



Impact of G protein-coupled receptor heteromers in endocrine systems



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ABSTRACT

The fine-tuning of endocrine homeostasis is regulated by dynamic receptor mediated processes. The superfamily of G protein-coupled receptors (GPCRs) have diverse roles in the modulation of all endocrine axes, thus understanding the mechanisms underpinning their functionality is paramount for treatment of endocrinopathies. Evidence over the last 20 years has highlighted homo and heteromerization as a key mode of mediating GPCR functional diversity. This review will discuss the concept of GPCR heteromerization and its relevance to endocrine function, detailing *in vitro* and *in vivo* evidence, and exploring current and potential pharmacological strategies for specific targeting of GPCR heteromers in endocrine health and disease.

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1. Introduction

G protein-coupled receptors (GPCRs) are pervasive to most physiological and endocrinological processes. Their necessity in maintaining endocrine homeostasis makes them a lucrative therapeutic target, with approximately 40% of current prescription drugs targeting a GPCR. Resulting pathophysiological disorders caused by GPCR dysfunction drives our need to dissect the basic science underpinning the complex modalities of GPCR regulation. Furthermore, understanding these processes is paramount to more targeted and efficacious next generation pharmaceuticals and to personalized medicine approaches (Ferre et al., 2014).

Homo/heteromerization of GPCR is now a widely accepted modality of how GPCRs regulate their physiological functions (Ferre et al., 2014; Gomes et al., 2016). The current evolved model of GPCR

signaling incorporates the ever-increasing complexity in GPCR signal pathways and mechanisms of regulation, to which homo/heteromerization has made a significant contribution. Although most studies have documented homo/heteromerization using heterologous cell lines, several studies have demonstrated the *in vivo* significance of these receptor-receptor interactions. Such complexity in receptor regulation provides key mechanism/s for the multiple and dynamic roles these receptors play *in vivo*.

This review will discuss the functional and *in vivo* evidence for GPCR heteromers in endocrine systems. We will describe the criteria for assessing and classifying GPCR heteromers, review their known functional impact from both *in vitro* and *in vivo* studies of these hetero-complexes on endocrine function.

2. Detecting and classifying GPCR heteromers-an overview

GPCR heteromerization is defined as a macromolecule complex composed of at least two receptor units, with biochemical properties that are demonstrably different from those of its individual components (Gomes et al., 2016). With an increase in the number of reports identifying GPCR heteromers, the resulting challenges in distinguishing the difference between receptors localised to the same cell undergoing functional cross-talk, versus receptors complexed as physiologically relevant heteromers became an important distinction. Thus, three consensus criteria were published by

Abbreviations: α 1BR, α 1B adrenergic receptors; AT1R, Angiotensin 1 receptor; BRET, Bioluminescence resonance energy transfer; B2R, Bradykinin 2 receptor; CRH, Corticotrophin releasing hormone; D2R, Dopamine D2 receptor; FRET, Fluorescence resonance energy transfer; FSHR, Follicle stimulating hormone receptor; GIPR, Gastric-inhibitory polypeptide receptors; GLP-1, Glucagon-like peptide-1; GPCR, G protein-coupled receptor; GHSR, Growth hormone secretagogue receptor; LHR, Luteinizing hormone receptor; μ OR, μ -opioid receptor; MC3R, Melanocortin-3 receptor; PLA, Proximity ligation assay; V1bR, Vasopressin 1b receptor.

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the International Union of Basic and Clinical Pharmacology to facilitate the classification of true GPCR heteromers (Kenakin et al., 2010). The first of these criteria concerns the requirement for evidence of physical receptor-receptor interactions in native or primary tissue. Traditionally, methods such as co-immunoprecipitation and resonance energy transfer techniques (Angers et al., 2000; Hounsou et al., 2015; Albizu et al., 2010; Pflieger et al., 2007; Ramsay et al., 2002) have been used to demonstrate receptor-receptor interactions and hence the proximal existence of heteromers. Recent technological advances in super-resolution and single molecule imaging present an innovative methodology for detecting GPCR heteromers. Techniques such as fluorescent correlation spectroscopy (Mazurkiewicz et al., 2015a; Zakrys et al., 2014), single particle tracking via total internal-reflection fluorescent microscopy (Kasai et al., 2011; Hern et al., 2010; Calebiro et al., 2013) and localization microscopy techniques such as photo-activated localization microscopy (Scarselli et al., 2012; Jonas et al., 2015) have been utilised to identify GPCR heteromers and homomers. Likewise, proximity ligation assays (PLA), also provide a mechanism for identifying heteromers in native tissues (Gonzalez et al., 2012).

