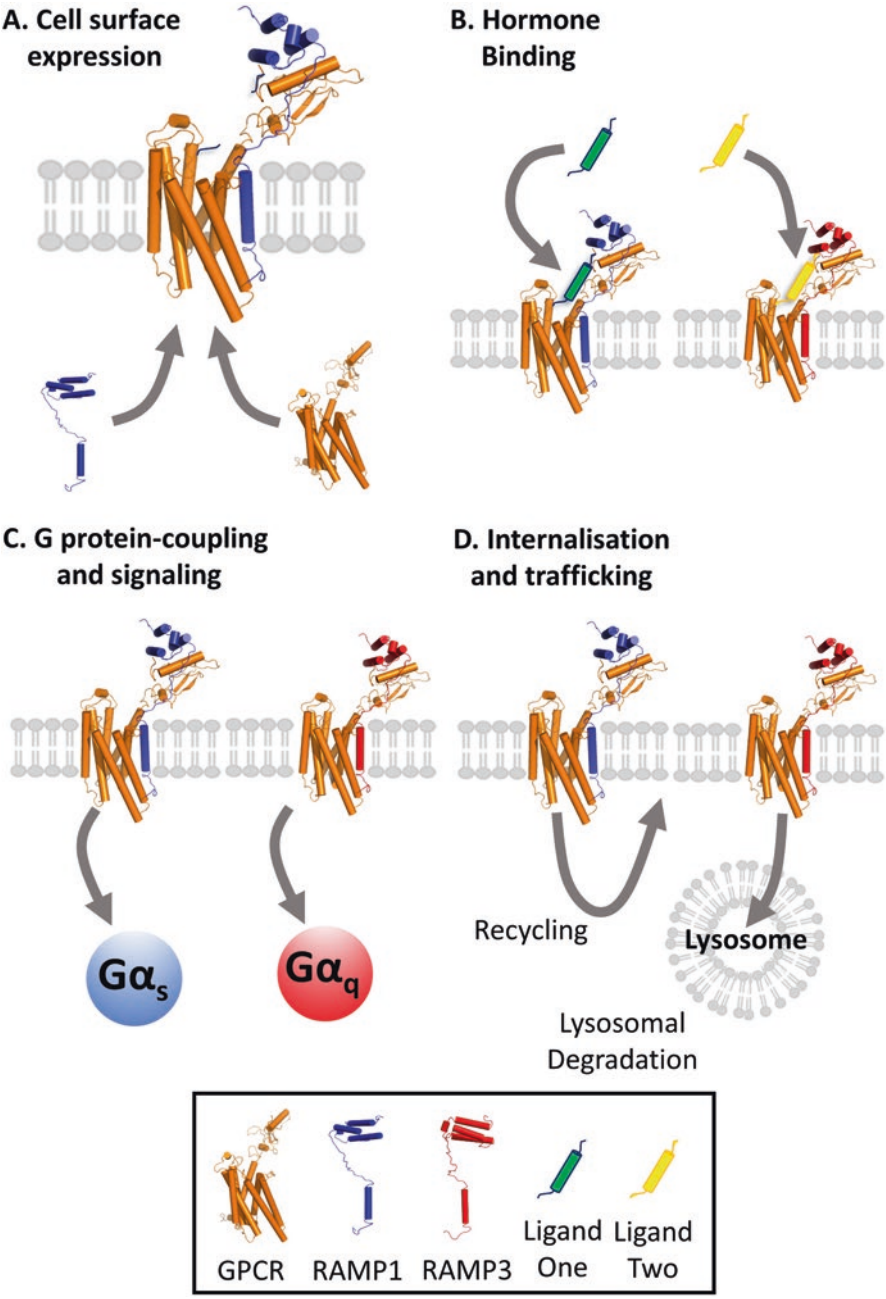

Fig. 11.1 Schematic illustrating the major roles of RAMPs in on GPCR function; (**a**) enabling the cell surface expression of the receptor, (**b**) RAMPs can change the pharmacological selectivity of a receptor allowing it to respond to multiple ligands, (**c**) RAMPs can influence G protein coupling altering the signaling properties of the receptor, (**d**) RAMPs can alter the trafficking of a receptor, directing whether the receptor is degraded or recycled to the cell surface

Table 11.1 List of RAMP interacting GPCRs

Receptor	Class	Interacting RAMP(s)	Effect on receptor
CasR	C	RAMP1 and RAMP3	Trafficking
CLR	B	RAMP1-3	Receptor trafficking, pharmacology
CRF_1	B	RAMP2	Trafficking
CTR	B	RAMP1-3	Pharmacology, signaling
Glucagon	B	RAMP2	Signaling
GPER	A	RAMP3	Trafficking
PTH_1	B	RAMP2	Not determined
PTH_2	B	RAMP3	Not determined
Secretin	B	RAMP3	Trafficking
$VPAC_1$	B	RAMP1-3	Signaling
$VPAC_2$	B	RAMP1-3	Signaling

CasR Calcium sensing receptor, *CRF_1* corticotropin releasing factor receptor 1, *GPER* G protein-coupled estrogen receptor, *PTH* parathyroid hormone receptor, *VPAC* vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide

structures of the RAMP alone or in complex with the receptor, except that the C-terminal tail bends towards CLR, forming contacts.

The crystal structures of RAMP1 and RAMP2 in complex with the CLR ECD have revealed how RAMPs interact with this receptor. The RAMP and CLR ECD form a heterodimer with 1:1 stoichiometry. The interface is formed between the N-terminal α-helix of CLR and helices 2 and 3 of RAMPs 1 and 2 where there is a conserved patch of hydrophobic residues (Fig. 11.2). This interaction is driven primarily by hydrophobic and hydrogen bonding interactions [18, 19].

11.4 Structural Basis of Peptide Ligand Interactions with RAMPs

The availability of peptide bound crystal structures of the AM_1 and CGRP receptor ECD have given important insights into how RAMPs influence ligand binding at the ECD [16]. The CGRP receptor ECD was crystallized with the CGRP analog fragment (D31, P34, F35) $CGRP_{27–37}$ that has enhanced affinity [20], while the AM_1 receptor was crystallized with a C-terminal fragment of AM.

In other class B GPCRs the C-terminus of the peptide forms an α-helical structure that binds within a groove in the receptor ECD formed between the N-terminal α-helix and loops 3 and 4 [11, 21] (Fig. 11.2). In contrast AM and the CGRP analog are largely unstructured, mostly forming contacts with the binding groove of the CLR ECD, but they have a β-turn structure at the C-terminus which enables the C-terminal residue of CGRP (Phe) and AM (Tyr) to form contacts with a single residue within the RAMP, W84 in RAMP1 and E101 in RAMP2 [16] (Fig. 11.2). The mode of peptide

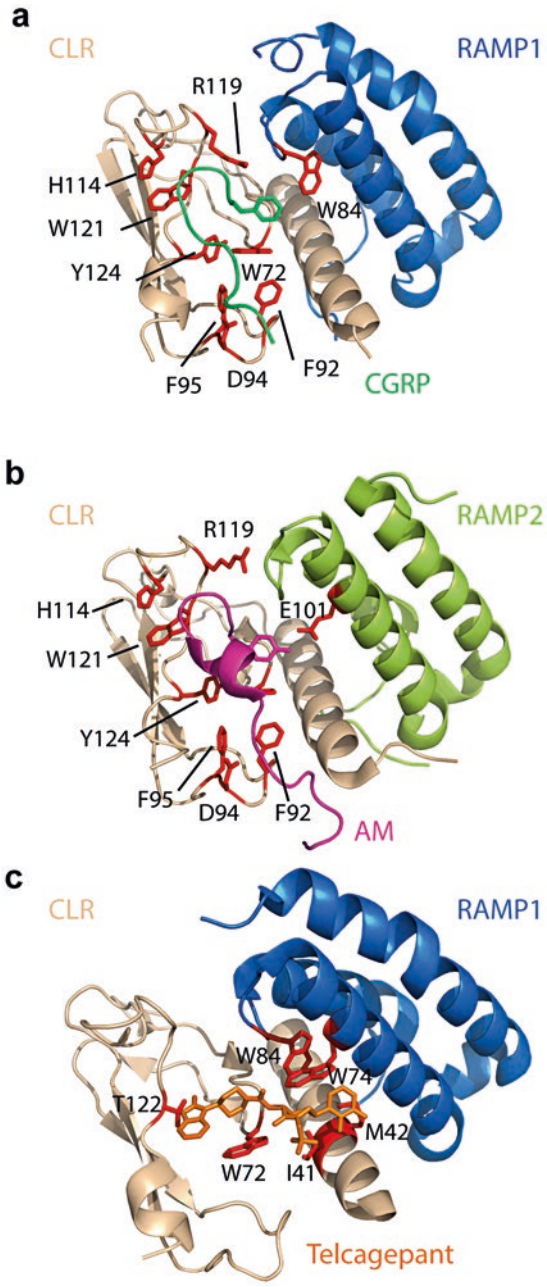

Fig. 11.2 (**a**) The CGRP analog (D31, P34, F35) $CGRP_{27–37}$ bound to the CGRP receptor ECD, with important residues for ligand binding shown as sticks (PDB ID 4RWG). (**b**) AM bound to the AM_1 receptor ECD with important residues for ligand binding shown as sticks (PDB ID 4RWF). (**c**) The CGRP receptor ECD with the small molecule drug telcagepant bound, receptor residues important for binding are highlighted as sticks (PDB ID 3N7S)

binding to the RAMP is slightly different between the CGRP and AM_1 receptors. In RAMP2 the equivalent residue to W84 is F111, with a smaller side chain that cannot make contacts with the peptide, instead contact is formed between residue E101 and the AM residues K46 and Y52.

This unique mode of binding is also supported by mutagenesis studies in both the ECD and the full length receptor, where swapping W84 in RAMP1 and E101 in RAMP2 to Ala led to a reduction in peptide affinity at the isolated ECD or cAMP potency at the full length receptor [16]. Mutation of the C-terminal residue of CGRP and AM to Ala also led to large reductions in affinity [15]. While there are currently no structures of the AM_2 receptor ECD, mutagenesis data suggests that AM interacts with RAMP3 in a similar manner to RAMP2. The equivalent residue to E101 (E74 in RAMP3) is also deleterious to AM potency and binding when switched to Ala or Phe, therefore it is likely that the hydrogen bond formed between the Glu residue with the C-terminal Tyr of AM is conserved [22, 23].

While RAMP1 and RAMP2 have different amino acids that form contacts with the peptide, this does not completely account for the differences in the pharmacology observed between the CGRP and AM_1 receptors. Exchange of RAMP1 and 2 residues located within the peptide binding groove failed to switch the peptide selectivity of the receptor, although it did lead to a reduction in affinity for the cognate ligand [16]. This was also observed in a previous study of the AM_1 receptor ECD [17].

Residue swaps of the C-terminal residue between AM and CGRP (Tyr and Phe) were performed to determine the influence of this residue on the receptor selectivity of the receptor. The presence of the C-terminal Tyr residue was able to enhance the affinity of the CGRP analog (D31, P34, F35) $CGRP_{27–37}$ at the AM_1 receptor while retaining wild-type affinity for the CGRP receptor ECD [16]. Introducing a C-terminal Phe residue into $AM_{37–52}$ reduced affinity for the AM_1 receptor ECD but did not lead to any enhancement of affinity at the CGRP receptor ECD. This demonstrates that the C-terminal Tyr is important for hydrogen bonding with E101 in the AM_1 receptor, but that it does not contribute to selectivity versus the CGRP receptor [16].

These data indicate that RAMPs may not influence receptor selectivity solely by forming direct contacts with the peptide. Instead subtle structural differences induced by the different RAMPs may also contribute to receptor selectivity. The conformation of the CLR ECD is subtly altered between the CGRP and AM_1 receptor structures and the position of RAMP1 and RAMP2 is slightly different. RAMP2 is shifted closer to the peptide binding groove of CLR while within CLR, ECD loop 2 moves slightly and the side-chain of CLR residue R119 (located towards the top of the peptide binding groove) occupies a different position between the two structures (Fig. 11.2).

The CTR shares a high level of homology with the CLR (approximately 60%), and within the ECD many of the key residues involved in peptide binding are conserved despite it having a distinct ligand binding profile than the CLR. The recent publications of the crystal structure of the CTR ECD bound to salmon CT [24] as well as mutagenesis and modeling studies [25, 26] provide evidence that the peptide binding site and the mode of peptide binding resembles that of CGRP and AM at the CGRP and AM receptors, with the presence of a β-turn at the C-terminus of the peptide. This suggests that this feature could be unique to the CT peptide family.

As CTR alone forms a distinct receptor, this allows the role of residues within CTR to be examined in CTR alone but also in the presence of RAMPs to see what influence they have on ligand binding. Interestingly more CTR residues appeared to become involved in amylin interactions when RAMPs were present [26]. Molecular dynamic simulations suggested that the presence of a RAMP may enhance the flexibility of the CTR ECD enabling additional contacts to be formed with the ligand [26].

Like the CGRP and AM receptor ECDs, the CTR, AMY_1 and AMY_2 receptor ECDs show similar pharmacology to the full length receptor, with the same rank order of potency observed. This highlights the fact that RAMP interactions with the ECD are a critical determinant of receptor selectivity.

There appear to be some key differences in peptide binding of AMY receptors compared to the CGRP and AM receptor ECDs; mutation of the C-terminal residue of the peptide antagonist AC413 to Ala resulted in a modest decrease in affinity for the AMY_1 receptor ECD but no change at the CTR and AMY_2 receptor ECDs. This is in contrast to the results observed with CGRP and AM at the CGRP and AM_1 receptors and also with salmon CT at the CTR, where the C-terminal residue was critical for peptide affinity [15, 25, 26].

Residue swaps of the C-terminal residue between amylin (Tyr) and CT (Pro) revealed that the addition of the Tyr to CT did not enhance affinity at AMY receptors, instead reducing the affinity at all receptors [25]. Introducing a C-terminal Pro into amylin and AC413, interestingly led to an enhancement in affinity. In the crystal structure of the CTR ECD bound to salmon CT, the C-terminal Pro makes a stacking interaction with residue W79 of the CTR ECD [24] explaining the importance of this residue. In the isolated ECDs of the AMY_1 and AMY_2 receptors mutation of the RAMP1 residue W84 and the RAMP2 residue E101 to Ala did not result in any reduction in affinity for amylin or the AMY receptor antagonist AC413. While there was evidence in the full length AMY_1 receptor that the W84A mutation reduced amylin and CGRP potency [27], the mutant receptor also had significantly reduced cell surface expression which may explain the discrepancy.

The binding sites of the AMY receptor ECDs share a high level of similarity with that of CGRP and AM_1 receptors, yet exhibit distinct pharmacology. It is not clear what gives rise to the different pharmacological profiles of these receptors, but mutagenesis and structural data indicates that conformational changes in the ECD induced by RAMPs likely play a role.

11.5 Structural Basis of Small Molecule Interactions with RAMPs

The CGRP receptor is an important drug target for migraine and several small molecule antagonists have been developed against it. Crystal structures have been determined of the CGRP receptor ECD in complex with two small molecule antagonists telcagepant and olcegepant [18] (Fig. 11.2). The small molecule binding site largely

overlaps with the binding site of the CGRP analog, with contacts formed by the CLR ECD in addition to RAMP1, where the residues W74 and W84 form key interactions [18]. This is also supported by the mutagenesis data as changing these residues to Ala resulted in reduced affinity [28].

11.6 Interactions of Peptides with the Receptor TM Domain

While RAMP interactions with the ECD are the most important factor in influencing ligand binding, interactions with the TM domain and extracellular loops (ECLs) of the receptor must also be considered, given the known role of these regions in peptide binding to class B GPCRs [29]. A study examining the CLR ECL region in AM interactions with the AM_1 and AM_2 receptors, found that several residues had differential effects between the two receptors. AM induced cAMP signaling was abolished at the AM_1 receptor with the mutants L195A, C212A and P353A however at the AM_2 receptor some degree of function was retained, other mutants had more pronounced effects. A271L, Y277A, Y278A, N279A and C282A, all had significant effects on signaling at the AM_1 receptor but had no effect at the AM_2 receptor, in contrast Y367A reduced potency at the AM_2 receptor but no effect at the AM_1 receptor [30]. Modelling of the two receptors, suggested that the different RAMPs may induce different conformations of ECL3 [30]. RAMP2 and RAMP3 may enhance the binding of AM by making the binding pocket carry a more negative charge.

This provides evidence that RAMP2 and RAMP3 can alter the roles of residues within the TM domain on ligand binding, suggesting that their influence on ligand binding extends beyond the ECD. It is unclear however if these interactions with the TM contribute to selectivity between the different receptor subtypes. This is however in line with earlier studies showing that the short RAMP C-terminus can also influence ligand interactions with CTR [31, 32].

It is not clear what part of the CLR TM domain is the main interaction site for the RAMP single TM helix. Models generated of the AM_1 and AM_2 receptors place the RAMP TM helix between TM6 and TM7 [30], although this has not been validated experimentally there is evidence from the secretin receptor that RAMP3 forms contacts with TM6 [33].

11.7 Trafficking

RAMPs are not capable of reaching the cell surface alone, but can act to traffic receptors to the cell surface. CLR requires the presence of RAMPs to be expressed at the cell surface. RAMP1 possesses an endoplasmic reticulum (ER) retention motif QSKRT in the intracellular C-terminus [34], when an interaction is formed with CLR this retention motif is apparently masked and leads to trafficking of the

receptor complex to the cell surface [34]. This sequence is partially conserved in RAMP2 and RAMP3 suggesting a common mechanism across all three RAMPs.

While CTR does not require RAMPs to be trafficked to the cell surface, it allows RAMPs1-3 to reach the cell surface where they form AMY receptor complexes. This presumably occurs through a similar mechanism as the CLR, although this has not been confirmed experimentally.

RAMP dependent effects on receptor expression have been reported with other family B GPCRs; the secretin receptor can interact with RAMP3, trafficking it to the cell surface. While this interaction does not appear to alter the function of the receptor, co-expression of RAMP3 can rescue a receptor mutant that is normally trapped intracellularly [33]. Greater cell surface expression of the CRF_1 receptor was observed when co-expressed with RAMP2 [35].

There is evidence that RAMPs are also able to traffic other receptors to the cell surface. The class C calcium sensing receptor when expressed alone stays within the ER, but when co-expressed with RAMP1 or RAMP3 it is transported forward, allowing it to be expressed at the cell surface as a mature glycoprotein [36]. This was the first report to suggest that RAMPs were capable of interacting with other classes of GPCRs. Subsequently an interaction with the family A GPCR GPER and RAMP3 was reported, where it appears to alter localization of the receptor in vivo [37].

Several other GPCRs have been demonstrated to traffic RAMPs to the cell surface; The PTH_1 and PTH_2 receptors can traffic RAMP2 and RAMP3 respectively [38]. These associations do not appear to have any effect on receptor function, so whether these receptor-RAMP heteromers are physiologically relevant is yet to be determined. Contradictory results have been observed with $VPAC_2$ receptor. It was initially reported not to interact with RAMPs [38], however a subsequent study found it was capable of trafficking all three RAMPs to the cell surface [35]. Trafficking of all three RAMPs by the $VPAC_1$ receptor and RAMP2 by the glucagon receptor has also been reported [38, 39]; these interactions appear to influence receptor signaling. It is unclear why some GPCRs preferentially couple with different RAMPs and the structural basis of this has yet to be determined

11.8 Internalization

After stimulation with their cognate ligand, the CGRP, AM_1 and AM_2 receptors are internalized [40, 41]. The internalization process is β-arrestin and dynamin dependent indicating that internalization occurs through the clathrin-coated pit endocytotic pathway [40, 41]. At the CGRP receptor, CLR is phosphorylated but not RAMP1. However both receptor components are internalized and co-located with β-arrestin [41]. The recycling of receptor complexes was found to be inefficient, with a significant amount of receptor located in the lysosomal compartment [40].

RAMP3 has a type 1 PDZ motif (DTLL) at its C-terminus, permitting interactions with other proteins during the process of internalization. Bomberger and colleagues

demonstrated that this allows interactions to be made with the Na^+/H^+ exchange regulatory factor (NHERF), which blocked internalization of cells expressing the AM_2 receptor but not those expressing the CGRP and AM_1 receptors [42].

Co-expression of N-ethylmaleimide sensitive factor (NSF) with the AM_2 receptor led to the recycling of the receptor after internalization rather than degradation, but it had no effect on the recycling of the CGRP and AM_1 receptors [43]. The interaction with NSF is also mediated through the PDZ motif present in RAMP3 [43]. It is unclear what are the physiological consequences of these differences in receptor internalization. These studies have not been followed-up and it is not clear whether RAMPs can impact these effects on GPCRs beyond CLR.

11.9 Signaling

The binding of a hormone to its GPCR is thought to facilitate conformational changes in the receptor that leads to the activation of intracellular G proteins or other proteins (eg. β-arrestin) and subsequent downstream signaling events [44, 45]. Evidence from studying the interactions between CLR and RAMPs suggests that RAMPs may act as allosteric modulators, altering the conformation of the associated GPCR. It follows that RAMPs may therefore directly or indirectly modulate intracellular signaling [16, 25, 26, 30].

RAMP controlled signaling has been best described for the CLR/RAMP heterodimers; the AM_1, AM_2 and CGRP receptors. This is because in the absence of a RAMP, CLR cannot bind hormones and is not expressed at the cell surface [5]. Thus RAMPs are essential for CLR signaling. CLR is usually associated with activation the $G\alpha_s$ subunit of the G protein, which activates adenylate cyclase to increase cAMP [46]. Coupling to $G\alpha_{i/o}$, which typically inhibits cAMP formation and is traditionally identified by sensitivity to pertussis toxin and $G\alpha_{q/11}$, which is associated with the activation of PLCβ and the accumulation of inositol phosphates has also been reported [47]. However, it can be difficult to assign a particular G protein to a specific cellular response, given the potential diversity of the signaling repertoire and the overlapping nature of intracellular signaling pathways. For example, studies of the $G\alpha_{q/11}$ pathway are frequently undertaken in the presence of cAMP accumulation. Although no specific evidence for CLR-mediated signaling through β-arrestin has been described for CGRP, AM_1 or AM_2 receptors, the CGRP receptor is known to recruit β-arrestin, which is involved in receptor internalization and can act as a scaffold for downstream signaling [41, 48, 49]. Given the differential effects of RAMPs on the internalization of CLR discussed earlier, it is plausible that β-arrestin signaling may display similar differences. Indeed, direct comparisons of how RAMPs may change CLR signaling via different pathways have not been deeply studied.

The interpretation of signaling data at other RAMP interacting GPCRs is more complicated. Unlike CLR, other RAMP partners are functionally expressed at the

cell surface. This results in the potential for both GPCR monomers and GPCR/RAMP heterodimers in transfected cell models. This is particularly relevant for CTR, which robustly couples to $G\alpha_s$ or $G\alpha_q$ [1, 50]. The formation of AMY_1 (CTR/RAMP1) and AMY_3 (CTR/RAMP3) receptors has been well described to enhance amylin mediated cAMP accumulation, but has much smaller effects on the activation of Ca^{2+} mobilization and ERK phosphorylation [51–53]. This suggested that RAMP1 and RAMP3 maybe enhancing $G\alpha_s$ coupling efficiency. However, the binding of amylin to AMY_1 was not affected by co-transfection with Gα subunits, whereas amylin binding was enhanced by co-transfection of $G\alpha_s$ at AMY_2 and by either $G\alpha_s$ or $G\alpha_q$ at AMY_3 [52]. Thus, the G protein present affects amylin-mediated CTR signaling in a RAMP dependent manner, but it is unclear if the interaction with G proteins is indirect through conformational changes in CTR or direct through interactions with the functionally important RAMP C-terminus [31, 32].

Perhaps the best evidence for RAMP-dependent signaling comes from the co-expression of RAMP2 with the CRF_1 receptor. Here, $G\alpha_i$ and $G\alpha_q$ coupling were enhanced, resulting in increased maximal Ca^{2+} and $GTP_\gamma S$ binding. Basal coupling of $G\alpha_i$ was also enhanced [35]. Similar findings have been observed for the glucagon receptor, $VPAC_1$ and $VPAC_2$. Co-expression of glucagon receptor and RAMP2 results in the enhancement of cAMP accumulation. The authors suggest that RAMP2 reduces glucagon receptor coupling to $G\alpha_i$ in this model [39]. RAMP2 enhances the maximum $VPAC_1$ stimulated inositol phosphate accumulation [38]. RAMP1 and RAMP2 have been shown to enhance $G\alpha_i$ coupling of $VPAC_2$, resulting in increased basal activity [39]. A separate study suggested that RAMPs optimize G protein coupling to $VPAC_2$ [54]. However, in another study no interactions between RAMP and $VPAC_2$ were observed, suggesting that these effects may depend upon cellular background [38].

11.10 Physiological Role of RAMPs

11.10.1 Tissue Expression

There is evidence for the widespread expression of RAMPs across many tissue types (reviewed in [10]). These early studies have relied on mRNA expression, without giving insight into what receptor complexes are actually expressed at the cell surface. Unfortunately the lack of availability of reliable antibodies against native RAMPs and GPCRs have slowed efforts to determine the precise composition of receptor complexes that are expressed across different cell types [55]. Limited access to a well characterized anti-RAMP1 antibody has allowed co-localization with CLR and CTR protein to be performed in human brainstem and trigeminal ganglia [56–58]. This suggests that both CGRP and AMY_1 receptors are present in these tissues. Further studies are required to co-localize RAMP1 with other GPCR partners.

11.10.2 Animal Models

Animal models have given insight into the physiological role of RAMP-receptor complexes, although these have primarily focused on complexes with the CLR. Knock-outs of all three RAMPs have been generated in mice.

RAMP1 knockout mice exhibit higher blood pressure with no change in heart rate [59]. αCGRP has a potent relaxant effect in the arteries of wild-type (WT) mice, however this effect was absent in the RAMP1 knockout animals [59]. Enhanced serum levels of proinflammatory cytokines in response to lipopolysaccharide were observed in the knockout animals. Administration of αCGRP and βCGRP suppressed TNF-α and IL-12 production in bone marrow-derived dendritic cells from WT mice but not those from RAMP1 knockout mice. A RAMP1 knockout mouse has also been generated by a different group to investigate the role of CGRP signaling in asthma. In this model, no differences in blood pressure were observed compared to WT controls. In response to ovalbumin challenge, mice lacking RAMP1 had reduced airway resistance and inflammation compared to WT, reduction of CLR also induced airway resistance, indicating that this response occurs through a CGRP receptor complex [60].

The over-expression of human RAMP1 in primary cultured rat trigeminal ganglia neurons and rat aortic vascular smooth muscle cells using adenoviral gene transfer, showed enhanced cAMP signaling in response to CGRP, suggesting that the RAMP1 is the limiting factor in receptor signaling [61, 62]. Findings in the trigeminal ganglia were validated in vivo using a mouse model overexpressing human RAMP1 in the nervous system. In this model greater plasma extravasation was observed in response to CGRP administration compared to WT animals [62]. Metabolic effects were also observed with this model, consistent with the actions of amylin interacting with the AMY_1 receptor [63]. These animals exhibited a phenotype of lower body weight and fat mass, higher oxygen consumption and body temperature. In addition to weight loss, amylin-induced meal ending satiation and sympathetic nerve activity in brown adipose tissue were enhanced in these mice [64, 65]. These data clearly demonstrate the potential for altered RAMP expression to lead an altered physiological response.

RAMP2 knock-out mice have been generated by two separate groups [66–68]. Embryonic lethality was observed, with defects in vascular development [67, 68]. Knockouts of the other component of the AM_1 receptor, CLR and the ligand AM were also embryonic lethal, supporting the role of this receptor in vascular development. In contrast RAMP3 knockout mice are viable. Despite RAMP3 also forming a receptor for AM, it is not able to compensate for the loss of RAMP2, indicating distinct physiological roles for the different AM receptor complexes [66]. RAMP3 knockout mice appear normal until old age where they exhibit a lower body weight [66]. RAMP2 heterozygous mice are viable, but a range of endocrine related phenotypic abnormalities are observed. These include fetal loss and postnatal lethality, enlarged pituitary glands, skeletal abnormalities with lower bone mineral density and delayed bone development [69]. Mice heterozygous for

CLR do not feature all of these phenotypic abnormalities. Therefore some defects could arise from disrupted RAMP2 association with other GPCRs, suggesting that these interactions also play an important physiological role.

The physiological consequences of RAMP interactions with other GPCRs have also been investigated using animal models. As previously discussed the CRF_1 receptor interacts with RAMP2 in vitro, altering the signaling properties [35]. The interaction appears to be relevant in vivo. In RAMP2 heterozygous mice adrenocorticotrophin hormone release in response to CRF is reduced [35]. GPER interacts with RAMP3 in vitro and in RAMP3 knockout mice the subcellular distribution of GPER is abnormal. In mice with a genetic background prone to heart disease, administration of the GPER agonist G-1, resulted in a reduction in cardiac hypertrophy and perivascular fibrosis that was RAMP3 and sex dependent [37]. This demonstrated for the first time that RAMP complexes with GPCRs outside class B can have physiological relevance. These interactions must be considered when interpreting the phenotypes of RAMP knock-out animals.

11.11 Concluding Remarks

In summary, RAMPs are a fascinating small family of GPCR accessory proteins that can have several impacts on the function of these receptors. It is still unclear how many of the reported interactions occur in physiological systems and this will be important to determine in ongoing studies. In particular RAMPs offer interesting possibilities for developing highly selective drugs against particular RAMP-GPCR heterodimer pairs and therefore it is essential to determine the functional role of each of these.

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Chapter 12
Class C GPCR: Obligatory Heterodimerization of $GABA_B$ Receptor

Qing R. Fan, William Y. Guo, Yong Geng, and Marisa G. Evelyn

Abstract $GABA_B$ receptor is the first known G-protein coupled receptor (GPCR) that requires heterodimerization for function. It is comprised of a heterodimeric assembly of GBR1 and GBR2 subunits, each of which consists of an extracellular domain, a seven-helix transmembrane domain, and a cytoplasmic tail. Many regulatory mechanisms exist to limit transmembrane signaling to complete heterodimers. While the extracellular domain of GBR1 is responsible for ligand recognition, the transmembrane domain of GBR2 is required for G-protein activation. In addition, GBR2 facilitates the cell surface expression of GBR1 through coiled-coil interactions in the cytoplasmic region. Lastly, structures of a GBR1:GBR2 ectodomain heterodimer in the resting and active states demonstrate that the ligand-binding subunit GBR1 undergoes domain closure upon agonist binding, while the modulatory subunit GBR2 remains constitutively open. Receptor activation requires the formation of a novel heterodimer interface between membrane proximal domains. In this chapter we review the discovery of $GABA_B$ receptor, the critical role of heterodimerization to receptor function, the conformational state of the heterodimer at rest, and the structural mechanism of ligand-dependent receptor activation.

Keywords $GABA_B$ Receptor • Heterodimer • G-Protein Coupled Receptor • Subunit Interaction • Crystal Structure • Surface Expression • Ligand Recognition • Activation Mechanism

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12.1 Discovery of $GABA_B$ Receptor

The function of brain circuitry entails both excitatory and inhibitory signals. Inhibitory signals are primarily mediated by the neurotransmitter γ-aminobutyric acid (GABA). GABA acts through two different classes of cell surface receptors: the ionotropic $GABA_A$ receptors [1], and the metabotropic $GABA_B$ receptor [2, 3].

$GABA_A$ receptors were discovered on the neurons of the mammalian central nervous system (CNS) [4, 5] as well as the neurons and axons of the peripheral nervous system [6–8]. These receptors are ligand-gated ion channels that mediate fast synaptic inhibition [1]. The existence of $GABA_A$ receptors along the axon in sympathetic ganglia [8] led scientists to propose that GABA receptors may also be present on the terminals of such neurons, and that their activation would suppress neurotransmitter release [9]. The search for additional GABA-binding sites on sympathetic neuron terminals led to the discovery of a novel type of GABA receptor in 1979, the $GABA_B$ receptor, that displays ligand-binding properties distinct from those of $GABA_A$ receptors [9–13]. Its activity is unaffected by $GABA_A$ agonists such as 3-aminopropanesulphonic acid, and $GABA_A$ antagonists including bicuculline. In addition, it stereospecifically recognizes the β-p-chlorophenyl derivative of GABA, (R)-baclofen, which is not active at $GABA_A$-binding sites.

$GABA_B$ receptor was subsequently found to be widely distributed in the CNS [12, 14, 15]. It is a G-protein coupled receptor (GPCR) that produces slow and prolonged inhibitory activity through the second messenger $G_{i/o}$ protein [2, 3]. In response to GABA, it regulates the activity of Ca^{2+} and K^+ channels through Gβɣ, and inhibits the function of adenylyl cyclase through $G\alpha_{i/o}$ [16–23] (Fig. 12.1). Specifically, activation of presynaptic $GABA_B$ receptor blocks neurotransmitter release through the suppression of voltage-gated Ca^{2+} channels [18, 19]. Presynaptic $GABA_B$ receptor can function as an autoreceptor on GABA-containing nerve terminals [24] or as a heteroreceptor on the terminals of other neurons, including glutamatergic terminals [16]. In addition, stimulation of postsynaptic $GABA_B$ receptor activates G-protein-activated inwardly rectifying K^+ channels (GIRKs) to induce hyperpolarization of neurons [17, 20].

12.2 Isolation of GBR1 Subunit and Its Isoforms

Biochemical isolation of $GABA_B$ receptor was first made possible in 1997 with the availability of a high-affinity $GABA_B$ antagonist, CGP64213 [25]. Studies using radiolabeled [^{125}I]-CGP64213 identified two highly conserved $GABA_B$ receptor isoforms in the vertebrate nervous system, GBR1a and GBR1b. Cloned GBR1a and GBR1b proteins have molecular masses of approximately 130 kDa and 100 kDa respectively, similar to native $GABA_B$ receptors in cortex, cerebellum and spinal cord cell membranes revealed by photoaffinity labeling [25]. These two isoforms differ by the presence of two complement control protein modules, termed CCPs or

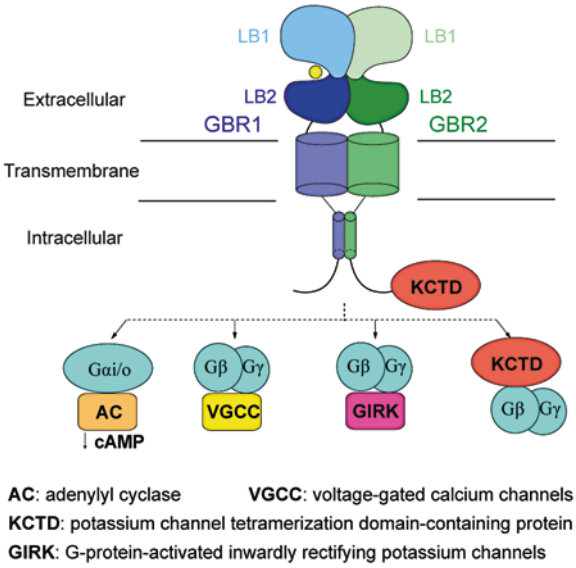

Fig. 12.1 Schematic representation of $GABA_B$ receptor and its signaling pathways

sushi domains, located at the GBR1a N-terminus [26, 27]. GBR1a and GBR1b have identical pharmacological profiles, independent transcriptional regulation, and different spatial and temporal distribution [16, 25, 28–30]. While both GBR1a and GBR1b traffic into dendrites, the sushi domains also selectively target GBR1a into axons [16, 31]. In addition, GBR1a is predominantly associated with presynaptic $GABA_B$ receptor function, while GBR1b mostly contributes to postsynaptic inhibition [16, 28].

12.3 Discovery of an Obligatory Heterodimer

Cloned GBR1 proteins exhibit different ligand-binding and functional properties, when compared with those of native $GABA_B$ receptor. Although the rank order of potencies of $GABA_B$-specific ligands is the same at both recombinantly expressed GBR1 and native $GABA_B$ receptor, recombinant GBR1 has 100-fold lower affinity for agonists [25]. In addition, heterologously expressed GBR1 fails to mediate activation of effector channels in *Xenopus* oocytes [25].

The discovery of GBR2 in 1998 provided an awaited explanation for this discrepancy [32–37]. The GBR2 gene was discovered by data-mining expressed-sequence-tag databases for GBR1a/b homologs [32–34, 37]. Sequence analysis indicates that GBR2 is the closest known relative to GBR1, with 35% identity overall. Immunoprecipitation experiments indicated that GBR1 and GBR2 associate with each other [32–36]. Yeast two-hybrid screening revealed that the two polypeptides form heterodimers through their intracellular C-terminal tails [34, 35]. The

two proteins also co-localize at individual neurons and in transfected cells [32, 33, 35–37]. Finally, co-expression of GBR1 and GBR2 yields a receptor that binds agonist with the same high affinity as native receptor, and reconstitutes $GABA_B$ functional activity [32–36]. Consequently, $GABA_B$ receptor became the first known heterodimeric GPCR [38].

In fact, $GABA_B$ receptor functions as an obligatory heterodimer of GBR1 and GBR2 subunits [32–37]. It was found that individual GBR1 and GBR2 subunits fail to induce [^{35}S]GTP-ɣS binding when expressed in mammalian cells, whereas co-expression of GBR1 and GBR2 results in robust, agonist-dependent stimulation [34]. Furthermore, although neither GBR1 nor GBR2 is coupled to GIRKs or voltage-gated Ca^{2+}-channels in *Xenopus* oocytes, the combination of GBR1 and GBR2 strongly activates these effector channels upon agonist binding [32–35].

In addition to the critical interaction between these two principal subunits, $GABA_B$ receptor associates with a subfamily of the potassium channel tetramerization domain-containing (KCTD) proteins through the GBR2 cytoplasmic tail [39, 40]. These KCTD proteins act as auxiliary subunits of $GABA_B$ receptors to enhance agonist potency, accelerate agonist response, and promote receptor desensitization by uncoupling Gβγ from effector channels [40–42] (Fig. 12.1). Though outside the scope of this chapter, the role of KCTD proteins in affording functional diversity to $GABA_B$ signaling pathway is a fascinating and important area of research.

12.4 Heterodimeric Interaction and Subunit Function

GPCRs are divided into four main classes (A, B, C and F) based on sequence homology [43]. Most GPCRs, such as rhodopsin and β_2-adrenergic receptor, belong to class A; they contain a seven-helix ligand-binding transmembrane domain and can function as monomers [44–46] (Fig. 12.2).

$GABA_B$ receptor belongs to the class C GPCR family, which also includes the metabotropic glutamate receptors (mGluRs), calcium-sensing receptor (CaSR), and several taste and pheromone receptors [47, 48]. Each of these receptors is characterized by a large extracellular domain, in addition to the canonical seven-helix transmembrane domain (Fig. 12.2). This extracellular domain has sequence homology to bacterial amino acid-binding proteins and contains the class C receptor ligand-binding site within a Venus Flytrap (VFT)-like module [48]. Another unique feature of class C GPCRs is that they require dimerization for function. The mGluRs and CaSR function as disulfide-tethered homodimers [49–56], while $GABA_B$ and taste receptors function as heterodimers [32–36, 57, 58].

$GABA_B$ receptor subunits work in-concert to carry out receptor function. Each subunit is composed of three domains, the extracellular domain, the transmembrane region and the cytoplasmic tail. Studies using chimeric receptors indicate that both the GBR1 and GBR2 ectodomains are necessary for full agonist-induced activation of the receptor [59–61]. The GBR1 ectodomain is solely responsible for ligand-recognition [62], as GBR2 ectodomain does not bind GABA or any other known ligand [63]. Nevertheless, GBR2 increases agonist-binding affinity of GBR1 [33, 34, 61, 64–66]. The GBR2

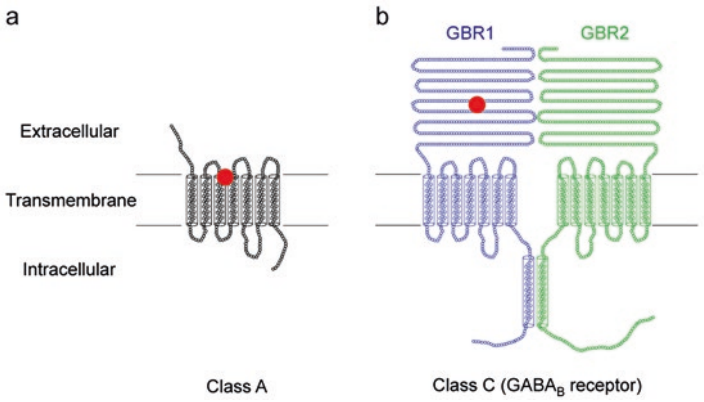

Fig. 12.2 Schematic representation of a (**a**) class A and (**b**) class C ($GABA_B$) GPCR

transmembrane domain contains the interaction sites for G-protein coupling and selectivity. Mutations in either the second or third intracellular loops of GBR2 abolish G-protein activation [59, 66–69]. Although GBR1 is not required for G-protein coupling, its transmembrane domain enhances coupling efficiency [59, 61, 68]. In addition, GBR2 facilitates the cell surface expression of the intact heterodimeric receptor [70–72].

The obligatory nature of the $GABA_B$ heterodimer is not limited to allosteric effects on ligand affinity or facilitation of appropriate trafficking, but appears to be a pervasive requirement that spans the lifecycle of this receptor. Further underscoring this is the redundancy indicated by association of the two subunits, that involves participation of all three domains of each subunit.

Interaction between the GBR1 and GBR2 extracellular domains can be detected by time-resolved fluorescent resonance energy transfer (TR-FRET) techniques and sucrose gradient sedimentation [64, 65]. Direct binding between GBR1b and GBR2 ectodomains with a 1:1 stoichiometry has been demonstrated by native gel shift assay, gel filtration chromatography and titration isothermal calorimetry [73].

Intersubunit FRET reveals an asymmetric rearrangement of the GBR1 and GBR2 transmembrane domains, and indicates that receptor activation does not involve within-subunit structural changes [74]. Taken together, these results imply a reorientation between interacting $GABA_B$ subunits on the membrane during receptor activation.

Sequence analysis indicates that a GBR1:GBR2 interaction likely occurs through the coiled-coil domain within the intracellular region of each subunit, as confirmed by yeast two-hybrid screening [34, 35]. Direct binding between the coiled-coil domains of GBR1 and GBR2 is measured by native gel shift assay, analytical ultracentrifugation and isothermal titration calorimetry [73, 75].

The remainder of this chapter reviews insights gained from a peek at the heterodimeric interactions of the ectodomain and intracellular coiled-coil regions. It should be noted, however, that structural studies of the full-length receptor in multiple functional states will be required to uncover the nuanced regulation and function of this obligatory heterodimer.

12.5 Heterodimer Interface

Crystal structures have been determined for the extracellular domain of human GBR2 ($GBR2_{VFT}$) [73], and for a heterodimeric GBR1b:GBR2 ectodomain complex ($GBR1b_{VFT}$:$GBR2_{VFT}$) in three functional states, in the apo form, bound to six different antagonists and bound to two different agonists [76] (Fig. 12.3). Agonists included the endogenous ligand GABA and clinical drug baclofen. Each complex consists of a non-covalent heterodimer of $GBR1b_{VFT}$ and $GBR2_{VFT}$, indicating that the two ectodomains "dance cheek-to-cheek" – a heterodimer orientation wherein the protomers are joined side by side and facing opposite directions.

The extracellular domains of GBR1 and GBR2 have similar overall structures that are consistent with their sequence homology. Each subunit has a VFT-like module consisting of two lobe-shaped domains (LB1 and LB2) connected by three short loops. Both the LB1 and LB2 domains have an αβ-fold composed of a central β-sheet

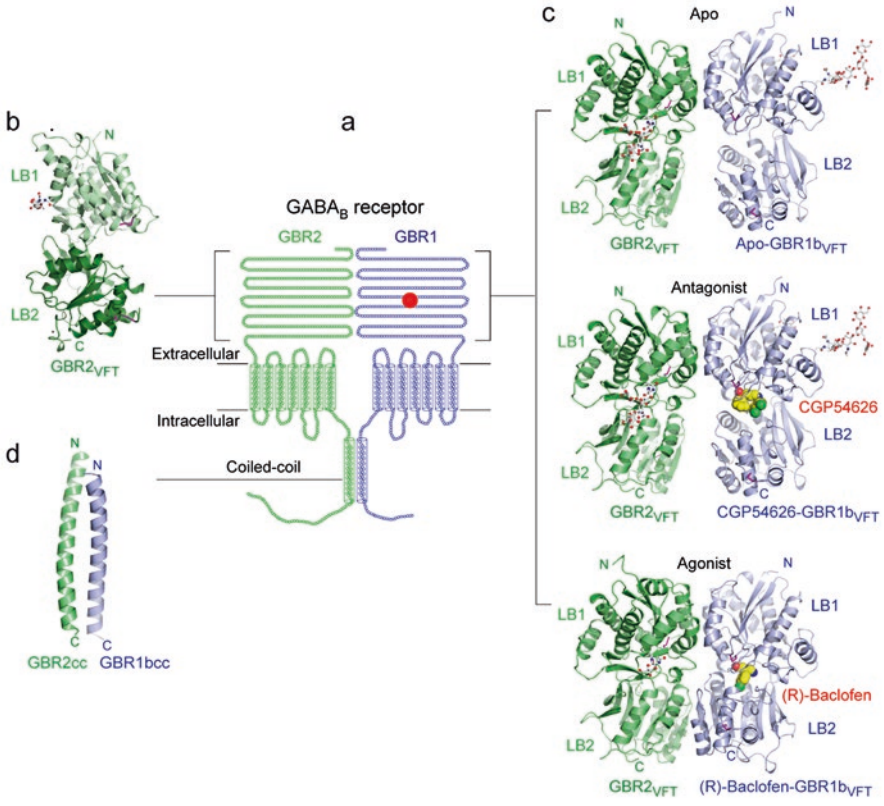

Fig. 12.3 Structures of human $GABA_B$ receptor. (**a**) Schematic diagram of $GABA_B$ receptor. (**b**) $GBR2_{VFT}$ structure. (**c**) $GBR1b_{VFT}$:$GBR2_{VFT}$ complex structures in the apo, antagonist-bound or agonist-bound states. (**d**) $GBR1b_{CC}$:$GBR2_{CC}$ complex structure. The observed carbohydrates are shown as ball-and-stick models in *gray*. Disulfide bridges are in *magenta*. The ligands are displayed as space-filling models

flanked by α-helices. The bi-lobed architecture of $GABA_B$ subunits is similar to that found in mGluRs [77–79], natriuretic peptide receptors [80–83], ionotropic glutamate receptors [84–86], and bacterial periplasmic amino acid-binding proteins [87]. However, both GBR1 and GBR2 lack the cysteine-rich region (CR) that connects the VFT module to the transmembrane domain in other class C GPCRs.

The structures of $GBR1b_{VFT}$:$GBR2_{VFT}$ heterodimer reveal the specific interactions between subunits in the resting and active conformations [76]. In the resting state, subunit association is primarily mediated by the LB1 domains of the two subunits (Fig. 12.4). This LB1-LB1 interaction is mediated by the B and C helices of both subunits. It features three deeply buried tyrosine residues (Y113 and Y117 of GBR1b, and Y118 of GBR2) that are critical for heterodimer interaction and receptor activation. This dimer interface is largely conserved in the active conformation, indicating that the LB1-LB1 interaction mostly serves to facilitate dimerization.

Agonist binding induces the formation of a novel heterodimer interface between the LB2 domains [76] (Fig. 12.4). The heterodimer contacts consist primarily of hydrogen bonds. This reliance on hydrophilic interactions to form a distinct subunit interface in the active state allows the receptor to readily dissociate upon returning to its resting state. The additional interaction between LB2 domains in the active state is consistent with calorimetry measurements showing that GBR2 ectodomain has higher affinity for agonist-bound, as opposed to antagonist-bound, GBR1b ectodomain [73]. Together, these indicate that GBR2 enhances agonist-binding affinity of GBR1 through stabilization of the agonist-bound conformation of GBR1.

The cytoplasmic region of each $GABA_B$ receptor subunit features a coiled-coil motif instrumental to cell-surface expression of the receptor heterodimer [70–72, 88]. The crystal structure of the core GBR1b:GBR2 coiled-coil heterodimer (GBR1cc:GBR2cc) consists of a left-handed parallel coiled-coil that is stabilized by a hydrophobic core and networks of hydrogen bonds [89] (Fig. 12.3).

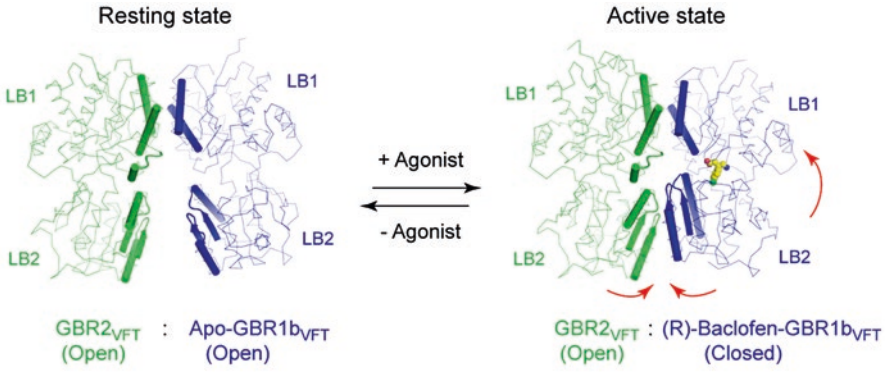

Fig. 12.4 Agonist-induced conformational changes. The C traces of apo and (R)-baclofen-bound $GBR1b_{VFT}$:$GBR2_{VFT}$ structures are displayed. The structural elements involved in heterodimer formation are illustrated using ribbons (LB1-LB1: B and C helices; LB2-LB2: F and G helices, f and g strands and connecting loops). The ligands are show as space filling models (This figure is adapted from Supplementary Fig. 14 of Ref. [76])

Less is known of the structural nature of the transmembrane portions of the heterodimer, as the transmembrane domain structure for $GABA_B$ receptor is yet to be solved. Like other GPCRs, sequence analysis indicates that the transmembrane domain of each $GABA_B$ subunit consists of seven transmembrane helices connected by extracellular and intracellular loops. Among the class C GPCRs, only the crystal structures for the transmembrane domains of two mGluRs, mGluR1 and mGluR5, have been solved [90, 91]. A comparison of the transmembrane structures from class A, B, C and F GPCRs indicates that the overall helical bundle structure is conserved among the four classes. While the mGluR5 transmembrane domain is monomeric in the crystal, mGluR1 forms a dimer through transmembrane helix I [90, 91]. It remains to be determined whether the transmembrane domains of $GABA_B$ subunits form a dimer, and what within- and between-subunit conformational changes are triggered by receptor activation.

12.6 Heterodimer Trafficking

The intracellular region of $GABA_B$ receptor contains elements that control receptor trafficking [71, 88], such that surface expression of a functional $GABA_B$ receptor requires heterodimerization. While GBR2 can reach the cell surface, GBR1 is trapped inside the cell when expressed alone [93]. Surface expression of individual GBR1 is prevented by the presence of an arginine-based endoplasmic reticulum (ER) retention signal (RSRR) [70–72] and a di-leucine internalization motif (EKSRLL) [71, 88], both of which are located within its cytosolic domain. Mutation or deletion of the ER-retention signal facilitates surface expression of GBR1 [70–72]. Mutation of the di-leucine motif alone is not sufficient to release GBR1 from intracellular retention; however, it further increases the plasma membrane expression of various GBR1 mutants lacking the ER retention signal [71].

Recent studies have identified PRAF2 as an ER gatekeeper for $GABA_B$ receptor [92]. This ER membrane protein binds to the GBR1 subunit, and prevents its progression to the Golgi apparatus. GBR1 is only released after GBR2 displaces bound PRAF2, allowing the combined subunits to be trafficked to the cell surface [70–72]. Disruption of the hydrophobic coiled-coil interface through mutations in either subunit impairs surface expression of GBR1, indicating that the coiled-coil interaction is required to defeat ER retention of GBR1 [71, 89].

The GBR1cc:GBR2cc complex structure reveals the mechanisms by which the trafficking signals located within GBR1 are inactivated [89]. The di-leucine internalization motif of GBR1 directly interacts with GBR2 as part of the coiled-coil structure, and is buried at the heterodimer interface. In contrast, the ER retention signal of GBR1 is not part of the core coiled-coil structure; rather it is located adjacent to its C-terminal end. Structural observations, together with thermodynamic measurements, indicate that assembly of the $GABA_B$ coiled-coil heterodimer blocks access to the ER retention signal by steric hindrance.

12.7 Ligand Recognition

Malfunction of $GABA_B$ receptor has been implicated in various neurological and mood disorders, including spasticity, epilepsy, anxiety and drug abuse [2, 3, 94]. Ligands of $GABA_B$ receptor have a diverse array of pharmacological effects. $GABA_B$ agonists invoke muscle relaxation, antinociception, and suppression of drug craving [2, 3, 94]. Baclofen, a selective $GABA_B$ receptor agonist, is used clinically to treat muscle spasticity associated with multiple sclerosis and spinal cord injury [2, 3, 94]. $GABA_B$ antagonists can suppress absence seizures and improve cognitive performance in animal models [2, 3, 94]. GS39783, a $GABA_B$ positive modulator, also displays anxiolytic effects [95]. Because of the varied pharmacological applications of GABAergic ligands, a mechanistic understanding of $GABA_B$ receptor-ligand interactions may assist the design of novel compounds with therapeutic prospects.

The extracellular domain structures of $GABA_B$ receptor reveal the molecular basis of agonist and antagonist recognition by $GABA_B$ receptor [76] (Fig. 12.5). The ligand-binding site is located at the interdomain cleft of GBR1 subunit. Agonist-bound GBR1 has a closed cleft, and the bound agonist is inaccessible to bulk solvent. In contrast, antagonist-bound GBR1 has an open cleft, and bound antagonist remains solvent accessible.

The residues that control ligand-binding specificity and affinity have been identified by a combination of structural analysis and alanine scanning mutagenesis of the ligand-binding site [63, 73, 76, 96–98]. It was found that the LB1 and LB2 domains play different roles in the recognition of agonist and antagonist [76]. Known agonists and antagonists of $GABA_B$ receptor are derivatives of GABA, and have the general structure of a γ-amino acid. Structural studies indicate that the two ends of these known GABA analogues are secured by a common set of LB1 residues through hydrogen bonds. The LB1 domain is primarily responsible for anchoring the antagonists, while the LB2 domain plays a supporting role by contacting selective antagonists and enhancing their potency. In contrast, the LB2 domain is directly involved in binding the two ends of each agonist molecule, and both LB1 and LB2 domains are required for agonist recognition.

Mutations of ligand-binding residues in the LB1 and LB2 domains confirm their distinct roles in ligand recognition [63, 73, 76, 96–98]. Alanine substitutions of LB1 residues Trp 65, Ser 130, Ser 153, His 170 and Glu 349 of GBR1b essentially eliminate antagonist binding, and substantially reduced agonist-dependent G_i protein activation. Mutations of two key LB2 residues, Tyr250 and Trp 278, have much less effect on antagonist binding, but are detrimental to agonist-induced receptor response.

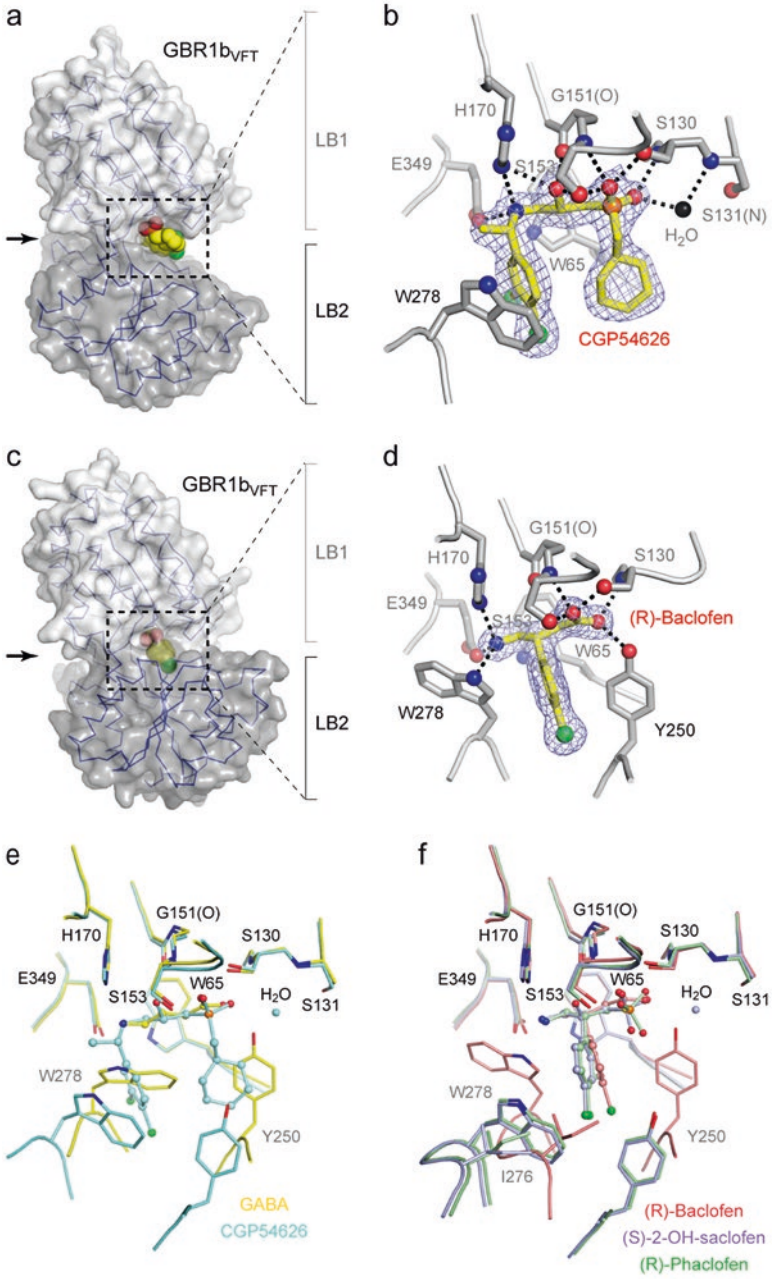

Fig. 12.5 Ligand recognition by GBR1b$_{VFT}$. (**a**, **c**) Molecular surface of GBR1b$_{VFT}$ bound to antagonist CGP54626 (**a**) or agonist (R)-baclofen (**c**). Ligand is displayed as a space-filling model. (**b**, **d**) Specific contacts between GBR1b$_{VFT}$ (*gray*) and CGP54626 (*yellow*) (**b**) or (R)-baclofen (**d**), viewed in the direction of the arrow in **a** or **c**. Mesh represents the final 2Fo-Fc electron density map contoured at 1σ. Hydrogen bonds are represented by *black dashed lines*. (**e**) Comparison of the binding sites of agonist GABA and antagonist CGP54626. (**f**) Comparison of the binding sites of agonist (R)-baclofen and two related antagonists (S)-2-OH-saclofen and (R)-phaclofen (This figure is adapted from Fig. 4 of Ref. [76])

12.8 Ligand Response

The different ligand-bound $GBR1b_{VFT}$:$GBR2_{VFT}$ complex structures also provide an explanation for the mechanisms of action by agonists and antagonists [76]. The first step of $GABA_B$ receptor activation is extracellular domain closure of the GBR1 subunit. Each agonist facilitates GBR1 closure by directly interacting with both LB1 and LB2 domains. In comparison with the agonists, each antagonist contains a bulky substituent that blocks domain closure of GBR1. For example, the potent antagonist CGP54626 possesses two large substituents, one at each end of the molecule; it inhibits $GABA_B$ activity with a dissociation constant (K_D) of 4 nM [94].

An interesting case study involves the different functional properties of three structurally analogous ligands, (R)-baclofen, an agonist, versus 2-(OH)-saclofen and phaclofen, two antagonists. The largest substituent in all three ligands is a β-chlorophenyl ring at the central position of the molecule. Their major difference, and the determining factor of agonist versus antagonist action, lies in the geometry of the α-acid motif at one end of each molecule [76]. While the α-carboxylic acid group of (R)-baclofen has a planar geometry, the α-acid groups of both antagonists assume a tetrahedral geometry that is incompatible with the active-state conformation of Tyr250. Furthermore, the α-substituent pushes the β-chlorophenyl ring towards the γ-amino end of each antagonist, thereby generating potential steric interactions with LB2 residues Ile 276 and Trp 278 to prevent GBR1b domain closure.

In summary. Antagonists function by confining the receptor to the open configuration of the inactive state, while the agonists stabilize its active state by facilitating GBR1 domain closure.

12.9 Activation and Inactivation Mechanisms

The active state of $GABA_B$ receptor exhibits a structural asymmetry, wherein the ligand-binding subunit GBR1 is closed, and the modulatory subunit GBR2 remains open. Domain closure of GBR1 subunit is sufficient to activate $GABA_B$ receptor; the receptor can be locked in a constitutively active state through the introduction of a pair of cysteines that maintain the ectodomain of GBR1 in a closed form through a disulfide bridge [99]. Conversely, ectodomain structures of individual GBR2 suggest that GBR2 adopts a constitutively open conformation [73]. In fact, introduction of a large glycan at the interdomain cleft of GBR2 to prevent domain closure does not affect receptor function, indicating that GBR2 closure is not required for $GABA_B$ receptor activation [73].

The crystal structures of $GABA_B$ receptor extracellular domain reveal the heterodimer arrangement in different functional states and the conformational changes associated with receptor activation [76]. The apo and antagonist-bound $GABA_B$ structures represent the resting and inactive states of the receptor. The agonist-bound

complex corresponds to the active state. These structures show that both $GABA_B$ subunits adopt an open conformation at rest, and only GBR1 undergoes domain closure in the active state.

Comparison of the inactive and active structures of $GABA_B$ extracellular domain identifies a unique receptor activation mechanism that involves a specific association between membrane-proximal domains [76]. The heterodimer interface between the membrane-distal LB1 domains of $GABA_B$ subunits is largely conserved in the resting and active states. The membrane-proximal LB2 domains, however, form of a novel heterodimer interface upon receptor activation. Agonist binding is coupled with a decrease in separation between the GBR1 and GBR2 ectodomain C-termini, suggesting that signal transduction across the membrane may additionally involve a rearrangement of the heterodimer interface between transmembrane domains.

Formation of the LB2-LB2 heterodimer interface is both necessary and sufficient for $GABA_B$ receptor activation as demonstrated by functional analysis [76, 100]. Studies using an N-glycan wedge scanning approach found that introduction of a large glycan into the LB2 domain of either subunit renders the receptor inactive, likely by preventing the relative movement between LB2 domains upon agonist binding [100]. Conversely, disulfide crosslinking experiments showed that the receptor can be locked into a constitutively active state by introduction of a disulfide bond across the LB2-LB2 interface (GBR1b-T198C and GBR2-Q206C) that was engineered based on the active $GABA_B$ structure ([76]).

Several lines of evidence suggest that, in the absence of ligand, $GABA_B$ receptor exists in a dynamic equilibrium between the resting and active states, as has been observed for other GPCRs such as mGluRs [77] and β2-adrenergic receptor [101] (Fig. 12.6). First, the apo-$GBR1b_{VFT}$:$GBR2_{VFT}$ complex adopts the resting-state conformation in the crystal structure [76]. Second, the cysteine mutant pair GBR1b-T198C and GBR2-Q206C forms disulfide-tethered heterodimer in the absence of ligand, and the resulting covalently-linked receptor displays constitutive activity [76]. This implies that the apo receptor can also assume the active-state configuration, wherein these residues come into close contact with one another to form a disulfide bond. Third, the double cysteine mutant receptor loses its constitutive activity under reducing conditions, an indication that the receptor has returned to its favored resting conformation [76]. Together, these results indicate that $GABA_B$ receptor spontaneously oscillates between the resting and active states. Furthermore, the ability of the $GABA_B$ receptor to adopt the active conformation in the absence of agonist is associated with its basal activity.

In the conformational equilibrium of $GABA_B$ receptor, antagonist maintains the resting conformation of the receptor, while agonist stabilizes its active conformation. Agonist binding to $GABA_B$ receptor causes VFT closure in the GBR1 subunit, an expansion of the heterodimer interface, and a decrease in the separation between the membrane-proximal LB2 domains [76]. Similar conformational changes have been observed for the activation of other class C receptors. For CaSR, agonist-induced VFT closure leads to the formation of a novel homodimer interface between the LB2 and CR domains [102]. For mGluRs, single-molecule FRET studies indi-

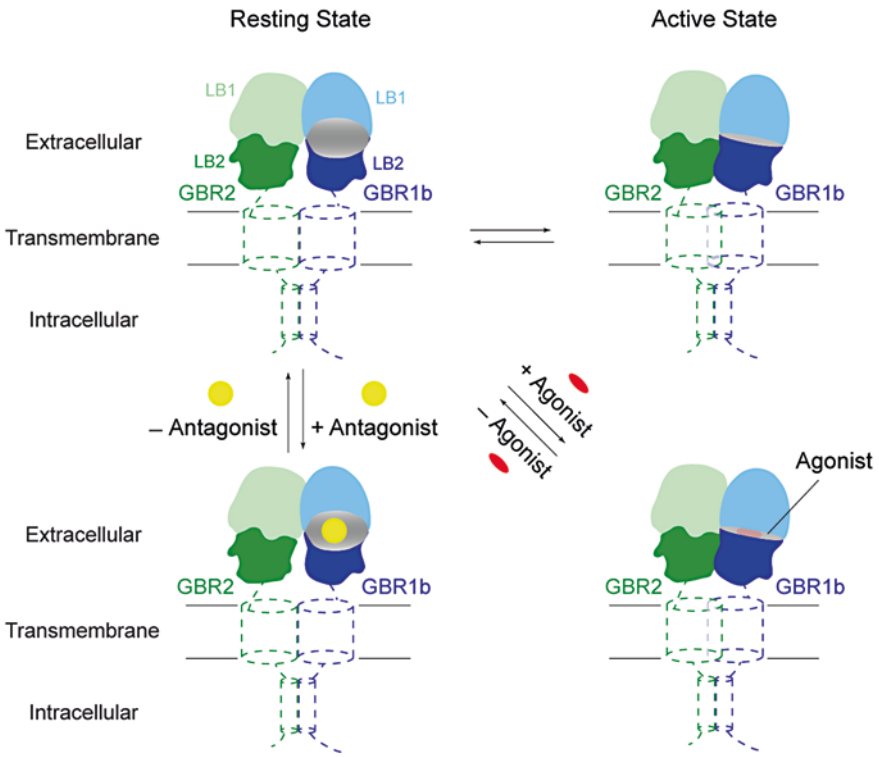

Fig. 12.6 Schematic diagram showing the proposed conformational equilibrium of full-length $GABA_B$ receptor (This figure is modified based on Supplementary Fig. 14 of Ref. [76])

cate that the LB2 domains of mGluRs come into close proximity in the active state [103]; disulfide crosslinking experiments suggest that a specific association between the CR domains is sufficient to trigger receptor activation [104]. The combination of agonist-induced domain closure and specific associtiation of membrane proximal domains may be a universal mechanism shared by all class C GPCRs during ligand-mediated receptor activation. An understanding of the specific heterodimeric nature of $GABA_B$ receptor signal transduction across the plasma membrane, and its implications on more general mechanisms shared across class C family members, requires structural studies of the full-length receptor, in multiple functional states and in complex with downstream signaling proteins.

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Chapter 13
Class C GPCRs: Metabotropic Glutamate Receptors

Veronika Hlaváčková, Laurent Prézeau, Jean-Philippe Pin, and Jaroslav Blahos

Abstract Metabotropic Glutamate receptors (mGluRs) belong to Family C GPCRs. Interestingly enough receptors for Gamma-amino butyric acid, the GABAb receptors from this family, were first discovered putative heterodimers.

The mGluRs are also putative dimers, both homodimers and, as revealed recently, also heterodimers. The heterodimerization may occur between different receptors (e.g. mGluR1/5, mGluR2/3), but also between splice variants resulting from alternative transcription of one gene (mGluR1a/b). This leads to previously unexpected diversity both physiologically, and potentially pharmacologically.

The fact that mGluRs form dimers has crucial functional relevance. The Glutamate binding side is constituted by extracellular portion called Venus flytrap domain (VFT). Upon agonist binding, VFTs of each subunit changes shape. To transfer the activation of the VFT portion further toward the cell, the heptahelical domains are forced to change their relative position, which within the dimer leads to consecutive activation of single heptahelical domain. This heptahelical domain than in turn activates G-proteins, most likely in similar manner as in case of GPCRs from other families (that may be functional in monomeric state). The mechanistic explanation of this complex activation process might be such that change of the shape of VFT within hypothetical monomeric mGluR structure would bind agonist, change its shape, but this would not be possible to transfer further on the transmembrane region. The activation process of Family C GPCRs requires allosteric cooperation within the transmembrane heptahelical regions and the change in relative position between the two subunits that precedes activation of single subunit heptahelical domain.

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Abbreviations

3,5-DHPG	3,5-Dihydroxyphenylglycine
5-HT2A	5-Hydroxytryptamine 2A receptor
AA	Arachidonic acid
$A_{2A}R$	Adenosine 2A receptor
AC	Adenylylcyclase
Ala	Alanine
AM	Allosteric modulator
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Acc	Active closed-closed conformation
Aco	Active closed-open conformation
Aoo	Active open-open conformation
ATP	Adenosine triphosphate
$BRET^2$	Bioluminiscence resonance energy transfer
cAMP	Cyclic adenosinmonophosphate
CaSR	Calcium sensing receptor
CFP	Cyan fluorescent protein
CHO	Chinese hamster ovary cells
CNS	central nervous system
CRD	Cysteine-rich domain
CT	C-terminus
Cys	Cysteine
D2R	Dopamine 2 receptor
DAG	Diacylglycerol
DCG-IV	(2S,2′R,3′R)-2-(2′,3′-Dicarboxycyclopropyl)glycine
e1, e2, e3	extracellular loop 1, 2, 3
ECD	Extracellular domain
EPSC	Excitatory postsynaptic current
Era	Nuclear estrogen receptor a
ERK	Extracellular signal-regulated kinase
FRET	Fluorescence resonance energy transfer
GABA	Gamma-aminobutiric acid
GB1, GB2	subunit 1, 2 of GABAB receptor
GIRK channel	G protein-coupled inwardly-rectifying potassium channel
Glu	Glutamate
GPCR	G-protein coupled receptor
HD	Heptahelical domain
His	Histidine
i1, i2, i3	intracellular loop 1, 2, 3

IP3	Inositol 3,4,5 – triphosphate
L-AP4	L-2-amino-4-phosphonobutyric acid
LB1, LB2	Ligand binding domain 1, 2
LBD	Ligand binding domain
LTD	Long-term depression
LTP	Long-term potenciation
mGluR	metabotropic glutamate receptor
NAM	Negative allosteric modulator
NMDA	N-Methyl-D-aspartic acid
PAM	Positive allosteric modulator
PiP2	Phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase C
SRET	sequential BRET-FRET trasnfer
TMD	Transmembrane domain
Trp	Tryptophan
TRPC1	Transient receptor potential channel 1
VFT	Venus Fly-Trap domain
WT	Wildtype
YFP	Yellow fluorescence protein

13.1 Instead of Foreword: Introduction to the mGluRs

Glutamate is the major excitatory neurotransmitter involved in most brain processes. It mediates neurotransmission through the activation of two types of receptor molecules, ionotropic and metabotropic receptors. In contrast to the ionotropic receptors that are responsible for the fast change in the membrane potential, the metabotropic glutamate receptors (mGluRs) modulate variety of neural processes via G-proteins and other signalling molecules.

The mGluR-family consists of eight members, mGluR1-mGluR8. They are further classified into three groups according to their sequence similarities, signal transduction mechanisms and agonist selectivity (Table 13.1). Receptors within the same group share about 70% sequence identity, while 45% identity is found between the groups [1–5].

13.1.1 Metabotropic Glutamate Receptors and Their Role in the Central Nervous System (CNS)

Expression of different mGluRs receptors and even of their splice variants varies between brain regions and neuronal populations, as illustrated by their location in the hippocampus. While group I receptors are expressed in majority of hippocampal

Table 13.1 Summary of mGlu receptor subtypes

Group	Receptor type	G-protein coupling	G-protein target	Intracellular messenger	Specific agonist	Cloning[a]
mGluR I	mGluR1	Gq, Gs, Gi/o	↑PLC, AC	↑IP3, Ca^{2+}, cAMP	3,5-DHPG	1991[1,2]
	mGluR5	Gq	↑↓PLC	↑↓IP3, Ca^{2+}		1992, 1993[3,4]
mGluR II	mGluR2	Gi/o	↑AC	↑cAMP	DCG-IV	1992[5]
	mGluR3					
mGluR III	mGluR4	Gi/o	↓AC	↓cAMP	L-AP4	
	mGluR6					1993[6]
	mGluR7					1994[7,8]
	mGluR8					1995[9]

[a]References: [1]Masu, Nature; [2]Houamed, Science; [3]Abe, JBC; [4]Minakami, BBRC; [5]Tanabe, Neuron; [6]Nakajima, JBC; [7]Okamoto, JBC; [8]Saugstad, MolPharm; [9]Duvoisin, J Neurosci

neurons, group II receptors predominate in principal cells of CA2, CA3 and CA4 regions and are almost absent from pyramidal cells of CA1. Group III mGluRs are confined to the mossy fiber projection field in CA3 stratum lucidum. The role of mGluRs and glutamate-dependent synaptic plasticity in establishing memory was proposed, and yet another type of synaptic modulation mediated through mGluRs, long-term potentiation, is found in CA1 region of hippocampus [6–22]

The abundant expression of group I mGluRs in cerebellum, ventral tegmental area and nucleus accumbens sustained the role of these receptors in the regulation of motor activity [23]. In mGluR1–deficient mice a severe motor coordination and spatial learning deficits occurred [24]. Increase in the dendritic calcium concentration mediated by mGluR1 is important for the induction of long-term depression (LTD) at parallel fiber cell to Purkinje cell synapses. LTD is believed to be one of the mechanisms of cerebellar motor learning. Moreover, there is evidence that the two types of mGluRI receptors (1 and 5) play a distinct role in the development of human cortex and this is regulated by differential expression of the two receptors in specific periods of the prenatal development [6, 25].

Together with another L-AP4 sensitive receptor – mGluR7- mGluR4 is further found predominantly in the olfactory system and cerebellum, though with different cellular expression than mGluR7 [22, 26]. Another Gi/o-coupled receptor – mGluR6 – was uniquely found on ON bipolar cells in retina [27]. Lastly, mGluR8 was found in olfactory bulb, as well as in retinal cells, in the cortex and also in different brain regions during the development, but not in hippocampus or cerebellum [28].

Various mGlu receptor types are expressed besides neurons also in glial cells, as illustrated by the predominant expression of mGlu3 in astrocytes, leading to multiple functions (regulation of metabolites transport, regulation of glutamate uptake, etc.) [29–31].

13.1.2 Cellular Distribution, Signaling, and Functions of mGluRs

Modulation of many neuronal functions by mGluRs is related to their subcellular distribution, which is different depending on the brain regions considered and during brain development processes [32]. MGluRs of group I are mostly expressed at postsynaptic terminals, peri- and extrasynaptically, mediating long-term changes [7]. Interestingly, mGluR5 is found also intracellularly on the nuclear membrane where it was proposed to modulate gene expression via an activation of different kinases [33–35]. Group II mGluRs are expressed on both pre- and postsynaptic membranes, whereas Group III mGluRs (6, 7 and 8) are expressed presynaptically and modulate the neurotransmitter release from presynaptic terminals [6, 24, 25, 34–40].

Receptors from Group I also modulate functional state of other neurotransmitter receptors including ionotropic and metabotropic glutamate receptors, as well as several types of ion-channels [41, 42]. The functional and physical networks between the ionotropic and metabotropic glutamate receptors are often mediated by intracellular scaffolding proteins that are involved in the process of synaptic plasticity [43].

MGluR1 and mGlu5 activates the Gq-protein leading to stimulation of Phospholipase C (PLC) and production of inositol triphosphate (IP3) and diacylglycerol (DAG) production from phosphoinositol 3,4,5 triphosphate (PiP2) degradation. But mGlu1 couples also Gs- and Gi/o-proteins, which increase or decrease the activity of the adenylyl cyclase (AC) respectively. AC catalyses the production of the second messenger cyclic AMP (cAMP) from ATP. Residues in the i2 loop and C-terminus are responsible for the coupling selectivity to different G-proteins [44–47]. Group II and III mGluRs are supposed to be coupled to inhibitory $G_{i/o}$-proteins that negatively regulate AC.

In addition, glutamate is able to trigger a variety of direct and indirect mechanisms in various types of cells including the stimulation of cyclic AMP or cyclic guanosine monophosphate responses, the activation of other types of phospholipases (D and A_2) and release of arachidonic acid (AA) [28, 29, 48]. These receptors also regulate gene expression by activation of various types of extracellular signal-regulated kinases (ERKs) or Src kinases [34, 49, 50]. Finally, mGluRs are able to up- or down-regulate several types of cation channels either the voltage-sensitive or ligand-activated [41, 51]. Some examples of the physiological interference between mGluRs and other receptors are summarized in Table 13.2.

Beside the direct or indirect interaction between mGluRs and ionotropic receptors, mGluRs can interact with different proteins, protein complexes or even transcription factors and modulate either physiological, or pathological functions, as summarize in Table 13.2 (more detailed in Sect. 13.3) [52].

The multiple functions of mGluRs in the CNS foreshadow their crucial role in several neurological disorders such as pain, epilepsy, Alzheimer's and Parkinson's disease, pathology associated with ischemia, schizophrenia, anxiety and drug addiction.

Table 13.2 Examples of some functional interactions of mGluRs and other receptors

Receptor type	Receptor/channel	Effect	System[a]
mGluR1a	P/Q-type Ca^{2+} channels	Inhibition	Cerebellar Purkinje cells[1]
mGluR1a	ERα	Modulation of sexual receptivity	Hypothalamic neurons[2]
mGluR1	TRPC1	Propagation of slow EPSCs	Cerebellar Purkinje cells[3]
mGluR1	NMDA	LTP	Dentate gyrus[4]
mGluRI	NMDA, AMPA	Internalization	Hippocampal exc. synapses[5]
mGluR5	D2R	Inhibition of synaptic recruitment of mGluR5	Striatum[6]
mGluR5	$A_{2A}R$	Inhibition of D2R-mediated motor activity	Striatum[7]
mGluR II	GIRK channel	Activation	Cerebellar interneurons[8]

[a]References: [1]Kitano, JBC 2003; [2]Dewing, Jneurosci 2007; [3]Kim, Nat 2003; [4]Naie and Manahan-Vaughan, EJN 2005; [5]Snyder, Nat Neurosci 2001; [6]Mao and Wang, J Neurosci Res, 2016; [7]popoli Neuropsychopharm 2001; [8]Knoflach, JP 1998

Growing number of selective compounds for mGluRs make possible studies of the physiological and pathological roles of these receptors in the nervous system and simultaneously represent promising instruments for treatment of neurological and psychiatric disorders in which glutamatergic neurotransmission is abnormally regulated [53].

13.1.3 MGluR Structural Features, Similarities and Differences with Other GPCRs

The existence of mGluRs has been foreseen since 1970s. MGluR1 was cloned using the functional expression screening strategy in *Xenopus* oocytes [9, 14]. The topology deduced from the primary sequence shows four structural characteristics. The hydrophilic amino terminus is preceded by variant peptide of predominantly hydrophobic amino acid residues that constitutes a signal peptide. During protein synthesis, this sequence allows the nascent polypeptide to properly fold and enter the endoplasmic reticulum (ER). The N-terminal or extracellular domain, is composed of Venus Fly-trap like domain (VFT) and cysteine rich region that is followed by seven hydrophobic transmembrane domains and an intracellularly located C-terminus (Fig. 13.1). VFT shares high sequence homology among mGluR receptors, which results in a challenge to develop specific mGluR-type ligands. On the contrary, the C-terminal domains (C-tails) vary in the length and the residue composition. The C-termini specifically interact with different intracellular proteins

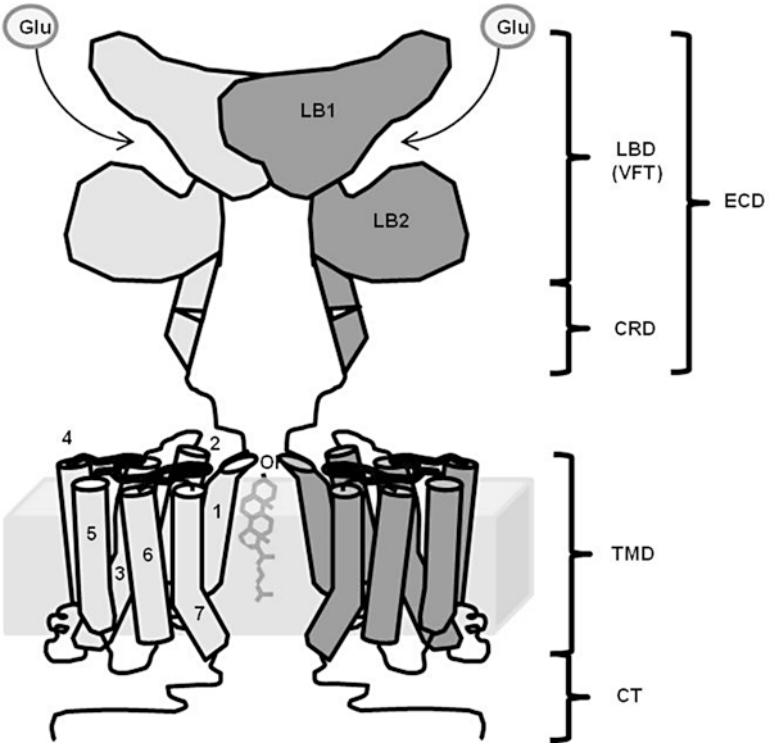


Fig. 13.1 Schematic representation of dimeric mGluR. Glutamate binds into a cleft formed by two lobes (LB1 and LB2) of each ligand binding domain (LBD). The extracellular domain (ECD) is composed of LBD and cysteine-rich domain (CRD) whose C-terminal linker connects to the first transmembrane helix (1). The transmembrane domain (TMD) is composed of seven transmembrane helices (indicated by number). These are interconnected by 3 extracellular loops and 3 intracellular loops. Second extracellular loop (bold) covers the extracellular inter-helical cavity. According to recent crystallographic studies the protomers face to each other by helix I and interacts with 6 cholesterol molecules. C-terminus (CT) is located intracellularly

that regulate the receptor properties into certain extent, inter-connect specific intracellular pathways and in some cases they are adjusted by alternative splicing between receptor variants, resulting in receptors with distinct properties, albeit arising from one gene. For example, the mGluR1 can be transcribed in several variants that combine in the receptor complexes, as will be discussed later in this chapter.

13.1.3.1 N-Terminal Part of the Receptor

Most of the receptors from class C GPCRs possess a large (approximately 600 residues) extracellular domain (ECD). The ECD is composed of a ligand binding domain called VFT that is related to bacterial leucin/isoleucin/valin binding protein [54] and cysteine rich domain (CRD). The VFT has the same bilobal tertiary structure as the

bacterial binding proteins [55, 56] and is responsible for the glutamate binding. Ligand binding site of mGluR1 is well defined [55]. The authors indicated, among others, that the tyrosine 74 is important for the ligand binding. Several polar amino acids, such as serine 165, threonine 188, tyrosine 236 and aspartate 318 contribute to the ligand recognition within all the mGluRs. These residues are conserved among all members of mGluR subfamily. Next, five polar residues (Tyr 74, Ser 164, Glu 292, Gly 293 and Arg 323) are preserved only in the group I mGluRs and may be responsible for the ligand preference of the mGluR1 and mGluR5. The binding of glutamate to VFT is allosterically modulated by interaction with calcium (Ca^{2+}), or chloride (Cl^{-}) and exerts positive cooperativity with glutamate binding to the adjacent VFT [57–59]. In mGluR1 there are 3 conserved cysteine residues in the first third of the ECD of mGluRs. Among them, cysteine 140 has been proposed to function as a disulfide bridge between the two VFTs [60].

VFT is followed by CRD. The rigid structure of CRD was well documented using crystallographic studies. CRD consists of three β-sheets, each composed of two antiparallel β-strands. The domain carries 9 cysteine residues; eight of them form intra-domain disulfide bridges that hold the entire domain as a compact structure [61]. The remaining cysteine residue also forms disulfide link but its partner is located on LB2 of the VFT. Its mutation results in impaired receptor function, although the VFT retains its binding properties, and the recombinant receptor has preserved the expression pattern [62].

13.1.3.2 Transmembrane Domain (TMD)

Each CRD is followed by a seven transmembrane region domain, also called heptahelical domain (HD) as it is formed by seven membrane-spanning alpha-helices. The helices are interconnected by three extracellular (e1-3) and three intracellular (i1-3) loops forming a compact bundle. All intracellular domains of mGluRs play role in G-protein activation. Among the three intracellular loops, the i2 loop is responsible for recognition of the C-terminus of Gqα-subunit and thus encodes the Gα-protein selectivity [45–47].

Recently, crystallographic studies helped us disclose important structural determinants of HD of mGluR1 and 5 in comparison to receptors of class A and B in terms of function, dimerization and determination of allosteric binding site [63, 64]. One important structural determinant for mGlu receptors is a long second extracellular loop that is covalently linked to the top of TM III domain via a disulfide bridge forming a hairpin structure and covering the top of the HD. TM1 on one side and TM IV on the opposite form borders of the HD, whereas bundle of TM II, III, V-VII domains form a core of the HD as well as an allosteric binding site. In comparison to class A and B receptors, the position of helix V and the extracellular part of helix VII is shifted to the center of the HD thus a narrower top of the receptor is formed. Together with the spread e2 loop, these properties restrict to easily access the allosteric binding site. On the other hand, proline 833, or 820 within the HD of mGluR1 and 5, respectively, bends outward its intracellular half opening the intracellular cavity of the transmembrane core.

Interestingly, as in class A GPCRs, similar stabilizing structural motifs were found among members of class C. First, a salt bridge formed between conserved lysine $^{3.50}$ in TM3 and glutamine $^{6.35}$ in TM6 stabilizes the inactive conformation of HD by interacting with highly conserved serine in i1 loop, thus impeding the activation of a receptor (analogous to E(D)RY sequence in class A GPCRs). Mutation of any of these three residues increases the constitutive activity of the receptor. Next, side chains of highly conserved lysine $^{7.51}$ and phenylalanine $^{7.48}$ in the TM7 fulfill the space made by movement of TM6 outside the bundle (analogous to NPxxY(x)F motif of class A GPCRs). Finally, two hydrogen bonds are formed between the indole ring of tryptophan $^{6.50}$ in TM6 and leucine$^{5.44}$ and glycine$^{5.48}$ in the TM5 that stabilize the HD of mGluRs.

Apart from an allosteric binding site for cations within VFT, which binding facilitates activation of the receptors, HD provides another space for pharmacological modulation of the receptor activity by allosteric modulators (AM). Binding of positive or negative (mostly inverse agonist) allosteric modulators (PAMs, NAMs, respectively) stabilizes HD in its active or inactive conformation, respectively. Neutral modulators prevent binding of PAMs or NAMs. It is interesting to note that truncated receptor without the N-terminal VFT and CRD, possessing only HDs and C-termini behaves in comparable manner as rhodopsin-like receptors in terms of G-protein coupling and regulation by ligands. PAMs or NAMs act on this truncated receptor as full agonists or inverse agonists respectively [65]. Recently, an allosteric binding site for the inverse agonists of mGlu1 receptors was nicely revealed in crystallographic studies [66]. Because of the relatively closed domain structure, the allosteric binding site is very narrow with a small entrance for a ligand. The very same pocket in mGluR5 accommodates positive, neutral and negative modulators. The modulators share common stretched structure with two or more phenyl rings on both sides of an alkyne linker and as such perfectly fit to the narrow binding cavity. Substituents of these rings determine whether an AM will exert negative ($-OCH_3$), neutral ($-OH$ or Cl^-) or positive (F^-) effect [67]. Residues within TM3-7 form the hydrophobic nest for the NAM mavoglurant. Most of these residues were already mapped using a mutational analysis for another NAM MPEP [68, 69]. Among the 22 amino acid residues of the allosteric binding site, the conserved tryptophan $^{6.50}$ is very important determinant in binding of AMs. A single mutation of this conserved tryptophan causes loss of the MPEP binding in mGluR5. Interestingly, in such a mutant some of the AMs exert positive effect, whereas they act as negative AMs on the wild-type mGluR5 suggesting that the tryptophan side chain interacts with a negative AM to stabilize the receptor HD in an inactive conformation [70].

13.1.3.3 Intracellular C-Terminal Domain

The receptors end with an intracellularly located C-terminus. There are several splice variants that have been cloned from a rodent or human brain for mGluR1, 4, 5–8. Except mGluR5 the splicing occurs within the intracellular C-termini. Splice variants of mGlu1 receptor are called -1a, -1b, -1d, -1e and –1E55. The first sequenced mGluR1 (mGluR1a) bears a long C-terminus (318 amino acids).

A shorter variant, mGluR1b, lacks last 292 amino acids but contains a short unique sequence [22]. The long C-terminus of mGluR1a is replaced by 26 amino acids in mGluR1d [71]. The additional splice variants mGluR1e and –1E55 are only composed of the extracellular domain and thus, if expressed, it could be secreted or attached to the membrane by a lipid modification [72].

C-termini of the mGluRs assemble with different enzymes, scaffolding or cytoskeletal proteins that participate in regulation of the receptor neurotransmission. The existence of different splice variants will then generate an even greater variety of interacting proteins for a certain receptor as well as to provide more options for physiological modulation. Moreover, splicing is also responsible for triggering specific targeting and trafficking of different receptor variants, including ER retention signal. These structural features and their functional consequences will be discussed in more detail in Sect. 13.3.1.

There are specific sequences for phosphorylation or binding phosphatases in mGluR1a, 5a and b, and mGluR7b. Besides, SUMOylation enzyme Ubc9 binds mGluR8b, while Calmodulin binds mGluR7a, and Tamalin interacts wiht mGluR8a and b, within their intracellular C-termini [73].

Regulation of certain variants of group I mGluRs is mediated by intracellular scaffolding proteins called Homer proteins [74, 75] that serve as a link between the receptors and other scaffolding and several targeting proteins [52]. There are several splice variants of Homer proteins, the long one has PDF motive mediated interaction with the long mGluR1a and both variants of mGluR5. The structure of the long C-termini enables the interconnection of membrane mGluR1a and mGluR5 receptors with intracellular receptors (IP3R or ryanodine receptor) and other scaffolding and cytoskeletal proteins (Shank, actin) and thus participates in organizing the postsynaptic density domains bringing modulatory mGlu receptors in proximity of ionotropic glutamate receptors.

13.1.4 MGluR Dimers: Several Interfaces Between the Dimers Were Mapped

Crystals of ECDs were found to form oligomers [61]. From biochemical assays we know that mGluRs exist as intermolecular disulfide-linked dimers within ECD. They form dimers already in the ER [76] and as such, they are trafficked to the cell surface, which is required for their function [57]. All mGluRs form homodimers, but some can also form dimers in combination with another splice variant of the same receptor subtype or even with another mGluR, generating numerous heterodimer combinations, as will be discussed in next chapter [77–80]. Finally, mGluRs may form higher ordered oligomers with other GPCRs, like 5-HT2A.

In some GPCR dimers the TM helices are involved in dimerization or oligomerization. It is usually mediated by TM1 or TM4 [81, 82]. In others, such as crystallized rhodopsin dimers or complex formed by mGluR2 and 5-HT_{2A} serotonin

receptor, the receptor interface is formed mainly by TM5 [83, 84]. On the basis of FRET studies it was also suggested that the transmembrane helix V is one of the TMDs that might be involved in a formation of the inter-receptor interface in mGluR homodimers. The observed FRET change was the largest between the i3/i3 loops, which interconnect the TM5 and TM6, in comparison to i1/i1 or i2/i2 loops. As such, the helix V might be involved in the process of HD activation [85]. These results propose a large conformational rearrangement between i3 loops or TM5 and TM6 movement in a receptor dimer during receptor activation rather than a distance between certain intracellular parts as suggested. Locking the TM4/TM5 interface causes an impairment of agonist activation of such a receptor dimer whereas locking the TM6 interface produces constitutively active receptor. Based on these results it is suggested that transmembrane helices are rearranged during the activation and the interface between the two HDs in a dimer is different in an active *versus* inactive dimer [64].

Recent dimer of HDs in the mGluR1 crystallographic studies revealed another interface formed by TM1 domains. Interesting finding of a cluster of 6 cholesterol molecules in this interface suggests its role in dimerization and/or in the process of activation [66]. Indeed, the affinity of mGluRs to glutamate alters depending on fluidity of the membrane [86].

13.2 Dimeric Structure of mGluRs Is Crucial for Activation Mechanism

13.2.1 Conformational Changes of the Ligand Binding Domain Lead to the Activation of the mGluRs

As mentioned above, binding of glutamate, or an orthosteric agonist promotes conformational changes of one, or both adjacent VFTs. Each VFT may reach open (o) or closed (c) conformations (intra-protomer conformation), and their relative position may be in two different states – resting (R) and active (A) (Fig. 13.2). R and A conformations are modulated through the dimer interface. Each VFT is composed of two domains, lobe 1 (LB1) and lobe 2 (LB2), and flexibility of the linker between them allows adapting open or closed states. Structure of three crystals of the mGluR1 ligand-binding domain was identified [55, 61]. To date, six different conformations were observed in resolved crystal structures of mGluR1, 3 and 7. These are called resting open-open (Roo), resting closed-open (Rco), resting closed-closed (Rcc), active open-open (Aoo), active closed-open (Aco) and active closed-closed (Acc) (Fig. 13.2). First three conformations occur in the absence of an agonist [56]. The symmetrical conformation Acc was crystalized in the presence of glutamate and gadolinium trivalent cations (Gd^{3+}). Gadolinium ions interact with a cluster of negatively charged amino acids, mainly Glu 238 [3]. Neutralization of this residue by

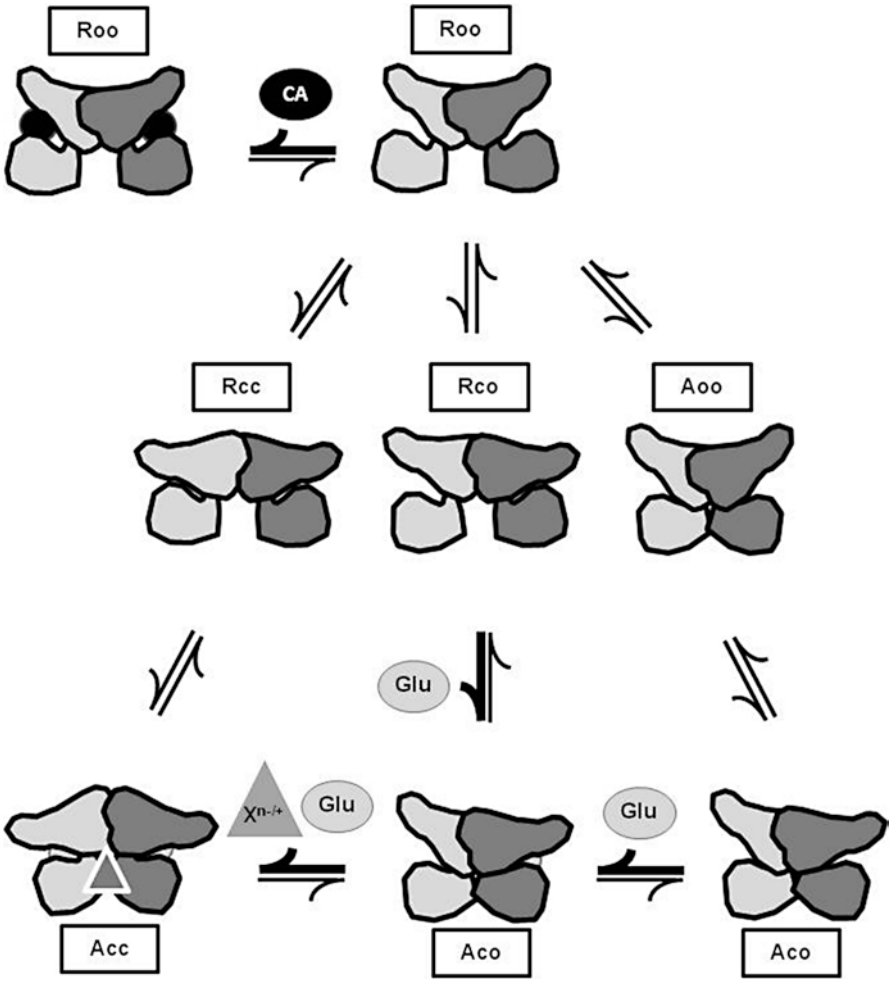


Fig. 13.2 Schematic representation of different conformational states of a dimeric LBD of mGlu receptors. Ligand-binding domain (LBD) of mGluR1 can gather different conformations that exist in equilibrium. Roo (Resting open open) represents inactive state, both LBD within a dimer are opened and can be further stabilized by competitive antagonist (CA). Other resting conformations Rco (Resting closed open) and Rcc (Resting closed closed) exist in dynamic equilibrium with Roo conformation without presence of an agonist. The Rcc (Resting closed closed) conformation could count for a desensitized receptor dimer with reduced affinity to an agonist. Active conformation is represented by Aco (Active close open) and Acc (Active close close) forms that are stabilized upon glutamate (Glu) and modulation by ions ($X^{n-/+}$). The Aco conformation could also exist without ligand suggesting an intermediate structure of the partially active receptor on the way to its resting state (Roo). To fulfil the overview of the A and R states of a dimeric LBD we have to mention the last Aoo (Active open open) conformation, where both protomers get closer to each other in the absence of a ligand, or as a not-preferred intermediate conformation

His or Ala substitution causes an increase in the basal activity of the receptors and also facilitates the activation of mGluR1 receptor [87].

The close relationship between the conformational state and the distance of two 7TMs can be sorted as follows: Rcc > Rco > Roo > Aoo > Aco > Acc. The Rcc conformation in principle restricts to activate TMD because the distance between the two CRDs and thus HDs in a homodimer is too large (126 Å), whereas in Acc conformation the CRDs and adjacent HDs have distance 46 Å. It is generally presumed that the mechanism of activation is based on a dynamic equilibrium between these conformational states, that is regulated by presence of high glutamate and/or PAMs, is in favour of Acc state (Fig. 13.2). The Rcc conformation found in crystals of mGluR3 was suggested to represent the desensitized receptor conformation with a weaker responsiveness to an agonist [61].

Similar to gadolinium ions, physiological concentrations of the extracellular calcium (0,8-1,5mM) can enhance the glutamate sensitivity of mGlu1 receptor [88]. In cerebellar Purkinje cells, extracellular calcium causes increase of the sensitivity of mGluR1 to glutamate [58]. It was shown that the effect is regulated by $GABA_B$ receptors that were co-immunoprecipitated with mGluR1 from cerebellar neurons, and in the absence of GABA, calcium was able to promote the action of mGluRs [89].

It was reported that a closure of at least one VFT is necessary to allow the dimer of ECDs to reach the active state, albeit partial [90]. Kniazeff et al. have demonstrated that closing of both protomers (Acc) is required for a full activation of mGlu receptors [57]. This was further supported by the finding that the R75A/WT heterodimer, which can bind only one glutamate per dimer (R75A mutant has impaired glutamate binding), showed smaller rearrangement in comparison with the WT homodimer [85].

13.2.2 Transfer of the Active State from VFT to the Heptahelical Domain Region

The activation of the receptor proceeds in several steps (Fig. 13.3). First, an inactive Roo conformation that is characterized by long distance of C-termini of both LB2 domains in a ECD dimer is in a dynamic equilibrium with an Aco conformation. Upon glutamate binding, the two lobes (LB1 and LB2) of a VFT get closer, which leads to a capture of the bound glutamate that stabilizes this state. Binding of an agonist brings the two LB2 domains within the dimer into close proximity and their C termini, as well as Cys254 (in mGluR1), get closer. The distance between the two cysteines in LB2 was estimated to be 46.9 Å, 23.5 Å and 16 Å for Roo, Aco and Acc conformation, respectively [62]. Presence of chloride and/or calcium ions facilitates the glutamate binding and thus reaching the Acc conformation [58, 59].

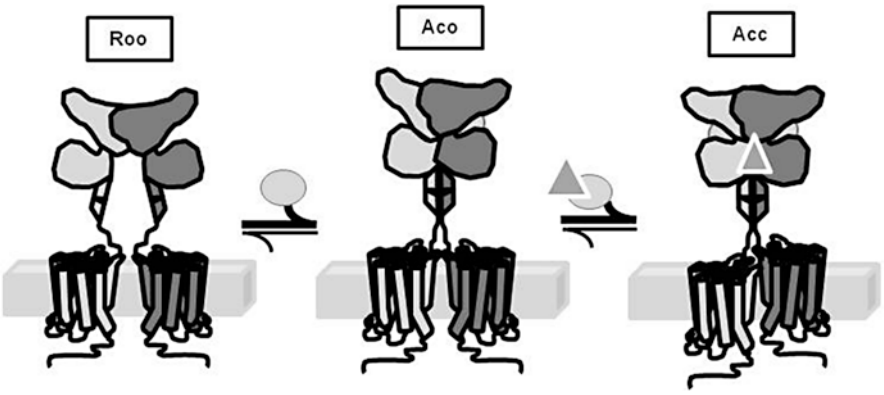


Fig. 13.3 Schematic representation of initiation of activation of a dimeric mGlu receptor. Glutamate (*grey circle*) causes closure of the ligand-binding domain where it binds. Large conformational shift of the LB2 domains toward each other leads to a rearrangement of the rigid CRDs. These move to each other and thus enable HDs to come to close proximity. High concentration of calcium (*dark grey triangle*) can facilitate glutamate binding by the adjacent subunit. Alternatively change into Acc conformation requires high concentrations of glutamate because of the negative cooperativity. An active HD changes the conformation gain. Transmembrane helices uncover the intracellular loops that couple Gα-protein.