The second consensus criteria requires there to be heteromer-specific properties, be it a change in the pharmacology of the receptors via G protein specificity or allosteric binding properties, or ligands that are heteromer-specific. This is demonstrated via classical biochemical, pharmacological and cell signaling techniques to determine changes in ligand binding, G protein-dependent and G protein-independent signal activation. The third criteria necessitates the requirement for the direct physiological evidence for the importance of the identified heteromer. Methodology used to determine this include RNA interference or *in vivo* studies to introduce genetic modifications in receptor protomers participating in the heteromer. If the transmembrane interface is known, expression or incubation of cell permeable peptides corresponding to the transmembrane region have been employed *in vitro* and *in vivo*. Thus, confirming the physiological requirement for the heteromer (Gomes et al., 2016; Kenakin et al., 2010).

In practice, there are very few identified heteromers that fit all three criteria; with criteria three often the hardest to fulfil given that most functional heteromers have been identified using heterologous cell lines. Therefore, fulfilment of two out of three of the criteria are required for the acceptance of a GPCR heteromer. With respect to endocrine systems, most identified GPCR heteromers that fulfil all three inclusion criteria are neuroendocrine in nature. The functional significance of which will be discussed in further detail.

3. Impact of GPCR heteromers on receptor activity in endocrine systems

The canonical view of GPCR signaling has evolved from a ligand binding to a single monomeric receptor that activates a single heterotrimeric G protein, to one of growing complexity. GPCR homo/heteromerization provides a modality whereby receptors can mediate multiple functions via modulating receptor trafficking (both exo- and endocytosis), ligand specificity and functional selectivity. In obligatory heteromers such as the GABAB1 and GABAB2 receptors, (also observed with the sweet and umami taste receptors (Nelson et al., 2001, 2002; Xu et al., 2004)), the functional significance of heteromerization is required for cell surface expression of both receptors, as well as G protein-coupling (Kaupmann et al., 1998; Duthey et al., 2001; Pin et al., 2004), via transactivation of GABAB1-GABAB2 heteromers. However, identifying the functional significance of GPCR heteromers are not always essential to all functions mediated by a specific receptor. That said,

heteromer formation can increase the spectrum of ligand recognition and signal outcomes of a receptor. One common mode of regulation within a heteromer is via allosterism, the outcome of which can lead to distinct functional responses to that of the individual receptors. Broadly speaking, allosteric interactions within heteromers can lead to three different functional outcomes:

1. Ligand binding. Ligand binding within a heteromer results in either positive or negative cooperativity exerted on the neighbouring receptor(s) within the heteromer.
2. G protein recruitment. G protein selectivity may change or exert differential preferences between heteromeric and homomeric complexes.
3. G-protein independent mechanisms via β -arrestin recruitment. The heteromer may favour or acquire G protein-independent functionality via β -arrestin recruitment.

Many GPCR heteromers have been shown to have direct roles in endocrine homeostasis, impacting metabolism, reproduction, nutritional status and stress responses (Table 1). The functional significance of such heteromers has largely been dissected using heterologous cell lines, examples of which will be discussed below.

3.1. Metabolism and nutrition

Endocrine-mediated feedback for the control of satiety and appetite is essential for maintaining metabolic homeostasis. Key pathways that regulate appetite stimulation and feeding are mediated via the growth hormone secretagogue receptor (GHSR). GHSR is expressed within various hypothalamic nuclei involved in food intake and reward-seeking behaviour (reviewed by (Wellman and Abizaid, 2015)), and thus has been suggested to be a central player in regulating metabolic homeostasis in both a ligand-dependent (via Ghrelin) and -independent manner, via intrinsic basal GSHR activity (Wellman and Abizaid, 2015; Yin et al., 2014). Several studies have demonstrated GHSR to heteromerize with other GPCRs that also regulate metabolic status (Wellman and Abizaid, 2015). Indeed, heteromerization of GSHR and the melanocortin-3 receptor (MC3R) was identified by fluorescence resonance energy transfer (FRET) (Rediger et al., 2009), with immunohistochemical and *in situ* studies co-localizing GHSR and MC3R to the arcuate nucleus of the hypothalamus. The functional significance of the GHSR/MC3R heteromers examined *in vitro* via co-expression of GHSR and MC3R in COS-7 and HEK293 cells, showed that GSHR/MC3R heteromers may enhance MC3R-dependent cAMP accumulation yet decrease basal and ligand-induced GHSR activity. Moreover, the enhanced MC3R activity observed was dependent on the decrease in basal GHSR activity, suggesting positive and negative allosteric regulation between MC3R and GHSR protomers to create functionally asymmetric complexes (Rediger et al., 2011).