Transfer of the conformational change from the VFT into the TMD of the receptors that leads to activation of the coupled G proteins includes a relative movement of the CRDs. The importance of the CRD in the receptor activation was verified by two observations. Firstly, a mutation in any cysteine in whichever of the two CRDs leads to the loss of communication between the VFTs and TMDs. This loss of communication can be overcome by an application of PAM. Secondly; the covalent interaction between the two CRDs, when mediated by engineered cysteines in certain positions, is sufficient for the full constitutive activation of such mGluR mutants [91]. The CRD is a rigid structure that is maintained by covalent bonds of the cysteine bridge between the CRD and the LB2 [62]. This covalent bond allows transfer of structural change of VFTs on HDs in the receptor dimer and they sustain relative position rearrangement [61, 92] (Fig. 13.3). Using data from the crystalographic studies, it was estimated that the distance between the linkers connecting CRD and TMD is about 20 Å in the Acc conformation, suggesting close proximity of both CRDs in the active state. Furthermore, in the dimerization interface Trp 588 might participate in the activation of the HD by interaction with cholesterol molecules [61, 66].

Tateyama and Kubo (2006) reported that mGluR1 expressed in CHO cells activates either G_q or G_s-protein depending on distinct conformation of N-terminal as well as intracellular domains under specific receptor activation. As discussed above, receptors spontaneously change their conformations and agonists stabilize their active conformation. Receptor that was activated by glutamate and reached Aco

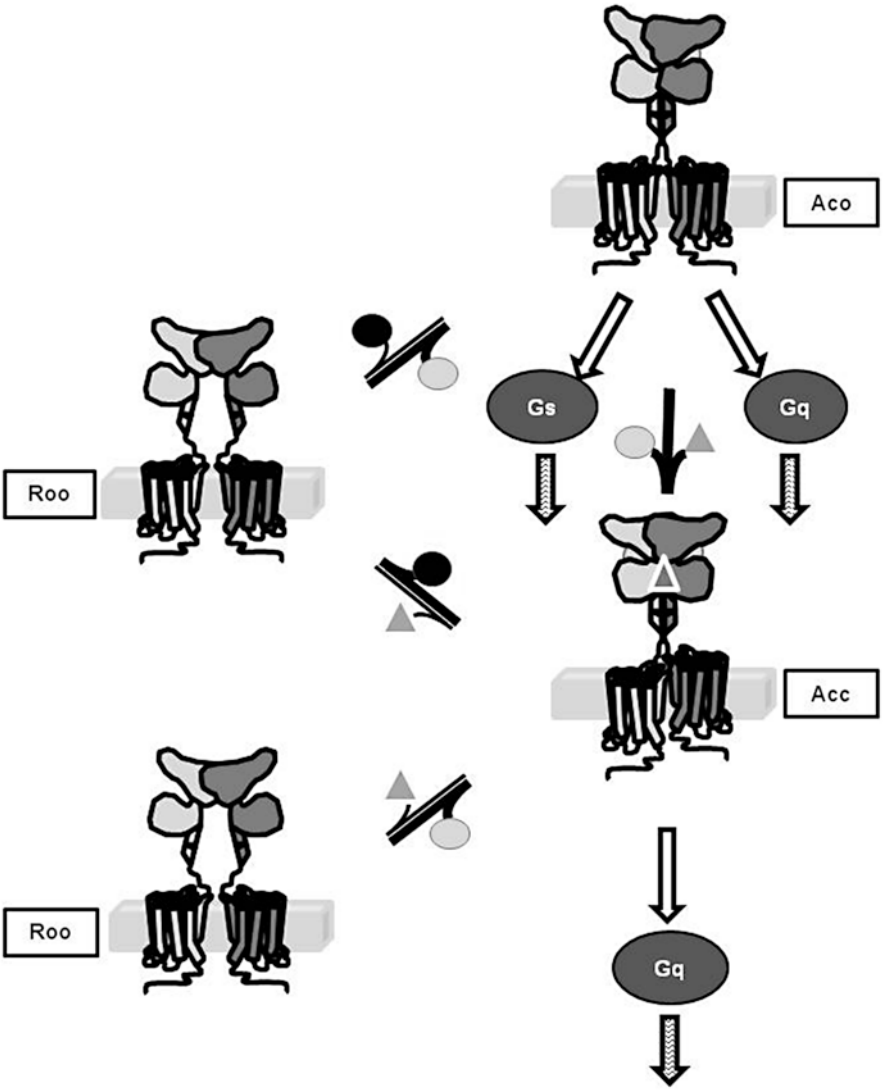


Fig. 13.4 Schematic representation of functional difference between Aco and Acc conformations of the mGluR1 and their preferential coupling to G-proteins. MGluR1a serves as a multiple regulator of the signaling depending on conformational states. Glutamate-induced Aco state activates both G_s and G_q pathways. High glutamate and/or Gd^{3+} leads to G_q but not G_s coupling. High concentration of Gd^{3+} induces a non-functional inactive state comparable to the one stabilized by a competitive antagonist. Coloring is the same like in previous figures

conformation can activate both G_s and G_q proteins, whereas receptor in fully active conformation (Acc) can stimulate only G_q proteins in the heterologous expression system (Fig. 13.4). It was further suggested that whether receptor activates G_q or G_s protein depends on the position of the intracellular portions [93].

13.2.2.1 In Activated mGluRs, Single Heptahelical Domain Within a Dimer Couples to G-Proteins

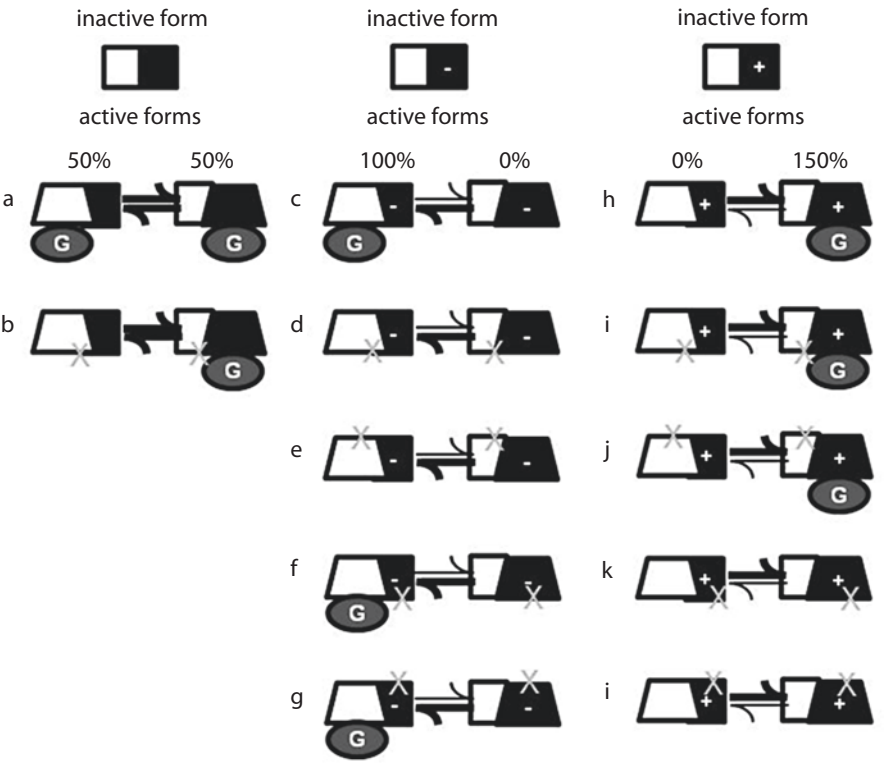
We used a series of mutations of mGlu1 receptor that allowed us (1) to control the subunit composition (such heterodimers bearded a specific set of mutations in each subunit). These mutants allowed us to control (2) the sensitivity to Glutamate, (3) the receptivity to allosteric modulators (4) the coupling to G-proteins. Thus by using functional assays and a set of pharmacological tools we were able to examine whether the receptors' HDs are activated symmetrically, as the ECDs dimers, or whether only one HD reaches active state and transfers the conformational change on G proteins.

We proposed that either of the two subunit's HD in a dimeric receptor can potentially activate G-proteins, but that only one of them is activated at the moment of a signal transduction and hence activates the coupled G-protein [94]. Our conclusion was built on several findings. Firstly, a single mutation within i3 loop (F781P) of a single HD of the two within the dimer caused decrease in the G-protein activation by the dimeric receptor. Secondly, binding of an inverse agonist within one HD did not influence the G-protein coupling of the activated dimer, as the receptor was able to fully activate G-proteins and only when both subunits were sensitive to NEMs, the receptor was blocked (Fig. 13.5). And thirdly, PAMs exert full effect on the Glutamate-activated receptors even in case when only one subunit's HD was receptive to the PAM. Moreover, when an inverse agonist binds into a subunit that is impaired in G-protein coupling, we observed enhancement of the transmission of the activation onto G-proteins. In accord with these observations is a result of fifth observation resulting from an experiment in which we employed a PAM and the i2 mutation non compatible with the G-protein activation. Binding of the PAM into the subunit with impaired G-protein activation leads to full inhibition of the signal transduction. This also is in line with the proposed mechanism of the mGluR activation that within the dimer only one HD reaches the active conformation that is needed to activate G-proteins [95]. Our studies about the receptor's activation using different receptor mutants are illustrated in the Fig. 13.5.

13.2.2.2 Allosteric Interaction Between VFT and TMD

Using a single mutation that disrupts ligand binding within VFT in combination with the i2 loop mutation disabling the G-protein coupling in it was shown that neither *cis*- nor *trans*-activation is preferred during the signal transduction from ECD to HD [96].

It is interesting to note, that apart of the downward signal transduction, lower stages of the receptor, or even receptor binding partners, can influence the conformational state of superior parts, or the receptor affinity. Thus, a lack of G-protein association can decrease the binding of an agonist, on the other hand the PAM binding within the HD leads to maintenance of the VFT in the active state by promoting the active state of HD [92].



Agonist	Inverse agonist	Positive allosteric modulator
a Full activity	c Full activity	h Enhancement
b Partial activity	d Full inhibition	i Enhancement
	e Full inhibition	j Enhancement
	f Enhancement	k Full inhibition
	g Enhancement	l Full inhibition

Fig. 13.5 Proposed model: one HD being turned on at a time. The receptor is represented as a dimer of HDs, one in white, the other in black. The inactive conformation of the HD is represented by a rectangle, whereas the active form is represented by a trapezoid. The presence of the mutations that prevent G-protein activation or agonist binding are indicated by **X**. The presence of an inverse agonist is indicated by dash (**c**–**f**). The presence of positive modulator is indicated by plus (**g**–**j**). The expected effect of negative and positive modulators, respectively, according to our model proposing that only one HD can reach the active state at a time, are indicated in Table below the illustrations. (**a**) Control condition, with either HD being activated. (**b**) One HD is mutated in its i3 loop such that only 50% of the dimers activate the G-protein. (**c**–**g**) An inverse agonist is supposed to prevent the black HD from reaching the active state, therefore the equilibrium between the two states will be shifted toward the white HD which will turn on and an inverse agonist will have no effect (**c**) or will fully block the active subunit (**d** and **e**). By preventing the black HD from reaching its active state, an inverse agonist will increase the probability that the white HD will reach its active state thus leading to an enhancement of the agonistic effect (**f** and **g**). (**h**–**l**) Positive allosteric modulator stabilizes active conformation of the black HD, leading to enhancement of the receptor activity (**h**–**j**) or full inhibition (**k** and **l**) due to the stabilizing of the active conformation in the impaired subunit (*black*) incapable of coupling a G-protein (**k**) or binding an agonist (**l**)

Table 13.3 Rearrangement of the intracellular loops during activation as measured by FRET by Tateyama et al

Subunit 1	Subunit 2	Glu-induced FRET
i1CFP	i1YFP	Decrease
i2CFP	i2YFP	Increase
ctCFP	ctYFP	n.c.
i1CFPctYFP	i1CFPctYFP	n.c.
i2CFPctYFP	i2CFPctYFP	n.c.

The mode of activation controls triggering of a specific signalling pathway. The mGluR1 can activate G_q-, G_s- and $G_{i/o}$-proteins depending on the way of receptor activation. Moreover, there is an evidence for voltage dependency of mGluR1a and mGluR3, suggesting that depolarization causes a conformational change of the receptor to its high- or low-affinity state, respectively [97], thus regulating the coupling to a certain G-protein. As discussed above, one HD effectively activates G_q-protein dependent pathway because of the fact, that a presence of one subunit deficient in G-protein coupling per receptor dimer did not completely impair the activity of the receptor [94, 96]. In contrast, the activation of the $G_{i/o}$-protein dependent pathway under similar conditions is attenuated suggesting possible involvement of both HDs in the $G_{i/o}$-protein coupling and activation [98, 99]. Negative cooperativity might play an important mechanism to control the proportion of receptors in Aco and Acc conformations, and thus selectivity towards intracellular signalization pathways [88].

13.2.2.3 Structural Rearrangement of TMD Is Crucial for the Activation of G-Proteins

Fluorescence resonance energy transfer (FRET) is a method widely used for studies of structural rearrangements of proteins. Tateyama and co-workers used FRET changes measurements between CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein) that they fused within several intracellular parts of the mGluR1 receptor; the intracellular loop 1, 2 and the extreme C-terminus (i1, i2, Ct, respectively) [100]. They observed significantly reduced FRET ratio in the mGluR1-i1-CFP/mGluR1-i1-YFP heterodimer upon the agonist addition, whereas there was significantly increased FRET efficiency in mGluR1-i2-CFP/mGluR1-i2-YFP dimer following the activation (Table 13.3). Co-transfection of mGluR1-i2-CFP/mGluR1-Ct-YFP led to a measurable FRET signal, but this did not change upon the receptor activation. The authors, according to these data, proposed a model of rearrangement of HDs in such a way that the i2 loops of both subunits are, upon activation, getting closer to each other, whereas the distance between i1 loops is increasing. The authors of this study suggested that the mGluR activation involves the rearrangement within the HD region leading to change in their relative position.

Indeed, the measured distances between the i1 and i2 loops in the dimeric receptor 3D structures derived from mGluR3 and rhodopsin comparing Roo and Aco

states are 54 vs. 66 Å and 55 vs. 42 Å for i1-i1 and i2-i2 distances, respectively [61]. Simulation of mGluR5 with or without a specific positive allosteric modulator indicated the movement of the intracellular part of TM5 towards the helix VI, suggesting that this outward movement facilitates the coupling of a G-protein [101].

13.2.2.4 Stepwise Mechanism of the Single HD Activation

More detailed study using FRET approach proved that the conformational changes within the single subunit HD follow the inter-subunit movements within the TMD [102]. As shown in Fig. 13.6, there were two main FRET sensors created that allowed these measurements. One allowed us to detect the inter-subunit changes and the other one the intra-subunit changes within the single HD that was called A-sensor in the study. The expression control system of C-termini of GABA B1 and B2 subunits, which was described prior to our study, was used to manipulate the formation of specific heterodimer composition [57]. Importantly, swapping the long, flexible C-tails of mGluR1a with the rigid C-termini of $GABA_B$ allowed us to measure FRET changes when one fluorophore was fused within the i2 loop and the other was fused with the novel C-tail of the same subunit.

Comparison of the kinetics of the FRET change evoked by an agonist revealed that the conformational change between the two subunits' HDs precedes the conformational change within a single subunit (Figs. 13.6, 13.7 and 13.8). This result is in accordance with conclusion of the previously reported study that was discussed above.

The deactivation kinetics of both intra- and inter-subunit sensors appeared to be approximately ten times slower than the activation kinetics in the FRET studies. The rates were in the range of 300 ms. This suggested that either the two deactivation processes occurred in a closely linked manner or, under the measuring conditions, the two processes were limited by agonist dissociation and the differences were too small to be detected [102].

Hence, a relative movement follows the activation of VFTs by binding of orthosteric ligands between the two HDs, which in turn results in the activation of single HD that transmits the conformational change on G-proteins.

13.3 Heterodimers of mGluRs

The mGlu receptors form covalently linked dimers. The former notion that mGluRs form strict homodimers was overcome recently. They can form heterodimers of two different mGluR subtypes (1 and 5, 2 and 4) when co-expressed in a heterologous system [77], and existence of some heterodimers mGluR1/5 was also shown *in vivo*, [78]. Moreover, recently it was proved that dimers of two distinct splice variants of the mGluR1 exist in either a heterologous system [79] or *in vivo* [79].

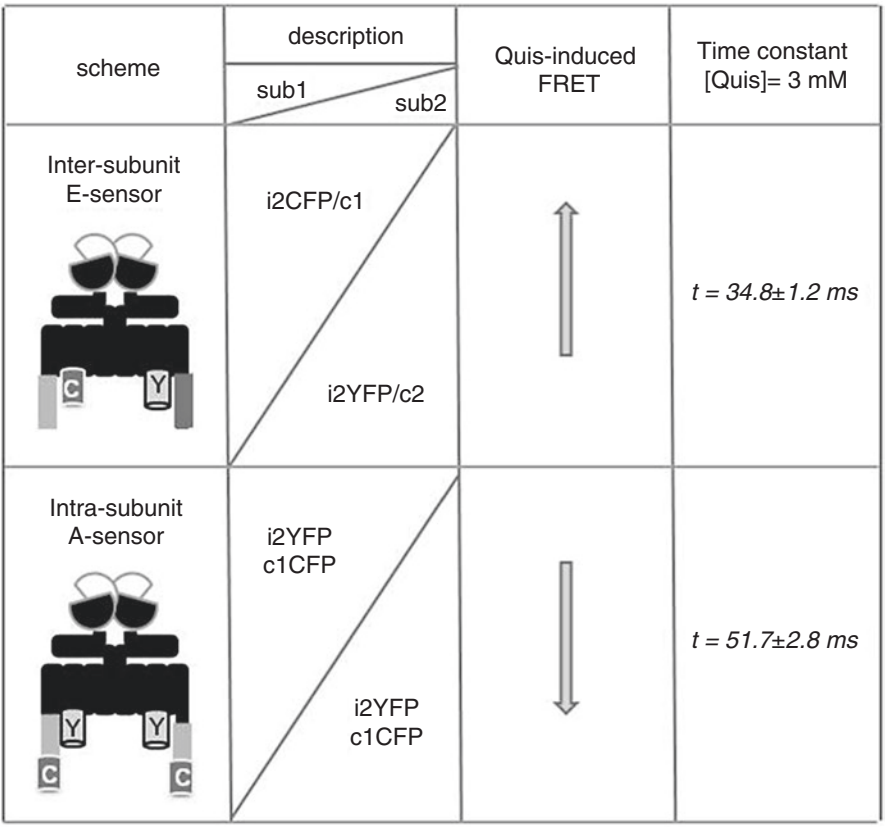


Fig. 13.6 Agonist-induced inter-molecular change within a dimer is faster than the intra-molecular change of a single subunit. Schematic illustrations of mGluR1 receptor fused with CFP or YFP as indicated, change in FRET signal upon quisqualate binding and dynamics of the FRET change upon stimulation with 3mM quisqualate (after Hlavackova SciSignal2012). In saturating conditions, it is expected that the receptor will be found in the Acc conformation. The inter-subunit rearrangement is so fast that it probably reflects binding of an agonist and subsequent conformational changes of the LBD, CRD and first approaching of the HDs to each other seam to happen almost at the same time. Intra-subunit FRET reflects either more complicated reorganization of one protomer in respect to another, or the time delay might represent the time necessary for a G-protein coupling that guide one of the HDs to switch to an active state. *c1* CT of GABAB1, *c2* CT of GABAB2, τ time constant

13.3.1 Heterodimers Among Splice Variants of mGluR1

What is the physiological relevance of the alternative splicing of mGluR mRNA? First, different length of C-terminus might influence the sensitivity to agonists [103]. Also, agonist potencies may differ to distinct splice variants of mGluR1 [104]. However, alternative splicing might have a different influence on the coupling

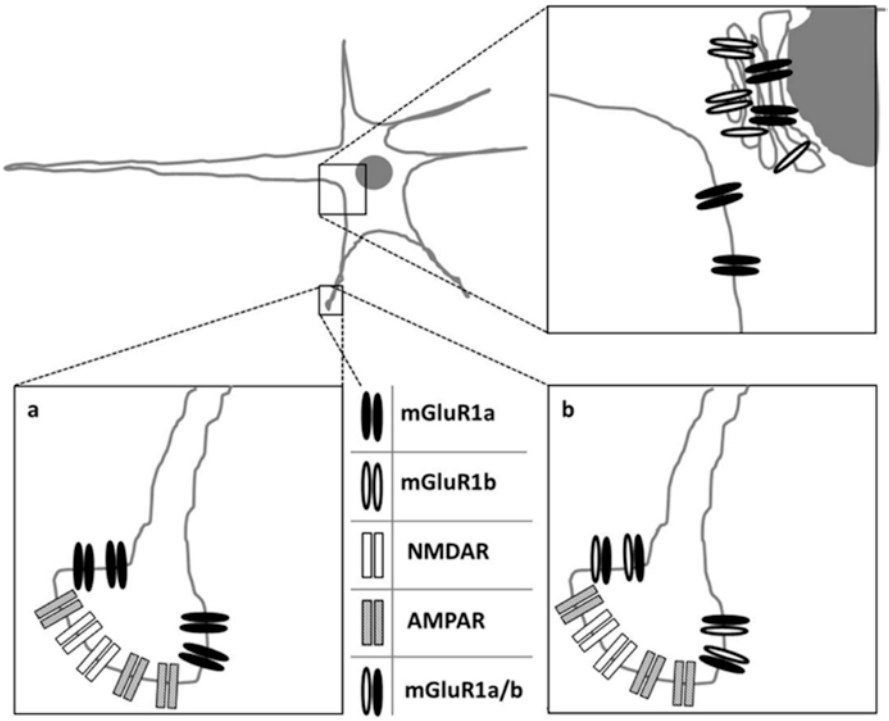


Fig. 13.7 Schematic representation of distribution of different splice variants of mGluR1. Most likely, under different conditions, the mGluR1 is targeted to the distal part of dendrites in (**a**) homodimeric (a/a) or (**b**) heterodimeric (a/b) form. The mGluR1b is believed to be unprocessed in soma either as a single subunit or as a homodimer. Both bands appear on immunoblots (Pickering et al. 1993; Techlovska 2014)

efficacy to the G-protein or the interactions with intracellular proteins that can result in triggering different signaling pathways. Unlike mGluR1a, mGluR1b triggers uniquely G_q-mediated signaling pathway [104–106]. Finally, our recent results support the hypothesis that the C-terminal domain influences its membrane targeting and a receptor density on the cell surface [79, 80].

The splice variants are differently targeted in the cell when expressed alone in transfected retinal neurons [107]. This targeting is regulated by the $RRK^{877–879}$ signal on the C-terminus. On the other hand immunogold staining showed that in the rat cerebellar Purkinje cells, both splice variants were found perisynaptically on the dendritic spines, and this was shown also for the mGluR1b in hypothalamus [15, 108]. Such controversial results led to further investigations. Interestingly, recent study shows that dimerization of two different receptor splice variants (mGluR1a and b) is necessary for correct targeting of the shorter variant to the distal parts of neurons using the same mechanism of masking the $RRKK^{877–880}$ signal [79, 80]. This finding shows that also in case of mGlu1b the $RRKK^{877–880}$ signal may be

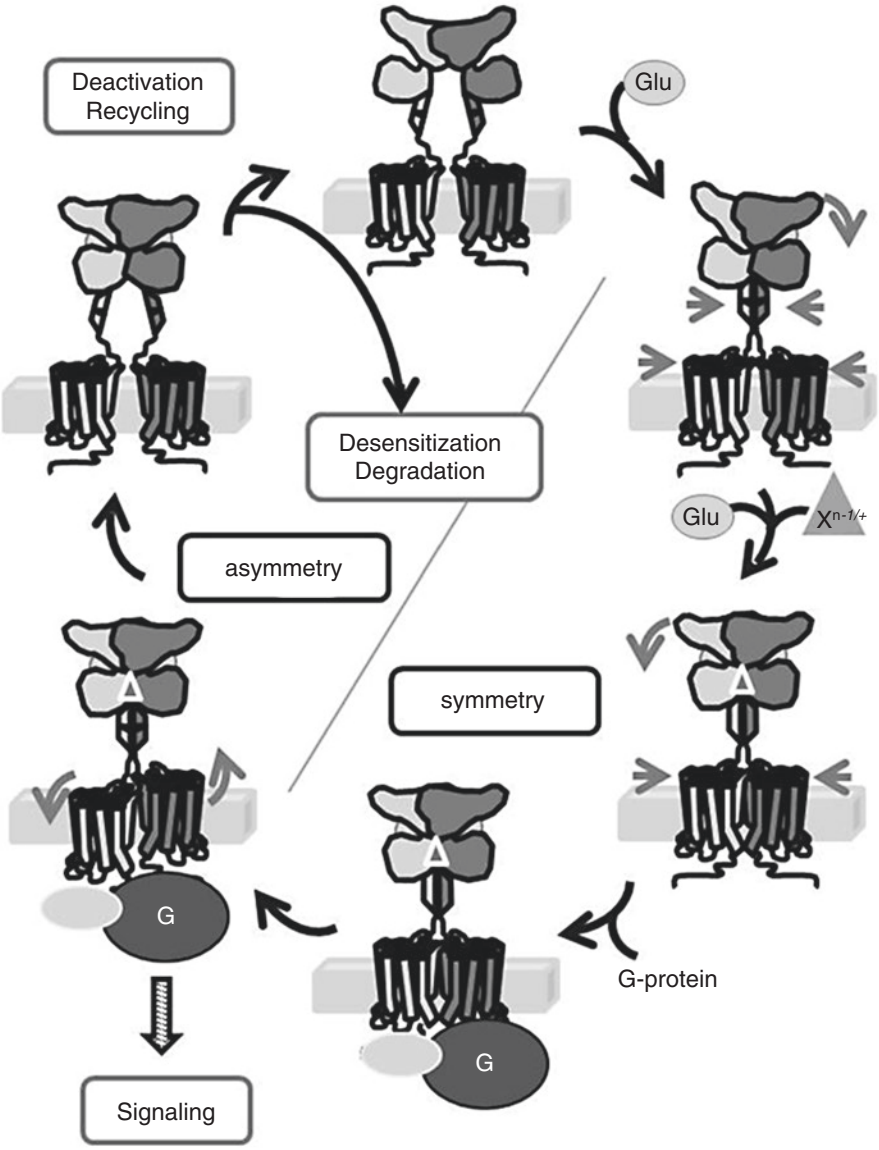


Fig. 13.8 Proposed mechanism of activation of the mGlu receptor. Binding of a single glutamate causes large conformational changes within ECDs leading to reciprocal movement of the two TMDs. Closure of the second LBD probably causes further reorganization, triggering the change in conformation within a whole dimer. In contrast to symmetrical action of ECDs and fast movement of the two HDs toward each other, only a single subunit, turned on at a time, activates a G-protein and triggers the signaling cascade leading to appropriate intracellular response. Which HD will be turned on depends probably on the position of the G-protein

masked by the dominant dendritic signal of mGluR1a, which in turn leads to the targeting of the mGluR1b to the distal parts of dendrites close to the postsynaptic density but accompanied with mGluR1a. Interestingly enough, the heteromeric formation of mGluR1a/b is reminiscent of that of $GABA_B$ receptor heterodimerization that is described in corresponding chapter. Briefly, GB2 subunit bears a similar motive of basic residues causing its retention within the Endoplasmic reticulum until it is released by a neutralization motive within the GB1 subunit. In mGluR1b, the retention within the ER is neutralized by the long C-tail of mGluR1a and the dimer is able to be transported from the ER to the plasma membrane. In one of our studies we showed that C-tails of mGluR1a and mGluR1b swapped with those in $GABA_B$ also lead to the heterodimerization of such recombinant subunits [79].

13.3.2 Heterodimers Between Different mGluRs

Because the expression of different receptor subtypes is often overlapping and the receptors of one group have similar dimerization features, one would expect that more or less related receptors would form dimers or higher ordered oligomers. E.g. mGluR1 and 5 are found on postsynaptic membranes in hippocampal neurons of CA1 zone where they play a crucial role in establishing LTP and LTD. Similarly, the expression of mGluR2 and mGluR3 overlaps in hippocampus and amygdala. Overlapping expression of the two receptors (mGluR1 and 5) in the retina suggested their potential functional interaction. On contrary, group III mGluRs appear to have a distinct distribution in the retina suggesting variety of modulation by mGluR 4, 7 and 8 in the visual system (for review see [109]).

An elegant study using time-resolved FRET analysis revealed the formation of functional heterodimers in cells co-expressing mGluR2 and 4. Additional analysis proved possible heterodimerization of receptors from the same group or alternatively between group II and III. The physiological impact of such dimers is yet to be discovered. Some preliminary electrophysiological studies indicate that such heterodimers might have distinct pharmacological properties than those of the different homodimers [78].

13.3.3 Heteromerization of mGluRs with Other Class C GPCRs

An intriguing observation of mGluR1a:CaSR heterodimers raised the question of physiological mechanism for the formation of such complexes in cells as well as its functional impacts. Both receptors co-localize in cerebellum and hippocampus and CaSR co-immunoprecipitates with mGluR1a from cells co-expressing both receptors [76, 110]. Alike mGluR or CaSR homodimers their heterodimers are also

held together by disulphide bonds [111]. Although the mechanism of triggering an alternative gathering is not yet clear, the homodimeric *versus* heterodimeric composition might respond to the extracellular concentrations of glutamate and/or calcium during neuronal activity.

13.3.4 Heteromerization of mGluRs with Other GPCRs

GPCRs from different classes may form complexes with mGluRs. It was reported that mGluR2 dimer forms complex with 5-HT2A-serotonin that triggers specific cellular responses upon an application of hallucinogenic drugs [83]. Similarly, using combined $BRET^2$ and FRET techniques (SRET) and a bimolecular complementation assay it was proved that mGluR5 is in close proximity to adenosine 2A and dopamine D2 receptor ($A_{2A}R$, D2R) forming higher ordered oligomers in a heterologous system. Moreover, these receptors co-localize on postsynaptic membranes of GABAergic striatopallidal neurons and the three receptors immunoprecipitate from the striatal lysates suggesting their possible direct interaction *in vivo* [112].

As reported previously, the presence of mGluR5 in close proximity might enhance the affinity of $A_{2A}R$ to adenosine [113]. These findings support studies showing close relationship between the synergistic stimulation of mGluR5 and $A_{2A}R$ and the inhibition of D2R-mediated motor activity [114] that is crucial for disclosing a proper pharmacological treatment of neuropsychiatric disorders such as Parkinson's disease [115].

13.4 Conclusion: Why Dimeric Structure of mGluRs?

If one subunit of mGluRs is able to activate a G-protein, what is the rationale for an existence of dimers of class C GPCRs? In some receptors, the asymmetry is given by a composition of the receptor dimer, like in case of the $GABA_B$ receptor, in which one subunit binds the agonists, and the other activates G-proteins. It remains to be explored what mechanism is responsible for the asymmetric functioning of a homodimeric receptor. It was reported that a single heterotrimeric G-protein interacts with a GPCR dimer [116]. Because the surface interface of the receptor dimer and a G-protein fit, it is likely that one HD will interact with the α-subunit, whereas the second HD will interact with the βγ complex [84]. It may well be, that such an asymmetric activation of HDs in a dimer is directed by the asymmetry of heterotrimeric G-proteins on one side and by the negative cooperativity of the ligand binding, as discussed above, on the other side.

Taken together, a large scissor movement within a dimeric ligand binding domain upon agonist binding involving either LB1-LB2 or LB2-LB2 domain movements, triggers series of consecutive conformational changes within CRDs that are transferred on a relative movement between the HDs, that in turn leads to an activation of

a single HD. We may speculate that fusion of the VFT and HD was phylogeneticaly selected as the best scenario with the result of construction of a receptor with two important features. These are binding of a small ligand within the VFTs by a receptor that is capable of activating, G-proteins aby a single HD, and/or other signaling pathways. The mechanism of transduction of the detected ligand binding then needs to reflect the conformational changes of the VFTs on the HDs, and again, from other receptors systems it is known that dimerization allows reflection of the activation by merely changing the relative position of the protomers. In this regard, the initial phase of the activation mechanism of Class C GPCR is reminiscent of that of receptors with kinase activity and the latest phase adopts the features of the Class A, or B GPCRs.

Dimerization of the distinct splice variants of mGluR1 or heterodimerization between mGluRs results in the receptors with unique features, even with the theoretical possibility of dual signaling by a set of such heterodimers.

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Part III
Assembly and Trafficking

Chapter 14
The Monomer/Homodimer Equilibrium of G Protein-Coupled Receptors: Formation in the Secretory Pathway and Potential Functional Significance

Arthur Gibert, Martin Lehmann, Burkhard Wiesner, and Ralf Schülein

Abstract In the last years, it became clear that the majority of the G protein-coupled receptors seem to be expressed not as exclusive monomers or homodimers, but in a monomer/homodimer equilibrium. Strikingly, monomers and homodimers of a specific receptor seem to interconvert dynamically. It is unclear at the moment, however, whether this equilibrium may undergo changes during receptor lifetime, how it is regulated and whether it has any functional significance. It was previously shown that G protein-coupled receptor homodimerization takes place in the endoplasmic reticulum and first data show that this may also hold true for equilibrium formation. This review addresses the limited available data for the monomer/homodimer equilibrium of these receptors. It also summarizes modern imaging methods which are useful to study these transient interactions at the plasma membrane and also in intracellular compartments.

Keywords G protein-coupled receptor • Dimer • Monomer/dimer equilibrium • FCCS • smTIRF • FRET

Abbreviations

2D-K_D	2D equilibrium dissociation constant
β_1-AR	β_1 adrenergic receptors
α_{1b}-AR	α_{1b} adrenergic receptor

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$A_{2A}R$	Adenosine 2A receptor
AT_2R	Angiotensin II type 2 receptor
B_2R	Bradykinin B2 receptor
CRF_1R	Corticotropin-releasing factor receptor type 1
D_1R	Dopamine D1 receptor
ECFP	Enhanced Cyan fluorescent protein
ER	Endoplasmic reticulum
ET_AR	Endothelin A receptor
EYFP	Enhanced Yellow fluorescent protein
FCCS	Fluorescence cross correlation spectroscopy
FCS	Fluorescence correlation spectroscopy
FPR	N-formyl peptide receptor
FRET	Fluorescence Resonance Energy Transfer
$GABA_BR$	γ-Aminobutyric acid receptor type B
GFP	Green fluorescent protein
GNRHR	Gonadotropin-releasing hormone receptor
GPCRs	G protein coupled receptors
ka	Constant rate for dimer formation
kd	Constant rate for dimer dissociation
LHR	Luteinizing hormone receptor
M/D	Monomer/homodimer equilibrium
M2	Muscarinic acetylcholine receptor M2
PAR1	Protease-activated receptor 1
smTIRF	Single molecule total internal reflection fluorescence microscopy
TSHR	Thyroid stimulating hormone receptor
V_2R	Vasopressin 2 receptor

14.1 Introduction

G protein coupled receptors (GPCRs) represent the largest protein family in eukaryotic cells and are the most important drug targets. In the last decades, cloning of these receptors and more recently the determination of their three-dimensional structure has revolutionized molecular pharmacology. In the beginning, it was thought that these receptors are expressed as monomers. Indeed it was shown initially for rhodopsin that a GPCR monomer represents the smallest functional unit which is able to induce G protein-coupling, desensitization, arrestin binding and signal transduction [1–3]. It was discovered along the years, however, that the vast majority of the receptors may form higher order structural states which are of physiological and pharmacological significance [4–6]. Specific receptor monomers may interact to form homodimers or higher order homomers. The formation of heteromers was also described. Whereas it seems to be clear that these GPCR interactions take place and are of functional relevance, there are many uncertainties concerning even very basic questions in this field:

1. What are the functional differences between GPCR monomers and their oligomeric states?

The vast majority of GPCRs are able to form homodimers and this interaction may influence various receptor properties. At this functional level, however, it is currently not comprehensively understood, what distinguishes GPCR monomer from a homodimer in terms of signaling modulation (e.g. G protein selectivity, ratio of receptors/G proteins involved, desensitization/internalization behavior). This also holds true for heteromeric interactions.

2. Which receptor domains mediate the formation of homomers?

Homodimerization of GPCRs involving different interfaces in the intracellular loops (ICL) transmembrane domain helices (TMH), e.g. at the region ICL2-TMH4 [7–11], TMH5–6 [12, 13], TMH4–5 [14] or at the N-terminal part [15] have been reported. If we assume that homodimerization (x−x) of a specific GPCR occurs via a defined interface, it is conceivable that the formation of higher order homomers (such as x−x/x or x−x/x−x constellations) occurs via different interfaces which are, however, unknown.

3. Which receptor domains mediate the formation of heteromers?

Similar to the situation described above, it is unclear whether heterodimerization of GPCR monomers (x and y) occurs via the same interaction interfaces as in homodimers or at different contact points. In both cases, the formation of x−y heterodimers will be observed. If different contact points are used, however, higher order heteromer constellations such as x−x/y−y or even x−x/y are possible. At the moment, the nature of the oligomeric state of GPCR heteromers is not comprehensively understood.

4. What is the functional significance of the monomer/homodimer equilibrium (M/D) of GPCRs?

It has been previously described that GPCRs may be expressed as a mixture of monomers and homodimers and that the two forms interconvert dynamically [16–20]. Such a concomitant presence of monomers and homodimers in a mixture did not attract much attention so far. It should be stressed, however, that these results challenge many findings, e.g. the interpretation of concentration response curves of GPCRs in cases where the homodimeric state influences G protein selectivity: since a mixture of monomers and homodimers is measured, the contributions of the monomers and dimers to second messenger formation are usually not clear. Moreover, this raises the question whether the M/D itself has any functional significance.

In the past decade, much progress was made to detect the individual oligomeric states of GPCRs using confocal laser scanning microscopy and quantitative methods to measure the M/D of GPCRs are also available. The main experimental obstacle to answer many of the above raised questions is, however, to express the individual oligomeric states of the GPCRs independently and to analyze their

functional and interaction properties. To this end, forced GPCR monomerization as well as forced homodimerization and heterodimerization are needed.

In this review we focus on the M/D of GPCRs. In the first part, we will summarize the available data addressing this equilibrium, its potential functional significance and its establishment during receptor transport through the secretory pathway. The second part will deal with modern imaging methods allowing not only to monitor GPCR interactions but also to quantify the M/D. This methodological section will be completed in the third part by discussing methods to obtain forced monomers and homodimers of GPCRS.

14.2 Detection and Functional Analysis of the M/D of GPCRs

The concept of the M/D of GPCRs is relatively new and has not yet attracted much attention with just a few publications touching upon this topic. This is somewhat surprising since these findings could challenge many of the findings described for GPCRs, in particular those where specific functions of the dimers were described. Indeed, most of the previous functional studies have been performed without taking a ratio of monomers and dimers into account making it difficult to rate the contributions of each form. To date a potential functional significance of the M/D remains unclear. Whereas many studies addressed the detection of the individual oligomeric forms of GPCRs, even at a single molecule level [21], monomer/homodimer ratios were calculated only in a handful of papers. The work for these GPCRs will be summarized in the following section.

14.2.1 Muscarinic Acetylcholine Receptor M1

Previous research on muscarinic receptors postulated that the muscarinic acetylcholine receptor M2 (M_2 receptor) forms oligomers [22], that the M_3 receptor forms covalent dimers [23] and that the M_1, M_2 and M_3 receptors are predominantly present as constitutive high affinity homo-dimers [24]. In the same study, heterodimers between the subtypes of the muscarinic acetylcholine receptors (M_1/M_2, M_2/M_3 and M_1/M_3) were observed. They were, however, only barely detectable, because their affinity was much lower than those of the homodimers [24].

The first analysis of a monomer/homodimer ratio of a GPCR was performed in 2010 for the M_1 receptor using single molecule total internal reflection fluorescence microscopy measurements (smTIRF; see also Sect. 13.3) [17]. In this study, it was not only shown that the M_1 receptor is expressed in a monomer/homodimer ratio at the plasma membrane but also that the interactions of the individual forms are transient meaning that monomers and homodimers interconvert dynamically. The half-

life of the M_1 receptor homodimer was calculated to be about 0.5 sec. at 23 °C and no evidence was obtained for the presence of higher order oligomers. Of note, only about 30% of the total M_1 receptors formed homodimers at the plasma membrane. This pilot study for the M1 receptor addressed for the first time the problem that the qualitative detection of GPCR dimers for a specific receptor is not enough. If monomers are present concomitantly, functional data should be taken with care as long as the functional properties of the oligomeric forms could not be differentiated experimentally. This is even more the case when receptors are measured in an overexpressing cellular system since more homodimers may be present under these conditions.

14.2.2 N-Formyl Peptide Receptor

Later on, similar observations were made for the N-formyl peptide receptor (FPR) again using smTIRF measurements [18]. The applied technique allowed not only the calculation of the M/D of this receptor, but also the 2D equilibrium dissociation constant (2D-K_D) and the constant rate for dimer formation (monomer association k_a) and homodimer dissociation (k_d). It was shown that the amount of FPR dimers at 37 °C = 41% which is the result of a 2D-K_D of 3.6 copies/μm^2 with a homodimer life time of 91 ms (k_d of 11.0 s^{-1}). Monomers are converted into homodimers every 150 ms (k_a of 3.1 [copies/μm^2]$^{-1}$ s^{-1}. Of note, ligand binding and receptor activation did not change the monomer/dimer ratio of the FPR which is consistent with other results (see below).

14.2.3 Beta Adrenergic Receptors

smTIRF measurements with SNAP-tagged receptors were performed to analyze the transient homodimerization of beta adrenergic receptors, namely β_1-AR and the β_2-AR [16]. Of note, the lifetime of both homodimers was about 4 s which is approximately 40 times longer than that of the FPR [18] and 6 times longer in comparison to the M1 receptor [17]. These results suggest that the lifetime of the homodimers of the various GPCRs may differ substantially. However, experiments were carried out at 20.5 °C in the case of the β_1-AR and β_2-AR but at 23 °C and 37 °C in the case of the M1 receptor and the FPR respectively. It is not excluded that these temperature differences also influence homodimer lifetimes. Interestingly, the monomer/homodimer ratios of the β_1-AR and β_2-AR were different although these receptors are homologous. In the case of the β_1-AR, 30% dimers were present whereas about 60% dimers were found for the β_2-AR. It could also be shown that receptor densities influenced the oligomeric state. Whereas the amount of homodimers of the β_1-AR was 30% at low densities (0.15–0.3 particles/μm^2) it outwent the monomer fractions at highest particle densities. Under the latter conditions, a small amount of tri- and

tetramers could also be observed. The 60% dimers found in the case of the β_2-AR at low densities transferred to a mixture of dimers (50%), trimers (30%) and tetramers (15%) at highest densities. Neither activation of the β_1-AR nor that of the β_2-AR influenced the oligomeric states of the receptors. These results are consistent with those obtained for the FPR [18]. Of note, the results for the β_2-AR are controversy since a recent study using smTIRF microscopy failed to detect homodimers suggesting that the receptor is expressed almost exclusively as a monomer [25]. The very different experimental setups may contribute to this discrepancy (intensity analysis vs. two-color incidence; live vs. fixed cells; stable vs. transient transfection; N-terminal vs. C-terminal labeling [25]. In contrast, in a fluorescence correlation spectroscopy (FCS) analysis using photocounting histogram, it was postulated that the β_2-AR is expressed almost exclusively as a homodimer [26].

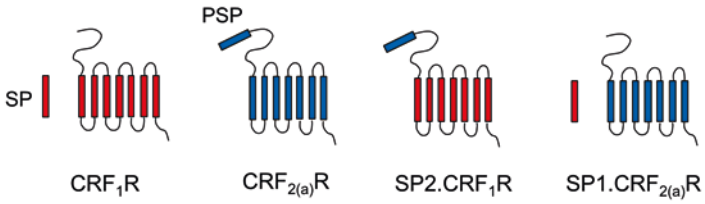
14.2.4 Corticotropin-Releasing Factor Receptors

Whereas the quantitative analysis of the M/D of GPCRs is now well established, it is completely unclear whether expression of a receptor in such a mixture has any functional significance. In the case of the corticotropin releasing factor receptor type 1 (CRF_1R) some recently published results pointed towards this direction. Analysis of the M/D of the CRF_1R by fluorescence cross correlation spectroscopy (FCCS) measurements at the plasma membrane showed that at 22% of the receptors form homodimers at 23 °C [20]. Similar results were obtained in this study using smTIRF experiments (29%). The authors used the FCCS technique to determine the M/D of other GPCRs, namely that of the protease-activated receptor 1 (PAR1), the endothelin B receptor (ET_BR), the vasopressin V2 receptor (V_2R), the luteinizing hormone/choriogonadotropin receptor (LHCGR) and the thyroid stimulating receptor (TSHR). The amount of homodimers of the latter receptors at the plasma membrane ranged from 7% (TSHR) up to 17% luteinizing hormone receptor (LHR). The advantage of the FCCS methodology is that it can also be used in intracellular compartments. It was shown that the M/D of the CRF_1R is established already in the ER and seems to be constant throughout the receptor's life cycle [20]. A similarly stable M/D at the plasma membrane and in the ER was also observed for the ET_BR in the same study. Of note, the equilibrium of the CRF_1R remained stable following receptor activation which is consistent with the results for the FPR and the adrenergic receptors (see above).

In the case of the CRF receptors, the functional significance of the individual oligomeric states was analyzed in detail and these studies are also helpful to derive hypotheses of the functional significance of the equilibrium, at least for the CRF receptors. It was shown that the $CRF_{2(a)}R$ subtype of the CRF receptors is expressed as an exclusive monomer due to the presence of a unique N-terminal domain, the so called pseudo signal peptide [27, 28]. Signal peptides mediate the first step of the intracellular transport of GPCRs, their insertion into the membrane of the

endoplasmic reticulum (ER) via the translocon complex [29]. Signal peptides are then cleaved-off from the mature receptor. In the case of the $CRF_{2(a)}R$, however, the signal peptide remains uncleaved and thereby prevents homodimerization of the receptor, most likely by the presence of a bulky glycosylation in the pseudo signal peptide [28]. In contrast to the $CRF_{2(a)}R$, the CRF_1R has a conventional and cleaved signal peptide and is able to form homodimers [28]. It could also be shown that the oligomeric state of the CRF receptor subtypes influences their signal transduction properties. Whereas the monomeric $CRF_{2(a)}R$ couples only Gs, the dimer forming CRF_1R is able to couple to Gs and, at higher occupancy, also to Gi leading to a bell-shaped concentration response curve for cAMP formation [28, 30]. All these properties could be transferred in signal peptide swap experiments: a CRF_1R containing the pseudo signal peptide of the $CRF_{2(a)}R$ was expressed exclusively as a monomer and was only able to couple Gs, a $CRF_{2(a)}R$ carrying the conventional signal peptide of the CRF_1R was able to form homodimers and to couple to both Gs and Gi (see Fig. 14.1 for a summary) [28]. The reason why a CRF receptor monomer is only able to couple to Gs is not understood. It can be speculated that binding of one ligand molecule to a monomer only enables Gs coupling whereas binding of two ligand molecules to a homodimer leads to an alternative conformation enabling both Gs and Gi interactions. A similar model was proposed for the TSHR [31]. Here, occupancy of both sites of the TSHR oligomer was necessary for coupling to Gs and Gq; occupancy of one side enabled only Gs coupling.

It should be stressed, however, that the properties of forced CRF_1R monomers were only compared as yet to receptors expressed in a M/D. The bell-shaped concentration response curve under these conditions may not only result from a situation where the monomer couples exclusively Gs and the homodimer in the mixture



Dimer formation	Homodimer	Monomer	Monomer	Homodimer
M/D equilibrium	yes	no	no	yes
G protein selectivity	Gs > Gi	Gs	Gs	Gs > Gi

Fig. 14.1 Summary of the available results for the dimerization of the CRF_1R and the $CRF_{2(a)}R$. The CRF_1R (*red*) possesses a conventional cleaved signal peptide (SP) and is able to form homodimers which are expressed with monomers in an equilibrium. It couples to Gs and Gi. The $CRF_{2(a)}R$ (*blue*) contains an uncleaved pseudo signal peptide (PSP) and is expressed exclusively as a monomer. It couples only to Gs [28]. All these properties were transferable in signal peptide swap experiments (constructs PSP-CRF_1R and SP-$CRF_{2(a)}R$)

both Gs and Gi. It may also be formed when the monomer couples exclusively Gs and the homodimer exclusively Gi. A forced homodimer of the CRF_1R is needed to address this question unambiguously. In any case, monomers and homodimers of the CRF_1R have different G protein coupling properties (Gs vs Gs/Gi or Gs vs Gi). Coupling to Gi may represent a mechanism to prevent an overshoot in the cAMP response at high occupancy. If so, it is likely that the position of the M/D is of functional significance: the more homodimers are present in the mixture, the easier this inhibition could be achieved.

14.2.5 Open Questions for the M/D of GPCRs

It is conceivable that the potential functional significance of the M/D of GPCRs can only be studied properly when the properties of forced monomers and forced homodimers were compared with those of receptors expressed in an equilibrium. This is experimentally challenging and some recent progress in this field will be summarized in the third part of this review. Several other questions for the M/D of GPCRs must be addressed in future studies:

(i) The observed amount of homodimers in the equilibrium is relatively low ranging from 7% for the TSHR [28] up to 60% for the β_2-AR [17]. Both smTIRFM and FCCS analyses used so far are single molecule techniques and cells with low receptor expression were consequently chosen for these kinds of experiments. Although this may converge to endogenous receptor expression, the influence of the receptor expression levels to the position of the M/D should be addressed in more detail. The position of the equilibrium may follow the law of mass order if free two-dimensional diffusion and unhampered interactions occur. In this case, 2D–KD values could be calculated out of the receptor densities. However, diffusion and interactions may be hindered due to unknown protein factors/scaffolds. Receptor titration experiments are needed to obtain at least an idea of whether a simple model of free diffusion/interaction applies. This is, however, difficult to address experimentally since higher receptor expression levels interfere with the applicability of the single molecule techniques.

(ii) Following activation by specific ligands, the monomer/homodimer ratio of all studied GPCRs remained stable (FPR, β_1-AR, β_2-AR, CRF_1R, see above). A constant equilibrium at a given receptor number may thus be a general feature of GPCRs. It should be addressed in future studies whether this holds true, in particular by using GPCRs for which older experiments suggested an influence of e.g. ligand binding on the dimeric state.

(iii) To date, only studies addressing the equilibrium of monomers and homodimers were performed but many GPCRs form heterodimers, too. It is conceivable that heterodimers are also present in an equilibrium, but in this case, the situation is more complex since monomers, homo-and heterodimers are present

in a mixture. In the case of the $CRF_{2(a)}R$, it was recently shown that this receptor, which is unable to form homodimers, is still able to form heterodimers with the dopamine D1 receptor [32]. These results indicate that the interfaces of homo- and heterodimerization are different which makes the situation even more complex. Although experimentally challenging, it would be interesting to see if and how the M/D of a GPCR changes if the possibility was given to form heterodimers, too.

(iv) The use of FCCS measurements paved the way to analyze also the M/D of GPCRs in intracellular compartments, too. In the case of the CRF_1R it was shown, that the monomer/homodimer ratio is similar in comparison to the situation at the plasma membrane [33]. Numerous reports have shown that dimerization of GPCRs takes place in the ER, i.e. immediately after synthesis of the receptors. In fact, dimerization in the ER was shown to be the prerequisite for efficient trafficking in some cases such as the γ-aminobutyric acid receptor type B ($GABA_BR$) [34]. Similar results were obtained e.g. for the α_{1b} adrenergic receptor (α_{1b}-AR) [35], where homodimerization promotes ER exit and for the α_{1D} adrenergic receptor (α_{1D}-AR) [36–42], where heterodimerization with the α_{1B}-AR was necessary for efficient transport [42]. Interactions leading to a decreased rather than to a facilitated ER exit of GPCRs were also described, e.g. for the dopamine receptor 3 [43, 44], the V_2R [45], and the gonadotropin-releasing hormone receptor (GNRHR) [46].

Taking these and many other reports into account, it is not surprising that the M/D is also formed in the ER. However, the finding that the equilibrium of the CRF_1R does not differ between the ER and the plasma membrane is not a matter of course. After integration into the ER membrane, folding of GPCRs is facilitated by molecular chaperones. The lectin chaperones calnexin/calreticulin play an important role in case of the frequently N-glycosylated GPCRs, and also for the CRF_1R [27]. Most likely, chaperone-assisted folding engages monomeric rather than dimeric GPCRs in the beginning and a higher monomer/homodimer ratio may thus be expected in the ER. However, the CRF_1R may fold very quickly leading to a non-observance of this phenomenon. In future studies, GPCRs with a slower folding dynamics should be considered when measuring the M/D in the ER. On the other hand, the result that the equilibrium of the ET_BR did also not differ substantially between the ER and plasma membrane [20] indicates that such a constant ratio may be a more general feature of GPCRs.

14.3 Optical Methods to Analyze GPCR Dimerization and Their Monomer/Dimer Equilibrium

The use of genetically encoded fluorescent fusion proteins, such as the green fluorescent protein (GFP) and related molecules [47, 48] have facilitated microscopical studies of GPCRs *in vitro*, in particular in live cells. Moreover, new protein labeling

techniques using small organic fluorophores and semiconductor nanocrystals became available [48, 49]. Both fluorescent protein fusions and small organic fluorophore have revolutionized the analysis of GPCR dimerization and most recently also led to the development of methods allowing the quantification of the monomer/dimer equilibrium of GPCRs. These optical methods will be briefly summarized in the second part of this review.

14.3.1 Fluorescence Resonance Energy Transfer (FRET)

Non-radiative energy transfer between two light-sensitive chromophores was already described in 1948 by Theodor Förster [50]. FRET could be detected between two fluorescent nanoparticles or molecules. Excitation energy of the donor fluorophore is not transferred as a photon to the acceptor fluorophore. Instead a dipole-dipole coupling mechanism is used (singulett-singulett transmission). During this process, the donor fluorophore reaches its ground state (S_0) without radiation while the acceptor fluorophore can transmit the absorbed energy in form of fluorescence. Figure 14.2 summarizes this process of non-radiative energy transfer schematically. The quantum yield of the energy transfer transition, the FRET efficiency, can be used to measure protein-protein interactions. Specifically, the FRET efficiency depends on the following parameters:

- The distance between the donor and the acceptor must be in a range of 1–10 nm.
- The spectral overlap of the donor emission spectrum and the acceptor absorption spectrum must be typically larger than 30%.

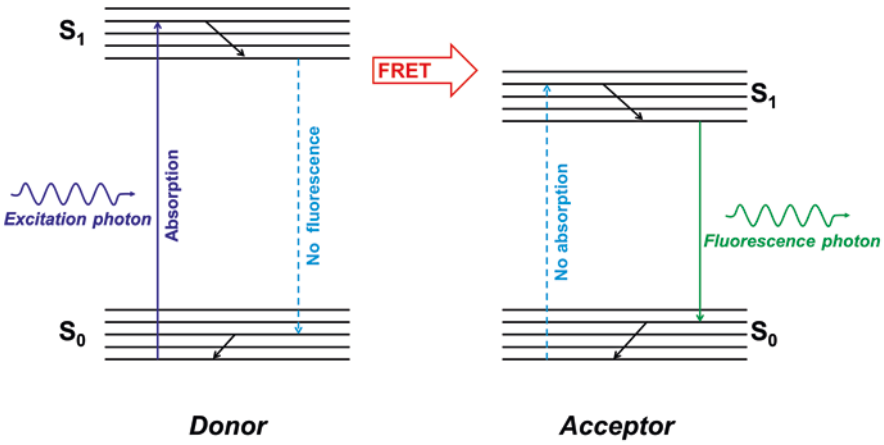


Fig. 14.2 Jablonski energy diagram illustrating the non-radiative energy transfer between two light-sensitive chromophores. Following donor excitation, energy is transferred in a singulett-singulett transmission from the donor to the acceptor. The donor fluorophore thereby reaches its ground state the acceptor fluorophore is excited and reaches its ground state by emitting fluorescence light

- The fluorescence quantum yield of the donor (Φ_D) should be greater than 0.1 and the attenuation coefficient of the acceptor (ε_A) should be greater than 10^3 L mol^{-1} cm^{-1}.
- The relative orientation of the donor and acceptor dipole moments must lie in parallel vibration planes.

The energy transfer efficiency (E) depends on the donor-acceptor separation distance (R) with an inverse 6th power law. The efficiency can be calculated via the donor fluorescence intensity in the presence and absence of the acceptor (I_{DA}, I_D) according to Eq. 14.1

$$E(\%) = \frac{1}{1+(R/R_0)^6} \times 100 = \left(1 - \frac{I_{DA}}{I_D}\right) \times 100 \tag{14.1}$$

R_0 is the Förster distance of the donor-acceptor pair, i.e. the distance at which the energy transfer efficiency is 50%.

In the case of GPCRs, very well suited fluorescent fusion proteins for photobleaching-FRET are the *enhanced cyan fluorescent protein* (ECFP) and *enhanced yellow fluorescent protein* (EYFP). Here, the Förster distance is 4.9 nm [51]. The energy transfer efficiency of ECFP and EYFP can be calculated from the spectra (e.g. from z-stacks obtained by confocal microscopy) shown in Fig. 14.3 using the spectral range of highest ECFP (436–489 nm) and lowest EYFP signals (<1%) as shown in Eq. 14.2 [28]:

$$E(\%) = \left(1 - \frac{\sum_{436}^{489} I_{DA}(\lambda)}{\sum_{436}^{489} I_D(\lambda)}\right) \times 100 \tag{14.2}$$

I_{DA} is the fluorescence intensity of ECFP (D, donor) in the presence of YFP (A, acceptor) and I_D is the fluorescence intensity of ECFP in absence of EYFP.

Dynamic protein–protein interactions lead to an increase and decrease of the FRET signal during the experiment and can usually be verified by conventional FRET experiments. In the case of static protein–protein interactions, however, it is crucial to exclude false–positive signals. This can be achieved by a photobleaching-FRET approach. Here, acceptor photobleaching causes an increase of the donor emission following the protein-protein interaction. It should be noted, however, that photobleaching may cause a significant cell damage and donor bleaching. To avoid this, a FRET-methodology could be used which calculates the increase in ECFP intensity only under the assumption of EYFP bleaching [28]. Taking the different quantum yields of ECFP and EYFP into account, the following formula (Eq. 14.3) can be used:

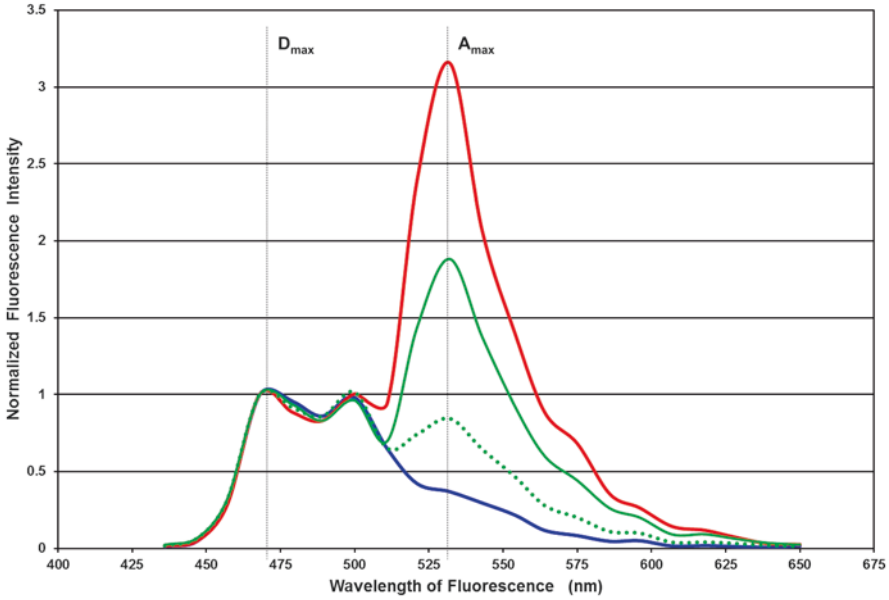


Fig. 14.3 FRET measurements of transiently transfected HEK 293 cells expressing ECFP alone (*blue curve*), or an ECFP-EYFP tandem fusion protein (*red curve*), or an ECFP-ECFP-EYFP triple fusion (*green solid line*), or an ECFP-ECFP-ECFP-EYFP quadruple fusion (*green dotted line*). Note that each donor fluorophor present in addition to the FRET pair significantly reduces FRET efficieny. Excitation wavelength = 810 nm (IR laser, two-photon technique); D_{max} = maximal donor fluorescence, A_{max} = maximal acceptor fluorescence. The curves were normalized at 468 nm (first maximum of ECFP)

$$E(\%) = \left(1 - \frac{\sum_{436}^{489} I_{DA}(\lambda)}{\sum_{436}^{489} \bar{I}_{DA}(\lambda) + \sum_{436}^{489} \bar{I}_{DA}(\lambda) \times \frac{\left[\sum_{436}^{650} \bar{I}_{DA}(\lambda) - \sum_{436}^{650} \bar{I}_{DA}(\lambda) \right] \times \frac{\phi_D}{\phi_A}}{\sum_{436}^{650} \bar{I}_{DA}(\lambda)}} \right) \times 100 \tag{14.3}$$

I_{DA} is the fluorescence intensity of ECFP in presence of EYFP, $\bar{I}_{DA}$ is the normalized fluorescence intensity of ECFP in presence of EYFP and $\bar{I}_D$ is the normalized fluorescence intensity of ECFP without EYFP. Φ_D and Φ_A are the fluorescence quantum yields of ECFP and EYFP respectively [33]. $\bar{I}$ describes the fluorescence spectra normalized to the maximal ECFP fluorescence at 468 nm.

If an approach without acceptor bleaching is used, it is necessary to assess for a direct excitation of the FRET-acceptor by the FRET-donor excitation wavelength

Table 14.1 Fluorescent fusion proteins for FRET measurements

Donor	Acceptor	Donor excitation	Acceptor emission	Reference
EBFP2	EGFP	383 nm	505 nm	[78]
ECFP	EYFP	440 nm	527 nm	[79]
Cerulean	Venus	440 nm	528 nm	[80, 81]
EGFP	EYFP	484 nm	527 nm	[82]
EGFP	DsRed	484 nm	583 nm	[79, 83]
EGFP	mCherry	484 nm	610 nm	[84]
NowGFP	mOrange	494 nm	562 nm	[85]
NowGFP	mRuby2	494 nm	559 nm	[85]
EYFP	mRFP	514 nm	607 nm	[86]
Venus	dtTomato	515 nm	581 nm	[87]
Venus	mCherry	515 nm	610 nm	[87]
Venus	mPlum	515 nm	649 nm	[88]

prior to the experiments. If such a direct acceptor excitation could not be excluded, it must be corrected in the spectrum. Otherwise, FRET efficiency will be overestimated or, in the worst case, a false-positive FRET signal may be recorded.

A critical issue with FRET experiments is the limited resolution of light microscopy. The calculated FRET efficiency represents a mean value resulting from a bundle of molecules rather than from single molecules. Of note, homodimerization of GPCRs occurs not only between the differentially labeled receptors but also between receptors tagged with the same fluorescent protein. In principle, the influence of acceptor homodimers can be eliminated by signal correction (direct excitation). An example is shown in Fig. 14.3 for ECFP and EYFP. Each donor fluorophore, which is present in addition to the FRET pair, significantly reduces the signal and consequently FRET efficiency. This influence could be used to derive a model for the M/D which could also be quantified for a specific GPCR by the help of additional controls [52]. The FRET methodology cannot distinguish dimers and higher order oligomers. Thus, a M/D could only be calculated, if no higher order oligomers were formed. The most commonly used FRET pair of fluorescent fusion proteins is ECFP and EYFP, Table 14.1 summarizes several useful alternatives.

When performed carefully, the FRET methodology is very well suited to prove GPCR interactions, predominantly on a qualitative level. The technique was used, for example, to analyze the homodimerization of the following GPCRs: endothelin A receptor (ET_AR) [53], ET_BR [53] and the CRF_1R [52]. Heterodimerization could be shown e.g. between the ET_AR and ET_BR [53], the dopamine D1 receptor (D_1R) and the dopamine D2 receptor (D_2R) [53], the angiotensin II type 2 receptor (AT_2R) and the bradykinin B2 receptor (B_2R) [54] and between the adenosine 2A receptor ($A_{2A}R$) and the D_2R [55]. Finally, FRET experiments were also used to prove that the $CRF_{2a}R$ is expressed as an exclusive monomer [28] confirming the FCCS experiments reported earlier.

14.3.2 Fluorescence Lifetime Imaging Microscopy (FLIM)

As described above, the Förster resonance energy transfer causes the transfer of the excitation energy from the donor to the acceptor in a non-radiative mechanism. The residence time of the donor fluorophore in the excitation state is thereby reduced. Measuring the fluorescence lifetime of the donor consequently also allows detection and quantification of the energy transfer efficiency and thereby the detection of protein-protein interactions. Analysis of the fluorescence lifetime is carried out using imaging procedures and fluorescence lifetime microscopy (FLIM). The fluorescence lifetime of a fluorophore is strongly dependent on the properties of its chemical environment. Analysis of the donor fluorescence lifetime in dependence of the energy transfer to the acceptor molecule is also known as FLIM-FRET.

FLIM is usually a confocal microscopy technique producing images reflecting differences in the exponential decay rates of the fluorophore fluorescence. Fluorescence lifetimes can only be determined in time slices using a pulsed laser source. The majority of the confocal microscopy setups use either pulsed IR laser (two-photon excitation, emissions lines from 780 nm to 930 nm), or a pulsed laser diode (440 nm) or a pulsed white light laser (emission lines from 470 nm to 670 nm).

The most frequently used fluorophore pair for FLIM is ECFP and EYFP; other useful combinations are listed in Table 14.1.

The photon-excited donor fluorophore will drop to the ground state through a number of different (radiative and/or non-radiative) decay pathways based on decay rates. One of these pathways must include the spontaneous emission of a photon yielding the donor fluorescence. In the complete description, the fluorescence emitted will decay with time according to Eq. 14.4:

$$I_D(t) = I_{D0} \times e^{-t/\tau_M} \tag{14.4}$$

$I_D(t)$ represents the fluorescence intensity of the donor at the time t following excitation, I_{D0} the highest fluorescence intensity at the beginning of the measurements and τ_M the fluorescence lifetime (the mean time in which the donor molecule remains in the exited state).

The fluorescence lifetime of the donor will decrease in the presence of the acceptor, due to the non-radiative energy transfer from donor to the acceptor. A few examples for fluorescence lifetime measurements are shown in Fig. 14.4. In most cases, fluorescence lifetime decrease follows a single exponential function. However, a double exponential function is also possible and can be described by Eq. 14.5:

$$I_D(t) = I_{D1} \times e^{-t/\tau_1} + I_{D2} \times e^{-t/\tau_2} \tag{14.5}$$

$I_D(t)$ represents the fluorescence intensity of the donor at the lifetime t following excitation, $I_{D1} + I_{D2}$ the highest fluorescence intensity of the first and second component and τ_1 and τ_2 the lifetimes of the first and the second exponential function.

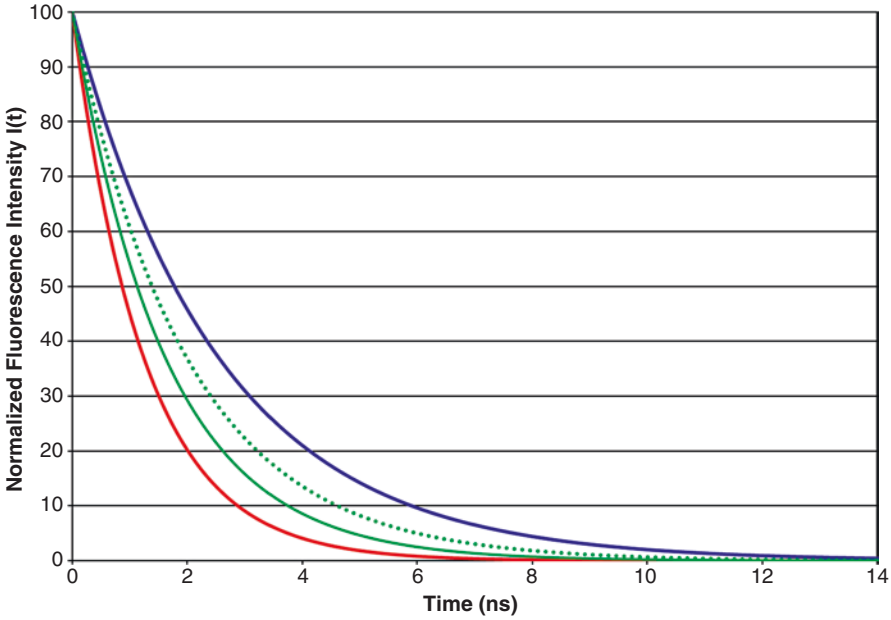


Fig. 14.4 FLIM measurements of transiently transfected HEK 293 cells expressing ECFP alone (*blue line*; τ_M = 2.57 ns) or an ECFP-EYFP tandem fusion protein (*red line*; τ_M = 1.25 ns) or an ECFP-ECP-EYFP triple fusion (*green line*; τ_M = 1.63 ns) or an ECFP-ECFP-ECFP-YFP quadruple fusion (*green dotted line*; τ_M = 2.00 ns). Fluorescence lifetimes are shown by exponential decay curves. Note that the fluorescence lifetime of the tandem fusion ECFP-EYFP is shorter (the decay is faster) than ECFP alone due to the FRET transmission caused by the proximity of the fluorophores. This effect is decreased in the presence of additional donor fluorophores (constructs ECFP-ECFP-EYFP and ECFP-ECFP-ECFP-EYFP). Excitation wavelength: 810 nm (IR laser, two-photon technique)

The donor fluorescence lifetime τ_M is calculated by Eq. 14.6 from the arithmetic mean of τ_1 and τ_2 taking the variables I_{D1} and I_{D2} into account.

$$\tau_M = \frac{\left(I_1 \times \tau_1\right) + \left(I_1 \times \tau_2\right)}{I_{D0}} \tag{14.6}$$

Analogous to FRET measurements, FLIM analyses can be used to calculate the energy transfer efficiency which serves as a parameter for protein-protein interactions [56] (Eq. 14.7).

$$E(\%) = \left(1 - \frac{\tau_{DA}}{\tau_D}\right) \times 100 \tag{14.7}$$

τ_{DA} is the mean fluorescence lifetime (τ_M) of the donor in presence of the acceptor and τ_D the mean fluorescence lifetime (τ_M) of the donor in absence of the acceptor.

The obvious advantage of the FLIM technique is the absence of problems with spectral overlap which makes acceptor bleaching redundant. Furthermore, by increasing the recording time, even weakly expressed proteins can be detected in live cells. An obvious disadvantage of FLIM is, however, that the recording time is in the range of seconds to minutes. It is thus not possible to distinguish between transient or stable interactions.

In summary, FLIM or FLIM-FRET are attractive, relatively novel method to detect GPCR interactions [57, 58]. The technique was used, for example, to analyze the homodimerization of the $A_{2A}R$ [57, 59] and the CRF_1R [28]. Heterodimerization could be shown e.g. between the D_2R and the serotonin 5-HT1A receptor (5-HT1AR) [60]. Finally, it was also shown by FLIM measurement that the $CRF_{2a}R$ is expressed as an exclusive monomer [28]. Similar to FRET measurements, the FLIM technique cannot differentiate dimers and higher order oligomers.

14.3.3 Fluorescence Correlation Spectroscopy (FCS)

Fluorescence correlation spectroscopy (FCS) allows the detection of single molecules and is a therefore a very powerful method to determine concentration, mobility, and interaction of optically labeled GPCRs or other proteins in live cells. The basis of FCS is the measurement of light fluctuations emitted by very low concentrated diffusing fluorophores [61]. These fluctuations are detected in a small confocal volume, the size of which is determined by the wavelength of the laser line and the microscopic lens. The recorded fluorescence trace alone is not very meaningful and must be transformed using an autocorrelation function $G(\tau)$ which is based on the comparison of the fluorescence intensities at time t and at time $(t + \tau)$ (Fig. 14.5). The basic formula for the correlation function is (Eq. 14.8):

$$G(\tau) = \frac{\langle \delta F(t) \rangle \times \langle \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2} \tag{14.8}$$

$\langle\rangle$ indicates the time average and $\delta F(t) = F(t) - \langle F(t) \rangle$ the fluctuations around the mean intensity.

More detailed information addressing FCS were published recently [62, 63]. By fitting the autocorrelation function (two or three dimensional), several parameters can be derived such as the number of molecules or the duration of stay of a single molecule in the confocal volume [61].

Similar to FRET or FLIM, two different fluorophores are needed for FCS measurements to study protein-protein interactions. Useful fluorophore pairs for FCS starting from an excitation wavelength of 440 nm are listed in Table 14.1. The

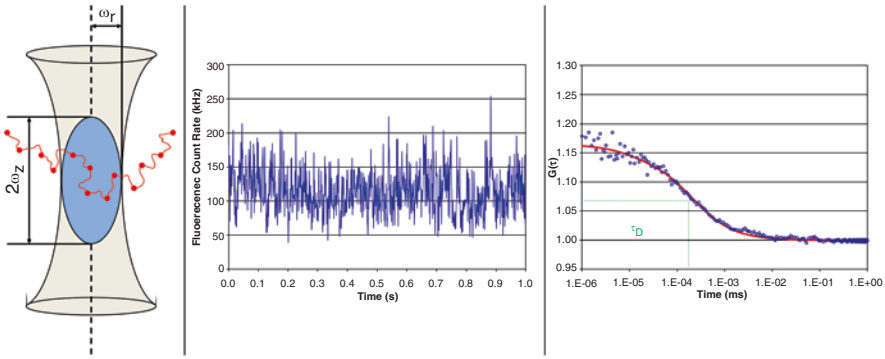


Fig. 14.5 FCS measurements of transiently transfected HEK 293 cells expressing the mCherry fluorescence protein. *Left panel:* Schematic depiction of the confocal volume size in *Z*- and *XY*–direction (*blue*). A fluorophore diffusing through the confocal volume is shown in red. *Middle panel.* Fluorescence fluctuations of EGFP in the confocal volume over time. *Right panel.* Autocorrelation function derived from fluorescence fluctuations shown in the upper panel (*blue circles*, *lower part*). Fitting of this function (*red line*) allows the calculation of the diffusion time τ_D and the number of molecules at $G(\tau)_{max}$

overlap of the emission spectra should be as low as possible to minimize crosstalk. In case of a significant crosstalk, corrections must be carried out [64].

The use of different fluorophores in FCS measurements allows to correlate the diffusion of one fluorophore (e.g. EGFP) to the other (e.g. mCherry) and to derive a cross correlation function (fluorescence cross correlation spectroscopy, FCCS). The signal of the first fluorophore at a time *t* is correlated to the signal of the second fluorophore at a time $(t + \tau)$. The cross-correlation of two fluorophores indicates their spatial association since a random co-diffusion through the small confocal volume is unlikely.

In the case of cross correlation, Eq. 14.8 [65] is transferred into the following form [64] (Eq. 14.9):

$$G_{rg}(\tau) = \frac{\langle \delta F_r(t) \rangle \times \langle \delta F_g(t+\tau) \rangle}{\langle F_r(t) \rangle \times \langle F_g(t) \rangle} \tag{14.9}$$

r and *g* are the indices for the red (e.g. mCherry) and green (e.g. EGFP) fluorescent signals respectively.

FCCS measurements for a tandem fusion of EGFP and mCherry (EGFP-mCherry; obligatory heterodimer) and co-transfected EGFP and mCherry (i.e. non-fused) are shown in Fig. 14.6. A clear cross-correlation is only seen the case of the heterodimer.

Instead of using two different fluorescent fusion proteins, a single photo-convertible fusion protein may be used for FCS experiments. In this case, 50% of the fluorophores must be photo-converted prior to the measurements to assess for cross correlation of the two signals. The usability of photo-convertible Kaede and

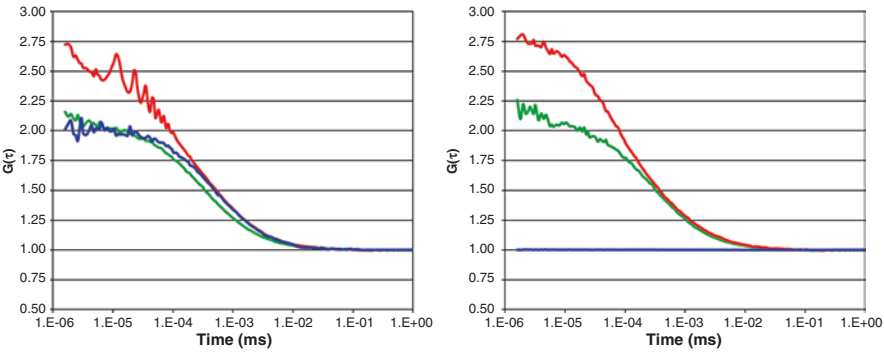


Fig. 14.6 *Left panel.* FCCS measurements of transiently transfected HEK 293 cells expressing an EGFP-mCherry tandem fusion protein. *Green* and *red lines* represent the autocorrelation curves for EGFP and mCherry respectively. The *blue line* represents the cross correlation curve. Note that cross correlation could be detected due to the proximity of the fluorophores in the fusion protein. *Right panel*. FCCS measurements of transiently transfected HEK 293 cells co-expressing non-fused EGFP and mCherry. Auto and cross correlation curves are depicted as described above. Note that no cross correlation could be detected in this case

Kikume- Green-Red fusions to study GPCR interactions in live cells by FCCS was recently demonstrated [66].

The advantage of the FCS technique is that it works with very low fluorophore concentrations which are in the range of endogenous GPCRs expression. The monomer/dimer ratio of GPCRs could be calculated accurately. Another advantage is that measurements can also be carried out in intracellular compartments such as the ER [28]. However, similar to FRET and FLIM measurements, the FCS technique cannot differentiate dimers and higher order oligomers. The technique was used, for example, to analyze the homodimerization of the following GPCRs: CRF_1R, ET_BR, V_2R, PAR1, TSHR, LHR [20, 67]. In the case of these receptors, the monomer/dimer ratio was also determined [20]. Moreover, FCS measurements were used to prove that the $CRF_{2(a)}R$ is expressed as an exclusive monomeric GPCR [20].

14.3.4 Single Molecule Total Internal Reflection Fluorescence Microscopy (smTIRF)

The total internal reflection fluorescence microscopy is a method allowing a resolution in Z-direction which is usually below 200 nm. The method relies on a light beam irradiating a glass-water transition (optical interface). If the light beam hits the transition at a shallow angle (critical angle θ), it is totally reflected. Behind the glass, in the fluid, an evanescent light field is formed, the intensity of which decreases exponentially with a total penetration depth less than 200 nm. Fluorescent molecules within this light field could be excited to emit fluorescence light if they are able to absorb the irradiated wavelength. Due to the limited dimensions of the

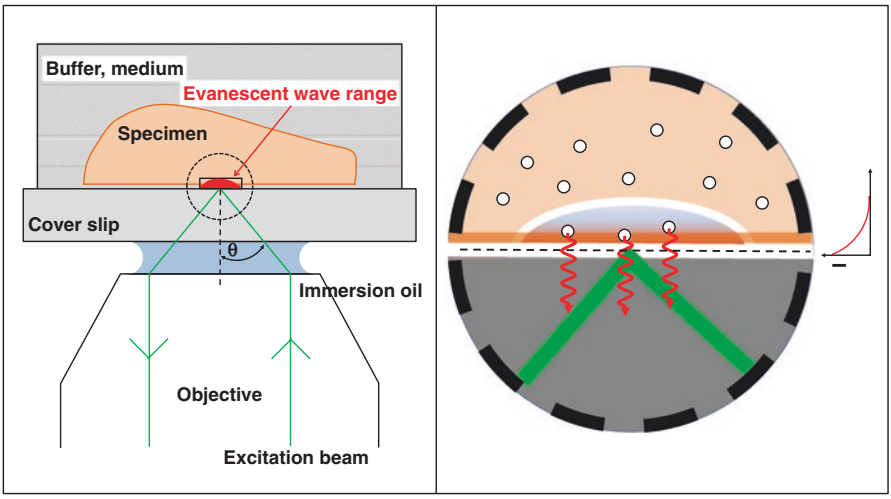


Fig. 14.7 Principle of total internal reflection fluorescence microscopy. *Left panel*. The excitation beam (*green line*) is totally reflected if it reaches the optical transition (coverslip/buffer or medium) within a critical angle θ. It creates an evanescent light field behind the glass (*red area*). *Right Panel*. Magnification of the specimen region. The intensity of the evanescent light field decreases exponentially (*right diagram*). Fluorophores (*white dots*) present in this field may be excited to emit fluorescence light

light field, detection of fluorescence signals is very accurately. The principle of the method is summarized in Fig. 14.7, detailed descriptions of this techniques and its use in cell biology were published previously [68–73].

The resolution limit of smTIRF measurements is determined by the excitation wavelength, the microscopical lens and the molecular brightness of the fluorophore. To reach a single molecule level, the following experimental strategy is recommended. First, the fluorescent protein alone (i.e. in its non-fused form) is immobilized by antibodies and photobleaching experiments are carried out leading to a stepwise decrease in the intensity of the fluorescent dots. This usually allows the determination of the fluorescence intensity representing single molecules [20, 74]. Thereafter, GPCRs or other proteins labeled with the same fluorescent fusion protein are visualized in longer time series and photobleaching experiments are repeated with these fusions. The obtained data are analyzed using a suitable software, e.g. GMimPro [75]. Knowledge of the fluorescence intensity of a single fluorescent molecule, trace analysis of the fusion proteins together with their discrete photobleaching steps then provide information on the number of monomers, dimers or oligomers of the fusion proteins present in the sample [28]. The advantage of smTIRF measurements is that monomers, dimers and higher order oligomers can be distinguished and that only one fluorophore is required. An obvious disadvantage of the technique is that measurements are limited to the plasma membrane. Figure 14.8 depicts the method schematically.

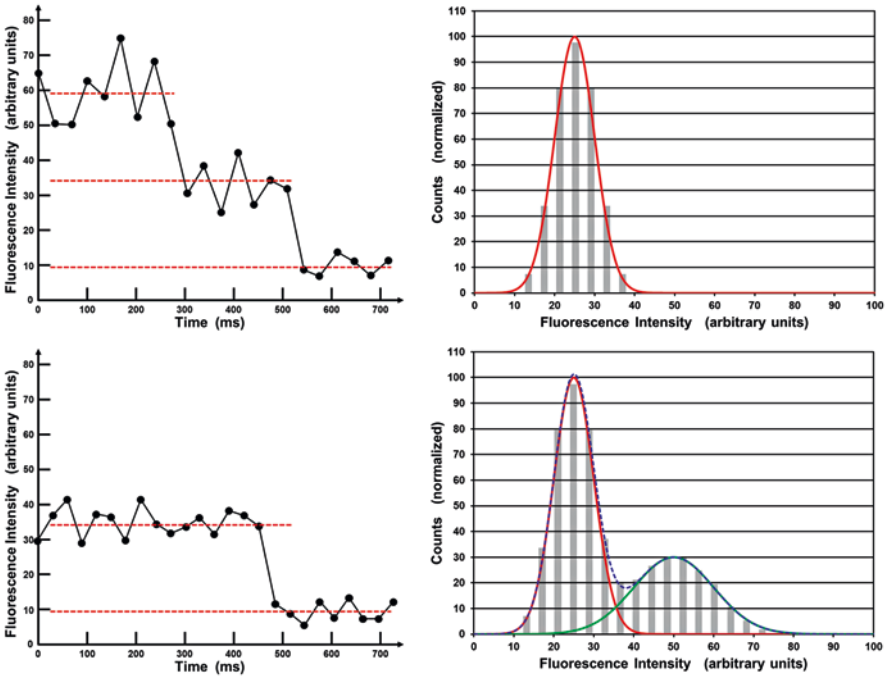


Fig. 14.8 smTIRFM measurements of transiently transfected HEK 293 cells expressing a CRF_1R.YFP fusion protein. *Left panel*. Comparison of intensity traces of moving fluorescent spots representing dimeric CRF_1R.YFP (*upper trace*) and monomeric CRF_1R.YFP (*lower trace*). Photobleaching shifts the fluorescence intensity of monomeric CRF_1R.YFP immediately to background levels. In contrast, photobleaching of the dimeric form shifts the fluorescence intensity to the monomeric form prior of reaching background levels. *Right upper panel*: Normalized fluorescence intensity distribution histogram of purified, antibody-immobilized YFP on a poly-L-lysine-coated coverslip (*upper graph*). Best fitting is achieved by a Gaussian function (*red curve*) with a mean value of 25 ± 5 a.u. *Right lower panel.* Normalized fluorescence intensity distribution histogram of CRF_1R.YFP. The best fitting is achieved by a two Gaussian function. The mean of the first component represents monomeric CRF_1R.YFP (25 ± 5 a.u.; *red curve*), the mean of the second component represents dimeric CRF_1R.YFP (50 ± 10 a.u.; *green curve*). The *blue dotted curve* represents the combination of both components. For GPCRs, monomers, dimers and higher order oligomers can be distinguished using such measurements (in the case of CRF_1R.YFP, no higher order oligomers are formed). Fluorescence intensity distribution histograms are also suitable to determine the monomer/dimer ratio of a specific GPCR

The technique was used, for example, to analyze homodimerization of the following GPCRs: CRF_1R, [20], monotropic gamma-aminobutyric acid B receptor ($GABA_BR$) [16] and the muscarinic M1 receptor (M_1) [17].

Table 14.2 Comparison of optical methods to analyze GPCR dimerization

	FRET	FLIM	FCCS	smTIRF
Number of fluorophores required	Two	Two	Two[a]	One
Analysis of intracellular compartments	Yes	Yes	Yes	No
Concurrent analysis of different cellular compartments/ regions	Yes	Yes	No	Yes[b]
Analysis of static protein-protein interactions	Yes	Yes	Yes	Yes
Analysis of dynamic protein-protein interactions	Yes	Yes[c]	Yes	No
Differentiation between dimers and oligomers	No	No	No[d]	Yes
Determination of the monomer/dimer equilibrium	Yes[e]	Yes[e]	Yes[c]	Yes

[a]One fluorophore if a photoconvertible fluorescent protein was used (50% switch) [66, 89]
[b]Limited to the membrane behind the cover slip
[c]Relatively large time frame needed
[d]Possible when using a photon counting histogram with defined calibrations parameters
[e]The presence of higher order oligomers must be excluded and expression levels of the fluorophores must be the same
[f]The presence of higher order oligomers must be excluded

14.3.5 Summary: Optical Methods to Analyze GPCR Dimerization and Their Monomer/Dimer Equilibrium

As outlined above, each of the optical methods has its specific advantages and disadvantages. It must also be taken into account that the expenses to setup the individual systems differ substantially. Whereas conventional laser scanning microscopes could be easily modified to carry out FRET and FLIM measurements, the costs to establish FCS and the smTIRF analyses are much higher. The capabilities of the individual methods are summarized in Table 14.2.

14.4 Methods to Obtain Forced Monomers and Homodimers of GPCRs

All GPCRs analyzed so far are expressed in a M/D at the plasma membrane (except for the monomeric $CRF_{2(a)}R$). These findings challenge many findings for GPCRs in particular in cases where it was proposed that dimerization alters the functional properties of the receptors. If a mixture of monomers and dimers was present, the individual contributions of the monomers and dimers to a functional effect cannot be separated. Moreover, the question raises whether the M/D itself has any functional significance.

Whereas the detection of GPCR monomers and homodimers and the quantification of their equilibrium are well established using the various optical methods described above, the functional analysis of the individual oligomeric states is experimentally still very challenging. To address a potential functional significance of the GPCR M/D, forced GPCR monomerization as well as forced homodimerization are

needed and the properties of the monomers and homodimers must be compared to the characteristics of receptors present in the equilibrium. As mentioned above, forced monomers and homodimers are also needed to analyze the interaction interfaces of GPCR homodimers and higher order homomers and to resolve the nature of the heteromeric states of GPCRs. To obtain forced GPCR monomers, some progress was made in recent years. In the case of the $CRF_{2(a)}R$, a so far unique domain at its N-terminal tail was detected, namely its pseudo signal peptide (see above), which prevents homodimerization of the receptor and causes its expression as an exclusive monomer [27–30]. The homologous CRF_1R could be monomerized by the fusion of this pseudo signal peptide, too [28]. The mechanism by which the pseudo signal peptide prevents homodimerization is not clear but it was discussed that glycosylation of this protruding N-terminal domain impairs monomer contact. It is also not known whether fusion of this pseudo signal peptide could monomerize unrelated GPCRs or whether this is unique in the CRF receptor family. Few other studies addressed homodimer-separation, mainly by specific amino acid substitutions at the protomer interfaces. In the case of the melanocortin-receptor 4 (MC4R), for example, homodimerization could be inhibited by a domain-substitution approach using the non-interacting cannabinoid-1 receptor [76].

Even less studies addressed forced dimerization of GPCRs as yet. However, in one exemplary study using the thyrotropin-releasing hormone receptor (TRHR), a chemically induced dimerization (CID) approach was used and it could be shown that receptor phosphorylation (desensitization) and receptor internalization increased under these conditions [77]. It remains to be determined whether this CID strategy will be also successful in the case of other GPCRs.

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Chapter 15
Probing Self-Assembly of G Protein-Coupled Receptor Oligomers in Membranes Using Molecular Dynamics Modeling and Experimental Approaches

Thomas P. Sakmar, Xavier Periole, and Thomas Huber

Abstract G protein-coupled receptors (GPCRs) transduce chemical signals across membranes and mediate many fundamental cellular signaling pathways. The "signalosome" is the basic signaling unit and comprises and an agonist ligand-receptor complex bound to a heterotrimeric guanine nucleotide-binding regulatory protein (G protein) in a biological membrane. Although in many cases, a monomeric GPCR is competent to transduce signals, the role of receptor dimerization and higher-order self-assembly and how receptor oligomers affect pharmacology and cellular physiology has emerged as one of the most intriguing and challenging problems in the field. Here we review recent insights gained from a multidisciplinary research approach using computational coarse grain molecular dynamics (CGMD) simulations and experiments facilitated by molecular and chemical biology approaches. One particular focus of recent work has been to define the contact sites between receptor dimers.

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Abbreviations

2-D	two-dimensional
3-D	three-dimensional
5-HT_{1A}R	serotonin$_{1A}$ receptor
A_{1A}R	adenosine A1A receptor
A_{2A}R	adenosine A2A receptor
AFM	atomic force microscopy
β_1-AR	β_1-adrenergic receptor
β_2-AR	β_2-adrenergic receptor
BC	benzylcytosine
BG	benzylguanine
BRET	bioluminescence resonance energy transfer
CGMD	coarse-grain molecular dynamics
DAFT	docking assay for TM components
DHA	docosahexaenoic acid
DOPC	1,2-dioleyl-*sn*-glycero-3-phosphocholine
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphocholine
D_2R	dopamine D2 receptor
ECL	extracellular loop
FCS	fluorescence correlation spectroscopy
FPR	*N*-formyl-peptide receptor
FRAP	fluorescence recovery after photobleaching
G protein	heterotrimeric guanine nucleotide-binding regulatory protein
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
GPCRs	G protein-coupled receptors
H	helix
ICL	intracellular loop
LHR	luteinizing hormone receptor
mGlu1	metabotropic glutamate receptor 1
meta-I	metarhodopsin I
meta-II	metarhodopsin-II
NSOM	near-field scanning optical microscopy
OR	opioid receptor
PIP	phosphatidylinositol bisphosphate

PMF	potential-of-mean-force
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
PUFA	polyunsaturated fatty acid
ROS	rod outer segment
RLuc	*Renilla* luciferase
$S1PR_1$	sphingosine 1-phosphate receptor 1
SDPC	1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine
SDPE	1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphatidylethanolamine
SM	sphingomyelin
SMLM	single-molecule localization microscopy
SPT	single-particle tracking
TM	transmembrane
US	umbrella sampling
WHAM	weighted histogram analysis method

15.1 Introduction

G protein-coupled receptors (GPCRs) mediate transmembrane (TM) signaling upon binding to a stimulatory ligand known as a pharmacological agonist. The formation of GPCR-agonist ligand complex results is a characteristic series of conformational changes known generically as receptor activation. The hallmark of GPCR activation is the outward movement and rotation of TM helices and rearrangement of stabilizing hydrogen-bond networks within the core of the receptor and also predominantly at the cytoplasmic surface. These changes in receptor conformation allow the engagement of a specific heterotrimeric guanine-nucleotide binding regulatory protein (G protein), and G protein binding actually increases agonist-ligand binding affinity, showing allosterism in GPCR-mediated signaling. Once the G protein α subunit disengages from the receptor, the agonist-bound state persists and the receptor becomes a substrate for phosphorylation by cellular GPCR receptor kinases (GRKs). A phosphorylated GPCR, sometimes in complex with G protein βγ subunits depending on the specific receptor in question, then engages with β-arrestin. The process of receptor phosphorylation, which is a requirement for pharmacological desensitization, and β-arrestin binding initiates both receptor internalization, also known as sequestration, and non-canonical signaling, including mitogen-activated protein (MAP) kinase pathways.

The degree and complexity of GPCR mediated signaling in a particular cell type depends on the expression pattern of GPCRs and downstream signaling candidates. Not all GPCRs will behave the same in different tissue types as judged by cell-based assays of cells in culture, for example. There are accepted to be 826 different GPCRs encoded in the human genome [1]. Of course, not all of these GPCRs are expressed in every cell type. However, a variety of cell types and tissues have been studied with the aim of determining GPCR cellular expression patterns, in part to validate

receptors as drug targets in tissues involved with particular disease states. A detailed discussion of this matter is beyond the scope of this chapter, but RNA-Seq methods are being used extensively to shed light on this important issue, at least in terms of mRNA levels that correspond to specific GPCR types. Cell surface expression levels are more relevant than mRNA levels to determine receptor functionality and human "protein atlas" programs are also underway to determine specific expression and association patterns of GPCRs and receptor-interacting proteins.

The complex of an agonist-bound receptor with its cognate G protein in a biological membrane bilayer has been termed a "signalosome," which can be assumed to be the basic functional TM signaling unit [2]. In fact, at least for the dim-light visual pigment, rhodopsin, which is found in the retinal rod cell, a single monomer rhodopsin is capable of activating its G protein, transducin, at a rate that is judged to be consistent with full functionality. At the same time, there is overwhelming evidence that GPCRs, including rhodopsin, form dimers and higher-order structures in membranes and that the functional activity of receptor oligomers can vary from their monomeric counterparts. Adding to the complexity is the fact that one type of GPCR can assemble into dimers and higher-order oligomers and complexes with another type of GPCR. The physical association of two (or more) types of GPCRs is termed a "heterodimer" (or "hetero-oligomer"), as opposed to a "homodimer" (or "homo-oligomer").

Quantitative understanding of the functional effects of receptor oligomerization has been limited because the techniques employed to identify and characterize receptor assembly in cell membranes have been primitive. For example, it might not be surprising that to over-express a particular GPCR in a cell type in tissue culture might lead to the appearance of homodimers of the expressed receptor. Standard site-directed mutagenesis experiments to attempt to understand the mechanism of dimerization of expressed receptors can be unsatisfactory because of a variety of potential pitfalls. To determine receptor-receptor interaction state in total cell extract might not provide a good picture of what is happening at the plasma membrane, where functional signaling assays take place. A number of novel cell-based assays have been developed or adapted over the past decade to attempt to overcome the inherent difficulties of measuring the association of receptors in bilayers, including Förster resonance energy transfer (FRET), fluorescence recovery after photobleaching (FRAP), bioluminescence resonance energy transfer (BRET), and single-molecule fluorescence methods like single-molecule tracking with green fluorescent protein (GFP)-labeled expressed receptors where diffusion rates should be proportional to mass.

The aim of this chapter is to discuss GPCR oligomerization in the context of the development of two related general approaches to enable quantitative studies of receptor interactions and their functional consequences – one computational and the other experimental. The computational modeling approach involves coarse grain molecular dynamics (CGMD) in order to study the organization membrane protein systems (see [3]). The experimental approach involves the ongoing development of chemical biology and single-molecule imaging approaches to facilitate quantitative studies of receptor-receptor interaction (see [4]).

15.2 Membrane Dynamics and Oligomerization of GPCRs

Until the past decade or so, GPCRs were described as monomeric units that could form a ternary complex with a ligand and a G protein to initiate transmembrane signaling. In line with the notion that the basic unit of signaling might consist of a monomeric GPCR, firm experimental evidence showed that either monomeric rhodopsin or monomeric β_2-AR purified and reconstituted into high-density lipoprotein particles is capable of activating G protein [5–7]. Monomeric rhodopsin solubilized in detergent micelles is also able to activate G protein at the diffusion limit [8].

Nonetheless, evidence for the existence of the assembly of GPCRs into monomers or higher order structures is overwhelming. Oligomerization of purified receptors reconstituted into lipid vesicles has been reported [9–11]. In addition, the occurrence of GPCR homo- or hetero-oligomerization has been extensively documented in a variety of biological membranes [12–15]. Also, in native tissues atomic force microscopy (AFM) revealed that rhodopsin molecules assemble into higher order structures in the native disc membrane [16, 17]. Finally, fluorescence techniques have demonstrated the oligomerization of β-adrenergic receptors on cardiac myocytes [18, 19] and oxytocin receptors in mammary tissue [20].

Whether or not there are general functional principles that underlie receptor oligomerization remains unclear, but many receptor-specific effects have been documented [21, 22]. For example, for some receptors oligomerization is a prerequisite for surface expression [23–25]. Dimerization of GPCRs has also been implicated as a regulatory mechanism for ligand binding, G protein activation, and arrestin recruitment. In this model, the stoichiometry of ligand, receptor, and G protein (or arrestin) in the tertiary signaling complex can deviate from the classic 1:1:1 ratio [26]. Promotion or inhibition of oligomerization by ligands has frequently been reported, implying the possibility of targeting the dimerization interface for therapeutic purposes [22, 27]. In addition to receptor-specific factors that drive self-assembly, it appears that GPCR oligomerization depends on the composition, thickness, and curvature of its membrane environment [9, 28].

15.3 Experimental Approaches to Probe GPCR Oligomerization

Selected experimental methods for probing GPCR oligomerization are summarized in Fig. 15.1. Most of the methods employ FRET between pairs of fluorescent probes attached to individual receptors. Compared with the methods based on fluorescently-labeled antibodies or protein tags, using fluorescent ligands for imaging GPCR oligomers has certain important advantages. First, it is possible to image endogenously expressed GPCRs in native tissue because there is no need to overexpress a modified receptor expression construct. Second, fluorescent ligands are smaller than

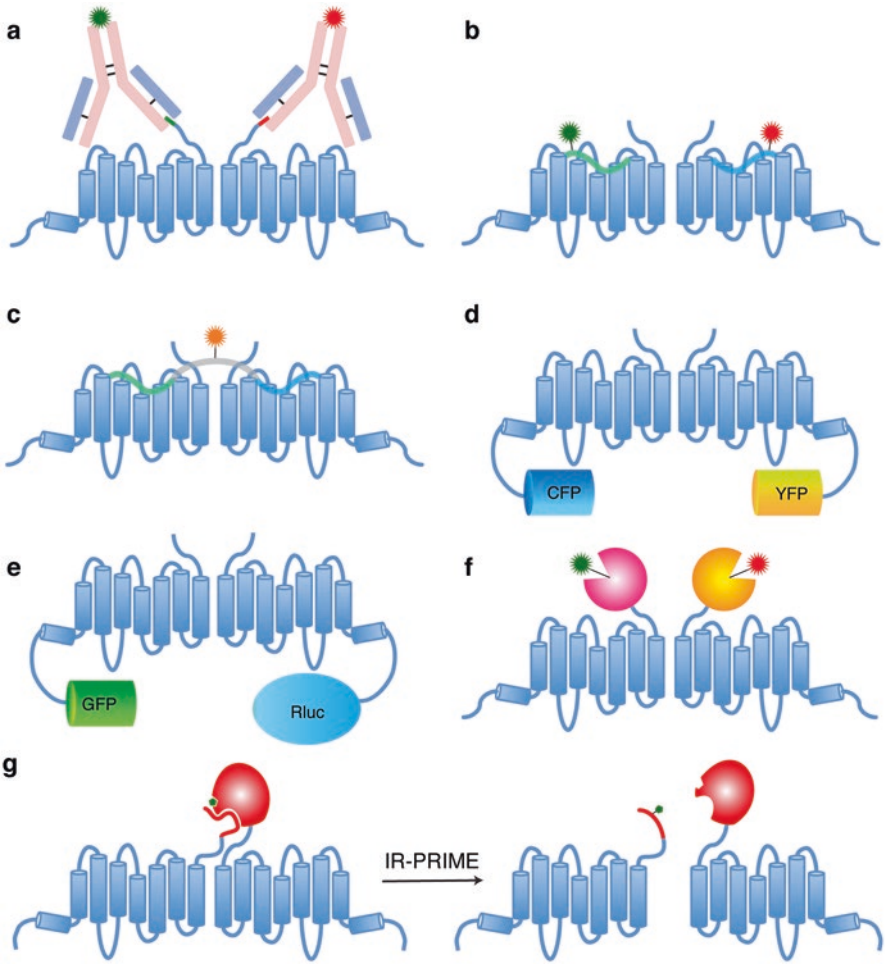

Fig. 15.1 Experimental strategies to detect GPCR dimers in living cells. (**a**) FRET between fluorescently labeled antibodies. (**b**) FRET between fluorescent ligands. (**c**) A fluorescent bivalent ligand. (**d**) FRET between fluorescent proteins. (**e**) BRET between luciferase and a fluorescent protein. (**f**) FRET between fluorophores conjugated to the orthogonal SNAP-tag and CLIP-tag. (**g**) Interaction-Dependent Probe Incorporation Mediated by Enzymes (ID-PRIME) (From Tian et al. with permission [4])

antibodies or protein tags. However, the scarcity of validated fluorescent ligands and bivalent ligands obviously limits the utility of this approach.

The use of luciferase and bioluminescence resonance energy transfer (BRET) has been a key methodological driver of studies of GPCR oligomerization. Bioluminescence resonance energy transfer exploits the non-radioactive energy transfer between the luminescence of *Renilla* luciferase (RLuc) (donor) and a fluorescent protein (acceptor) to detect intermolecular interactions [29, 30]. BRET does

not require extrinsic excitation, which eliminates some negative practical aspects of FRET such as photobleaching, cross-excitation of the acceptor, background autofluorescence, and cellular damage due to photo damage. The ratiometric nature of BRET generally enables quantitative measurement of protein-protein interaction with good reproducibility, and BRET has been used to examine receptor oligomerization in a number of systems [31–34]. BRET as well as FRET studies using a variety of tagged GPCR constructs has provided a large body of evidence suggesting that oligomerization is a fundamental aspect of the regulation of GPCR signaling [12, 13, 35, 36].

In particular, a number of chemoenzymatic receptor-tagging technologies are based on self-labeling chemistries in fusion tags that catalyze auto covalent modification. The original example of these useful self-labeling proteins is the SNAP-tag derived from the mammalian O^6-methylguanine-DNA methyltransferase, which utilizes O^6-benzylguanine (BG) derivatives as a substrate [37]. A SNAP-tag can be fused to a given GPCR and subsequently labeled with a synthetic dye linked to benzylguanine [38, 39]. Engineering of the useful O^6-methylguanine-DNA methyltransferase has yielded the CLIP-tag, which accepts benzylcytosine (BC) derivatives [40]. The convenient mutual orthogonality of the SNAP and CLIP modules also enables simultaneous labeling with different fluorophores in the same cellular milieu. Due to the modular designs of benzylguanine and benzylcytosine substrates GPCRs have been labeled with a broad spectrum of synthetic dyes [41, 42], with a lanthanide [43], as well as with quantum dots [44]. The SNAP-tag and CLIP-tag modules are slightly smaller (19-kDa) than GFP (27-kDa), and they also offer more flexibility in terms of the choice of fluorescent reporters [45].

Fluorescent protein tags, fluorescently-labeled antibodies, and self-labeling protein tags have each been employed in real-time tracking of GPCR localization to provide insights about oligomerization in cell membranes [46–48]. Control experiments for such studies are not straightforward. For example, one study based on the segregation of tagged proteins showed that even fluorescent proteins with mutations intended to favor the monomeric state had a tendency to oligomerization – but the SNAP-tag caused the least perturbation to the localization of the labeled protein [49], suggesting that SNAP-tagged GPCRs might be most amenable to studies of receptor localization and oligomerization.

In the GPCR field especially, the SNAP-tag and CLIP-tag technology has facilitated the application of time-resolved FRET techniques based on lanthanide emitters. The luminescence of lanthanide/macrocycle complexes, like Terbium cryptate or Europium cryptate, has a large Stokes shift as well as a significantly longer luminescence lifetime (~ milliseconds) than that of the intrinsic fluorescence arising from biomolecules (<10 nanoseconds). Based on these properties, lanthanide emitters can be paired with far-red fluorophores to achieve highly sensitive FRET measurements where background is minimized [50]. Previously, lanthanide labels were typically conjugated to proteins through reactions with cysteines or lysines, which limited their usefulness [51]. However, commercially available lanthanide-labeled SNAP and CLIP substrates are now available and provide a general approach for attaching lanthanide probes to GPCRs [43, 52].

Time-resolved FRET is useful in probing the binding events between SNAP-tagged GPCRs and fluorescently labeled ligands, which has been adapted to high-throughput format for screening receptor-ligand interactions useful for drug discovery [52, 53]. Time-resolved FRET has also been used to assess the homo-oligomerization state of the family A metabotropic glutamate receptor and the family C γ-aminobutyric acid (GABA) receptor [43, 54], and has also been adapted to study chemokine receptors [55], β-adrenergic receptors [19], and the muscarinic acetylcholine receptor [56].

The lateral mobility of GPCRs was initially investigated by fluorescence recovery after photobleaching (FRAP) using receptors tagged with a fluorescent protein or labeled with a fluorescent ligand. In a FRAP experiment, a defined area of the cell membrane containing the labeled receptor is photobleached. Then the fluorescence intensity recovers over time because other labeled molecules diffuse into the previously bleached area. In addition to FRAP, fluorescence correlation spectroscopy (FCS) has also been used to investigate the mobility of different GPCRs in the membrane of live cells [57]. However, both FRAP and FCS only measure average diffusion times and therefore cannot account for local heterogeneities in the cell membrane or provide direct information about receptor stoichiometry.

Proximity-dependent enzymatic labeling methods, like ID-PRIME or BioID, report protein interactions based on the physical proximity of binding partners. While there is only one example in the published literature applying ID-PRIME to detect GPCR oligomerization [58], this strategy has great potential for understanding GPCR signaling networks. Apart from fluorescence techniques, GPCR oligomerization can, at least in theory, be profiled by chemical crosslinking, mass spectrometry and other classical proteomics methods.

15.4 Single-Molecule Approaches to Probed GPCR Oligomerization

An elegant single-molecule surface-tracking study for SNAP-tagged GPCRs revealed different oligomerization levels of β-adrenergic and $GABA_B$ receptors [59]. In a single-molecule FRET study, SNAP-tagging was used to understand the conformation dynamics of metabotropic glutamate receptor dimers [60]. The mutual orthogonality of the SNAP-tag and CLIP-tag as noted above was used to probe the heterodimerization between different GPCRs, such as the cannabinoid and the orexin receptors [61], metabotropic glutamate receptor subunits [62], the dopamine D2 and the ghrelin receptors [63], and the dopamine D2 and D3 receptors [64].

The single-molecule methods commonly used to study membrane receptor oligomerization include single-molecule photobleaching, single-molecule FRET, single-molecule localization microscopy (SMLM), and single-particle tracking (SPT) [65]. Single fluorescent molecule video imaging reports the variations of the diffusion of

fluorescently-labeled receptors over time and space, but also shows whether diffusing receptors oligomerize and for how long [15, 66]. The first single-molecule demonstration of transient dimerization of a GPCR in living cells was achieved by Hern et al. utilizing an antagonist of the M_1 muscarinic receptor derivatized with Alexa Fluor 488 or Cy3B [67]. By tracking individual antagonist-bound receptors in the two channels corresponding to the two fluorophores, the transient correlated motion of two receptor molecules heterologously expressed in CHO cells was demonstrated. Dimers formed, but quickly dissociated again into monomers, with an average time constant of 0.7 s (at 23 °C). Whether the M_1 muscarinic receptor formed dimers in the absence of the antagonist at physiological temperatures remains unknown. Kasai et al. later fully characterized the monomer-dimer equilibrium of the *N*-formyl-peptide receptor (FPR) in CHO cells at 37 °C using a fluorescently-labeled agonist and an FPR-YFP fusion construct [68]. They observed no change in the monomer-dimer equilibrium upon ligand binding, with a typical association time of ~150 ms and a dissociation time of ~90 ms.

Calebiro et al. observed transient homodimers with lifetimes of about 4 s at 20 °C for β_1-AR and β_2-AR labeled via a SNAP-tag in CHO cells [59]. Similarly, ligand binding did not alter the equilibrium or affect the mobility of the receptors. Interestingly, β_2-AR seemed to have a higher tendency to form dimers than β_1-AR at a given expression level. Calebiro et al. also characterized $GABA_B$ receptors, which are prototypical family C GPCRs. They found between $GABA_{B1}$ and $GABA_{B2}$ receptors to be in equilibrium between heterodimers and higher-order oligomers, with a preference for tetramers and octamers. An increase in the lateral mobility was observed after agonist binding, suggesting that the ligand can modulate interactions between the receptor and the actin cytoskeleton.

All these studies highlight the dynamic nature of receptor-receptor interactions and suggest that transient dimer or oligomer formation might be a general mechanism for GPCRs. Kasai et al. even proposed that dynamic homodimers must be crucial for some GPCR functions, which remains to be verified, and that downstream signaling through G proteins, kinases, or arrestins might be differentially induced by monomers and dimers [66]. Single-molecule imaging strategies as noted above might be suitable for addressing these biologically-relevant questions.

Determining the stoichiometry of higher order oligomers by traditional single-molecule imaging can become very challenging. Larger oligomeric assemblies of GPCRs were first described in the context of AFM images of rows of rhodopsin dimers in the retina [16, 17, 69]. AFM is however not suitable for most GPCRs, whose expression level under physiological conditions is orders of magnitude lower than rhodopsin. Super-resolution imaging techniques have demonstrated their ability to provide relative or absolute quantitative information about protein copy numbers in oligomers and clusters [70, 71]. These methods have recently been applied to investigate the organization of GPCRs and arrestins in the cell membrane.

Visualizing β_1-AR and β_2-AR clusters in cardiomyocytes, where receptor activation controls contraction, was first performed using near-field scanning optical microscopy (NSOM) using receptors fused to a fluorescent protein. It was found that these receptors were organized in nanodomains with a diameter of ~150 nm and

did not reorganize upon agonist binding [18, 72]. NSOM is nonetheless particularly difficult to implement in living cells. More recently, SMLM was used to re-evaluate the molecular density of the β_2-AR in cardiomyocytes, HeLa cells, and CHO cells [73–75]. It was found that the receptor indeed preassembled in clusters typically 100–200 nm in size in cardiomyocytes and that the distribution did not significantly change upon addition of ligands, corroborating the findings of the NSOM studies. However, no clustering was observed in HeLa or CHO cells, which was consistent with the findings of earlier tracking studies [59] and with the fact that the β_2-AR is fully functional as a monomeric entity [5]. The absence of significant clustering was further confirmed in CHO and HEK cells by colocalization analysis [76]. Similarly, no significant cluster formation in CHO cells was found for the HIV entry co-receptor CCR5, another rhodopsin-like family A GPCR, although it did accumulate to high densities in the filopodia of these cells [77]. On the other hand, the luteinizing hormone receptor (LHR) was shown to mostly form oligomers in HEK cells [78]. In this case, however, thanks to an experimental localization precision of less than 10 nm, analysis of the spatial arrangements of the molecular localization coupled to molecular modeling led the authors to postulate possible structural arrangements for trimers and tetramers.

15.5 Computational Approaches to Study Oligomerization of GPCRs

In general, computational approaches can create models of receptors and their dynamics in membrane systems, but are not directly informative concerning receptor function. Recent reviews have reported molecular dynamics simulations for the study of GPCRs [79–81], and more general computational methods for predicting the structure of GPCRs and their interactions with ligands, including issues related to allosterism and biased signaling [82]. Additional reviews have covered the different length and time scales relevant to GPCR signaling [83, 84], interactions between GPCR and membrane components membranes [85, 86], and simulations of biomembranes at both atomistic and coarse grain (CG) resolutions [87, 88].

In the field of computational modeling, CGMD has become an important tool to study biomolecular processes [89–91], including the lateral organization of membrane proteins [87]. In particular the Martini model CGMD approach [92] has proven to be a very powerful tool to study GPCR signaling. The Martini CG force field was developed to compute simulations of biological systems and was used initially on simple lipidic systems. More recently it has been applied to complex systems such as mimics of realistic biological membranes with up to 62 different lipid types and proteins [93–97]. Self-assembly simulations of GPCRs were designed with the aim of studying systems where multiple receptors coexist [98–104]. A typical simulation might consist of a large number of GPCRs (2–144) embedded in a preformed lipid bilayer and regularly spaced to maximize their

dispersion in the membrane, often built up from the repeat of a unit cell containing a single receptor. From that dispersed configuration, it is possible to follow the time course of the assembly of the receptor oligomers. Unfortunately, on the time scale accessible to the Martini CGMD simulations it is mainly forward reaction – namely the association of the receptors – that is observed. Only limited events of dissociation have been reported, although it is assumed that the reaction is reversible, even though the supramolecular organization of the receptors described by CGMD simulations on time scales up to 100 μs is most likely not representative of biological time scales and the complex dynamics in under actual physiological conditions. Nevertheless, the simulations provide valuable information about receptor behavior in lipid bilayers, including their propensity to oligomerize, the TM interacting interfaces that are accessible for protein-protein contacts, and the kinetics of the contact interactions. A comparison of simulations performed under different environmental conditions, for example different lipid composition that affect membrane thickness and viscosity, provide significant insights related to lipid/receptor interplay and can provide information about how to design validating experiments [97–104].

In the search for relevant protein-protein contacts, Wassenaar et al. developed a protocol called "docking assay for TM components" (DAFT) [105] to predict effectively the most accessible contact interfaces of TM helices and proteins. One application of DAFT showed that for a simple system such as single TM helical protein (glycophorin A) with a unique interface, the method is able to reliably predict the dimerization interface and the effect of a loss-of-function mutation. DAFT was also able to predict the trimeric arrangement of the GCN4-derived peptide MS1, a TM helix. For more complex systems such as GPCR oligomerization, where multiple interfaces may form, DAFT has limitations and cannot identify interfaces with populations reflecting their relative thermodynamic stability.

The lack or limited number of sampled binding/unbinding events in self-assembly simulations in general, but with GPCRs in particular, by definition, precludes the use of thermodynamic quantities such as the relative binding free energies of specific interfaces [99]. Another point to consider when interpreting the populations of interfaces obtained from self-assembly simulations is the possibility of a strong kinetic contribution, a point recently demonstrated by Provasi et al. [103]. In summary, self-assembly simulations have revealed important contribution of lipid/protein interactions that drive self-assembly and will continue to be of great use. The combination of self-assembly simulations of multiple receptors revealing cooperative features and supramolecular organizations [98–100, 103, 104] and the use of DAFT to generate more systematic information on accessible interfaces will be of interest in both GPCR [88] studies and other TM proteins [106, 107].

15.6 Insights About Supramolecular Organization from Computational Studies

An area of great interest for GPCR research is the search for structural keys to the supramolecular organization and pattern of rhodopsin oligomerization as a model for the self-assembly pattern of other GPCRs. One approach is to use the potential of mean forces (PMF) between two receptors using the umbrella sampling (US) technique [108]. It calculates discrete reaction coordinates, the distance between receptors, into bins that are explored independently using a biasing umbrella potential to maintain the distance close to the value at each bin (umbrella window) and assures overlap between consecutive bins. The deviation from the ideal distribution of the distance reflects the stability of the system in a particular window and the knowledge of the biasing potential allows the generation of unbiased distributions corresponding to each umbrella window. These are combined afterward using the weighted histogram analysis method (WHAM) [108–111]. Filizola and co-workers and Periole and co-workers have used this approach on opioid receptors (OR) and rhodopsin, respectively, to explore particular interfaces.

Studies concerning the supramolecular organization of rhodopsin [98], a light-sensitive receptor involved in the visual phototransduction, were motivated by the report of an highly ordered organization of the photoreceptor in rod outer segments (ROS) disc membrane from atomic force microscopy (AFM) images [16]. Experimental work that was informed by the CGMD results was used to show a correlation of the spatial distribution of rhodopsin in model membranes with photoactivation propensity [9]. Of particular interest was a correlation of rhodopsin's spatial organization and photoactivation efficiency to membrane thickness. These results strongly suggested that the hydrophobic mismatch between the receptors and the lipid bilayer governs the degree of association and also activation efficiency.

15.7 Rhodopsin Oligomerization Pattern

A first set of Martini CGMD simulations [98] described the self-assembly of 16 rhodopsin receptors embedded in a lipid bilayer with a range of membrane thicknesses defined by experimental data [9]. The receptors were observed to self-assemble spontaneously in perfect agreement with the experimental data from Botelho et al. A maximal dispersion of the receptor was observed for an intermediate lipid thickness matching the hydrophobic thickness of rhodopsin. The correlation between the hydrophobic mismatch and the assembly propensity of the receptor was not a complete surprise to either experimental or computational approaches as hydrophobic mismatch had been pointed out as a driving force for membrane protein association in general [112] and for rhodopsin in particular [9]. In addition, the Martini CGMD simulations matched quite well with the FRET signal reported earlier.

An exciting novel finding of the CGMD study was the demonstration of non-homogeneous membrane deformation around the protein. The membrane bilayer adapted to the protein/membrane interface variably at different regions of the protein surface. In addition, the protein/membrane interface heterogeneity varied with the hydrophobic thickness of the membrane. Of particular note was that the regions on the protein surface where the membrane deformed the most in response to hydrophobic mismatch strongly correlated with the location where the protein was seen making protein-protein contacts upon self-assembly. Based on the assumption that protein/membrane hydrophobic mismatch drives integral protein assembly by reducing the membrane deformation [112], these results suggested that protein contacts would form favorably at specific locations of the protein surface and that these locations might vary with the membrane thickness. Inhomogeneous membrane deformation around rhodopsin was also confirmed by atomistic resolution MD simulations [113].

In a follow-up study Periole et al. [99] probed more specifically the relative strength of different interfaces in rhodopsin dimers. This work was intended to build a hypothetical model of rhodopsin organization in its native environment, the ROS disc membrane, depicted by AFM images [16] as a highly ordered set of rhodopsin dimers in a row-of-dimers orientation. The details of the interfaces could not be resolved from the AFM data and the nature of the dimer interface remained highly debated. A row-of-dimers orientation model was built based on the images, showing a symmetric TM4/5 rhodopsin dimer [114]. This apparent static arrangement of rhodopsins initially seemed to contradict earlier biophysical experiments [115].

Later Periole et al. [99] probed rhodopsin dimerization interfaces using two complementary approaches: self-assembly simulations [98], and calculation of the potential of mean force (PMF) as a function of receptor separation distance. The simulations confirmed the preferential linear arrangement of rhodopsins when embedded in a membrane bilayer and revealed a few preferential interfaces. The interfaces involving TM1 and helix 8 (simultaneously) or TM5, combined symmetrically and asymmetrically, were predominantly observed. The interfaces involving the other surface of rhodopsin, around TM4 and TM6, did not participate in a significant manner. The limited formation of interfaces centered on TM4 and TM6 was due to the presence of an energy barrier to their formation. This energy barrier resulted from the trapping of lipids at the interface and the stabilization of a metastable state where lipids lubricated the interface. The interfaces forming in the self-assembly simulations (TM1/H8 and TM5) did not show an energy barrier to their formation. The PMFs led to the identification of two types of interfaces: some *weak,* with an energy barrier to spontaneous formation, and others *strong,* with a deep minimum at the interface and no energy barrier to complex formation.

Interestingly, the PMFs demonstrated a striking stability of the symmetric TM1/H8 dimer interface compared with the other interfaces probed. This dimer interface had previously been observed in two-dimensional (2-D) [116] and 3-D [117] electron microscopy and X-ray crystallography of opsin, rhodopsin, and metarhodopsin I (meta-I) and II (meta-II) [118–120]. These data motivated a study in which we were able to show that the symmetric TM1/H8 interface existed in the native ROS disc

membrane based on a combination of chemical cross-linking experiments, partial proteolysis and high-resolution liquid chromatography–mass spectroscopy [121]. Many other GPCRs have also been crystalized with the TM1/H8 interface [122–124].

Based on the TM1/H8 rhodopsin dimer interface it was possible to construct a row-of-dimers model that fulfilled the structural features extracted from the AFM images. In addition to the TM1/H8 dimer interface, this model was consistent with the existence of lubricated interfaces, centered on TM4 and TM6 that would stabilize interfaces between two dimers in a row. From a biophysical perspective on protein/membrane interplay, the stability of the TM1/H8 interface in the rhodopsin dimer is of primary importance, because it has a smaller protein burial than the other potential dimer orientations. This observation challenges the use of buried accessible surface area as a predictor of the strength of membrane-embedded protein-protein interfaces.

15.8 The Opioid Receptor (OR) Oligomerization Pattern

Filizola and co-workers, inspired by the Martini CGMD simulation technique [98] used metadynamics [125] to enhance sampling and published a series of studies probing GPCR interfaces. They emphasized the δ, μ, and κORs according to earlier studies [126]. They characterized the binding profile of the δOR at the TM4 (also referred to as TM4/3) interface calculating the first PMF of a GPCR interface [127]. They subsequently compared it to the PMF of the TM4/5 interface allowing them to rationalize cross-link experiments [128]. The receptors were embedded in a POPC lipid bilayer with 10% cholesterol and for the model of δOR they used a similar approach to that developed for rhodopsin [98, 129]. The simulations gave the TM4 (or TM4/3) interface of δOR as preferred compared with the TM4/5 interface. In a later study, Johnston and Filizola extended their work on OR using PMFs to look at interfaces of μOR and κOR as found in the crystallographic structures [130]. These include the TM1/H8 for both receptors and the TM5/6 for μOR. The TM1/H8 interface used contacts of TM1 on the side of TM2, similar to the case of rhodopsin. The results show that the symmetric interface involving TM5/6 is more stable than the one involving TM1/H8 when compared with the other interfaces and other receptors. The TM1/H8 interface of μOR has a similar affinity as the TM1/H8 interface of other GPCRs (β_1-AR and β_2-AR, described below and using the TM7 side of TM1 for contacts).

In related work on OR, Provasi et al. [103] used the self-assembly approach to look at the interfaces preferentially formed by three receptors from the opioid subfamily using PMFs: δOR, μOR and κOR. They investigated homomeric complexes for the three receptors and heteromeric between the pairs δOR/μOR and δOR/κOR. As noted by the authors, although this approach does not allow the calculation of free energies and thereby prevents the comparison of the relative strength of the interfaces formed (based on their populations) as with PMFs [99], it reveals the interfaces accessible on the time scale simulated. In all five systems the opioid receptors formed filiform (elongated) structures using the *small* sides of the receptors centered either on TM1/2 or TM5 alone and combined with TM4 or TM6. Significant

populations of symmetric interfaces were observed only in the cases of TM1/2/H8 and TM5. The frequency of appearance of the interfaces depends on the receptors simulated but their confidence intervals were broad and the differences might not be significant. This lack of significance of the different populations might be important since they do not reflect the results from the PMFs determined previously [130]. For instance, the symmetric TM5/6 interface for μOR is given to be more stable than the TM1/2/H8 one, but it is not significantly more populated. According to the PMFs, an even larger population would be expected for the TM1/2/H8 interface of κOR. Also of note was the absence of the TM4 (or TM4/3) interface for the δOR, while the TM4/5 was present. TM3 and TM7 were not involved in the interfaces.

15.9 The β_1- and β_2-Adrenergic Receptor Oligomerization Pattern

The β_1- and β_2-adrenergic receptors (AR) are of particular interest because these two receptors are highly similar (67% sequence identity) but do experience distinct association patterns in the membrane [19, 59]. Johnston et al. [131] compared their interfaces centered on TM3/4 and on TM1/H8. The comparison of the PMFs of both receptors and for both interfaces demonstrated that the two receptors behave identically within the error bars, at odds with fluorescence recovery after photobleaching (FRAP) experiments giving a more stable dimer for β_2-AR [19]. The PMFs also showed that for both receptors the interface TM1/H8 is stronger than the TM4/3 interface. The similarity of the PMFs for the two receptors contrasts with a later study using the CGMD technique in which significant differences were observed between β_1-AR and β_2-AR interactions with the bilayer environment, consistent with experimental observations of oligomerization patterns of β_1-AR and β_2-AR [100]. In this study, Mondal et al. show that the association between the β_1-AR and β_2-AR receptors reduces the energy penalty from residual hydrophobic mismatch, which they suggest is a major driving force towards the determination of protein contact upon oligomerization [100]. Ghosh et al. performed a self-assembly simulation of sixteen copies of β_2-AR in a model 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) lipid bilayer [102]. The analysis of the simulation showed an overall similar behavior as previously described for other receptors [98–100, 103]. Notably the receptors form a string-like cluster, and the contact between receptors involves TM1, H8, TM5 and TM6.

15.10 Cholesterol Effect on Receptor Oligomerization

Sengupta and co-workers have used the Martini CGMD simulation approach to study the interaction of cholesterol with GPCRs and rationalize its effect on their assembly. These studies follow the extended work of the Chattopadhyay group on the subject. Cholesterol is a significant component of biological membranes and has

been shown to play a critical role on membrane proteins [132] and on GPCR supramolecular organization and function [133–135] but its mechanism of action is still a matter of debate. Cholesterol might act directly on GPCRs through specific interaction with the receptors [136–139] or indirectly through modification of the membrane bilayer mechanical properties [135, 140–142]. In a first study, Sengupta and Chattopadhyay [143] characterized the interaction pattern of cholesterol with the serotonin$_{1A}$ receptor (5-HT$_{1A}$R). Cholesterol is found to interact with 5-HT$_{1A}$R on timescales from ns to μs at preferred occupancy sites. This pattern of interactions is in line with the concept of "non-annular" binding of cholesterol to 5-HT$_{1A}$R [136]. Non-annular binding refers to lipids that do not frequently exchange with other lipids in the bulk membrane. In a subsequent work Prasanna et al. [101] studied the interaction of cholesterol with β_2-AR, a receptor also known to have interactions with cholesterol [122, 123]. Here, the authors used self-assembly Martini CGMD simulation of a pair of receptors embedded in a POPC lipid bilayer with 0, 9, 30, or 50% of cholesterol. The analysis of these simulations led the authors to conclude that the presence and increase of the cholesterol content in the membrane systematically affects the interface the receptors use to assemble. An interesting aspect of Prasanna et al. results is the apparent contrast of the proposed mechanism of cholesterol stabilization of β_2-AR dimer at the TM1/H8 interface by the simulations with the one that could be suggested by the inspection of the β_2-AR structure [122, 123]. From the crystal structure one could speculate that cholesterol stabilizes the TM1/H8 interface by its location at the interface to act as "glue". This behavior has been reported for other systems [144]. Other recent studies have also found cholesterol at the TM1/H8 interface of GPCR dimers [97, 124].

15.11 The Docosahexaenoic Acid (DHA) Effect on Receptor Oligomerization

Guixà-González et al. [104] have recently used the Martini CGMD approach to investigate the mechanism by which DHA might affect the oligomerization of adenosine A_{2A} and dopamine D_2 GPCRs. DHA is an ω−3 polyunsaturated fatty acid (PUFA) 22-carbons long with six double bonds that has been shown to be essential for a proper brain function [145–147], Similarly, earlier studies reported the importance of DHA for vision by affecting the function of the visual photoreceptor rhodopsin [148–154]. Neuronal and rod cell membrane have extremely high DHA content: 50% and 60% of PUFA in neuronal cells and ROS, respectively. The reported effects of DHA on membrane biophysico-chemical properties (fluidity) combined with mounting evidence of the role of GPCR oligomeric states to their function, led the authors to hypothesize that DHA might affect GPCR function by contributing to GPCR oligomeric state stability. The existence of A_{2A} and D_2 receptors oligomers [155–157] and the relevance of their balance to

neuropsychiatry [64, 158–160] combined with the low level of DHA in patients with mental [147] and neurological disorders [145, 146] made them perfect candidates for this study. Guixà-González et al. concluded that DHA has a kinetic effect on the receptor assembly. The model membranes used in the protein self-assembly simulations were complex mixtures aimed at reflecting general brain lipid profiles.

15.12 Lipid Interactions with Rhodopsin

Grossfield and co-workers have used the Martini CGMD simulation approach to look at lipid distribution and interactions around rhodopsin [81]. This study followed several publications on the subject using an atomistic resolution [148–150, 161–164] and was inspired by a large set of experimental data [9, 133, 134, 141, 151–153, 165–177]. These works characterized the effect of the various lipid components specific to the ROS membrane and known to affect the function of rhodopsin. These effects are often measured by following the meta-I-meta-II equilibrium, the last two photo-intermediates of rhodopsin. Only meta-II is able to bind its cognate G protein transducin. The meta-I-meta-II equilibrium is sensitive to ROS membrane properties such as its lipid head group composition (PC, PE and PS), PUFA (DHA in particular) and cholesterol content. These effects are nicely summarized in Horn et al. [81]. In short, meta-II (active rhodopsin) is favored by an increase of the negative curvature of the membrane (provided by PE and DHA) and an acidic membrane surface (provided by PS). Cholesterol favors meta-I, but its content decreases as the disks age (become functional) and it stabilizes the disk membrane by compensating PE and DHA negative curvature.

Horn et al. [81] built a Martini CG model similar the one they used previously in atomistic MD simulations: a single receptor embedded into a 2:2:1 molecular ratio of SDPC:SDPE:chol [148–150, 164]. This particular composition aims at mimicking the ROS lipid composition [173, 178–182]. Two systems were simulated: one with rhodopsin (based on the PDB ID: 1 U19 ([183])) and one with opsin, representing the activated receptor (based on the PDB ID: 3CAP ([119])). In the activated receptor a conformational change involves the movements of the cytoplasmic side of TM5 (inward) and TM6 (outward) from the helical bundle. They performed 16 independent simulations of both systems each 1.6 μs, which represents an increase of system size by factor ~ 3 and of simulation length by about 10 when compared to the atomistic data. In their analysis Horn et al. demonstrated at many occasions the high degree of convergence of the sampling performed, illustrating one of the powers of Martini CGMD simulations: statistical significance of complex systems.

15.13 Evidence for Specific Lipid Binding Sites Mediating Dimerization

Direct, specific and localized interactions of cholesterol molecules at the surface of receptors have been reported in Martini CGMD studies for rhodopsin [81], 5-HT_{1A} [143] and β_2-AR [101]. These interactions are in line with reports using atomistic MD simulations [150, 162, 184–187], receptor crystal structures [122–124] and experimental data. In their study on β_2-AR, Sengupta and co-workers [101] suggested that cholesterol, through occupying certain locations on the protein surface with higher frequency, blocks interfaces from engaging in protein-protein contact. Given the ubiquitous presence of cholesterol in biomembranes, this blocking behavior of cholesterol is of general importance for membrane protein complex formation. It would therefore be important to consolidate this observation by finding other occurrences of this behavior, maybe for other GPCRs. It is notable that this "blocking" behavior is complementary to "gluing" behavior reported recently in the case of cardiolipin acting as a glue between the proteins constituting the respiratory chain complexes [144, 188]. A gluing behavior of cholesterol would also be consistent with the finding of its tight binding at the interface of GPCR dimer structures [122–124].

15.14 Several Factors Contribute to GPCRs Self-Assembly into Linear Aggregates

Studies of GPCRs using the Martini CGMD approach show that receptors form dimers and higher-ordered structures and actually assemble with a predominance of linear arrays (filiform) structures [98–100, 103, 104] with the appearance of some small branched structures on very long time scale [104]. The most straightforward explanation is the preference of the receptors to interact through the "small" sides of the receptors: centered either on TM1 or TM5 (Fig. 15.2). This behavior has been most clearly demonstrated in the case of rhodopsin [99]. It was shown that the interfaces involving TM1/H8 and TM5 (TM5, TM4/5) were found highly involved in the receptor interfaces formed in self-assembly simulations. The PMFs as a function of the receptor distance proved them to be much more stable than the other interfaces [99]. The interface involving TM4 (noted TM4/3 in other studies) and TM6 (TM6/7 might be a better representation) were not observed upon self-assembly. The PMFs of these interfaces revealed an energy barrier to their formation and a metastable state in which lipids lubricate the interface TM4. Linear aggregates are formed, most likely because the short sides can form direct contacts whereas the larger sides remain lubricated.

In their study of β_1-AR and β_2-AR, Mondal et al. rationalized the filiform organization of β_2-AR observed in self-assembly simulations on the basis of the presence of residual hydrophobic mismatch on the "small" sides of the receptors,

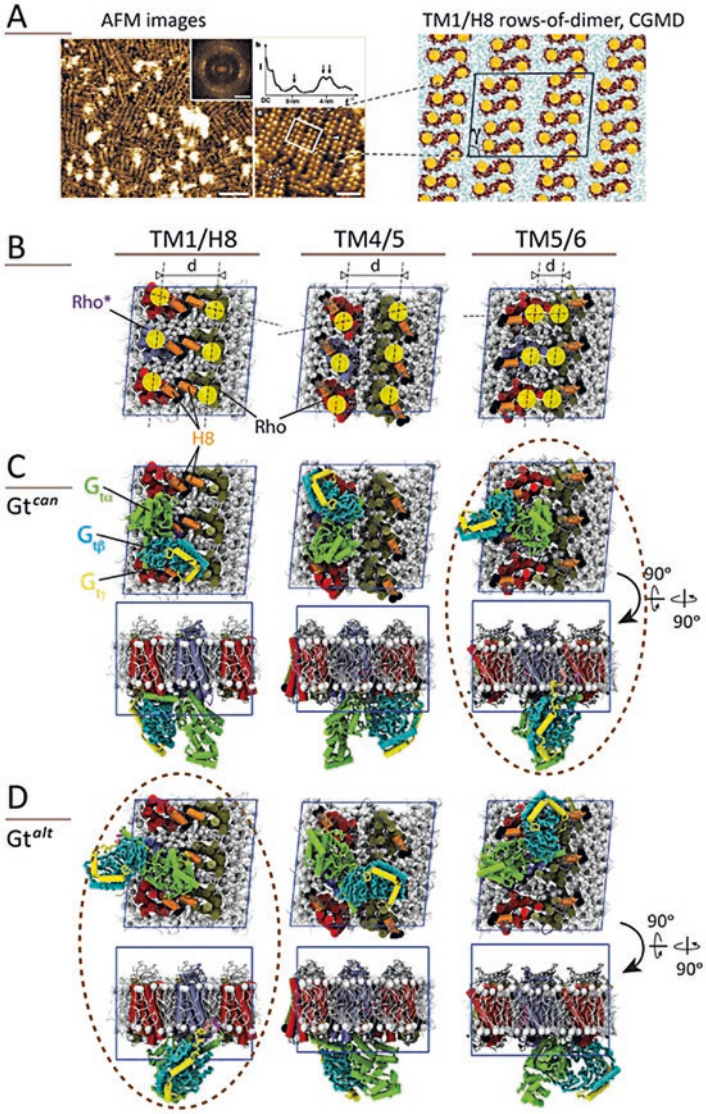

Fig. 15.2 Rhodopsin supramolecular organization in the Rod Outer Segment (ROS) membrane. (**a**) AFM images (*left*) revealing the row-of-dimer organization of rhodopsin in a native-like environment. Taken from [16]. The images were used to extract structural restrains used to build a first model of the rhodopsin dimer and dimer of dimers [114]. It corresponds to the TM4/5 model shown in (**b**). Results from CGMD simulations (*right*) were used to build an alternative model based on the TM1/H8 dimer interface. Taken from [99]. (**b**) Models of the rows-of-dimer organization of rhodopsin according to different dimer interfaces: the TM1/H8, TM4/5 and TM5/6 interfaces corresponding to the most stable in the PMFs [99], the earlier model build from the AFM images [114] and a potential alternative interface, respectively. (**c**–**d**) Binding mode of transducin (cognate G protein of rhodopsin) to the three row-of-dimer models shown in (**b**) using (**c**) the canonical orientation (built from the β_2-AR-Gαs complex structure [197]), and (**d**) an alternative orientation (From Periole with permission [3])

TM1/H8 and TM5, and not on the other larger sides [100]. In the case of opioid receptors, Provasi et al. [103] also observed filiform structures mainly involving the small sides of the receptors: centered on TM1 (TM1/2/H8) and TM5 (TM4/5 and TM5/6).

15.15 The Row-of-Dimers Organization of Rhodopsin in the ROS Disc Membrane

The proposal that rhodopsin in the ROS disc membrane is arranged into a highly-organized supramolecular structure is at the same time compelling, yet controversial (Fig. 15.2) [115]. One key element of the model suggests that transducin, which is tethered to the membrane due to post-translational myristoylation and isoprenylation, can carry out essential a one-dimensional search for light-activated rhodopsin by sliding along the membrane track demarcated by the rhodopsin dimer rows [69, 114, 189–194]. One direct consequence of the highly symmetric supramolecular organization of rhodopsin that is not often considered in the functional models is that it leaves only one side of rhodopsin exposed to the membrane. The exposed face of rhodopsin exposed to transducin is then entirely determined by the conformation of the functional dimer, emphasizing the importance of the search for the main dimer interface for rhodopsin and potentially other GPCRs. In such organization the search of transducin for an activated receptor is radically simplified.

According to the row-of-dimers model, the stronger dimer interface, TM1/H8, should be the main dimer interface (intra) and the weaker ones, TM4 and TM6, should exist in between the dimers (inter) in a row (Fig. 15.2b). The existence of the TM1/H8 contact as the main dimer interface was supported by its strength [99] and its presence in the ROS membrane [121]. In addition, this model satisfies the structural restraints based on the earlier AFM study (Fig. 15.2) [99]. However, this model differs from the model proposed earlier by the authors of the AFM images, which utilizes the TM4/5 interface for the intradimer contact and TM2 and TM6 for the interdimer contacts [69, 114]. In addition, functional models have also been derived from this particular arrangement [69, 114, 193]. In searching for potentially stable interfaces for rhodopsin [99], the TM5/6 did not appeared as a potential candidate. It was only recently observed in an experimental structure of μOR [195], and was shown to be more stable than the TM1/H8 interface for μOR based a PMF study using Martini CGMD simulations [130]. It was also reported to spontaneously form in self-assembly Martini CGMD simulations of homo- and hetero-dimeric interfaces of ORs [103]. Also of potential interest is its involvement in a rearrangement of the interface of a Family C GPCR [196].

15.16 Concluding Statements Concerning GPCR Oligomerization

Although dimerization or oligomerization of GPCRs does not seem to be a general requirement for ligand recognition or signaling, it might represent a mechanism for the cell to modulate receptor mobility at the cell surface, receptor intracellular trafficking, or receptor signaling functions. The plasma membrane can be envisioned as a complex dynamic heterogeneous distribution of lipids and proteins in which signaling from cell surface receptors is often highly compartmentalized. In the dynamic bilayer environment, GPCRs might exist in signaling microdomains, such as caveolae or lipid rafts, where specific protein-protein or protein-lipid interactions prevail. In this context, the lateral mobility of receptors is a key parameter describing how they might move in and out of such microdomains and encounter other identical or different receptors to form transient or stable dimers or oligomers. Ongoing work will continue to involve the use of both computational and experimental approaches as highlighted above.

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Chapter 16
Interaction of Membrane Cholesterol with GPCRs: Implications in Receptor Oligomerization

Durba Sengupta, G. Aditya Kumar, and Amitabha Chattopadhyay

Abstract G protein-coupled receptors (GPCRs) are the largest family of proteins involved in signal transduction across cell membranes, and represent major drug targets in all clinical areas. Oligomerization of GPCRs and its implications in drug discovery constitute an exciting area in contemporary biology. In this review, we have highlighted the role of membrane cholesterol and the actin cytoskeleton in GPCR oligomerization, using a combined approach of homo-FRET and coarse-grain molecular dynamics simulations. In the process, we have highlighted experimental and computational methods that have been successful in analyzing different facets of GPCR association. Analysis of photobleaching homo-FRET data provided novel information about the presence of receptor oligomers under varying conditions. Molecular dynamics simulations have helped to pinpoint transmembrane helices that are involved in forming the receptor dimer interface, and this appears to be dependent on membrane cholesterol content. This gives rise to the exciting and challenging possibility of age and tissue dependence of drug efficacy. We envision that GPCR oligomerization could be a game changer in future drug discovery.

Keywords GPCR • GPCR oligomerization • Membrane cholesterol • Actin cytoskeleton • Homo-FRET • Coarse-grain simulation

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16.1 G protein-Coupled Receptors (GPCRs)

G protein-coupled receptors (GPCRs) are cellular nanomachines that comprise the largest and most diverse group of proteins in mammals, and are involved in transfer of information from outside the cell to the cellular interior [7, 40, 49]. GPCRs are typically seven transmembrane domain proteins and include >800 members which are encoded by ~5% of human genes [63]. Cellular signaling by GPCRs involves their activation by ligands present in the extracellular milieu, and the subsequent transduction of signals to the interior of the cell through concerted changes in their transmembrane domain structure [12]. GPCRs regulate physiological responses to a variety of stimuli that include endogenous ligands such as biogenic amines, peptides, glycoproteins, lipids, nucleotides, Ca^{2+} ions and various exogenous ligands for sensory perception such as odorants, pheromones, and even photons. As a consequence, GPCRs mediate multiple physiological processes such as neurotransmission, cellular metabolism, secretion, cellular differentiation, growth, inflammatory and immune responses. Since GPCRs play a central role in cellular signaling and are implicated in pathophysiology of several disorders [17, 20], they have emerged as major drug targets in all clinical areas [9, 16, 20, 21]. It is estimated that ~50% of clinically prescribed drugs and 25 of the 100 top-selling drugs target GPCRs [50, 57].

16.2 Role of Membrane Cholesterol in GPCR Function

Since GPCRs are integral membrane proteins, interaction of membrane lipids with them constitutes an important area of research in contemporary biology. In particular, membrane cholesterol has been reported to have a modulatory role in the function of a number of GPCRs. Extensive work has been carried out in case of GPCRs such as the serotonin$_{1A}$ receptor, the β_2-adrenergic receptor, and opioid and cannabinoid receptors. In all these cases, membrane cholesterol has been shown to regulate receptor function, dynamics and oligomerization. Details of these effects have been described in previous reviews [13, 22, 31, 34, 46, 52]. We therefore prefer to direct interested readers to these reviews.

Current understanding of the mechanistic basis of GPCR-cholesterol interaction appears to indicate that specific effects in terms of cholesterol binding to certain regions (sequences) of the receptor play a role in these changes [33, 36], although global membrane effects cannot be ruled out [37]. Some of these regions (such as the cholesterol recognition/interaction amino acid consensus (CRAC) motif) have been identified in GPCRs [23]. The involvement of these regions in regulation of GPCRs by membrane cholesterol is being investigated for various types of GPCRs by experimental [32, 48] and simulation approaches [51]. Detailed molecular dynamics simulations have revealed that cholesterol binding on GPCRs is weak and dynamic, with an occupancy time ranging between ns and μs. The emerging model regarding the energy landscape of cholesterol association with GPCRs corresponds

to a series of shallow minima interconnected by low energy barriers [52]. A consequence of such interactions is the conformational plasticity exhibited by GPCRs induced by membrane cholesterol [44].

16.3 GPCR Oligomerization: Pitfalls of Commonly Used Hetero-FRET Approach to Monitor Receptor Oligomerization

GPCR oligomerization is an interesting and exciting aspect of contemporary receptor biology since it is believed to be an important determinant for GPCR function and cellular signaling [1, 18, 26, 29, 38, 53]. Such oligomerization is implicated in proper folding of receptors, thereby providing the framework for efficient and controlled signal transduction. The potential implications of oligomerization are far reaching, specially keeping in mind the role of GPCRs as major drug targets [14]. Evidence of GPCR dimers or higher-order oligomers has been reported in the last few years [1, 11, 24, 35, 60] and implicated in receptor trafficking, signaling and pharmacology.

Oligomerization of GPCRs in live cell membranes has been studied extensively utilizing fluorescence resonance energy transfer (FRET) approaches such as hetero-FRET (FRET between two different fluorophores) and bioluminescence resonance energy transfer [26]. The major intrinsic complication of hetero-FRET measurements arise from the use of receptors conjugated to two different probes, and a lack of control in their relative expression levels, the so-called 'Bystander FRET' [6, 8, 28]. A source of possible error is the often misunderstood inverse sixth power distance dependence of FRET [6, 41]. Another complication arises from the 'bleed-through' problem [6, 41]. This is usually manifested by the emission of one fluorophore being detected in the photomultiplier channel for the second fluorophore, due to broad bandwidths and asymmetrical spectral profiles.

In contrast, homo-FRET (FRET between two identical fluorophores) is a simpler variant of energy transfer because it takes place between like fluorophores and therefore requires only a single type of fluorophore. Fluorophores with a relatively small Stokes' shift will have a greater probability of undergoing homo-FRET. In addition, homo-FRET measurements can provide an estimate of higher-order oligomerization [62], which is a serious limitation with hetero-FRET measurements. This is important, specially in the microheterogeneous membrane environment, where multiple types of oligomeric clusters can coexist. Importantly, homo-FRET is manifested by a reduction in fluorescence anisotropy, a parameter that is largely independent of the concentration of fluorophores [58]. Homo-FRET leads to depolarization of the emission because of the lack of correlation between the orientation of the initially photoselected donor and the secondarily excited molecules [25].

16.4 Role of Membrane Cholesterol and the Actin Cytoskeleton in GPCR Oligomerization: Homo-FRET Approach

In view of the advantages of homo-FRET, we previously utilized this approach to explore the oligomerization state of the serotonin$_{1A}$ receptor [11]. Homo-FRET was assayed by the increase in fluorescence anisotropy upon progressive photobleaching of the receptor, in which fluorescence depolarization due to energy transfer was prevented by photobleaching of FRET acceptors [59]. Our results showed that the initial anisotropy of serotonin$_{1A}$ receptors tagged to enhanced yellow fluorescent protein (EYFP) in control cells was significantly low (~0.22) compared to the fundamental anisotropy (r_o) of EYFP (0.38; from [4]) (see Fig. 16.1a). The observed depolarization of emission was attributed to energy transfer (homo-FRET) between receptor molecules of the oligomers. Fig. 16.1a shows that there is a steady increase in fluorescence anisotropy of serotonin$_{1A}$-EYFP receptors with progressive photobleaching, which is expected for a system undergoing homo-FRET.

We utilized a previously developed theoretical formalism for deducing the type of oligomers from such anisotropy enhancement upon photobleaching data [62]. This formalism relies on the difference between the extrapolated and predicted (0.38) anisotropy values at 100% photobleaching limit for predicting oligomeric state, such that, larger the difference greater the fraction of higher-order oligomers (see Fig. 16.1b). In other words, with increasing oligomerization, the extrapolated anisotropy shows higher deviation from the predicted (fundamental) anisotropy. The predicted variation of fluorescence anisotropy with increased photobleaching for a homogeneous distribution of monomers, dimers, trimers, and tetramers (assuming an anisotropy of 0.38 for monomers) is shown in the inset of Fig. 16.1b. Due to experimental limitation of achieving very high degree of photobleaching (low signal-to-noise ratio), we compared the linearly extrapolated anisotropy with the predicted anisotropy to infer the presence of higher-order oligomers (see Fig. 16.1b). On the basis of the observed increase in fluorescence anisotropy upon progressive photobleaching (Fig. 16.1a), and the analysis of data based on the difference between the extrapolated anisotropy and the predicted anisotropy (Fig. 16.1b), we proposed the presence of constitutive oligomers of the serotonin$_{1A}$ receptor [11].

Utilizing this approach, we explored the role of membrane cholesterol and the underlying actin cytoskeleton on the oligomerization status of the serotonin$_{1A}$ receptor. Our results showed that actin cytoskeletal destabilization led to a reduction in the initial anisotropy and increase in the difference between the extrapolated anisotropy and the predicted anisotropy compared to control conditions (Fig. 16.1a, b). This suggested increased contribution from higher-order oligomers under such condition. In contrast, cholesterol depletion led to an increase in initial anisotropy, and reduction in the difference between the extrapolated anisotropy and the predicted anisotropy relative to control (Fig. 16.1c). These results show that cholesterol depletion effectively reduced the population of higher-order oligomers. Taken together, these results showed the presence of constitutive oligomers of the serotonin$_{1A}$ recep-

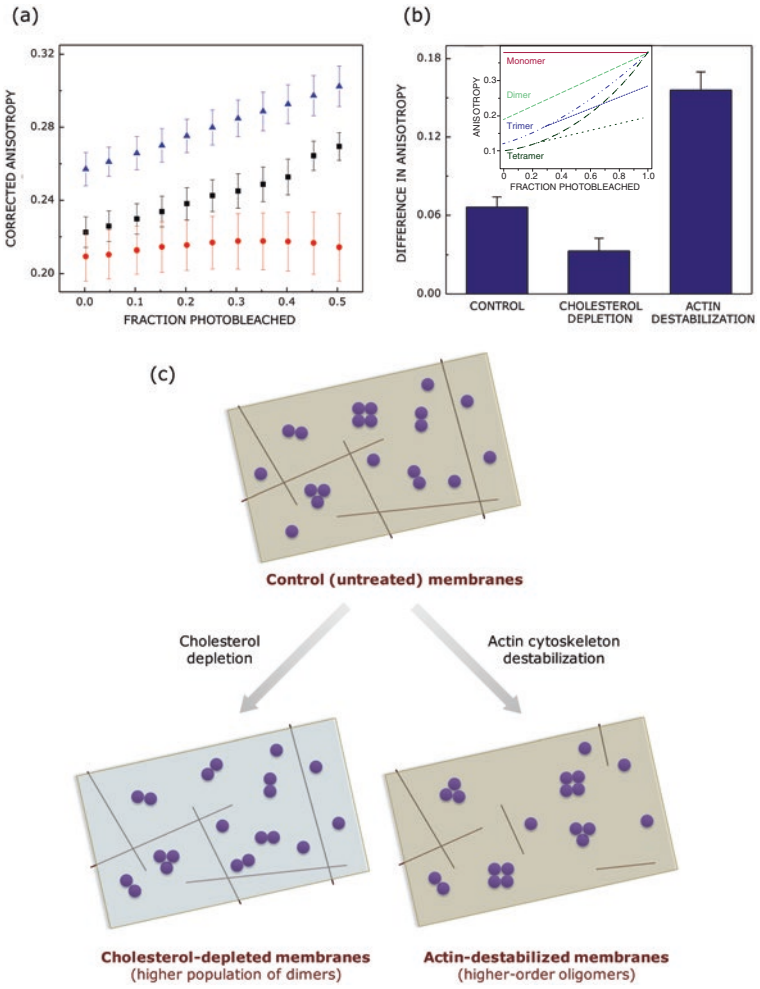

Fig. 16.1 GPCR oligomerization: the role of cholesterol and the actin cytoskeleton. (**a**) Fluorescence anisotropy enhancement profiles of EYFP-tagged serotonin$_{1A}$ receptors upon photobleaching. Anisotropies corrected for microscopic aperture-induced depolarization upon photobleaching as a function of the photobleached fraction of fluorescently tagged receptors are plotted for control (untreated) cells (■, *black*), cells treated with MβCD (▲, *blue*) and upon cytochalasin D treatment (●, *red*). (**b**) Difference between extrapolated (to complete photobleaching) and simulated (see *inset*) anisotropies in various conditions. The extent of receptor oligomerization can be determined using the difference between the anisotropy upon linear extrapolation of the photobleaching data (from (**a**)) to complete photobleaching, and the simulated anisotropy (from the *inset* which shows the simulation of the enhancement in anisotropy upon increasing photobleaching for a homogeneous population of monomers, dimers, trimers and tetramers using the formalism developed by Yeow and Clayton [62]). The magnitude of deviation of extrapolated anisotropy from simulated anisotropy is indicative of the extent of oligomerization. Adapted and modified from [11]. (**c**) A schematic representation of the effect of cholesterol depletion and destabilization of the actin cytoskeleton (shown as *rods* underlying the membrane) on oligomerization of the serotonin$_{1A}$ receptor (shown as *circles* on the membrane in *top view*). Receptors are present in heterogeneous oligomeric states in untreated (control) cell membranes. Depletion of cholesterol leads to an increase in the proportion of receptor dimers. Higher order oligomers of the serotonin$_{1A}$ receptor are observed upon destabilization of the actin cytoskeleton

tor and reorganization of higher-order oligomers in response to membrane cholesterol depletion, and actin cytoskeleton destabilization. These results are further supported by careful analysis of the organization of the EYFP-tagged serotonin$_{1A}$ receptor using time-resolved fluorescence anisotropy decay [35].

16.5 Computational Approaches to Explore GPCR Oligomerization

Computational methods, especially coarse-grain molecular dynamics simulations, have been effectively used to study the association of several GPCRs in molecular details. These simulations are limited to the sub-ms time regime, and have been able to capture the association, but not the dissociation of GPCRs. It should be noted that diffusion of GPCRs in cell membranes is slower than in model membranes, and association and dissociation of GPCRs could be diffusion-limited. Importantly, the factors that affect GPCR diffusion, such as membrane lipid composition, could be intricately linked to GPCR association. For instance, cholesterol depletion has been reported to induce dynamic confinement of the serotonin$_{1A}$ receptor in living cells [47].

16.5.1 Molecular Determinants of GPCR Dimers: Dependence on Protein-Lipid Interactions

Computational approaches represent powerful tools to analyze the molecular determinants of GPCR association. Coarse-grain simulations have been used to analyze the association of several GPCRs, such as the β_2-adrenergic receptor [42], rhodopsin [39] and the serotonin$_{1A}$ receptor [44]. Several protein-protein contact interfaces have been predicted in these receptors. Although the dimer interfaces are dependent on the receptor, the main sites at which association occurs appear to be common and are schematically shown in Fig. 16.2 for the β_2-adrenergic receptor. Two specific sites have been identified, involving predominantly transmembrane helices I/II and IV/V. Homo-interfaces, *i.e.*, a symmetric interface with the same site of both receptors, have also been reported. In addition, a role for transmembrane helix VI has been reported. Further, hetero-interfaces comprising of different transmembrane helices, but from the same sites on the receptor, have been reported. For instance, a I/II-IV/V interface has been reported in case of the β_2-adrenergic receptor [42]. Interestingly, the dimer interfaces in the opioid receptor family (μ, δ and κ subtypes) suggested a similar dimer interface across the different subtypes [45]. For the hetero-dimers, similar sites of dimer interface have been reported in the A_{2A} adenosine-D_2 dopamine receptor complex [15]. Since several interfaces have been reported, the next question that arises is related to the relative energetics of these interfaces. Coarse-grain simulations have been used to analyze the energetics of these

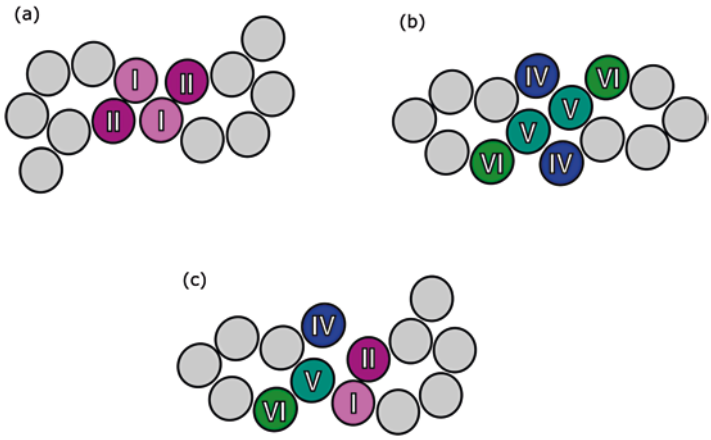

Fig. 16.2 A schematic representation of the predominant GPCR dimer interfaces observed in simulations of the β_2-adrenergic receptor. It appears that the predominant sites at which receptor association takes place are common to many receptors. The interacting helices at the receptor dimer interfaces are shown in panels (**a**–**c**)

interfaces. Importantly, the energetics of these dimer interfaces is of the order of kT. The values should be treated as qualitative due to the nature of the coarse-grain force-field and the free-energy calculations. Nonetheless, these results point toward several alternate dimer structures that could interconvert at room temperature.

From the studies on single component lipid membranes, we were able to identify a few specific interfaces. The dimerization of the β_2-adrenergic receptor was addressed by systematically varying the cholesterol concentration in the membrane [42]. The main interfaces described above, namely helices I/II and IV/V were observed (Fig. 16.3a–c). However, an intrinsic cholesterol dependence was observed in the simulations. In the absence of cholesterol, the dimer interface comprised only of transmembrane helices IV and V. At high cholesterol concentrations, transmembrane helices I and II were observed at the dimer interface. At intermediate concentrations, hetero-interfaces comprising of these two sites were observed. Interestingly, the two homo-interfaces have been observed in the crystal structure of a related receptor, the β_1-adrenergic receptor (Fig. 16.3d, e), although the lipid dependence is not clear in the crystal structure [19]. We believe that these interfaces are of similar energetics, and the membrane composition tunes the interactions of the receptors to modulate dimerization. In addition, a mixed bilayer of POPC/cholesterol has been used to analyze oligomerization in the β_2-adrenergic receptor [30]. However, these authors were not able to discern differences in the oligomerization pattern, possibly due to a lack of sampling.

A more comprehensive study was performed for the serotonin$_{1A}$ receptor, in which more subtle changes were observed relative to the β_2-adrenergic receptor [44]. Similar to the β_2-adrenergic receptor, cholesterol was found to modulate the dimer interface, although the structural and dynamic determinants of the dimer interfaces were found to vary. The time-course of dimer formation of two seroto-

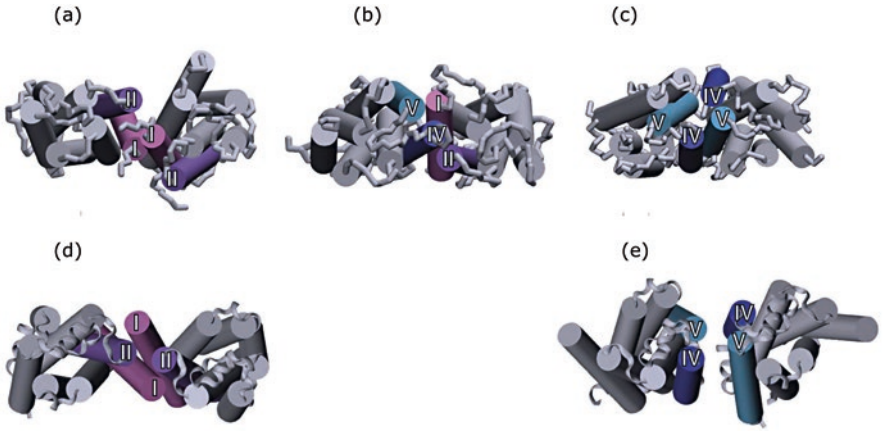

Fig. 16.3 Representative dimer interfaces of the β_2-adrenergic receptor in membrane bilayers of varying cholesterol concentration: (**a**) high (50 mol%) (b) intermediate (30 mol%) and (**c**) in the absence of cholesterol. Panels (**d**) and (**e**) represent dimer interfaces of the β_1-adrenergic receptor obtained from crystallography (PDB: 4GPO) (Adapted and modified from [42])

nin_{1A} receptor monomers at varying cholesterol concentration is shown in Fig. 16.4. The most striking feature was that the number of dimers formed at low cholesterol concentrations was higher than that at high cholesterol concentrations. Additionally, it was observed that a 'tight' dimer interface comprising only of transmembrane helix I was formed in the absence of cholesterol. In the presence of cholesterol, the flexibility of this dimer interface was higher and it formed a transmembrane helix I/II-I/II type of an interface. Additionally, several dimer interfaces comprising of transmembrane helices IV,V and VI were observed.

For the hetero-dimers, a unique modulation of the dimer interface has been reported in adenosine A_{2A}-dopamine D_2 receptors [15]. Polyunsaturated lipids, such as omega 3 fatty acids have been observed to modulate the dimer interfaces. A clear distinction in the kinetics of dimer formation was observed at high and low concentrations of omega 3 fatty acids. A comparison of the dimer interfaces of the opioid receptors (μ, δ and κ subtypes) suggested a similar dimer interface across the different subtypes, although an effect of the interfacial lipids was observed [45]. Preliminary results suggest a similar cholesterol dependence for the adenosine-dopamine receptor pairs (Prasanna et al. unpublished observations). The modulation of dimer interface upon varying cholesterol and other lipids that was hinted at with experimental studies, appears to be better resolved with simulations.

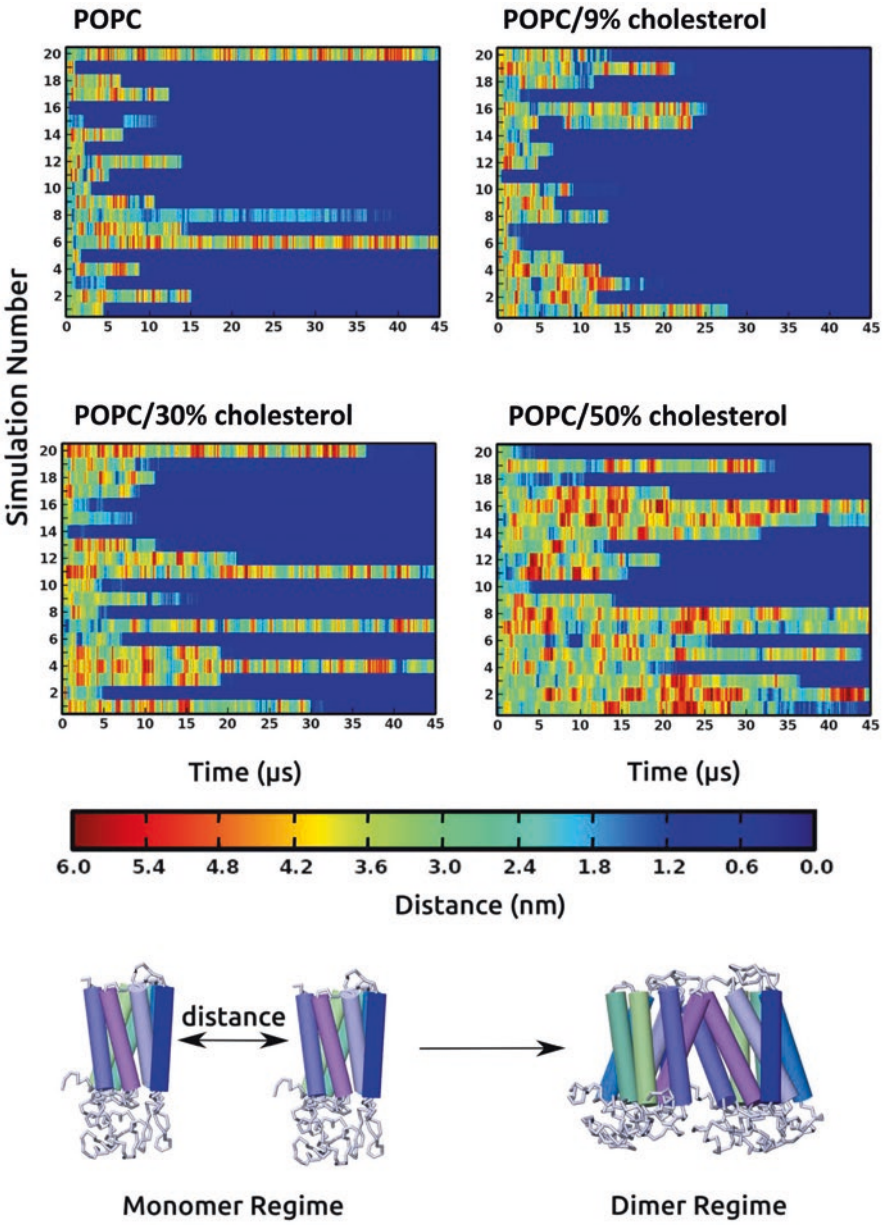

Fig. 16.4 Dimerization of the serotonin$_{1A}$ receptor is dependent on membrane cholesterol content. A schematic representation showing the minimum distance between two receptors in POPC membranes with increasing cholesterol content. Coarse-grain molecular dynamics simulations show that receptor dimerization is dependent on membrane cholesterol concentration. As apparent from the figure, lower number of dimers are observed at higher cholesterol concentrations. The entire range of distances between the receptor monomers is color coded and shown as a scale bar. The *bottom panel* shows the two receptors in monomer and dimer regimes (Adapted and modified from [44])

16.5.2 How Does Cholesterol Influence GPCR Dimerization?

The exact molecular mechanism of membrane cholesterol modulation of GPCR dimers is not clear [33, 36]. A recurrent theme pertains to the presence of lipid microdomains due to the presence of cholesterol. A more likely alternative view is that cholesterol modulates the energetics of the dimer interfaces, by destabilizing or stabilizing certain dimer conformations. For example, it has been proposed that membrane cholesterol interacts with the receptor and helps in forming a cholesterol-mediated dimer ([61]; see Fig. 16.5a for a schematic representation). On the other hand, unfavorable membrane perturbations due to hydrophobic mismatch have been proposed as non-specific mechanisms of GPCR dimer formations ([31]; see Fig. 16.5b).

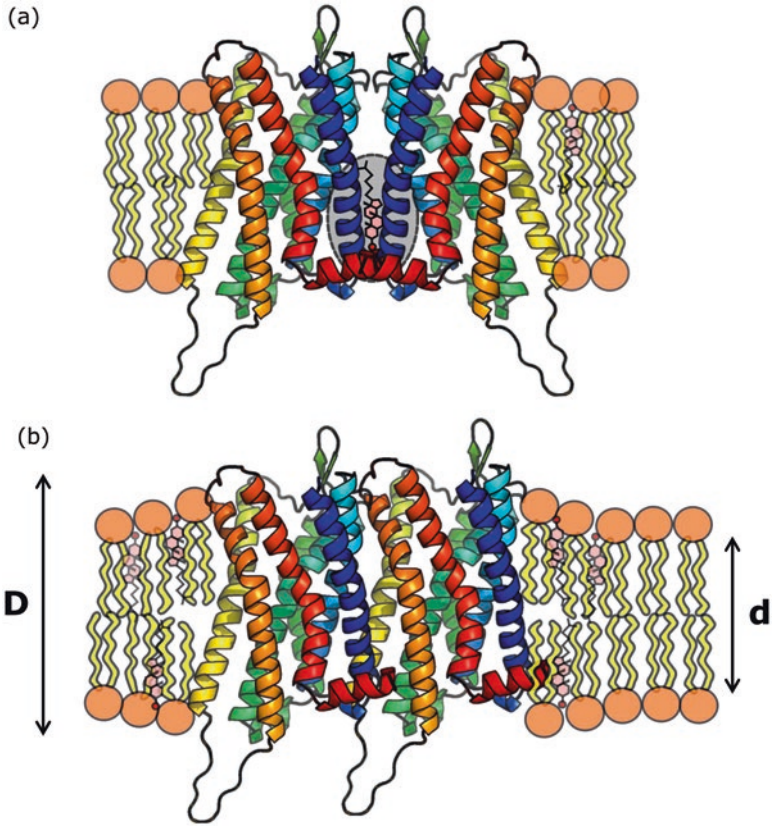

Fig. 16.5 Cholesterol-mediated mechanisms of modulation of GPCR dimerization. (**a**) Cholesterol may directly associate with GPCRs at inter-receptor (or inter-helical sites). (**b**) Hydrophobic mismatch between the length of the hydrophobic stretch in the receptor transmembrane region 'D' and membrane hydrophobic thickness 'd' can be induced by local concentration of membrane cholesterol

To test which of these mechanisms help to explain the cholesterol-mediated GPCR dimer interfaces in β_2-adrenergic receptor, we monitored both cholesterol binding sites and changes in the local membrane thickness. A direct correlation was observed between the occupancy of cholesterol at transmembrane helix IV and the dimer interface comprising of transmembrane helix IV [42]. We would like to suggest that cholesterol interaction sites are better represented as 'high-occupancy sites' or 'hot-spots', rather than binding sites [51, 52]. Along with the cholesterol interactions, the transmembrane helices that occur at the dimer interface (such as transmembrane helices I and IV) display large perturbations in their vicinity [43]. Taken together, these results suggest that non-specific effects direct the formation of dimers, while direct interaction of cholesterol with the receptor dictate the relative populations of the possible dimer species.

A similar effect is observed in case of the serotonin$_{1A}$ receptor dimer interface that could be related to both specific cholesterol interactions, and the non-specific effects [44]. An important difference observed with the β_2-adrenergic receptor was that the transmembrane helix I-II/I-II interface appears to be stabilized with increasing cholesterol concentration. This could be directly correlated to the cholesterol interaction sites that differ between these receptors. The most favorable cholesterol interaction site in the β_2-adrenergic receptor was at transmembrane helix IV, as opposed to several cholesterol interaction sites of comparable occupancies, including transmembrane helices I, V and VI in the serotonin$_{1A}$ receptor. This leads to the destabilization of the transmembrane helix IV/V interface in the β_2-adrenergic receptor in the presence of cholesterol, and an opposing stabilization of the flexible helix I-II/I-II interface in the dimer regime in the serotonin$_{1A}$ receptor. The take-home message is that cholesterol-mediated effects in GPCR dimers appear to be receptor-specific. A comprehensive approach involving both experimental and simulation inputs would help better understand this.

16.6 Future Perspectives: What Lies Ahead

Membrane cholesterol dependence of GPCR oligomerization opens up the important and interesting possibility of age and tissue dependence of drug efficacy. Cellular cholesterol is known to be developmentally regulated, also in a cell type/cell cycle dependent manner [27, 54, 55]. This could imply that the organization of dimers is age and cell type dependent. In addition, the tissue-dependent organization of GPCRs [5] could be important in the context of drug efficacy and specificity.

GPCRs act as crucial signaling hubs in higher eukaryotes. Although GPCRs represent the most predominant therapeutic targets, a large fraction of the GPCR receptorome remains unexplored from the drug discovery perspective [2]. It is estimated that ~150 GPCRs represent orphan receptors whose endogenous ligands and functions are yet to be established. These orphan receptors would be very useful in future drug discovery efforts. GPCR oligomerization and crosstalk incorporates

another dimension to this complex process of drug discovery. The exciting possibility of homo- and hetero-oligomerization of GPCRs provides tremendous diversity and potential to future drug discovery. Drugs that may prefer either GPCR dimers or monomers are already under consideration for novel drug development [21]. One such attempt involves development of drugs that block the activity of heterodimers of angiotensin II receptor type 1 (AT_1R) and chemokine type 2 receptor (CCR2) for the treatment of chronic kidney disease [3]. Similarly, post-synaptic heterodimers of adenosine A_{2A} receptor and dopamine D_2 receptor are believed to be crucial in the context of use of adenosine A_{2A} antagonists in the treatment of Parkinson's disease [10]. Knowledge of receptor oligomerization state under various pathophysiological conditions is of greater significance in the pharmacology of GPCRs since oligomerization gives rise to pharmacological diversity [56], opening new avenues for therapeutics.

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Part IV
Physiology and Therapeutic Potential

Chapter 17
Allosterism Within GPCR Oligomers: Back to Symmetry

Sergi Ferré

Abstract The Monod-Wyman-Changeux (MWC) model provided the most influential interpretation of allosterism within the frame of a symmetric oligomeric structure of regulatory enzymes. The initial studies of allosteric properties of G protein-coupled receptors (GPCRs) departed from these classical concepts of allosterism, considering GPCR monomers as main functional units. However, the phenomenon of GPCR homo- and heteromerization is becoming widely accepted. A new concept is that the pentameric structure constituted by one GPCR homodimer and one heterotrimeric G protein provides a main functional symmetric unit and oligomeric entities can be viewed as multiples of dimers. GPCR heteromerization opens up the possibility of allosteric interactions between different orthosteric ligands. Furthermore, the same properties of allosteric ligands demonstrated when considering GPCR as putative monomeric entities, mainly saturability, ability to separately alter the affinity and efficacy of the orthosteric ligand, probe dependence and functional selectivity, are also demonstrable with interactions between orthosteric ligands within the GPCR heteromer. A GPCR heterotetramer constituted by two molecularly different homodimers coupled to their cognate G protein and to adenylyl cyclase seems to constitute a common structure of a GPCR heteromer. Recent studies indicate that the canonical Gs-Gi interaction at the adenylyl cyclase level is a specific property of the GPCR heterotetramer. The evidence for GPCR oligomerization and the elucidation of symmetrical minimal functional units of GPCR homomers and heteromers, brings back the classical concepts of allosterism and promotes oligomerization and allosterism within GPCR oligomers as necessary elements in the research of GPCR physiology and pharmacology.

Keywords Allosterism • Symmetry • G protein-coupled receptor • Oligomerization • Heterotetramer • Adenylyl cyclase

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17.1 Classical Allosterism: Symmetry

'Allosterism' is currently defined as the process by which the interaction of a chemical or protein at one location on a protein or macromolecular complex (the allosteric site) influences the binding or function of the same or another chemical or protein at a topographically distinct site [1]. The prefix 'allo' in 'allosteric' has a Greek origin meaning 'other'. The term was introduced by Jacques Monod and Francois Jacob [2], to account for an 'allosteric inhibition', the situation where "an enzyme inhibitor is not a steric analog of the substrate". They largely based their assumptions in results obtained by their graduate student Jean-Pierre Changeux with the enzyme L-threonine deaminase, its substrate L-threonine and the regulatory inhibitor L-isoleucine [3]. Allosterism provided a new mechanism of regulatory proteins different to covalent modulation, such as phosphorylation. The introduction of these non-covalent modulations would initiate a revolution in Biochemistry, as Monod would anticipate when describing allosterism as "the second secret of life", after the genetic code [4].

The Monod-Wyman-Changeux (MWC) model was then introduced with the aim of bringing a general interpretation of allosteric effects within the frame of what it seemed a common oligomeric structure of regulatory, allosteric, enzymes [5]. The MWC model considered the quaternary organization and 'symmetry' properties of allosteric regulatory enzymes. At the outset of its description, the authors made explicit the distinction between two classes of allosteric effects, 'homotropic' and 'heterotropic', meaning interactions between identical or different ligands, respectively. It was specified that "few, if any, allosteric systems exhibiting only heterotropic effects are known. In other words, that homotropic effects are almost invariably observed with at least one of the two (or more) ligands in the system" [5]. Another significant conceptual distinction of Monod, Wyman and Changeux was between 'K systems' and 'V systems' [5] (although in their formulation they only addressed K systems). In any enzyme system, the effects are measured by two classical kinetic constants, Km and Vmax, as a function of the concentration of substrate and velocity of reaction. Two classes of independent effects could then be expected from allosteric systems. K systems demonstrate altered substrate affinity (Km) upon binding of the allosteric ligand, while V systems are those with altered catalysis (Vmax). Although the term 'cooperativity' had initially a less restricted use, it most commonly denotes the homotropic allosteric modulations of substrate affinity and can be either positive (as it is most often the case with regulatory enzymes) and negative (as it seems more common with membrane receptors, specifically with G protein-coupled receptors, GPCRs; see below).

The MWC model was described by a series of hypotheses, recently reviewed by Changeux [6]. Structurally, the MWC model implied that allosteric proteins are oligomers resulting from the assembly of 'protomers' associated in such a way that the molecule possesses at least one axis of symmetry. Protomers would be generally linked by a multiplicity of non-covalent bonds, conferring both specificity and stability on the association [5]. Two modes of association of the protomers could then

be assumed, 'isologous' and 'heterologous', where the bonding domains involve two identical or different binding sets, respectively. An isologous association confers a two-fold axis of rotational symmetry, giving rise to closed and even-numbered oligomers. As anticipated, isologous dimers and tetramers are very common among regulatory proteins [6], including hemoglobin, where four, distant O_2 hemes enveloped in a globin pocket interact cooperatively in an allosteric manner [7].

Functionally, the also called two-state concerted MWC model [5], which followed Changeux's Ph.D. thesis [3], represented the first elaborated model of the two-state mechanism of pre-existing conformational states, a consequence of a selective, rather than an instructive, effect of ligands [6, 8]. Oligomeric allosteric proteins have access to at least two reversible states, which are dependent on the conformational constraints imposed by the protomers. As pointed out by Changeux [6], "the notion that ligands selectively stabilize the state to which they preferentially bind and thereby mediate signal transduction via selection of conformational states arises directly from the formal description of the MWC model". *The affinity of the ligands, substrate and allosteric ligand, are altered with the transition of the protein from one to the other state and the molecular symmetry is always conserved*. Therefore, the presence of the allosteric ligand will be associated with a modification of the apparent affinity of the protein for the substrate, and conversely. As mentioned by Fenton [9], allosterism is then more strictly defined in functional terms as a comparison of how one ligand binds in the absence *versus* the presence of the second ligand. Cooperative ligand binding would then follow from the cooperative interactions between subunits and the MWC model would predict that in the systems in which an allosteric ligand modifies the apparent affinity of the substrate, but not in those systems where this affinity is not modified, the substrate should also exhibit cooperativity [5].

Recent structural and functional studies support the validity of the MWC model and agree with the allosteric interactions between regulatory and biologically active sites depending on conformational changes, but they have also shown that many regulatory proteins carry a variety of additional allosteric modulatory sites [6]. A significant fraction of these sites are localized at subunit interfaces, giving a frame for allosteric ligands to directly influence the quaternary organization of the protein [6]. A logical generalization would follow from allosterism of regulatory enzymes to membrane receptors, which would be equivalent to regulatory proteins which activated state would not imply a modification of the substrate (enzymes) but a signal transduction [10]. In fact, as Luigi Agnati indicated, instead of hemoglobin being awarded the rank of "honorary enzyme" conferred by Monod, it should as well be awarded the rank of "honorary receptor", since hemoglobin binds the ligand without inducing its modification [11].

17.2 Classical GPCR Allosterism: Departure from Symmetry

It soon became obvious that there was a parallelism between regulatory enzymes and membrane receptors, between substrate and orthosteric agonist, between Km and affinity, between Vmax and intrinsic efficacy. The orthosteric agonist (which binds to the same receptor site as the endogenous transmitter) has two main independent properties: affinity (the avidity to bind to the receptor) and intrinsic efficacy (the power of the bound agonist to induce a functional response). By binding to a non-orthosteric site, the allosteric ligand can then independently modify either of these properties. However, most research on GPCR allosterism developed independently from the concepts of classical allosterism, outside the frame of the MWC model and the symmetry construct, considering the GPCR monomer as the main functional entity.

A main reason that kept symmetry out of the scope of GPCR research was most probably the generalized assumption of interactions between GPCRs and G proteins in the frame of 'collision coupling' mechanisms. GPCRs, G proteins and adenylyl cyclase are the three elements of one of the most studied transmembrane cell signaling pathway [12]. In fact, this GPCR signal transduction is intrinsically allosteric as it involves the binding of an extracellular ligand and the "binding" of a G protein at a topographically distinct intracellular site [13]. In the classical and still generally accepted sequential model of GPCR-mediated signal transduction involving adenylyl cyclase activation, binding of one agonist molecule to one GPCR molecule leads, first, to G protein recruitment in its GDP-bound heterotrimeric Gαβγ form; second, G protein coupling triggers guanylyl nucleotide exchange, GDP for GTP, which induces rapid dissociation of Gα and Gβγ into free active subunits; third in its "active" GTP-bound state, Gα binds to adenylyl cyclase modulating its catalytic ability to produce cyclic adenosine monophosphate (cAMP) from ATP; finally, Gα GTPase activity induces hydrolysis of GTP to GDP, which terminates adenylyl cyclase signaling and promotes dissociation of Gα from adenylyl cyclase and reconstitution of the free heterotrimeric Gαβγ protein [12]. Explicitly, this classical model implies that the four components, GPCR, Gα, Gβγ and adenylyl cyclase, are freely mobile molecules able to couple by random collision. Collision coupling would be in line with the 'fluid mosaic' model of the plasma membrane, which considers the lipid bilayer as an isotropic milieu where membrane-embedded proteins diffuse in two dimensions and collide at random with each other [14].

It has been long recognized that agonists show 'binding heterogeneity', a dispersion of affinities for GPCRs in membrane preparations from transfected mammalian cell lines or from native tissues. The typical illustration is a competition experiment between a moderate concentration of a radiolabelled antagonist and increasing concentrations of the agonist, which results in a non-steep or even biphasic curve, apparently indicating the existence of two populations of GPCRs, with high and low affinities for the agonist. In the presence of a G protein, guanylyl nucleotides promote an apparent inter-conversion from the high to the low affinity sites, with

steepening and shifting to the right of the competition curve. In line with the classical view of GPCR signal transduction, a still common view of GPCR signaling holds that agonists promote the formation of a transient complex between the monomeric receptor and the G protein, with conformational changes in both molecules that lead to an increased affinity of the receptor for the agonist and for the heterotrimeric Gαβγ protein, which leads to G protein activation. The deep-rooted 'ternary complex' model of ligand-GPCR binding [15] proposes that the receptor-G protein complex accounts for the high affinity site for the agonist, while the G protein-uncoupled receptor accounts for the low affinity site. Guanylyl nucleotides would promote uncoupling of the receptor-G protein complex, converting all receptors into low affinity sites. According to the model, a non-steep or biphasic antagonist-agonist competitive-inhibition curve implies the existence of two populations of receptors, coupled and non-coupled to the G protein. Implicitly, this assumes a limited pool of G proteins (an assumption difficult to reconcile with the fact that the expression of G proteins in native cell systems outnumbers that of GPCRs [16]).

In apparent support for the 'monomer-ternary complex model', biphasic and guanylyl nucleotide-sensitive antagonist/agonist competitive inhibition curves were obtained with GPCR monomers (of β_2-adrenergic receptors, μ opioid receptors and rhodopsin) reconstituted in nanodisks, where they also activated G proteins [17–20]. In these studies, the proportion of receptor with high affinity for agonists increased from zero to 100% by increasing the G protein pool, indicating that the agonist binding heterogeneity was totally G protein-dependent, implying a G protein-mediated allosteric modulation of an orthosteric ligand in a non-symmetrical allosteric protein represented by the GPCR monomer. Experimental evidence therefore indicated that oligomerization, symmetry, was not a necessary condition for heterotropic allosterism within a GPCR.

"Monomeric" GPCR allosterism has been the focus of intense research, particularly related to the promising use of allosteric ligands in drug development, leading to the discovery of GPCR-specific allosteric-related phenomena. Those phenomena, now becoming classical properties of GPCR allosterism, are mainly 'saturability' or 'ceiling effect', 'the ability to separately alter the affinity and efficacy of the orthosteric ligand', 'probe dependence' and 'functional selectivity' or 'biased agonism' [1, 13, 21–23].

Saturability depends on the fact that the allosteric effect is the result of the binding of the allosteric modulator to a non-orthosteric site. When this site saturates, the allosteric effect reaches an asymptotic maximum value. In case of a negative allosteric modulator that selectively decreases the affinity of an orthosteric agonist, increasing concentrations will displace the concentration-functional response of the orthosteric agonist to the right but up to a limit, the ceiling effect. In contrast, a competitive orthosteric antagonist will theoretically produce a limitless displacement to the right. Following Fenton's description of allosterism [9], in line with the formal description of the MWC model for regulatory proteins but without considering oligomerization or symmetry, the ability to separately alter the affinity and efficacy of the orthosteric ligand, probe dependence and functional selectivity would reflect the ability of specific ligands and allosteric modulators (also including G

proteins, ß-arrestins and other signaling molecules as allosteric modulators) to simultaneously interact and stabilize specific states, conformations of the receptor. In this way, we can in principle conceive specific allosteric modulators that will preferentially associate and therefore stabilize a receptor conformation where the orthosteric agonist or the signal molecule bind with less affinity, leading to the respective apparent decrease in affinity or efficacy of the ligand; or a specific orthosteric agonist that prefers a receptor conformation also preferred by the allosteric modulator (probe dependence); or a specific orthosteric agonist that prefers a receptor conformation also preferred by a specific signaling molecule (functional selectivity).

Functional selectivity of GPCR ligands is becoming a particular objective of preclinical and clinical pharmacology. The main focus is finding compounds with predominant G protein- versus ß-arrestin-mediated signaling or the opposite, since there is evidence for their possible differential involvement in therapeutic *versus* side effects [24]. A recent clinically promising example is PZM21, a μ-opioid receptor agonist with functional selectivity for Gi/o-mediated signaling, associated to the analgesic effect, *versus* ß-arrestin-mediated signaling, associated with respiratory depressant and potentially addictive effects [25]. In our research group, we are extending the functional selectivity concept to G protein subtypes. We are finding evidence for differential affinity or efficacy of endogenous and synthetic ligands to dopamine D_2-like receptors and α_2-adrenoceptors depending on their specific coupling to the different Gi/o protein subtypes (Gi1, Gi2, Gi3, Go1 and Go2) [26, 27].

17.3 Allosterism Within GPCR Homomers: Back to Symmetry

Two concepts are gaining increasing acceptance in GPCR pharmacology, which are changing the landscape of GPCR allosterism: (1) pre-coupling of GPCRs with their preferred signaling molecules, and (2) GPCR oligomerization (reviewed in Ref. [28]). Accumulating experimental evidence suggests that GPCR activation commonly occurs without dissociation of the receptor from its G protein, without G-protein subunit dissociation and also with pre-coupling of the heterotrimeric G protein to adenylyl cyclase [28]. Experiments with resonance energy transfer (RET) techniques have provided clear evidence indicating that G protein subunits remain associated and pre-coupled during activation of many GPCRs [29–31]. The readout of experiments with RET donor and acceptor molecules fused to Gα and Gγ subunits indicates that G protein activation upon ligand binding to the receptors does not lead to dissociation, but implies a conformational change with rearrangement, reorientation, of its subunits [26, 27, 29, 30]. Levitzki and collaborators advanced this interpretation after finding successful signal transduction of a yeast pheromone receptor with fused G protein subunits [32]. Levitzki was also first to suggest that G proteins form stable complexes with adenylyl cyclase being independent of the

activation state of G proteins, mostly based upon co-purification of adenylyl cyclase from turkey erythrocyte membranes [33]. RET experiments have also supported these interactions [34, 35]. The structural model of G protein activation proposed by Bouvier's group [30] implies a significant modification of the quaternary structure of the heterotrimeric G protein, a reorientation without dissociation of the subunits still compatible with the existence of overlapping binding sites of Gα for adenylyl cyclase and Gβγ [36]. Their findings suggest that during G protein activation Gβγ is maintained in the heterotrimeric complex while being displaced away from its original site of association with Gα [30].

If pre-coupling is the norm, what about the two putative interconvertible populations of GPCR monomers coupled and not coupled to GPCR, conferring different affinity states for the agonists? Symmetry, oligomerization, cooperativity come to the rescue: a 'dimer-cooperativity model', which considers GPCR oligomerization, with minimal functional units as GPCR homodimers that allow a homotropic allosteric modulation, i.e., negative or positive cooperativity [37, 38]. There are no high and low affinity states of the receptor for the agonist, but an affinity of the agonist for the binding to the first protomer, which allosterically modulates the affinity for the second. In fact, the monomer-ternary complex model could never explain some radioligand binding data, such as an apparent modulation of antagonist binding by guanine nucleotides [39–41], biphasic antagonist/antagonist competition curves [42] or a downward concave Scatchard plots, which would indicate positive cooperativity [43]. All these data can be explained with the dimer-cooperative model, which does not assume a limited pool of G proteins. Instead, G protein can always be coupled and act as an additional allosteric modulator which increases or decreases the affinity of the agonist depending on its binding to guanylyl nucleotides. Recent studies by Redka et al. [44, 45] have unequivocally shown that although G proteins have a modulatory effect and impart sensitivity to guanylyl nucleotides, they are not required for the agonist binding heterogeneity *per se*, implying cooperativity and therefore dependence on GPCR oligomerization. These seminal studies were obtained by comparing the effect of G proteins and guanylyl nucleotides on antagonist/agonist competition experiments from monomeric acetylcholine muscarinic M_2 receptors (in solution or reconstituted nanodisks) and oligomeric M_2 receptors (preferentially in tetrameric form, in reconstituted vesicles). Significantly, these studies also showed that GPCR oligomers but not monomers resemble M_2 receptors in myocardial tissue in their qualitative response to guanylyl nucleotides [45]. Qualitative differences within the oligomeric versus the monomeric GPCR agree with symmetry. Every new element binding to an allosteric protein should constrain the possibilities of conformational states, which should determine differences in the preferred conformational state of the whole oligomer compared to the monomer when binding the available orthosteric and allosteric ligands.

Analysis of the dissociation kinetics of a tracer ligand in the absence and presence of a second ligand also represents a sensitive method to detect cooperative interactions between two topographically distinct binding sites. Thus, ligands that compete for the same site on a monomeric receptor should not influence one another's dissociation kinetics. In contrast, allosteric modulation between two

simultaneously bound and interacting sites, either within a receptor monomer or across a receptor homodimer or oligomer, should alter ligand dissociation [46]. This analysis has been used to demonstrate oligomerization of several GPCRs [43, 47–49]. Furthermore, negative cooperativity has also been demonstrated in living cells by measuring dissociation kinetics with fluorescent GPCR ligands [49]. GPCR homodimers could also be demonstrated in a native tissue (mammary glands) using ligands for oxytocin receptors separately fused to donor and acceptor Fluorescence RET (FRET) biosensors [50]. In the same study, the differential FRET obtained with labeled agonists and antagonists, with significantly lower FRET values obtained with agonists, indicated a stoichiometry of one agonist molecule per dimer consistent with negative cooperativity [50]. In summary, radioligand binding experiments and biophysical techniques have provided solid evidence for cooperativity as a common allosteric property of some ligands that bind to GPCR homomes and oligomers. In fact, cooperativity means a homotropic allosteric modulation, oligomerization, symmetry.

The analysis of the GPCR agonist binding heterogeneity therefore reveals that at least two GPCR protomers form part of a 'minimal signalosome unit'. An important additional concept that then arises from the new field of GPCR oligomerization is that *the pentameric structure constituted by one GPCR homodimer (with two orthosteric binding sites) and one heterotrimeric G protein provides a main functional unit and oligomeric entities can be viewed as multiples of dimers* [28, 38]. Additional evidence has been provided by application of RET and other biophysical techniques in living cells, such as fluorescence correlation spectroscopy and analysis of single fluorescence-labeled receptor molecules by total internal reflection fluorescence microscopy [51, 52]. Interestingly, although oligomerization of family C GPCR is well established, there is still resistance to accept homo- and heteromerization for family A and B GPCRs. However, recent crystallographic evidence has been reported for family A GPCR homodimers, including the chemokine CXCR4 receptor, the μ-opioid and κ-opioid receptor, the β_1-adrenergic receptors and rhodopsin (reviewed in [38]). Irrespective of the GPCR family, homodimers seem to be a predominant species [51, 52] with potential dynamic formation of higher-order symmetric oligomers, particularly tetramers [44, 45, 53]. Biochemical evidence implicates at least two distinct G protein regions in their interactions with the GPCR, localized in the Gα and Gγ subunits [54, 55]. The distance between these two regions in the Gαβγ heterotrimer is larger than the width of one GPCR protomer, indicating that for both contacts to take place simultaneously, one heterotrimeric G protein must contact two receptor protomers [54]. Altogether, there is evidence supporting a predominant pentameric structure of the GPCR homodimer-heterotrimeric G protein complex. Recently, the pentameric structure of two rhodopsin protomers and one heterotrimeric Gt has also been identified [56, 57].

The 'two-state dimer model', which is the most applicable model used to analyze radioligand binding experiments considering GPCRs homodimers as main functional units, provides very robust theoretical functions that yield very a significant fit with the experimental data [37, 38, 58]. Its robustness means that the calculated parameters are little dependent on changes in experimental conditions [58], which

is not the case for the 'two-independent-site model', the most commonly used model to analyze results from radioligand binding experiments. For instance, when trying to resolve complex radioligand binding data using the two-independent-site model, the values of the equilibrium dissociation constants and number of receptors obtained vary significantly depending on the concentration of the radioligand, which is not the case for the two-state dimer model [58]. In saturation experiments, the two-state dimer model provides the total number of radioligand binding sites (B_{max}; more specifically it calculates RT, the total number of dimers), the affinity of the radioligand for the first protomer in the unoccupied dimer (K_{DA1}), the affinity of the radioligand for the second protomer when the first protomer is already occupied by the radioligand (K_{DA2}) and an index of cooperativity of the radioligand (D_{CA}). A positive or negative value of D_{CA} implies either an increase or a decrease in affinity of K_{DA2} versus K_{DA1} and its absolute value provides a measure of the degree of increase or decrease in affinity (Fig. 17.1). In competition experiments, the model analyzes the interactions of the radioligand with a competing ligand and it provides the affinity of the competing ligand for the first protomer in the unoccupied dimer (K_{DB1}), the affinity of the competing ligand for the second protomer when the first protomer is already occupied by the competing ligand (K_{DB2}) or the radioligand (K_{DAB}) and an index of cooperativity of the competing ligand (D_{CB}). A positive or negative value of D_{CB} implies either an increase or a decrease in affinity of K_{DB2} versus K_{DB1} and its absolute value provides a measure of the degree of increase or decrease in affinity [37, 38] (Fig. 17.1).

The two-state dimer model assumes an initial symmetry during the first encounter of the ligand with the homodimer, i.e., the ligand has the same affinity for either protomer of a non-occupied homodimer. The apparent asymmetric pentameric structure of GPCRs (homodimer plus heterotrimeric G protein) might then seem incompatible with the two-state dimer model and the dimer-cooperativity model, especially if we also assume pre-coupling of GPCR, G proteins and adenylyl cyclase. Nevertheless, still within the frame of pre-coupling we can assume that G proteins are dynamically and symmetrically interacting with the GPCR before agonist binding and that the ligand modifies this interaction by stabilizing a conformation of the receptor that allows the α subunit to "tightly" bind the receptor and induce G protein activation [54].

In addition to ligand-binding properties, unique allosteric properties for each GPCR homodimer emerge in relation to intrinsic or signaling efficacy. Most experimental data agree with the model that proposes that ligand occupancy to the first protomer is enough to produce a significant G protein activation and functional response. When the ligand binds to the second protomer in the homodimer, it will often act as an allosteric modulator of the intrinsic efficacy of the ligand when bound to the first protomer, by potentiating or reducing the functional response, irrespective of the allosteric modulations at the binding level (reviewed in [38]). Furthermore, in addition to considering the allosteric modulations of an orthosteric agonist binding to the first promoter on the same ligand binding to the orthosteric site in the second protomer, a significant number of possible pharmacological allosteric modulations mediated by the homodimer

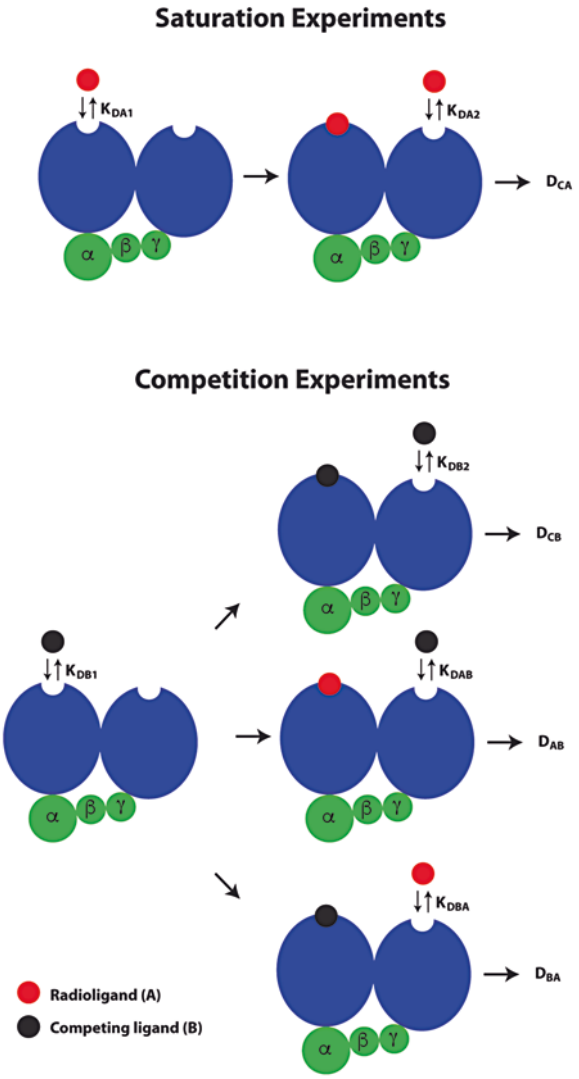

Fig. 17.1 Analysis of radioligand-binding experiments considering GPCRs as dimers: The two-state dimer model. For saturation experiments, K_{DA1} and K_{DA2} are the macroscopic equilibrium dissociation constants, which define the dissociation equilibria involved in the binding of a ligand to the receptor dimer. D_{CA} represents the *dimer cooperativity index*. $D_{CA} = 0$ implies no cooperativity, while positive and negatives values imply positive and negative cooperativity, respectively. For competition experiments, K_{DB1} and K_{DB2} correspond to the macroscopic equilibrium dissociation constants for the binding of the competing ligand to the first and second receptor in the dimer. K_{DAB}, is a value of the association and dissociation of the competing ligand on a dimer semi-occupied by the radioligand. Reciprocally, K_{DBA} is a macroscopic equilibrium dissociation constant of the radioligand binding to a receptor dimer semi-occupied by the competing ligand. D_{AB} and D_{BA} represent the corresponding dimer radioligand/competitor modulation indexes. D_{AB} or $D_{BA} = 0$ implies no modulation, while positive and negatives values imply positive and negative modulation, respectively. D_{CB} defines a dimer cooperativity index for the competing ligand. $D_{CB} = 0$ implies no cooperativity, while positive and negative values imply positive and negative cooperativity

appear when considering other ligands, not only orthosteric agonists and antagonists [38], but also positive and negative allosteric modulators and even bitopic (allosteric and orthosteric) ligands [59].

What would be the functional significance of homotropic allosterism, i.e., modulation of affinity (cooperativity) or modulation of efficacy of endogenous ligands by a GPCR homomer? In a framework of symmetric properties of both protomers, we would expect two different levels of ligand-mediated signaling that would depend on the concentration of the ligand. Negative homotropic allosterism could provide a mechanism that protects the biologic system against acute elevations of the endogenous ligand [11]. Positive homotropic allosterism, on the other hand, could provide an amplificatory mechanism, although to our knowledge no clear examples of positive cooperativity of endogenous ligands have been reported.

17.4 Allosterism Within GPCR Heteromers: Keeping Up with Symmetry

Although still fought with skepticism, the list of putative functional and pharmacologically significant GPCR heteromers keeps increasing. It must however be acknowledged that many candidates do not follow the consensus criteria recently established for their identification in native tissues [38, 60, 61]. GPCR heteromerization opens a new dimension of possible molecular and functional protein interactions within a signalosome. It also opens up the possibility of *heterotropic allosteric interactions between different orthosteric ligands*. The realization of this possibilities is leading to a profound modification of classical pharmacology. As briefly reviewed, when symmetry is maintained, GPCR heteromers provide the frame for biochemical interactions previously thought to be independent of intermolecular receptor-receptor interactions, on GPCR oligomerization, but on what it was previously labelled as interactions "via intracytoplasmic loops" or "at the second-messenger level" [62–64]. Second, the same properties of heterotropic interactions described for allosteric ligands when considering GPCR as putative monomeric entities, mainly saturability, ability to separately alter the affinity and efficacy of the (other) orthosteric ligand, probe dependence and functional selectivity, are also demonstrable in the heterotropic interactions between orthosteric ligands for the molecularly different protomers in a GPCR heteromer.

If the pentameric structure constituted by a GPCR homodimer and one heterotrimeric G protein provides a main functional unit, oligomeric entities should be viewed as multiples of dimers and GPCR heteromers would be mostly constitutes by heteromers of homodimers [38]. Recent studies are in fact providing strong support for what would be a symmetric solution of a complex heterogeneous macromolecular complex. This seems to apply particularly to heteromers that include a GPCR homodimer with preferential coupling to Gs/olf (Gs for short) proteins and another molecularly different homodimer with preferential coupling to Gi/o (Gi for short)

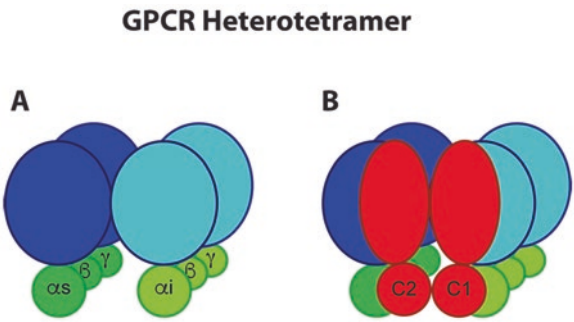

Fig. 17.2 The GPCR heterotetramer. (**A**) Constituted by two GPCR homodimers, one pre-coupled to Gs and and the other to Gi protein. (**B**) Gs and Gi coupled to the GPCR homodimers in the heterotetramer can simultaneously couple to the C2 and C1 catalytic domains of adenylyl cyclase (in *red*), respectively, providing the frame for the canonical Gs-Gi interaction at the adenylyl cyclase level

proteins. Such a 'GPCR heterotetramer' would sustain a functional pre-coupled macromolecular complex that includes two molecularly different GPCRs, their cognate G proteins and adenylyl cyclase and would provide the frame for a canonical interaction at the adenylyl cyclase level, the ability of a Gi-coupled GPCR to counteract adenylyl cyclase activation induced by a Gs-coupled GPCR [28] (Fig. 17.2). Recent studies using biophysical techniques and computerized modeling have provided experimental evidence for the existence of several GPCR heterotetramers that fulfill this scheme: the dopamine D1-D3 [65], the adenosine A_{2A}-dopamine D_2 [66, 67], the adenosine A_1-A_{2A} [68] and the A_1-D_1 receptor heterotetramer (in preparation). Using disrupting synthetic peptides with the amino acid sequence of different transmembrane domains of the receptors, we can now determine not only the interfaces involved in hetero- and homomerization in the heterotetramer [65, 67], but also the interfaces involved in the complex formation with adenylyl cyclase (in preparation). A very significant output of these studies is that the canonical Gs-Gi interaction at the adenylyl cyclase level is a specific property of a heterotetramer. In fact, it was already implicit that it should depend on an interaction between activated Gs and Gi proteins respectively and simultaneously interacting with the C2 and C1 catalytic domains of adenylyl cyclase [28, 69]. *Therefore, we are witnessing the fusion of an enzyme (adenylyl cyclase) with a complex GPCR oligomer, which might after all represent the main regulatory sensor of a macromolecular regulatory protein complex.* In addition, we are also obtaining conclusive evidence that indicates that symmetry is maintained (in preparation).

The A_{2A}-D_2 receptor heteromer constitutes one of the most studied and one of the few in the list of GPCR heteromers that fulfill the criteria for their functional significance in native tissues [61]. It will be used in this essay to elaborate on the generalization of the properties of heterotropic interactions between allosteric and orthosteric ligands in a putative GPCR monomer or a GPCR homomer to heterotropic interactions between different orthosteric ligands in a GPCR heteromer. Saturability can explain the relatively small shifts in radioligand-binding experiments caused by orthosteric-orthosteric ligand interactions in putative GPCR heteromers, which were

initially known as 'intramembrane receptor-receptor interactions' [62, 63, 70]. When trying to reach a consensus on nomenclature related to receptor oligomerization, it was proposed that this term be replaced by 'allosteric interaction in the receptor heteromer' [60]. However, with the new developments in this fast-growing field of GPCR oligomerization, particularly with the evidence of heteromerization of GPCR homodimers, it would be more appropriate and specific to be named 'heterotropic allosteric interaction in the GPCR heteromer'. In addition, we should distinguish between modulation of affinity or intrinsic efficacy. Although these allosteric interactions were initially believed to only involve orthosteric agonists, more recently we found evidence for orthosteric agonist-antagonist and also antagonist-antagonist heterotropic allosteric interactions in the GPCR heteromer. Thus, independent of its intrinsic efficacy, any orthosteric ligand for one of the protomers of a GPCR heteromer can be associated with a change in the affinity and/or intrinsic efficacy (in case of an agonist) of any orthosteric ligand for another molecularly different protomer of the same GPCR heteromer. This was recently observed with radioligand binding experiments in transfected mammalian cells and in the striatum (including human striatum) as well as with signaling experiments in tranfected cells. Basically, any orthosteric adenosine A_{2A} receptor ligand, agonist or antagonist, was able to decrease the affinity and intrinsic efficacy of any orthosteric dopamine D_2 receptor ligand, agonist or antagonist [67]. These constituted biochemical properties of the A_{2A}-D_2 receptor heteromer, since they depended on the integrity of the right quaternary structure of the heteromer, as demonstrated in transfected mammalian cells and striatal tissue by using disrupting mutations and peptides, respectively [67]. Surprisingly, these allosteric modulations disappeared upon agonist and antagonist co-administration. But results from radioligand dissociation experiments (as well as from experiments with double complementation of Bioluminescence RET biosensors) provided evidence for a tetrameric structure of the A_{2A}-D_2 receptor heteromer, constituted by A_{2A} and D_2 receptor homodimers, which could offer an explanation for this apparent contradiction [67]. The model assumes that occupancy of the A_{2A} receptor homodimer with either an orthosteric agonist or an orthosteric antagonist is associated with a conformational state that is associated with a reduced affinity and efficacy of D_2 receptor ligands, while simultaneous occupancy of the A_{2A} receptor homodimer by an orthosteric agonist and an orthosteric antagonist would not be possible with this conformational state (as indicated by differences observed in dissociation experiments of a radiolabelled A_{2A} receptor antagonist with agonists and antagonists). The model demonstrated an important heuristic value. As the model predicted, in the brain, under specific experimental conditions, A_{2A} receptor antagonists behaved as A_{2A} receptor agonists and decreased D_2 receptor function, and these effects were counteracted upon co-administration of both A_{2A} receptor agonists and antagonists (electrophysiological and locomotor activity experiments) [67]. Since under physiological conditions there is a tone of adenosine, this could in fact be the main mechanism by which the non-selective adenosine receptor antagonist caffeine and A_{2A} receptor antagonists produce psychomotor stimulant activation, by counteracting the functional effects that depend on D_2 receptor signaling by the A_{2A}-D_2 receptor heterotetramer.

The A_{2A}-D_2 receptor heterotetramer also provides a good example for the ability of an orthosteric ligand of a protomer in a GPCR heteromer to separately alter the affinity and efficacy of another orthosteric ligand for the other molecularly different protomer. In fact, this is also related to the concept of functional selectivity, since implies a further separation in the efficacy of activating different signaling proteins. We have recently shown that different intracellular Ca^{2+} levels exert a differential modulation of the A_{2A}-D_2 receptor heteromer-mediated adenylyl-cyclase and MAPK signaling in transfected mammalian cells and in striatal cells [66]. This depended on the ability of low and high Ca^{2+} levels to promote a selective interaction of the heteromer with the neuronal Ca^{2+}-binding proteins NCS-1 and calneuron-1, respectively. These Ca^{2+}-binding proteins differentially modulate (promote or disrupt) allosteric interactions within the A_{2A}-D_2 receptor heterotetramer, which therefore constitutes a unique cellular device that integrates extracellular (adenosine and dopamine) and intracellular (Ca^{+2}) signals to produce a specific functional response [66]. *These studies show that there are probably many possible additional molecules than can interact with the GPCR heterotetramer, adding more constraints to the possible landscape of its conformational states, which determine all possible allosteric interactions between ligands binding to all possible orthosteric and allosteric sites.*

Finally, as an example of probe dependence, out of six different screened selective A_{2A} receptor antagonists, we found a specific A_{2A} receptor antagonist, SCH442416, that displayed a selective homotropic allosteric modulation in the A_{2A}-D_2 receptor heterotetramer [42]. The allosteric property consisted on a strong negative cooperativity (as analyzed with the two-state dimer model) and was only present in cell preparations or in neuronal elements co-expressing A_{2A} and D_2 receptors. These results, in fact, gave the first clue about the tetrameric structure of the A_{2A}-D_2 receptor heteromer, which should include at least two A_{2A} receptor protomers to show homotropic cooperativity. Being a weak ligand for the A_{2A}-D_2 receptor heteromer, SCH442416 would not be useful in Parkinson's disease. Nevertheless, SCH442416 acts preferentially on presynaptic striatal A_{2A} receptors localized in cortico-striatal glutamatergic terminals, which in fact form heteromers with A_1 receptors [71]. By blocking presynaptic A_{2A} receptors, SCH442416 potently blocks cortico-striatal glutamatergic neurotransmission at doses that do not produce psychomotor activation, that do not block postsynaptic A_{2A}-D_2 receptor heteromers [42], which can have implications for the treatment of substance use disorders and other neuropsychiatric disorders [72]. At a more general level, these results underline the concept of using GPCR receptor heteromers-selective compounds to target specific cell types, for specific therapeutic purposes.

17.5 Conclusion

The possibility of finding the symmetry properties of allosteric regulatory enzymes in membrane proteins, including neurotransmitter receptors, was already explored by Changeux [3, 6]. He was particularly interested in ligand gated ion channels, since he was involved in the isolation and biochemical characterization of the nicotinic acetylcholine receptor [6, 8, 73]. In fact, a number of allosteric sites have been demonstrated in ligand gated ion channels, which are constituted by the assembly of subunits with symmetry axes perpendicular to the membrane plane [6, 8]. The initial studies of allosteric properties of GPCRs departed from the classical concepts of allosterism and there have been few attempts to explicitly evaluate those properties in terms of the MWC model [74, 75]. The here reviewed increasing evidence of oligomerization of GPCRs, with the elucidation of symmetrical minimal functional units of GPCR homomers and heteromers, brings back the classical concepts of allosterism and promotes oligomerization and allosterism within GPCR oligomers as necessary elements in the research of GPCR physiology and pharmacology.

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Chapter 18
Understanding the Physiological Significance of GPCR Dimers and Oligomers

Rory Sleno, Dominic Devost, and Terence E. Hébert

Abstract The significance of G protein-coupled receptor (GPCR) oligomerization has been a subject of intense study which still remains controversial. Functional class C GPCRs are well accepted as obligate dimers but scepticism still remains with regards to the generalizability of this phenomenon when considering other classes of GPCRs. Here, we focus on understanding the organization and relationships between receptor equivalents in oligomeric receptor complexes. We discuss receptor properties such as ligand binding cooperativity which can be best explained in the context of functional oligomeric entities, and the asymmetries in receptor structure and function created by oligomers as well as their implications for drug discovery.

Keywords G protein-coupled receptors • Dimerization • Signalling complexes • Allostery • Receptor organization

18.1 Introduction

GPCR dimers or oligomers have garnered significant interest in the last 20 years. The idea that homodimers existed was greeted with both excitement and scepticism-both of which continue to this day. Since the discovery that GABA-B receptors were obligate heterodimers in 1998–1999 (and other class C GPCRs since), many researchers became interested in how such heterodimers might generally alter our understanding of ligand specificity, allostery and downstream signalling outputs from GPCRs. The field remains lively, but apart from the certainty regarding class C GPCRs, the combination of excitement *and* scepticism remains regarding dimers in class A GPCRs [1–4]. We do not intend here to conduct an extensive review of the literature. Interested readers are directed to comprehensive recent reviews [5–7]

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and to the many other chapters in this volume. Rather, we will focus on examples where homo- and heterodimers help explain current observations regarding GPCR biology.

The question of GPCR stoichiometry *in vivo* is one that remains unanswered at present. It is clear that most if not all GPCRs can form homo- and/or heterodimers in heterologous expression systems. However, the jury remains undecided with respect to the functional roles of such dimers under native conditions in cells and tissues. We will discuss studies of the organization of GPCRs in the context of receptor oligomers which suggest that signalling may be driven or modulated by different asymmetric arrangements of receptors associated with their signalling partners. These latter considerations suggest an entirely new way of viewing GPCR oligomers and their associated signalling machinery as allosteric machines. We will also comment on the evolving picture of homo- and heterodimerization *in vivo*.

18.2 Monomers, Dimers and Oligomers, Oh My…

Reconstitution of GPCRs into proteoliposomes has shown that they can signal as monomeric proteins [8, 9]. However, it has also become clear in recent years that most if not all GPCRs can form dimers and possibly higher order structures (see [5–7, 10–13]) for review). Further, although most studies have suggested that dimers are fairly stable entities, several recent reports have suggested that dimers exist in an equilibrium with their monomeric forms. Using antibody crosslinking and fluorescence recovery after photobleaching (FRAP), it was demonstrated that dimerization of D2 dopamine receptors was a dynamic process, when one partner in the dimer was immobilized and photobleached, the other dimer partner remained mobile [14]. The same group also showed using bioluminescence resonance energy transfer (BRET) that β_2AR might become dissociated when the receptors are internalized [15]. However, some of the loss of BRET signal could also be attributed to conformational change rather than a loss of interaction *per se*. Using a combination of labelled ligands and total internal reflectance fluorescence (TIRF) microscopy, it was demonstrated that M1 muscarinic receptors are in an equilibrium between monomers and dimers where rapid interconversion was detected using single particle tracking [16]. Similar findings were obtained using single particle tracking of the N-formyl peptide receptor, again using a fluorescently-labelled ligand and TIRF microscopy [17]. It remains interesting to pursue such studies using heterodimeric receptors, rather than homodimers. This becomes important in light of recent studies demonstrating allosteric interactions between GPCRs in both contexts. As discussed below, it is especially critical in the case of receptor heterodimers.

Do these single particle tracking approaches exclude the possibility of receptor oligomers? Such data could be interpreted in different ways, one of which would imply allosteric interactions in the context of receptor oligomers, rather than a simple monomer:dimer equilibrium. Perhaps there is an equilibrium between dimers and larger structures. Using quantum-dot tagging of neurokinin-1 receptor, a recent

study demonstrated that a considerable plasticity occurs, depending on the presence or absence of agonist with respect to receptor clustering, suggesting an organizational plasticity dependent on cell state [18]. Another recent study using spatial intensity distribution analysis (SpIDA) showed that the distribution of M1 muscarinic receptors monomers versus dimers/oligomers was sensitive to agonist, the latter being more prevalent following agonist stimulation [19]. Other approaches using super resolution microscopy have also demonstrated a similar dynamic structural arrangement (reviewed in [20]).

However, the allosteric nature of ligand effects on binding in the context of a receptor oligomer was not directly considered in any of these studies. Interestingly, interactions between orthosteric and allosteric ligands can be detected in M2 muscarinic receptor monomers but those seen in receptor oligomers are richer and more complex, likely reflecting a broader functional role in the cell [21]. Ligand binding experiments provided initial evidence that GPCRs were multimeric proteins, with allostery providing a mechanism to explain cooperativity measured between different equivalents of ligand [22]. This was quickly overshadowed by the discovery that G proteins were critical allosteric regulators of receptors, turning the attention of the field towards receptor-G protein interactions. Later ligand binding studies explicitly suggested that oligomeric arrangements of receptors underlay cooperativity [23–26]. Indeed, allostery between ligand binding sites in receptor homodimers could be measured even when G protein partners were removed from receptor preparations [27, 28]. Cooperativity in ligand binding at the M2 muscarinic receptor dimer reconstituted with G protein was lost when a monomeric version of receptor was studied [29]. This argues that allostery is manifested between the receptors themselves through physical contact as part of a dimer. Certainly, G protein and effectors could also have allosteric impacts on ligand binding, as parts of larger complexes, with particular networks of allostery depending on which proteins interact during different phases of signal transduction.

18.3 Structural Asymmetries in GPCR Oligomers?

A number of studies have suggested that GPCRs can form higher order complexes in addition to homo- or heterodimers [25, 30]. Protein fragment complementation approaches (PCA) have been used to expand our knowledge regarding GPCR oligomerization. Reconstitution of split luciferase (*Gaussia* or *Renilla*) and split GFP constructs have shown that dimers of β_2AR [31] and D2 dopamine receptors [32] can be detected, complementing immunopurification and RET approaches, and most importantly, these approaches can be combined to detect and examine larger complexes. A number of investigators have used three partner PCA/RET to show that higher order complexes of GPCRs such as the A_{2A}-adenosine receptor homo- and hetero-oligomers with CB1 cannabinoid/D2 dopamine receptors [33–36] and CXCR4 multimers [37] can be detected.

FRET approaches have suggested similar higher order structures for the M2 muscarinic receptor and the β_2AR [38, 39]. Using spectral de-convolution and

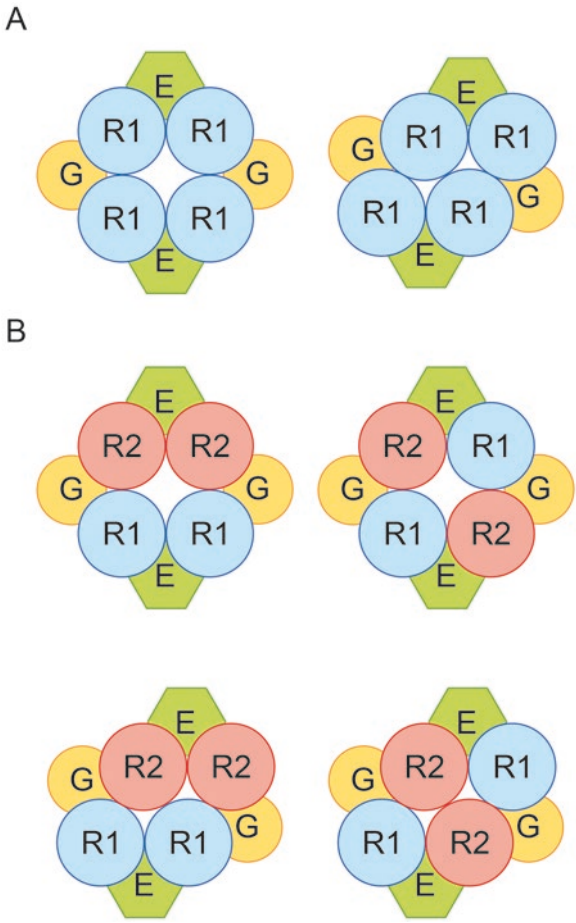

Fig. 18.1 *Asymmetric organization of receptor homo- and hetero-oligomers*. (**a**) Different views of receptor homotetramers in square or rhomboid configurations. In the rhomboid configuration, potential structural asymmetries with respect to organization of these complexes become evident. Thus, how receptors are organized and assembled with the interacting proteins might be controlled in the cell to produce distinct signalling architectures. (**b**) GPCR heterotetramers increase the organizational complexity further. The assembly of heterodimers and heterotetramers provides a much larger scope for the assembly of distinctly regulated allosteric signalling machines in either square or rhomboid orientations. Even in the "square" configuration (*top*), a number of asymmetries become possible with respect to how the signalling complex is organized, which again become greater in the "rhomboid" configuration (*bottom*). These differential arrangements may be manifested by ligand binding cooperativity between receptor equivalents and in how this information is transmitted to interacting proteins

fluorescence lifetime imaging, it was shown that M2 receptor homotetramers are likely to be in a rhomboid orientation, rather than a simple square array of receptor monomers ([38]), Fig. 18.1a, left panel). Such potential structural asymmetries may have dramatic impacts on signalling complex organization and thus functional

outputs. Homodimers or even homotetramers in a square array, have, by definition, fewer possibilities for asymmetric arrangements compared to rhomboid-shaped homotetramers, where structural asymmetries can be introduced with respect to how the entire receptor, G protein, effector complex is arranged (Fig. 18.1a, right panel). In the case of heterotetramers, the potential of either square or rhomboid arrangements for distinct allosteric interactions between receptors, G proteins and effectors is even greater (Fig. 18.1b). Thus, structural asymmetries in GPCRs may translate into steric constraints that play out into the organization of signalling complexes and ultimately in their function as allosteric machines. In fact, a recent study suggested that ghrelin receptor significantly alters D2 dopamine receptor signalling, via heterodimerization in brain regions which never see ghrelin, suggesting a function for the apo-receptor as a pure allosteric modulator, rather than a signalling receptor in these cells [40]. Similar findings were obtained with D1/D2 dopamine receptor dimers [41] although this has been disputed recently [42].

18.4 Asymmetries in GPCR Oligomers Translate into Signalling Consequences

GPCRs that signal as monomers do not have the allosteric possibilities for modulation inherent even in receptor dimers, relying more on canonical molecular crosstalk as part of regulatory pathways (Fig. 18.2a). One obvious functional advantage of dimers, more easily understood in the context of heterodimers, is that they can act on each other via bidirectional allosteric interactions, which may or may not depend on ligand occupation or the presence of particular signalling partners (Fig. 18.2b). However, more complex interactions may also result from structural asymmetries depending on the relative orientation and position of signalling partners. The first identified asymmetric receptor heterodimer was the GABA-B receptor, which consists of two subunits, one of which binds ligand and the other which transmits the signal to the G protein [43, 44]. This particular receptor complex is unique in that one subunit does not bind ligand but allosterically modulates the other and *vice versa* (reviewed in [45–47]).

Questions about such asymmetries have been raised for other classes of GPCRs as well. The Javitch group showed that the two-receptor equivalents in the context of a D2 dopamine receptor homodimer are organized asymmetrically with respect to their G protein partners [48] such that occupation by ligand of one receptor activates the receptor and occupation of the other modulates signalling allosterically. In the context of a homodimer this may not be so important as either receptor can serve each role and the asymmetry may not be detectable. However, such allosteric interactions may also be exploited pharmacologically to control dimer function [49].

This notion adds an entirely unappreciated wrinkle to signalling from heterodimers though, when we consider how asymmetry might play out in terms of allosteric receptor regulation. For example, we have shown that a heterodimer forms between the β_2AR and the oxytocin receptor [50, 51]. We further showed that the β_2AR/OTR

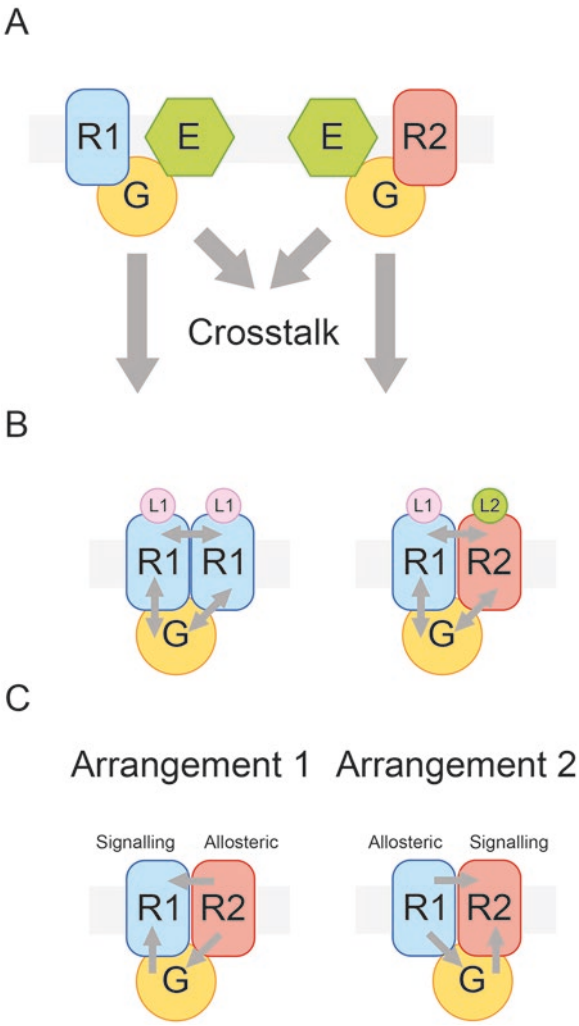

Fig. 18.2 *Allostery in GPCR monomers and oligomers*. (**a**) Monomeric GPCR signalling complexes have limited possibilities for inter-receptor allosteric modulation but can certainly regulate each other's activity via molecular crosstalk mediated by second messenger-activated protein kinases. (**b**) Allosteric communication in homo- and heterodimers with a shared G protein where information flow can go in a bidirectional manner between the different partners. However, the allosteric possibilities are greatest in the heterodimer. (**c**) Assembly of GPCR heterodimers which interact in distinct ways with a shared G protein can be assembled in different orientations such that in one case, R1 signals and R2 is a non-signalling allosteric modulator (whether occupied by ligand or not) of R1. The converse arrangement is also possible. Receptor homodimers might be asymmetrically organized with respect to their G protein and effector partners but this is unlikely to have functional consequences *per se* since cooperative effects between the receptor equivalents could be sensed in the same way

pair is an allosteric dimer in myometrial cells which express both receptors endogenously. Specifically, occupation of the β_2AR binding site by either agonist, antagonist or inverse agonist dampens signalling through OTR via mechanisms that cannot simply involve second messenger-mediated crosstalk. Similar results were seen for β_2AR-mediated signalling in the case of antagonist- or inverse agonist-occupied OTR. The presence of the OTR in either myometrial cells or in HEK 293 cells altered β_2AR signalling output providing credence to the notion that the dimeric complex forms a unique signalling entity.

We examined this possibility more directly demonstrating that the angiotensin II receptor type I (AT1R) and the receptor for prostaglandin F2α (FP) form heterodimeric complexes in both HEK 293 and vascular smooth muscle cells (VSMC), the latter where both receptors are again expressed endogenously. FP and AT1R in VSMC can be purified together using immunoprecipitation combined with photoaffinity labelling in HEK 293 cells as well [52]. Abdominal aorta ring contraction experiments revealed that PGF2α-dependent activation of FP potentiated Ang II-induced contraction whereas FP antagonists had the opposite effect. Similarly, PGF2α-mediated vasoconstriction was symmetrically regulated when using AT1R agonist and antagonist. Our data also showed that the PKC pathway is modulated in a distinct fashion by dual occupancy. Ang II-mediated vasoconstriction in the abdominal artery was potentiated by threshold concentrations of PGF2α, as was the effect of PGF2α by Ang II. However occupancy by two different FP antagonists also resulted in inhibition of Ang II-mediated contraction, an effect that cannot be explained by stimulation of second messenger-mediated crosstalk. Similar results were obtained with pre-treatment with L158,809, the AT1 antagonist on FP-mediated contraction [52].

However, asymmetrical responses in the heterodimers were observed for the binding to their respective agonists (in some cases regulated simply by the presence of the partner receptor), regulation of receptor-mediated MAPK activation and VSMC DNA and protein synthesis. With respect to signalling in VSMC, we showed that occupation of AT1R with an antagonist L158,809, strongly potentiated ERK1/2 activation by FP, an effect that was not reciprocated by occupation of FP with a specific antagonist AS604872 when measuring Ang II-mediated ERK1/2 signalling [52]. In order to further characterize the effects of stimulating the FP/AT1R dimer, we used [^{3}H]-thymidine incorporation as a DNA synthesis marker (indicative of proliferation), and [^{3}H]-leucine incorporation as a protein synthesis marker. VSMC were pre-treated with L158,809 to determine if it could potentiate [^{3}H]-thymidine incorporation following PGF2α stimulation. PGF2α alone elicited a small increase in [^{3}H]-thymidine, which could be inhibited by AS604872, but not by L158,809 pre-treatment. Similar results were obtained with [^{3}H]-leucine incorporation, and L158,809 had a slight potentiating effect on PGF2α-induced protein synthesis. Interestingly, AS604872, the FP antagonist, was as potent as L158,809 in inhibiting Ang II-induced [^{3}H]-thymidine and [^{3}H]-leucine incorporation [52], showing again the striking asymmetry in the regulation of cellular responses via the receptor heterodimer. Other studies have demonstrated the AT1 heterodimers with CB1 cannabinoid receptors, for example, also results in altered signalling

profiles compared to the parent receptors [53] and similar results have recently been shown for AT1R/apelin receptor heterodimers [8, 9]. These results clearly indicate that a full examination of the signalling profile are required to understand both symmetries and asymmetries for the FP/AT1R pair and likely for many receptor heterodimers.

These findings indicate that formation of the AT1R/FP dimer again created a novel allosteric signalling unit that showed both symmetrical and asymmetrical responses, depending on the signalling or phenotypic output measured. A similar picture may also emerge for the AT1R/purinergic P2Y6 receptor (P2Y6R) pair [54]. In this case, it is clear that the presence of P2Y6R affected AT1R signalling but the converse was not explored in detail. The AT1R seems to dimerize with many different GPCR partners, thus this has implications for the use of drugs to modulate one or both receptors in a putative dimer pair in the clinical setting.

This has tremendous implications for the formation and function of receptor heterodimers, in that multiple *asymmetrical* arrangements become possible depending on the relative orientation of each monomer to the G protein and possibly effector molecules. Thus, in one arrangement, protomer 1 is the *signalling receptor* and protomer 2 is an *allosteric modulator* that does not necessarily generate a signalling output of its own and the converse is true when the system is organized the other way around effectively generated two distinct signalling entities containing the same pair of receptors. However, in hetero-oligomers, structural asymmetries in receptor/G protein assembly may have dramatic consequences for signalling (Fig. 18.2c). As discussed, this notion greatly increases the potential organizational complexity of GPCR signalling and further suggests that determinants of signalling complex assembly (see below) will be of paramount importance in initially defining signalling specificity in a given tissue, cellular or subcellular compartment. Further, it suggests perhaps why heterodimers may have been difficult to detect *in vivo* since one receptor might in fact be silent with respect to signalling and thus missed in standard drug screens. That arrangement can be reversed if the complex is assembled or arranged differently- i.e. even with the same set of interacting partners, signalling output could be quite distinct. Not only are these considerations important for therapeutic efficacy, but may also predict and explain numerous off-target effects of currently used drugs. We need to assess the consequences for cellular signalling when receptors dimerize where one receptor may be silent with respect to signalling, the structural basis for such potential asymmetries in signalling and to understand mechanisms involved in how such complexes might be assembled.

18.5 What Does Structural Biology Tell Us About GPCR Dimers?

Several recent GPCR crystal structures including the CXCR4 chemokine receptor [55] and the μ- and κ-opioid receptors [56, 57] have demonstrated the presence of receptor dimers. In the case of CXCR4, ligand-bound dimers were detected in five

independent structures. The interface between the two monomers included TM V and VI in CXCR4, between TMI, TMII and TMVII in the κ-opioid receptor and between TMV and TMVI in the μ-opioid receptor (reviewed in [58]). These results highlight how the interface between receptor protomers varies depending on the homodimer studied. This likely suggests that heterodimer interfaces will also be as varied, if not more. This becomes an important issue in the context of receptor oligomers as well. For example, in a structure of oligomeric turkey β_1-adrenergic receptors two dimer interfaces were noted- one interface involving transmembrane domain (TM) 1, TM2, the C-terminal H8 and extracellular loop 1 and the other interface involving TM4, TM5, intracellular loop 2 and extracellular loop 2 [59]. A recent review has discussed what the implications of these structures are on our conception of the receptor/G protein interface [60]. While GPCRs can form dimeric or even oligomeric structures, many recent crystal structures (reviewed in [61]) showed little evidence for GPCR dimers, at least under the conditions required to purify and reconstitute them for structural studies. We would argue that the cellular context, loss when GPCRs are purified, likely plays a key role in dimerization *in vivo*.

18.6 Where and How Are Receptor Oligomers and Their Signalling Partners Assembled?

The potential organizational complexity of GPCR signalling is greatly increased in the context of asymmetric heterodimers and suggests that understanding the determinants of signalling complex assembly will be of paramount importance in defining signalling specificity at any given moment in particular tissues, cellular or subcellular compartments [13, 62]. This has tremendous implications for the formation of receptor heterodimers and hetero-oligomers, in that multiple asymmetrical arrangements might be possible depending on the relative orientation of each monomer to the G protein and possibly effector. Further diversity is added when we consider heterotetramers which can (1) have different numbers of each component subunit and (2) several distinct potential arrangements of those subunits. Important questions remaining include how and where heterotetramers can form, in what order subunits are added, in what stoichiometry and how signalling partners are added. As we have seen, receptor complexes can contain multiple receptors, what some authors have termed as receptor mosaics [63]. Also, if there are direct interactions between GPCRs and other receptor classes, might these structural asymmetries be important in their function as well?

There is evidence that receptor dimerization is required for efficient surface localization of a number of GPCRs including the β_2AR [64, 65] and the α_{1B}AR [66], (reviewed in [67]). More recently, it has been suggested that dimerization and/or oligomerization stabilizes GPCRs in their functional states, preserving their functional lifetimes [68]. GPCR dimers, both homodimers such as CCR7 [69] and heterodimers [70] likely act as hubs about which signalling complexes are organized.

Assembly of GPCR signalling complexes likely occurs during biosynthesis, rather than in response to agonist stimulation at the plasma membrane. GPCRs, in their various forms as monomers, dimers and oligomers, could certainly act as scaffolds for formation of specific hardwired signalling hubs. Such signalling complexes may be distinct for individual receptor monomers, homo- or heterodimers leading to a unique phenotypic output depending on the composition of such complexes. For example, a number of studies have demonstrated association, co-purification or co-immunoprecipitation of receptors with G proteins (reviewed in [71, 72]). Stable or meta-stable interactions of GPCRs with effector partners are one mechanism to assure rapid and specific signalling. Our own studies of the ontogeny of such signalling complexes suggests that complexes of either β_1AR and β_2AR [73] assemble with effector partners such as adenylyl cyclase [74, 75] and Kir3 channels [76–78]. Similar pre-assembled signalling complexes have recently been identified for M3 muscarinic receptors and $G\alpha_q$ [79]. A recent study demonstrated that PDZ ligand-bearing GPCRs may also lead to the formation of specific dimer-based complexes, depending on what PDZ proteins are recruited to each protomer [80].

Signalling complexes are likely formed during receptor biosynthesis and these interactions, as measured using different biochemical and biophysical approaches such as BRET or co-immunoprecipitation, have been shown to be insensitive to dominant negative versions of Rab1 or Sar1 (but not Rabs 2, 6 or 11) constructs [65, 74], which regulate anterograde receptor trafficking (reviewed in [81, 82]). Rabs and Sar1 are monomeric G proteins demonstrated to be important for vesicular transport to and from different cellular membrane compartments [83]. Interestingly, our data also showed that Gα subunits are assembled with nascent receptor/Gβγ/effector complexes either in ER export sites or in the Golgi since this interaction *was* blocked by dominant negative Sar1 and Rab 1 [65, 74]. Using this traffic block-based approach, we more recently showed that larger receptor oligomers can also be assembled in the ER. Using a combination of BRET and protein fragment complementation assays to study the interactions between two β_2AR constructs tagged with each half of split-Venus, we detected an interaction with β_2AR-Rluc suggesting the presence of a larger receptor homo-oligomer [84]. Interestingly, we also detected a larger array when we use two AT1R constructs tagged with each half of split-Venus with the β_1AR-Rluc or β_2AR-Rluc in a hetero-oligomeric context [84]. None of these interactions were sensitive to block with dominant negative versions of either Rab1 suggesting both homo- and hetero-oligomers also form in the ER as an early event in receptor biogenesis.

The role of pre-assembly has important implications for the formation of asymmetric, receptor-based signalling complexes. Asymmetry in the context of GPCR heterodimers can be viewed in multiple ways- either structural, functional or a combination of the two. Functional asymmetry can be defined as differences in signalling mediated by a receptor heterodimer, where occupancy of one receptor alters signalling via the other and this relationship may differ depending on how the receptors are stimulated- that is the asymmetry need not be necessarily reciprocal. To understand functional asymmetries, we first need to more extensively characterize signalling pathways downstream of putative heterodimers. Teasing out the

determinants of such assemblies will be critical for understanding what complexes are formed in a given context and may provide mechanistic insight into how asymmetric arrays are built. One simple way to use this system is to test the notion that the timing of synthesis, or the order of assembly, of key signalling components associated with a given GPCR heterodimer or hetero-oligomer determines which receptor becomes the signalling receptor and which becomes the allosteric modulator.

Such considerations are especially important in that GPCRs do not act as simple switches that turn single signalling pathways 'on' or 'off'. Instead, individual receptors or receptor complexes engage multiple signalling cascades and individual ligands can have differential efficacies toward specific subsets of these signalling effectors. Such ligand-biased signalling or functional selectivity, offers interesting opportunities to identify and develop compounds with increased selectivity and improved safety profiles. The mechanistic basis of biased signalling through GPCRs remains unknown. It has been assumed that different receptors "select" downstream signalling pathways in response to different ligands and how they might occupy the ligand-binding site and alter or stabilize unique receptor conformations. It may be possible that assembly of receptor homo- and hetero-dimeric/oligomeric complexes is a more likely basis for distinct cellular responses to particular ligands. We would argue that targetting assembly of signalling complexes might actually provide an even more "selective" set of biased assembly modulators than current approaches designed to find biased ligands. However, much work remains to identify the molecular determinants of signalling complex assembly in the interim, especially in the context of GPCR oligomers.

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Chapter 19
Heteromers Form Novel Signaling Complexes

Peter J. McCormick and Joaquin Botta

Abstract Heteromers of G protein-coupled receptors offer the potential for a vast array of signaling partners. Research over the last decade has focused on identifying different heteromer complexes and their signaling components in an effort to understand their cellular and physiological functions. Heteromer complexes may serve a modulatory role or form completely novel signaling platforms within the cell. This chapter reviews canonical and biased signaling pathways of GPCR heteromers with a focus on their signaling capabilities and cellular localization.

Keywords GPCR • G protein signaling • Heterodimer • Biased signaling

As GPCR homo- and hetero- dimers are becoming more widely accepted, research over the last decade has shifted to understanding why dimers exist. That is to understanding what their cellular and physiological functions actually are. A key aspect of dimerization is their ability to serve as either modulatory or completely novel signaling platforms within the cell. This chapter will highlight what has been discovered on the ability of heteromers to serve as scaffolds that influence cell signaling.

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19.1 G-Protein Signaling

The canonical G-protein signaling by GPCRs is thought to be a single receptor, coupling to one hetero-trimeric G-protein complex. Upon ligand binding, a series of conformational changes occur which lead to exchange of GDP for GTP in the alpha subunit of this heterotrimeric complex. The human genome encodes 21 Gα, 6 Gβ and 12 Gγ subunits. Depending on Gα subunit primary sequence similarity, heterotrimeric G proteins are typically grouped into four main classes: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ [1]. It was originally thought that one receptor coupled to one type of Gα subunit. However, it is now widely accepted that there is in fact a large amount of promiscuity between GPCRs and G-proteins [2–4]. The rules that govern these interactions are currently an intense area of research.

Upon exchange of GDP to GTP, there is thought to be a separation of alpha subunits from beta-gamma subunits. The alpha subunit, which is responsible for binding the nucleotide then goes on to interact with downstream effector proteins that propagate the signal to a variety of signaling pathways. When bound to GTP, the alpha subunit adopts structural conformations capable of interacting with specific downstream effectors. Direct evidence for these downstream actions comes from crystal structures of the Gα-effector complexes of $G\alpha_s$ bound to adenylyl cyclase (AC), $G\alpha_t$:PDEγ, $G\alpha_{13}$:p115-RhoGEF and $G\alpha_q$:GRK2 [5–8]. It is assumed that the beta-gamma subunits, which are not nucleotide bound are also now free to interact with effector proteins with several studies pointing to beta-gamma specific signaling outcomes [9–12]. Some of beta-gamma interactions overlap with those of the alpha subunits (adenylate cyclase and ion channels) while others appear more specific to beta-gamma, for example the activation of AKT in endosomes after the lysophosphatidic acid receptor-dependent association of $G\beta_1\gamma_2$ and Rab11a in these compartments. [13].

One of the first, and obvious questions that was posed upon the discovery of heteromers was the stoichiometry of receptors to G-proteins. If heteromers were composed of simply two different receptors, then presumably there would be two different heterotrimeric G-proteins bound or activated by this heteromeric complex. Navarro et al. have shown that heteromers consist of four different receptors coupled to two different G-protein complexes [14]. This complex matches other predictions of a rhombus configuration by Pisterzi et al. and Patowary et al. [15, 16]. Whether there is indeed one configuration for heteromers seems unlikely. In support of this using super-resolution microscopy Jonas et al. have shown that there are multiple configurations possible within a heteromer, suggesting that there may in fact be different active complex types [17]. For example, there could be a four receptor heteromer coupled to two G-proteins, or a two receptor heteromer coupled to a single G-protein. It remains to be understood whether such different conformations are responsible for driving different cellular pathways.

19.2 Biased Signaling

It is clear from structural data that GPCRs are not single on/off switches but instead have different ligand-modulated signaling states, and that higher order oligomers are important for activity [18–20]. The former is an important aspect of signaling from GPCRs termed, biased signaling. Originally coined by Kenakin and colleagues as "agonist trafficking", it was identified by the fact that different agonists led to different G-proteins trafficking to the receptor [21]. This concept was based upon observations such as the ability of different α_2 adrenoceptors to couple to G_s and/or to G_i after treatment with adrenaline, while oxymetazoline activates only G_i [22].

It now encompasses a much more broad appreciation that a ligand can stabilize a given receptor conformation that will preferentially lead to a certain downstream signaling pathway and/or a certain trafficking pathway.

Bias is thought to be achieved by influencing the receptor conformational landscape. As a protein, the receptor samples various folds and energy states. This principle has been demonstrated in a variety of studies over the last decade. This flexibility in the energy landscape can be taken advantage of by designing and employing ligands which can stabilize certain conformations of the receptor. Two examples illustrating the potential of biased ligands are the studies on the Angiotensin II type 1 receptor and the μ-opioid receptor.

The Sar^1, Ile^4, Ile^8-angiotensin II ([Sar^1, Ile^4, Ile^8]Ang II) agonist analog have shown AT1R-dependent mitogen-activated protein kinase (MAPK) signalling in a $G\alpha_{q/11}$-PLC/IP_3 independent manner [23]. This effector preference is driven by the selective recruitment of β-arrestin 2 to AT1R with no coupling to $G\alpha_{q/11}$ after [Sar^1, Ile^4, Ile^8]Ang II administration [24]. This biased system was validated and further explored by Rajagopal et al. using isolated cardiac myocytes expressing endogenous receptor levels and stablished the basis of a novel family of therapeutic agents for the treatment of cardiovascular disease with a dual functionality [25]. In one hand, molecules like [Sar^1, Ile^4, Ile^8]Ang II would block angiotensin II-mediated $G\alpha_{q/11}$ signalling pathways, thus acting as pathway-specific competitive antagonist and therefore retaining some of the effects of classical angiotensin receptor blockers. On the other hand, enhanced cardiac contractility and cytoprotection would be retained because of its β-arrestin-dependent nature [25, 26].

The μ-opioid receptor (MOR) is another well characterized target illustrating the promise of translating ligand bias into *in vivo* systems, in this case tipping the scale towards G protein-mediated signalling. Although its high efficacy as pain relieving agents, MOR agonist have been associated to several adverse effects including nausea, vomiting, constipation, respiratory depression, sedation and tolerance [27]. Studies in β arrestin 2 knockout mice (KO) showed enhanced morphine-induced analgesia and impaired MOR desensitization, suggesting the role of β-arrestin 2 in antinociceptive tolerance [28, 29]. Recently, TRV130 was devel-

oped aiming to promote the coupling of MOR to G proteins but not to β-arrestins, thus reducing gastrointestinal and respiratory dysfunction but with similar analgesic properties to. In fact, TRV130 was identified as a potent and selective MOR agonist with comparable G protein coupling to morphine and residual β-arrestin recruitment. In mice and rats, it exhibited potent analgesia with lower gastrointestinal and respiratory side effects when compared to morphine [30]. In humans, TRV130 was proven to be safe exerting central effects with reduced respiratory depression and nausea [31].

These examples reflect progress towards a new class of ligands with reduced side effects and enhance drug efficacy, achieving improved treatments and better serving patients' needs [32].

19.3 Biased Signaling and Heteromers

In the case of heteromers it must be considered that they are made up of at least two and possibly four or more functional targets. In addition, depending on the arrangement of the receptors, there are multiple interfaces within the overall heteromer that will have different mobilities and thus influence the adjacent receptors differently. The ability for a ligand to influence these mobilities and conformations will depend on the affinity of the ligand for the given protomer. The affinities are not equal in a heteromer pair. If one imagines a given conformational landscape of a given receptor and how a given ligand lowers the energy barrier to an activated conformation (depicted in Fig. 19.1). This same energy landscape can be raised or lowered via interaction with another receptor and *its* ligand (depicted in Fig. 19.1). Thus single receptors sample different energy states then heteromers. One of the functions of receptor oligomerization may be then to favor or stabilize certain receptor conformations. This concept is similar to the idea of allosterism [33, 34], where a molecule binding at a separate site on the receptor can alter affinity or efficacy of the orthosteric ligand.

It is this altering of protein conformation that can explain how receptor crosstalk at the level of cell signalling is achieved. Crosstalk can be defined as the ability of one receptor to influence the signalling of another. Crosstalk does not require protein-protein interactions as it can occur via changes in a downstream kinase or phosphatase. However, in the context GPCR heteromers, cross-talk is often a biochemical signature of the complex [36]. This cross-talk is not a simple shift in the time or dose requirements of a given ligand to elicit a certain efficacy in the presence of the heteromer. Instead, it is usually either an augmentation or decrease in the maximum efficacy of a given signalling pathway. How is this achieved? Through protein-protein interactions, the partner receptor is able to influence the conformational landscape and subsequently change a ligands ability to stabilize activated conformations. This influence most certainly is cell context dependent but how is still unclear.

Biased signalling is yet another area that heteromers seem to be influencing receptor signalling. In some cases, heteromer formation leads to a change in G-protein recruitment to a partner receptor. This is the case in the Dopamine recep-

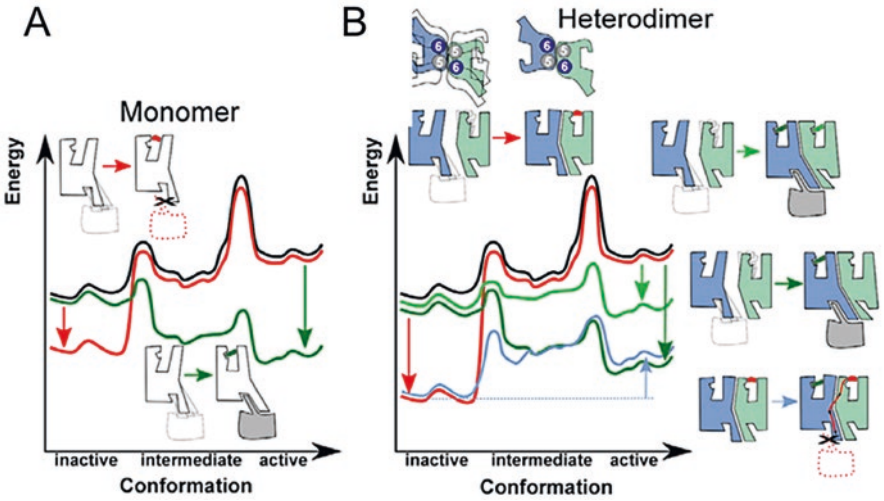

Fig. 19.1 Free energy landscapes diagrams illustrating the energy of monomeric and heterodimeric receptors towards their active conformation. The transition from inactive to active states depends on the energetic barriers between them and their energetic differences. (**a**) *Black, red and green lines* show the energy differences between unbound, inverse agonist/antagonist bound and agonist bound receptors. Upon ligand binding, inverse agonist/antagonist stabilize inactive forms of the receptor, while agonists stabilize intermediate conformations. In this state, the formation of the ligand-receptor-G protein ternary complex stabilizes active conformations. (**b**) Proposed model of allosteric modulation for TM5/TM6 heterodimer interfaces. In a ligand-free state (*black line*), the receptor TMs are in a dynamic conformational equilibrium. Antagonist binding (*red line*) stabilizes inactive conformations. In comparison to monomeric receptors, the energy of the antagonist bound receptors is lower in heterodimers because of the restricted mobility of the interacting TMs 5 and 6. In this low free-energy state, agonist binding to the second protomer cannot overcome the energetic barrier towards active conformations, leading to cross-antagonism. Agonist binding to both protomers results in reduced signalling efficacy (*light green line*), most likely because of a steric clash of the active TM5 and TM6 conformations in each protomer, leading to negative cross-talk. Single protomer agonist occupancy stabilize active conformations of TM5 and TM6 and G-protein recruitment (*dark green line*) (Figure adapted from Viñals et al. [35])

tor 1 (D_1R) – Histamine 3 receptor (H_3R), where D_1R, typically bound to G_s, now binds to G_i [37]. In other cases, there is one G-protein that appears to be the dominant signal despite the presence of two G-proteins. This is the case for the A1a-A2a heteromer where the two G-proteins, Gs and Gi are bound but the Gs protein seems to dominate over Gi [14]. As is the current case for single receptors, the rules that govern G-protein recruitment to heteromers is not understood.

19.4 Heteromers Modulation of Arrestin Signaling

A major breakthrough in our understanding of GPCR signaling was the discovery that the Arrestin molecule could, in addition to its role in attenuating signaling it could also serve as a signaling scaffold for an activated receptor [38, 39]. In addition

to altering G-protein recruitment, heteromer formation can also influence arrestin. The CXCR4/CXCR7 heterodimer is an example of this. Both receptors undergo activated after stromal cell derived factor-1 (SDF-1) or CXCL12 binding. However, while CXCR4 displays classical GPCR signalling properties [receptor activation/heterotrimeric G proteins recruitment/G protein or arrestin-mediated downstream effector activation], CXCR7 behaves as a "non" G protein coupled 7TM receptor because of its inability to activate G proteins, mainly signalling through β-arrestin-activated downstream pathways [40]. In addition to modulating $G_{i/o}$ coupling to CXCR4 [41], co-transfection of CXCR7 results in constitutive β co-transfection of CXCR7 results in constitutive ITEM-1″, "itemData": {"aut. Moreover, CXCR7 co-expression with CXCR4 potentiates CXCL2-driven effects in downstream signalling pathways involved in cell proliferation and migration in a β-arrestin 2-dependent manner, as these effects fade after siRNA knockdown [42].

The α_{2C}-adrenergic (α_{2C}-AR)-AT1 receptors heterodimers also illustrate how GPCR complexes can create new unique pharmacological targets with an important role in the physiology and pathophysiology of cardiovascular diseases. Using a series of *in vitro* complemented donor-acceptor resonance energy transfer (CODA-RET) approaches in combination with *in vivo* assays, Bellot et al. showed how dual agonist occupancy is translated into a different α_{2C}-AR-AT1R heterodimer conformation other than the single activated protomers [43]. Instead of changes in the effector recruitment levels, exposure to Ang II, norepinephrine (NE) or the combination of both ligands in cells expressing α_{2C}-AR-AT1R complexes stabilizes three different receptor-β-arrestin 2 conformations which lead to different receptor trafficking pathways. In addition, simultaneous α_{2C}-AR-AT1R protomer occupancy creates a new heteromer conformation capable of recruiting G_s proteins and thus triggering an heteromer specific G_s-cAMP-PKA signalling pathway that could be implicated in AngII and NE regulation of sympathetic activity [43].

Finally, and perhaps related to arrestin recruitment, heteromer formation can influence receptor trafficking.

19.5 Cellular Location and Signaling

As mentioned above, one aspect of signalling that has been recently discovered is the concept that receptors can signal from internal organelles, suggesting that this signal may be lead to cellular consequences somehow different then signals originating from the plasma membrane [44, 45]. As heteromers can alter signalling at the plasma membrane and alter trafficking, it seems likely that they will influence internal signaling as well. An example of protein-protein interactions in altering of signalling via sequestration or trafficking changes is the Calcitonin gene-related peptide receptor which complexes with accessory receptors that can alter trafficking and subsequently signaling. In the case of heterodimers this has been demonstrated with the incretin glucagon-like peptide-1 (GLP-1) receptor [46]. The internalization of the GLP-1 receptor could be altered or in some cases blocked by interaction with

other GPCRs. This change in internalization then alters downstream signaling. Using real-time single-particle tracking, Navarro et al. have shown that heterodimers between Adenosine receptors A_1 and A_{2A} (A_1R and $A_{2A}R$, respectively) lead to a change in mobility of the receptors in the plasma membrane [14]. It was observed that co-expression of $A_{2A}R$ with A_1R reduced the Brownian diffusion of A_1R, indicating restriction in its membrane motion. Similarly, the reciprocal assay also decreased $A_{2A}R$ mobility, illustrating how protein-protein interactions alter receptor trafficking. In the case of the previously mentioned heterodimer of CXCR4-CXCR7, it has been shown that ubiquitination can be triggered and that CXCR7 alters the internalization and degradation of CXCR4 [47].

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Chapter 20
Heteroreceptor Complexes Implicated in Parkinson's Disease

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Abstract Heteromerization alters GPCR recognition, G-protein activation, receptor signaling and trafficking, thus changing receptor protomer pharmacology and function. This review deals mainly with the A2AR-D2R and A1R-D1R heteroreceptor complexes, their balance with dopamine and adenosine isoreceptor complexes and their role in Parkinson's disease and its treatment. The major technique used for the visualization of the heteroreceptor complexes in the brain was the proximity ligation assay. A1R-D1R and putative A1R-D1R-D3R heteroreceptor complexes appear to exist in the direct pathway. Upon agonist activation the A1R protomer exerts a brake on the D1R protomer signaling of these complexes reducing the activity of the direct pathway with reduction of movement initiation. D1R-NMDAR and D1R-H3R-NMDAR heteroreceptor complexes in the striatal glutamate synapses integrate synaptic and volume transmission, where in the former complexes the D1R protomer enhances NMDAR signaling with enhancement of movements. A2AR-D2R and A2AR-D2R-mGlu5R heteroreceptor complexes with antagonistic

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receptor-receptor interactions exist in the dorsal striato-pallidal GABA neurons mediating motor inhibition. The A2AR and mGlu5R antagonists synergize to increase D2R protomer signaling by removing the A2AR and mGlu5R brakes on the D2R protomer signaling and heterobivalent compounds built of A2AR and mGlu5R antagonists may specifically and substantially remove these brakes reducing motor inhibition with development of antiparkinson actions.

Keywords GPCR heteroreceptor complexes • Receptor-receptor interactions • Dorsal striatum • Parkinson's disease • Heterobivalent drugs • Adenosine receptors • Fopamine receptors • Antiparkinson drugs • Oligomerization

20.1 Introduction

The receptor–receptor interaction field began with the studies on the neuropeptide/monoamine receptor–receptor interactions in membrane preparations in the early 1980s, which altered especially the affinity of the monoamine receptor subtypes [1]. It was proposed that their allosteric interactions in the plasma membrane took place in postulated heteroreceptor complexes of GPCRs that could involve the participation of adaptor/scaffolding proteins [2–8]. Now the receptor field in the CNS has expanded and includes not only the monomers but also homo and heteroreceptor complexes with receptor assemblies of unknown stoichiometry and geometry together with adaptor proteins [6, 8] as novel targets for treatment of neurological diseases, especially Parkinson's disease [9, 10]. Evidence was recently obtained that 5-HT2C receptors represent a mixture of monomer-homodimers and higher order homomers using spatial intensity distribution analysis [11]. The dynamic organization was indicated by the ability of 5-HT2C receptor antagonists to transform it into an organization dominated by monomers. Overall it appears that the integration of the signaling takes place in dynamic homo and heteroreceptor complexes and a theory was introduced that they play a major role in learning and memory and can form molecular engrams [7, 12, 13]. They will have a major impact on medicine in general, and especially in the neurosciences and be novel targets in neuropsychopharmacology.

Heteromerization alters GPCR recognition, G-protein activation, receptor signaling and trafficking, thus changing receptor protomer pharmacology and function. One emerging new concept in neuropsychopharmacology is that a direct physical receptor-receptor interaction can contribute to disease progression [6] and heterobivalent compounds may become novel drugs in treatment of neurological disorders by selectively targeting the heteroreceptor complexes [9, 10].

Static/non-dynamical human GPCR data derived from this and other interaction studies were integrated in a large-scale graph, called the GPCR heterodimer network [14] (*www.gpcr-hetnet.com*). This network provides global insight into adenosine and dopamine receptor heteromer connectivity, topology and organization in the context of the adenosine and dopamine receptor subfamilies and the GPCR network as a whole.

In the current review we will deal with the A2AR-D2R and A1R-D1R heteroreceptor complexes and the dopamine and adenosine isoreceptor complexes and their role in Parkinson's disease and its treatment [15–18]. The dopamine receptors are hub receptors and their complexes formed with NMDAR and histamine receptors will also be discussed in relation to Parkinson's disease [10, 19] as will be the role of GPCR-RTK complexes [20–22]. The major technique for the visualization of the heteroreceptor complexes in the brain was the proximity ligation assay [22–24].

20.2 A1-D1 and D1-D3 Heteroreceptor Complexes

The antagonistic A1R-D1R receptor-receptor interactions mainly exist in the striatoentopeduncular/nigral GABAergic neurons forming the direct pathway [3, 16, 25]. A1R-D1R heteroreceptor complexes were first observed with coimmunoprecipitation in cellular models [26] and then in the striatum [27]. The results in cellular models were validated with FRET/BRET technologies [25]. The allosteric antagonistic A1R-D1R interaction was seen as a reduction in the proportion of D1Rs in the high affinity state [3, 16, 25].

Studies at the network level demonstrated antagonistic A1R-D1R interactions in the modulation of GABA release in the direct pathway using microdialysis in the hemiparkinsonian rat [3]. These antagonistic interactions were also found in pharmacological behavioral work on motor functions using A1R agonists and antagonists [28, 29]. Also, using in situ PLA it was mapped the distribution of the A1R-D1R heteroreceptor complexes in the rat brain [24].

It is of substantial interest that A1R agonists counteract oral dyskinesias induced by levodopa [30]. As to the mechanisms involved it was early found in cell lines that D1R agonists could produce a disappearance of the A1R-D1R heteroreceptor complexes. The A1R agonist counteracted this disappearance [26]. It seems possible that such changes may also develop in the dorsal striatum in animal models of PD where A1R agonists may prevent a potential D1R agonist induced disruption of the A1R-D1R heteroreceptor complexes.

Such events may strongly contribute to the development of a D1R sensitization in the direct pathway after treatment with levodopa and D1R agonists since agonist activated A1R can no longer allosterically inhibit D1R recognition and signaling. Enhanced numbers of D1R homoreceptor complexes may develop and lead to an increased D1R signaling with increased transcription factor activation via the AC-PKA-pCREB pathway with increased formation of GPCR interacting proteins. This signaling pathway will also activate the DARPP-32 Thr34 leading to protein phosphatase-1 inhibition. Such molecular changes can reorganize multiple heteroreceptor and homoreceptor complexes in the postsynaptic membrane of the direct pathway leading inter alia to long term sensitized D1R signaling and increased firing. Dyskinesias may then develop since a pathological enhancement of the direct pathway activity exists initiating movements that also may not be properly matched by changes in the D2R regulated indirect pathway, which mediates motor inhibition.

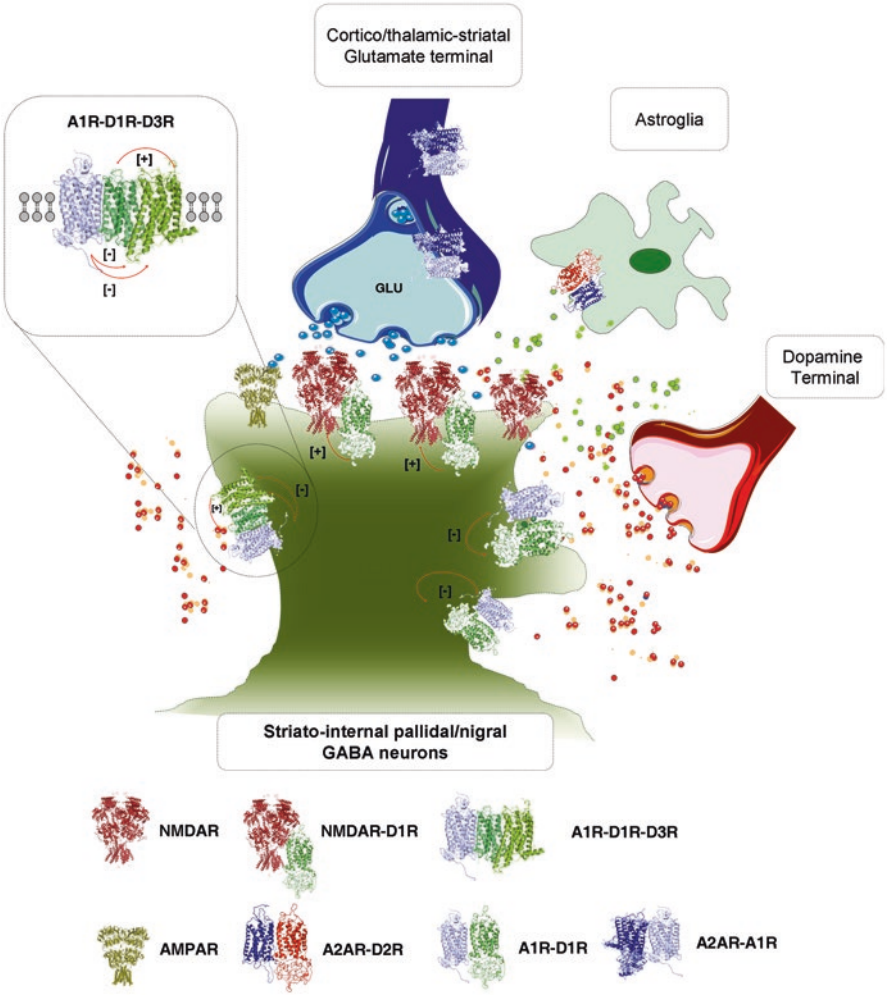

Fig. 20.1 Illustration of a number of relevant D1R and A1R heteroreceptor complexes in the dendritic regions of the striato-internal pallidal/nigral GABA neurons (direct pathway). They are located both in the glutamate synapses and in extrasynaptic regions. The incoming DA afferents mainly operate via volume transmission. The A2AR-D2R heterocomplexes may exist on the astroglia. The A1R-D1R-D3R and A1R-D1R postjunctional heterocomplexes may have a major role in the inhibitory modulation of postjunctional D1R signaling involving allosteric receptor-receptor interaction inhibiting the D1R protomer function. D1R-NMDAR heteroreceptor complexes exist in the glutamate synapses on the dendrites of the striato-internal pallidal/nigral GABA neurons. Through the allosteric receptor-receptor interactions D1R protmers appear to mainly enhance NMDAR signaling and thus the firing of the direct pathway [36] leading to increases in motor initiation. A2AR-A1R heteroreceptor complexes appear to exist on the glutamate afferents. In Parkinson's disease due mainly to the degeneration of the DA terminal networks especially the D1R hetero- and homoreceptor complexes in the striato-internal pallidal/nigral GABA neurons become reorganized with altered allosteric receptor-receptor interactions which contributes to the dysfunction of the striato-internal pallidal/nigral GABA neurons mediating motor initiation

It is of substantial interest that the composition of the D1R heteroreceptor complexes also likely changed in animal models of PD. Thus, a potential increase in the formation of D1R-D3R heteroreceptor complexes [31] in the direct pathway likely occurs based on increased expression of D3Rs [32]. D3R activation appears to enhance D1R signaling. This mechanism can likely contribute to development of D1R- induced dyskinesias via enhancing allosteric receptor-receptor interactions. It was postulated that A1R-D1R-D3R heteroreceptor complexes exist in the direct pathway in PD [33]. It remains to be demonstrated if A1 receptor agonist treatment can increase the formation of A1R-D1R and A1R-D1R-D3R heteroreceptor complexes and in this way at least in part exert its anti-dyskinetic actions in models of PD (Fig. 20.1).

Not only D3Rs are increased in the striatum from PD models rendered dyskinetic by 3, 4-dihydroxyphenyl-L-alanine (L-DOPA) treatment but also D1R-D3R heteroreceptor complexes. Such an increase is detectable in both rodent and non-human primate models of the disease [34]. Radioligand binding studies in rodents have shown that there is a lateralization in the interaction of dopamine with D1Rs and that dyskinesia correlates with a right/left striatal balance in D1R-mediated neurotransmission e.g., dyskinesia occurs when lateralization is lost [34, 35]. As heteromer formation leads to modification in receptor pharmacology, it is tempting to speculate that lateralization is due to a differential formation of D1R-containing heteromers in left vs. right striatum.

20.3 D1R-NMDAR Heteroreceptor Complexes

The discovery of the D1R-NMDAR heteroreceptor complexes was made by the laboratories of Fang Liu [36] and Missale [37, 38]. Two types of physical interactions were observed, one between the C-terminal of the D1R and NR1 subunit of the NMDA receptor, and another between part of the C-terminal of the D1R and the NR2A subunit of the NMDAR. Since many other proteins interact with D1R and NMDAR receptors, it seems likely that these interactions can develop independently of each other in D1R-NMDAR heteroreceptor complexes. Thus, the function of the D1R-NMDAR heteroreceptor can differentially change dependent on the physical interaction formed and its allosteric receptor-receptor interaction. It illustrates the dynamics of these heteroreceptor complexes where volume and synaptic transmission can be integrated through the receptor-receptor interactions.

The D1R interaction with the NR2A subunit decreases the NMDAR currents via reducing the recruitment of NMDAR to the plasma membrane not linked to changes in the phosphorylation cascades. Instead the D1R interactions with NR1 made possible the recruitment of calmodulin and PI3K to the NMDAR with activation of PI3K dependent signaling bringing down NMDAR mediated excitotoxicity. It is not clear, however, how these two types of states can be differentially induced.

The D1R-NMDAR heteroreceptor complex likely can exist in the glutamatergic synapse since D1R and NMDAR receptors can coimmunoprecipitate in postsynaptic

densities [37] (Fig. 20.1). It is of high interest that the synaptic scaffolding protein PSD-95 diminishes the D1R-NMDAR receptor-receptor interaction [39]. Nevertheless it was found that a TAT fusion peptide interfering with the D1R-NMDAR receptor interface reduces NMDAR dependent LTP associated with deficits in long-term memory [40]. However, extrasynaptic D1R-NMDAR heteroreceptor complexes also exist and can form a trap for lateral diffusion of D1R into the synapse and elsewhere [41]. They may also have an important functional role.

The D1R-NMDAR heteroreceptor complexes can be formed constitutively in the ER and then reach the plasma membrane. The D1R protomer in this complex does not internalize and therefore can maintain its signaling upon agonist activation [37]. NMDAR activation in fact increases the D1R presence on the plasma membrane via the D1R-NR1 interaction [42].

It therefore seems possible that the D1R-NMDAR heteroreceptor complexes in the direct pathway can participate in the development of the levodopa-induced dyskinesias upon chronic treatment. A reorganization of these complexes can take place that can contribute to a sensitization of D1R signaling and to a dominance of the D1R-NR1 interaction through a disappearance of the inhibitory D1R-NR2A interaction. In line with this view amantadine with NMDAR blocking activity can bring down levodopa-induced dyskinesias [43]. It should also be considered that also D1R-D3R-NMDAR heteroreceptor complexes may be formed which can further enhance D1R-mediated signaling with consequences also for NMDAR signaling in view of the increased presence of D3Rs in the direct pathway in animal models of PD [32].

20.4 D1R-H3R-NMDAR Heteroreceptor Complexes

Antihistamine drugs are one of the more consumed medications that produce benefits with little side effects. Since histamine receptors were identified in the CNS, there is hope that antihistamines may serve to combat some neurological diseases, specially those related with neurodegeneration. After identification of histamine H3 receptors in striatum we wanted to know whether these receptors could interact with dopamine receptors of the direct or of the indirect pathways. A direct interaction between H3R and D1R receptors was demonstrated by means of biophysical assays in a heterologous system. Interestingly, dopamine D1Rs in the presence of H3Rs did not couple to Gs but to Gi. This atypical coupling was one of the first examples of *in vitro* differential functional selectivity of a heteroreceptor complex [44]. In subsequent studies the occurrence of D1R-H3R heteroreceptor complexes was demonstrated in striatum. In another example of differential functional selectivity, it was showed that activation of H3Rs was linked to the p44 and p42 extracellular signal-regulated MAPK signaling pathway if the D1R was present. In fact, the Gi-mediated link to ERK 1/2 phosphorylation achieved by H3R agonists was lost in D1R KO mice. On the other hand, the ability of both D1R and H3R antagonists to block MAPK activation induced by either D1R or H3R agonists was also found in striatal slices. As often occurs, the D1R-H3R heteromer displays cross-antagonisms [19],

e.g., the antagonist of a receptor protomer also blocks the signaling originated in the partner protomer of the complex [45]. This common feature of heteroreceptor complexes makes unlikely the therapeutic use of antihistamines in Parkinson's disease, which in principle requires potentiating dopamine receptor-mediated signaling. In sharp contrast, the use of antihistamines to restore the dopaminergic balance in Huntington's disease deserves attention.

To answer the question of whether D1R, H3R and NMDAR receptors may form heteroreceptor complexes a convergent approach was used consisting of BRET, bimolecular complementation and *in situ* proximity ligations assays. It showed that multimeric complexes formed by one ionotropic and two metabotropic receptors are possible. Such a novel heteromer has been studied from a neuroprotection point of view and in relation with a disease that affects the functionality of NMDARs. Indeed, Alzheimer's disease is a neurodegenerative disease characterized by formation of ß-amyloid peptides, which interact and alter NMDAR function. Also the almost unique existing medication is an allosteric modulator of NMDARs. On the one hand, the multimeric complex is found in both wild-type and transgenic animals models of Alzheimer's disease. The results using H3R inverse agonist thioperamide as a neuroprotectant against ß-amyloid toxicity or NMDAR excitotoxicity proved promising in the APP/PS1 model of the disease [46].

20.5 D1R-D2R Heteroreceptor Complexes

Although there is controversy surrounding the full segregation of D1R and D2R receptors in GABAergic striatal neurons, there is a body of evidence indicating that some of these neurons express the two receptors in the nucleus accumbens. Furthermore, these receptors may form heteromeric complexes in cells co-expressing both receptors. One of the most important features of the complex is that it does not couple to Gs or Gi but to Gq. Therefore, signaling from dopamine receptors in D1R-D2R heteromers lead to calcium mobilization [47–51]. Actually this coupling to Gq could explain some findings that provide indirect evidence that dopamine receptor activation may engage Gq- and phospholipase C-mediated calcium pathways. Elucidation of the percentage of cells expressing D1R-D2R heteroreceptor complexes and of the relevance of such neurons in the basal ganglia functioning is necessary to assess the potential role of these heteromers in PD symptoms, neurodegeneration and/or levodopa-induced dyskinesia.

20.6 A2AR-D2R Heteroreceptor Complexes

They have a widespread distribution all over the dorsal and ventral striatum as demonstrated with the proximity ligation assay and seen as red blobs outside the nuclei [22–24]. They appeared to be located mainly in the dorsal and ventral striato-pallidal

GABAergic neurons but may also exist on striatal cholinergic interneurons and on the cortico-striatal glutamate terminals. BRET/FRET techniques and coimmunoprecipitation had earlier demonstrated these complexes in cellular models and they were found to be constitutive [52–55].

An antagonistic A2AR-D2R receptor-receptor interaction in these receptor complexes takes place at the level of D2R recognition, Gi/o activation and D2R protomer signaling [10, 54, 56–59]. The function of the D2R protomer is also changed by A2AR protomer activation through switching D2R protomer signaling from Gi/o into ß-arrestin mediated signaling [55]. It is of substantial interest that A2AR agonists counteracted D2R-mediated long-term depression and reintroduced long-term potentiation, showing their impact on neuroplasticity [60].

20.6.1 Coupling to Calcium/Calmodulin

The dopamine link to calcium-mediated signaling in striatal GABAergic neurons is still poorly described. The molecular interaction formed by calcium ion and calmodulin is key for different metabolic processes in a variety of cell types in different systems. Using BRET we found that calmodulin may form complexes with A2AR-D2R heteromers. Mutations analysis led to the identification of the proximal C terminus epitope of the A2AR as the motif involved in calmodulin binding to the heteroreceptor complex. Remarkably, calcium induced structural modifications in the A2AR-D2R-calmodulin receptor complex that correlated with modulation of both D2R and A2AR receptor-mediated MAPK signaling [61].

20.6.2 Models of Parkinson's Disease

20.6.2.1 The Hemiparkinsonian Rat Model

Increased antagonistic A2AR-D2R interactions were observed in terms of reduction of the affinity of the high affinity D2R agonist binding sites in dorsal striatal membrane preparations from hemiparkinsonian rats [62]. Such results introduced A2AR antagonists as potential antiparkinsonian drugs by targeting especially the A2AR protomer in a putative A2AR-D2R heteroreceptor complex removing the brake on D2R protomer signaling [9, 10, 56]. In line with this view A2AR antagonists significantly introduced locomotion after the use of subthreshold doses of D2likeR agonists [63].

The increases in antagonistic A2AR-D2R interactions reported above in the dorsal striatum in hemiparkinsonian models may reflect increases in the A2AR-D2R heteroreceptor complexes vs. D2R homoreceptor complexes. Thus, the absence of DA terminals and extracellular DA levels may lead to conformational changes in the

D2Rs favoring interactions with A2ARs and formation of A2AR-D2R heteroreceptor complexes.

The A2AR-D2R heteroreceptor complexes appear to exist mainly in the dorsal striato-pallidal GABAergic neurons (Fig. 20.2), which produce motor inhibition when activated. The inhibitory role of D2Rs in these neurons leads to removal of the brake on movements and thus to anti-parkinsonian effects. It is postulated that levodopa and D2R agonist treatment early on in treatment of PD can overcome the antagonistic A2AR-D2R interaction in the dorsal striatum in part through an increase in D2R homoreceptor complexes vs. A2AR-D2R heteroreceptor complexes and the antiparkinsonian actions develop.

Upon chronic treatment with levodopa and D2R agonists in PD, complications develop in terms of dyskinesias and wearing off actions. It was proposed that an upregulation especially of A2AR homoreceptor complexes might contribute to such complications. They may develop as a result of chronic levodopa and D2R agonist-induced increases through several mechanisms of phospho-CREB formation with activation of CRE in the A2AR promoter region [64, 65]. Also an internalization of the D2R structures can develop upon such chronic dopaminergic treatments. Thus, as a result the A2AR-D2R hetero- and A2A homoreceptor complexes can be in dominance over the D2R homoreceptor complexes which can help explain the reduction of the therapeutic effects of chronic dopaminergic treatments and the dyskinesia development. A proper downstate of the dorsal striato-pallidal GABAergic neurons may therefore no longer be reached with the dopaminergic treatments. In view especially of the increased brake on the D2R protomer signaling of the A2AR-D2R heteroreceptor complexes, the motor brake can therefore no longer be sufficiently removed which can also contribute to the dyskinesia development. The modest effects exerted by A2A receptor antagonist so far given alone or in combined treatments in clinical trials in PD suggest that also other mechanisms can participate in this process [66, 67].

20.6.2.2 The MitoPark Model

A genetic PD model, the MitoPark mouse model, was also used in the analysis of the antiparkinsonian actions of A2AR antagonists. In this model DA neurons undergo a slow and progressive degeneration due to the cell-type specific induction of mitochondrial dysfunction in midbrain DA neurons [68]. Progressive Parkinsonism develops in such mice with respiratory-chain-deficient dopamine neurons. Specifically they represent conditional knockout mice with disruption of the gene for mitochondrial transcription factor A (Tfam) in DA neurons.

These mice display gradual development of motor dysfunction, reproducing progressive stages of PD without the potential side effects of toxins used in other animal models [69]. As A2AR antagonist was used MSX-3, a prodrug of the water-soluble, highly specific A2AR antagonist MSX-2 [70]. It is hydrolyzed by cellular phosphatases and exhibits more than a 100-fold higher affinity to A2AR than A1R and is almost completely inactive at A2BR and A3R receptors [71].

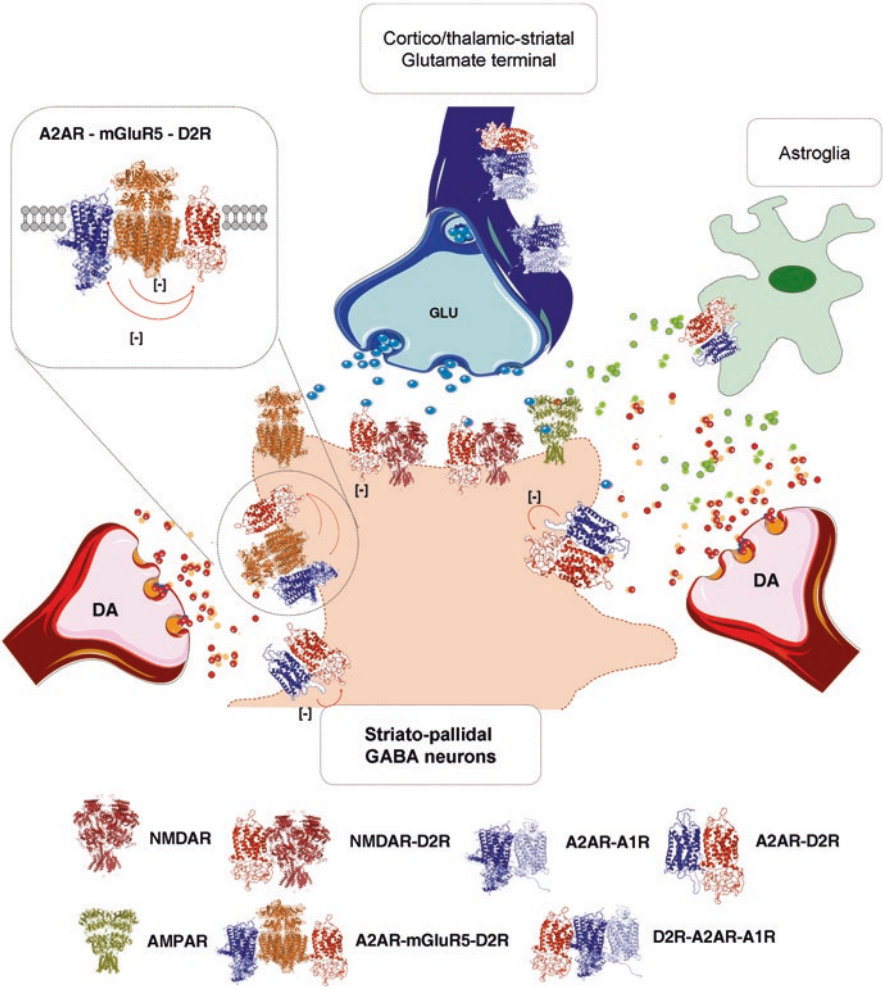

Fig. 20.2 Illustration of a number of relevant D2R and A2AR heteroreceptor complexes in the dendritic regions of the striato-pallidal GABA neurons. They are located both in the glutamate synapses and in extrasynaptic regions. The incoming DA afferents mainly operate via volume transmission. The A2AR-D2R heterocomplexes may exist also on the astroglia. The A2AR-mGluR5-D2R and A2AR-D2R postjunctional heterocomplexes may have a major role in the inhibitory modulation of postjunctional D2R signaling involving allosteric receptor-receptor interaction inhibiting the D2R protomer function. In the glutamate synapse NMDAR-D2R heterocomplexes exist inhibiting the postsynaptic NMDAR signaling through allosteric receptor-receptor interactions [99]. On the glutamate terminals A2AR-A1R and putative D2R-A2AR-A1R heterocomplexes may exist in which A2AR activation via allosteric receptor-receptor interactions with A1R can inhibit A1R signaling and thus remove its inhibitory effects on glutamate release. Upon D2R activation on the glutamate terminal, prejunctional A2AR signaling is instead reduced via an inhibitory allosteric receptor-receptor interactions with removal of its brake on prejunctional A1R signaling, which can contribute to the D2R inhibition of glutamate release. In Parkinson's disease due mainly to the degeneration of the DA terminal networks especially the D2R hetero and homoreceptor complexes in the dorsal striato-pallidal GABA neurons become reorganized with altered allosteric receptor-receptor interactions which contributes to the dysfunction of the striato-pallidal GABA neurons mediating motor inhibition

Locomotion was studied in the MitoPark mice during 8 weeks of daily treatment with saline,MSX-3, MSX-3 plus L-DOPA, or low and high doses of L-DOPA. The major result was that chronic MSX-3 treatment alone counteracted the gradual reduction of spontaneous locomotion (24 h after injection) found in saline controls and also in L-DOPA-treated animals [70]. The maintenance of locomotion by MSX-3 was clear-cut and was not observed with levodopa treatment. It was not related to neuroprotective actions since the striatal DA levels continued to be reduced. It is proposed that these antiparkinsonian actions of early MSX-3 treatment alone were related to a blockade of the reorganization of A2AR-D2R heteroreceptor complexes postulated to take place during the progressive degeneration of the nigro-striatal DA neurons [9]. It seems possible that the early blockade by the A2AR antagonist of the A2ARs being monomers or part of A2AR homo and heteroreceptor complexes can involve a blockade of the increased formation of A2AR-D2R heteroreceptor complexes (see above). As a result of their balance with other hetero and homoreceptor complexes, increased formation of e.g., D2R homoreceptor complexes can develop lacking the A2AR brake on D2R signaling which can help explain the maintenance of locomotion observed with A2AR antagonist treatment alone 24 h after injection of the antagonist.

Along these lines it is also possible to postulate a mechanism for the failure for low and high doses of levodopa to counteract the progressive reduction of spontaneous locomotion in the MitoPark model. The daily treatment with levodopa can lead to increased internalization of D2Rs and their activation can lead to increased expression of A2ARs (see above). As a result, the balance of the various A2AR and D2R homo and –heteroreceptor complexes can be altered in a different way 24 h later compared with A2AR antagonist alone treatment. In this case A2AR-D2R and A2AR-A2AR complexes may instead dominate over D2R monomers and D2R homoreceptor complexes. It becomes clear that by introducing the hypothesis that various A2AR and D2R homo and heteroreceptor complexes exist in a dynamic equilibrium with each other [8, 24] that is agonist/antagonist and disease regulated it becomes possible to explain the above experimental findings reported in models of PD.

Such a dynamic panorama likely exists also in the human striatum in PD but may here be even more complex. It becomes of high importance to understand how aggregations of alpha-synuclein producing synaptic dysfunctions in PD [72] will impact the dynamic panorama of mono-homo and heteroreceptor complexes in the synaptic and extrasynaptic plasma membranes of key motor circuits in PD. The hypothesis was introduced that this dynamic panorama of synaptic homo and heteroreceptor complexes together with adaptor/scaffolding proteins, ion channels etc. form the molecular basis of learning and memory [6, 8, 13, 24, 73].

20.7 A2AR-D2R-mGlu5R Heteroreceptor Complexes

A number of studies in cellular models and in brain indicate the existence of A2AR-D2R-mGlu5R heteroreceptor complexes which appear to exist mainly extrasynaptically on the spines of the dorsal striato-pallidal GABAergic neurons receiving

glutamate synapses from the cerebral cortex and the thalamus [56, 74, 75] (Fig. 20.2). They are likely inter alia in balance with A2AR-D2R heteroreceptor complexes and the corresponding D2R, A2AR and mGlu5R homoreceptor complexes. The A2AR-D2R-mGlu5R heteroreceptor complexes have a special significance since through their allosteric receptor-receptor interactions A2AR and mGlu5R can synergize to bring down D2R affinity, Gi/o coupling and signaling cascades over the MAPK and pCREB pathways [56, 76, 77]. In line with these neurochemical results mGlu5R antagonists increase locomotion and exert antiparkinsonian effects especially after cotreatment with A2AR antagonists [15, 78, 79].

It should therefore be considered that with the degeneration of the DA nerve terminal networks in PD reduced D2R activation takes place in and around the glutamate synapses on the striato-pallidal GABAergic neurons. Reduced D2R activation on the glutamate terminals will increase synaptic glutamate and ATP release and potentially also glial ATP release with ATP broken down to adenosine. These events may not only increase the brake on D2R-mediated signaling in already formed A2AR-D2R-mGlu5R heteroreceptor complexes but also increase the formation of such complexes not only postsynaptically (major location) but also presynaptically. Thus, it seems possible that increased extracellular levels of glutamate and adenosine can produce conformational changes in the mGlu5R and A2AR receptors which can enhance physical interactions with the D2Rs at least when not properly activated by DA due to the progressive degeneration of the DA terminals. As a consequence D2Rs are recruited into such complexes from other D2R complexes like the D2R homoreceptor complexes. It seems possible that a reorganization of the D2R complexes into A2AR-D2R and A2AR-D2R-mGlu5R complexes can be blocked by early treatment with A2AR and/or mGlu5R antagonists including the use also of negative allosteric modulators. If so, such treatments can delay the introduction of levodopa and D2R agonist treatments. It will be of interest to test this hypothesis.

With the onset of the dopaminergic treatments the PD patients will have several to many years of relief from parkinsonian symptoms especially at the level of motor function. With time, however, wearing off effects and dyskinesias will develop upon the chronic treatment with levodopa and D2R agonists, especially since it is difficult to fully avoid intermittent treatment [80]. According to our view one mechanism for the wearing off of the antiparkinsonian actions can be a progressive increase over time in the brake on the inhibitory D2R signaling and a progressive increase of the excitatory input to the striato-pallidal GABAergic neurons, which leads to increased motor inhibition. The molecular mechanisms can involve increased internalization of activated D2Rs with increased breakdown and reduced recycling via endosomes to the plasma membrane. An increased gene expression of A2AR and mGlu5R receptors through a panorama of agonist activated D2R signaling pathways can be another mechanism. As a result the balance of the various D2R, A2AR and mGlu5R homo and heteroreceptor complexes can change. A2AR-D2R, A2AR-D2R-mGlu5R and A2AR-mGlu5R heteroreceptor complexes will dominate with brake on D2R protomer signaling and D2R homoreceptor complexes become reduced. As a result the wearing off takes place and the antiparkinsonian effects of levodopa and D2R agonists become diminished with reduced on-time.

This hypothesis offers one explanation for the wearing off effects found in the clinic with dopaminergic treatments. It may be that continuous treatment delays this change in the panorama of the above homo and heteroreceptor complexes.

The chronic treatment with levodopa and D2R agonists also in parallel leads to dyskinesias [81, 82], which can also be found with D1R agonists [83]. There are reports that A2AR and mGlu5R antagonists can reduce dyskinesias [78, 79]. It seems that the balance between the activity in the D1R regulated direct (striato-internal pallidal/nigral GABAergic neurons) pathway initiating movements (Fig. 20.1), and the D2R regulated indirect (striato-pallidal GABAergic neurons) pathway mediating motor inhibition (Fig. 20.2), plays a major role. As discussed above there may develop with chronic dopaminergic treatment an increased brake on D2R signaling in parts of the striato-pallidal GABAergic system via a reorganization of the balance between A2AR-D2R-mGlu5R heteroreceptor complexes (dominance) and other heteroreceptor complexes containing one or two of these receptor protomers. As a result movements may be initiated from the direct pathway in the presence of increased motor inhibition from the indirect pathway. Dyskinesias will therefore develop due to distortion of movements since certain movements will be differentially inhibited due to reduced inhibition and/or increased activation in parts of the striato-pallidal GABAergic system. In this case A2AR and mGlu5R antagonists will remove the brake on D2R signaling and dyskinesias should become reduced due to increased inhibition of the striato-pallidal GABAergic neurons.

In another case, however, the balance between the direct and indirect pathways can be disturbed in another way also leading to dyskinesias. Thus, the chronic treatment with dopaminergic drugs can lead to a sensitization of the D2Rs by complete removal of the brake on the D2R protomer signaling and/or a reorganization of the homo-and heteroreceptor complexes that favor an exagerated D2R protomer signaling. In this case the loss of motor inhibition can produce dyskinesias since the movements initiated from the direct pathway cannot be properly accompanied by a correct inhibition from the indirect pathway. To counteract dyskinesia development the balance between the activity of the D1R-regulated direct pathway and the D2R-regulated indirect pathway appears to be fundamental.

Overall it would be of high interest to study how heterobivalent compounds targeting the A2AR-D2R-mGlu5R heteroreceptor complexes would improve motor function in models of PD. A combined blockade of A2AR and mGlu5R receptors achieved through use of heterobivalent compounds with A2AR antagonist and mGlu5R antagonist/negative allosteric modulator pharmacophores may specifically and substantially remove the brake on extrasynaptic D2R protomer signaling in the dorsal striato-pallidal GABAergic neurons mediating motor inhibition.

20.8 Cannabinoid CB1R-CB2R and A2AR-CB1R-D2R Heteroreceptor Complexes

Endogenous cannabinoids act on two specific receptor types, namely CB1R and CB2R. CB1Rs are considered the most abundant GPCR in CNS neurons. Accordingly, it was hypothesized that those receptors could form heteroreceptor complexes with A2AR and D2R receptors. A novel technique, a sequential BRET/FRET (SRET) was instrumental to detect trimeric complexes, and the A2AR-D2R-CB1R heteromer was the first one identified by the technique [84]. The assessment of quaternary structure changes induced by the use of mutant receptors and correlating them with the functionality of mutated receptors showed that the quaternary structure of the oligomeric complex is required for proper function and that conserved epitopes in intracellular domains of all three receptors are necessary for the link of the heteromer-specific signaling to the MAPK signaling pathway. The *in vitro* findings were confirmed in striatal slices of wild type and CB1R KO mice [85]. In one of the first studies looking for GPCR heteromer expression in CNS diseases we showed in a rodent and in a non-human primate model that the trimeric complex was present in the striatum of naïve and parkinsonian animals but that it was disrupted in parkinsonian animals chronically treated with L-DOPA [67, 86]. Thus, A2R-CB1R-D2R heteroreceptor complexes are putative targets for treatment in Parkinson's disease but not when dyskinesia appears [87].

Whereas dopamine receptor agonists or adenosine receptor antagonists had potential in the therapy of Parkinson's disease, the potential of CB1Rs as therapeutic targets in CNS diseases was undermined by (i) the fact that natural and synthetic agonists of the receptors are psychotropic and (ii) rimonabant, a selective CB1R antagonist approved for the treatment of obesity, was retired due to serious side effects (e.g. suicide) [88, 89]. CB2R is now considered a good alternative to CB1R receptors in the treatment of CNS diseases with a neuroinflammatory component. Consensus is still lacking concerning the degree of neuroinflammation in the pathogenesis of nigral neurodegeneration in Parkinson's disease but CB2R upregulation in microglial cells was shown in postmortem samples of parkinsonian patients [90]. Unfortunately, identification of heteroreceptor complexes in glia is still lacking in contrast to the case in neurons. Thus, there is a need to identify and characterize heteroreceptor complexes in glial cells. The expression of CB2Rs in neurons of the CNS is restricted to a few areas and a few specific neuronal populations. Interestingly, in the globus pallidus there is a high degree of colocalization of the two receptors that are able to form heteroreceptor complexes in neurons [91–93]. One of the features of CB1R and CB2R heteroreceptor complexes is a negative cross-talk on intracellular signaling and neurite outgrowth and the often-found cross-antagonism [92]. Such findings in the output neurons of the basal ganglia highlights the potential for involvement of cannabinoid receptors in Parkinson's disease and, more specifically of CB2R ligands, which may regulate neurotransmission with few side effects and without the risk of psychotropic actions. Expression of cannabinoid receptor complexes was studied in a non-human primate model of Parkinson's

disease. The heteromeric complex was found in both naïve and MPTP-treated macaques but its expression markedly diminished in dyskinetic animals rendered dyskinetic by chronic L-DOPA treatment. L-DOPA-induced disruption of CB1R–CB2R heteroreceptor complexes should be considered in developing drugs acting on cannabinoid receptor heteromers [91].

20.9 Adenosine A1-A2A Heteroreceptor Complexes

Due to the above-mentioned antagonistic interactions between adenosine and dopamine, which are in part mediated by A2AR-D2R heteroreceptor interactions, there are various A2AR antagonists in clinical trials with Parkinson's patients (www.clinicaltrials.org). Actually one of them has already been approved to use in these patients in Japan (marketed as Nouriast^{R}). Striatum is the region in the mammalian body with higher enrichment in A2AR expression. The receptors are not only expressed in GABAergic neurons of the indirect pathway but also in other striatal cell types. One specific location for A2AR expression is the presynaptic membrane of glutamatergic neurons innervating the striatum. We first showed that a high percentage of these presynaptic A2ARs (Figs. 20.1 and 20.2) are forming heteromers with A1Rs and that the A1R-A2AR heteroreceptor complex works as a concentration sensing device, at low adenosine concentrations A1Rs are activated and Gi-mediated signaling prevails and at high adenosine concentrations A2ARs are activated and Gs-mediated signaling prevails. The consequence is that adenosine enhances or depresses glutamate release depending on its concentration. Usually neither A2AR nor A1R-A2AR receptors in glutamatergic terminals are considered when assessing the therapeutic potential of anti-parkinsonian drugs. One aspect that is still forgotten in drug discovery is that drugs are acting on a variety of heteroreceptor complexes. Accordingly A2AR antagonists are blocking A2ARs expressed either as monomers or in complex with other receptors. From a molecular point of view the A1R-A2AR complex has been instrumental to know a putative stoichiometry of the receptors and of the interacting G proteins [94, 95]. Navarro et al. [95] have proposed a tridimensional structure formed by two A1R, two A2AR and two G proteins. The four receptors form a rhombus shaped structure and the G proteins underneath the receptor heterotetramer are different, i.e. one Gi and one Gs.

20.10 Putative A1R-A2AR-D2R Heteroreceptor Complex

Previous work indicated the existence of A2AR-D2R heteroreceptor complexes also on the striatal glutamate nerve terminals [15, 16]. Of special interest was the finding that in the DA nerve terminal denervated dorsal striatum the A2AR agonist CGS 21680 induced a markedly enhanced increase of the extracellular striatal glutamate levels vs the increase found in the dorsal striatum of controls [63, 96]. These results can be explained by the marked disappearance of the extracellular DA levels

in the denervated dorsal striatum. This reduces the D2R protomer activity with a loss of the reciprocal inhibitory D2R-A2AR interaction [97] and also of the D2R Gi/o mediated inhibition of the adenylyl cyclase, activated by the A2AR protomer via its Gs signaling [15, 16]. As a result the marked increase in A2AR agonist induced release glutamate release develops on the hemiparkinson model.

In view of the existence also of A1R-A2AR heteroreceptor complexes on the striatal glutamate terminals (see above) it is postulated that A1R-A2AR-D2R heteroreceptor complexes can be formed on the striatal glutamate terminals in balance with the A1R-A2AR and A2AR-D2R heteroreceptor complexes (Fig. 20.2). In view of the increase in the A2AR protomer activity upon loss of DA activation of the D2R in the parkinsonian model increased inhibition of the A1R protomer Gi/o mediated signaling can develop through antagonistic allosteric A2AR-A1R interactions and the increased A2AR Gs mediated signaling at the adenylyl cyclase. Thus, a reduction of A1R protomer signaling can also contribute to the marked increase in the striatal glutamate release induced by A2AR agonists on the denervated side in the hemiparkinson model.

20.11 Putative A2AR-D2R-FGFR1 Heteroreceptor Complexes

Heteroreceptor complexes formed by A2AR and the tyrosine kinase fibroblast growth factor receptor 1 (FGFR1) receptors, were demonstrated in the striatum and shown to enhance striatal neuronal plasticity through facilitatory A2AR-FGFR1 receptor-receptor interactions [20, 22]. The formation of striatal A2AR-D2R-FGFR1 heteroreceptor complexes was therefore postulated to take place under certain conditions in view of a possible balance between FGFR1-A2AR and A2AR-D2R with A2AR-D2R-FGFR1 heteroreceptor complexes as an intermediate structure (73). In this way it becomes possible for D2R agonists to modulate the FGFR1 signaling over the A2AR and thus structural plasticity, especially in the striato-pallidal GABAergic neurons. Such interactions are likely diminished in PD in view of the reduction of the D2R signaling. Chronic levodopa and D2R agonist treatment should instead increase these interactions leading to alterations in FGFR1 receptor-mediated signaling, especially in the striato-pallidal GABAergic neurons with changes in their structural plasticity and their function. It is also of substantial interest that D2R activation appears to increase the formation of D2R-Epidermal growth factor receptor heteroreceptor complexes in neuroblastoma cells [98].

20.12 D2R-NMDAR Heteroreceptor Complexes

The D2R has multiple ways to silence the striato-pallidal GABAergic neurons and thus reduce motor inhibition. In an extrasynaptic position it inhibits the L-type voltage dependent calcium channels. In a position within the striatal glutamate synapse

it interacts with the NR2B subunit of the NMDAR to bring down NMDAR-mediated ion channel signaling in the D2R-NMDAR complex [99]. Via extrasynaptic volume transmission DA [33] may diffuse into the synaptic cleft to inhibit the firing of the striato-pallidal GABAergic neurons. It is unknown to which extent the synaptic mGlu5R known to enhance NMDAR signaling can modulate the synaptic D2R-mediated signaling in a postulated D2R-mGlu5R-NMDAR heteroreceptor complex [45] (Fig. 20.2). It seems possible that D2R-NMDAR heteroreceptor complexes can in part be formed in the perisynaptic plasma membrane and diffuse in the membrane into the synaptic part provided they are not trapped by adaptor proteins and have become immobilized.

NR2B antagonists were shown to exert antiparkinsonian-like effects in animal models of PD [100] likely through blocking the NMDAR in the glutamatergic synapse of the striato-pallidal GABAergic neurons. It would be of interest to know if this action can be enhanced by A2AR and mGlu5R antagonists and/or D2R agonists. It would be of value to know if combined reduction of the excitatory synaptic NMDAR signaling together with enhancement of inhibitory D2R signaling in receptor complexes of the synaptic and perisynaptic regions in the striato-pallidal GABAergic neurons would give substantial increases in motor benefits in PD models.

20.13 Conclusions

Based on the current knowledge it becomes clear that the two major efferent pathways of the dorsal striatum the D1R rich direct GABA pathway mediating the initiation of movements and the D2R rich indirect GABA pathway mediating motor inhibition are regulated by distinct and dynamic heteroreceptor complexes of various types. In the direct pathway different types of D1R heteroreceptor complexes play a major role in integrating the synaptic and volume transmission signals while in the indirect pathway the major role is played by different types of D2R heteroreceptor complexes. The motor symptoms of PD develop in part because the reduced D1R signaling in the heteroreceptor complexes of the direct pathway leads to reduced firing of these neurons and movements can no longer be appropriately be induced. The other major mechanism is the reduced D2R signaling in the heteroreceptor complexes of the indirect pathway, which leads to increased firing in the striato-pallidal GABAergic neurons and motor inhibition.

It is proposed that a disbalance in the activity of the direct and indirect pathways produced by chronic levodopa and DA receptor agonist treatment causes the development of dyskinesias in PD patients undergoing such treatments. The hypothesis is given that the molecular mechanism involved is the reorganization of the different types of D1R and D2R heteroreceptor complexes in the direct and indirect efferent pathways, respectively. This reorganization with disturbed allosteric receptor-receptor interactions involves a marked change in the balance of the various D1R and D2R homo and heteroreceptor complexes in the direct and indirect

efferent pathways from the dorsal striatum with significant consequences for motor function. The reorganized heteroreceptor complexes may demand the pulsatile administration and full agonist activity of levodopa to produce the firing patterns of the direct and indirect efferent pathways leading to a disbalance of their activity and thus to dyskinesias [101, 102]. Due to the disbalance, the D1R rich direct pathway with exaggerated activity aims to induce movements in the presence of motor inhibition due to an abnormal activity of the indirect pathway that leads to dyskinesias.

20.14 Summary

- The demonstrated modest anti-parkinsonian effects of A2AR antagonists in animal models and clinical studies have given support to the concept that blocking antagonistic allosteric A2AR-D2R interactions in heteroreceptor complexes can contribute to the development of novel therapies of PD.
- The A2AR protomer in the A2AR-D2R heteromer in the dorsal striato-pallidal GABAergic neurons with antagonistic A2AR-D2R interactions may be a significant target for A2AR antagonists with regard to antiparkinsonian effects. This is true also for the A2AR mono-homoreceptor complexes in these neurons.
- One example of a possible chronic levodopa induced reorganization of the D1R heteroreceptor complexes of the direct pathway is the formation of a postulated A1R-D1R-D3R heteroreceptor complex. The receptor-receptor interactions in such a complex with D3R enhancing D1R-mediated signaling may produce deficits in the inhibitory A1R brake on D1R recognition and signaling. As a result, the D1R sensitization can produce an exaggerated motor drive in the direct pathway leading to a disbalance with the indirect pathway and dyskinesias develop.
- Another example of a possible chronic levodopa induced reorganization of the D2R heteroreceptor complexes of the indirect pathway is an increased development of A2AR-D2R-mGlu5R heteroreceptor complexes. An increased brake on D2R protomer recognition and signaling may here develop through increased activation of the participating A2AR and mGlu5R protomers that takes place through their ability via antagonistic allosteric receptor-receptor interactions to inhibit D2R protomer signaling [17, 33]. As a result exaggerated activity will develop in the striato-pallidal GABAergic neurons resulting in abnormally high motor inhibition interfering with the movements induced via the direct pathway and dyskinesias develop.
- The increased formation of A2AR-D2R-mGlu5R heteroreceptor complexes can also be one mechanism for the wearing off of the antiparkinsonian effects with levodopa and DA agonist treatments. The brake produced by A2AR and mGlu5R receptors is difficult to overcome and motor inhibition in part remains. Early treatment with A2AR antagonist and mGlu5R antagonist/negative allosteric modulator treatment may be an important strategy to remove the brake on D2R signaling in these heteroreceptor complexes in PD and thus motor inhibition.

- It seems likely that an effective brake on D1R signaling can also develop in the D1R heteroreceptor complexes of the direct pathway after chronic treatment with levodopa in some patients with PD. The A1R-mediated inhibition of D1R protomer signaling in novel A1R-D1R heteroreceptor complexes can e.g., become too strong in PD and movements cannot be properly initiated. Such events can also contribute to the wearing off of the therapeutic effects of chronic levodopa in PD.
- The integration of signaling of D1R-NMDAR with A1R-D1R and putative A1R-D1R-D3R heteroreceptor complexes in the direct pathway and its functional consequences on motor initiation is of high interest to be studied in health and disease like PD.
- This is true also for the integration of signaling of D2R-NMDAR with A2AR-D2R, A2AR-D2R-mGlu5R and A2AR-CB1R-D2R heteroreceptor complexes in the indirect pathway mediating motor inhibition.
- Development of heterobivalent compounds with high specificity in targeting the above heteroreceptor complexes offer a novel exciting strategy for treatment of PD restoring the balance of signaling in the various types of homo and heteroreceptor complexes and in the firing of the direct and indirect pathways. It gives a promising future for drug development in Parkinson's disease.

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