Heteromers of the Class B GPCR family have been suggested to have roles in glucose homeostasis. Ligand-dependent BRET screening of the glucagon receptor family heteromeric and homomeric interactions in HEK293 cells, showed glucagon-like peptide-1 (GLP-1)-dependent heteromer formation between GLP1 and gastric-inhibitory polypeptide receptors (GIPR). This heteromeric association was GLP-1-specific, with reversal of GLP-1/GIPR association observed with titration of GIPR. Functionally, a change in the activity of the GLP-1R was also observed in terms of calcium response, and β -arrestin recruitment (Schelshorn et al., 2012). There has also been recent evidence for functional cross-talk between *G α s* and *G α q*-coupled receptors in the regulation of long chain fatty acid mediated incretin secretion. Recent findings have shown that GPR40-mediated GLP-1 release by colonic

Table 1

Examples of G protein-coupled receptor (GPCR) heteromers that have been identified in distinct endocrine systems (see text). Abbreviations: α 1B adrenergic receptors (α 1BR); Angiotensin 1 receptor (AT1R); Beta1-adrenergic receptor (β 1R); Bradykinin 2 receptor (B2R); Corticotrophin releasing hormone receptor (CRHR); Dopamine D2 receptor (D2R); Dopamine D4 receptor (D4R); Follicle stimulating hormone receptor (FSHR); Gastric-inhibitory polypeptide receptor (GIPR); Growth hormone secretagogue receptor (GHSR); Glucagon-like peptide-1 receptor (GLP-1R); G protein-receptor 83 (GPR83); Luteinizing hormone receptor (LHR); Melanocortin-3 receptor (MC3R); somatostatin receptor 2 (SST2); somatostatin receptor 5a (SST5a); Vasopressin 1b receptor (V1bR).

GPCR heteromer	Endocrine system/axis	References
GHSR/MC3R	Hypothalamic-gastrointestinal axis	Rediger et al., 2009, Rediger et al., 2011
GHSR/D2R	Hypothalamic-gastrointestinal axis	Kern et al., 2012
GHSR/GPR83	Hypothalamic nuclei-metabolism	Müller et al., 2013
GHSR/SST5a	Insulinotropic action in pancreas	Park et al., 2012
GLP-1R/GIPR	Insulinotropic action in pancreas	Schelshorn et al., 2012
LHR/FSHR	Hypothalamic-pituitary-ovarian axis/pre-ovulatory ovarian follicle	Mazurkiewicz et al., 2015b, Feng et al., 2013
D4R/ α 1BR	Melatonin secretion from pineal gland	González et al., 2012
D4R/ β 1R	Melatonin secretion from pineal gland	González et al., 2012
AT1R/B2R	Renin-angiotensin system in blood pressure regulation	AbdAlla et al., 2001, Quitterer et al., 2004
V1bR/CRHR	Hypothalamic-pituitary-adrenal axis/adrenal chromaffin cells	Murat et al., 2012
D2R/SST2	Growth hormone-secreting pituitary tumors	Colao et al., 2007, Rocheville M1 et al., 2000

enteroendocrine cells requires high cAMP levels, achieved via the bile acid receptor TGR5 or GPR119-dependent $G_{\alpha s}$ activity (Hauge et al., 2014, 2016). Although these studies suggest functional cross talk at the level of G-protein dependent signaling between $G_{\alpha q}$ - and $G_{\alpha s}$ -coupled GPCRs, whether this is mediated via heteromerization remains to be demonstrated.

Heteromers of vasopressin 1b receptor (V1bR) and corticotrophin releasing hormone receptors (CRHR) have also been demonstrated to be functionally important for mediating the biological functions of their respective hormones. In HEK293 cells, V1bR/CRHR heteromers were shown to act to synergistically enhance the G protein-dependent signal responses. Such enhancement of vasopressin and CRH function could underpin their importance in mediating metabolic responses via adrenocorticotrophin hormone release and insulin secretion (Murat et al., 2012).

3.2. Reproduction

The gonadotropin receptors follicle-stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) are essential for reproduction (Jonas et al., 2014; Peltoketo et al., 2010). Expressed in specialised cellular compartments of the testis and ovary, FSHR and LHR coordinate gonadal steroidogenesis and the production and/or maturation of germ cells (Jonas et al., 2014; Peltoketo et al., 2010). Both FSHR and LHR have been shown to form homomers *in vitro* (Jonas et al., 2015; Rivero-Müller et al., 2010; Guan et al., 2009, 2010; Urizar et al., 2005; Tao et al., 2004; Horvat et al., 2001). Moreover, using a functional complementation approach, we have shown that transgenic co-expression of ligand-binding deficient LHR and signal deficient LHR could rescue the infertile phenotype of LHR knockout males (Rivero-Müller et al., 2010), thus demonstrating that homomerization is a physiologically relevant mode of LHR function. Recent studies have also shown that the FSHR and LHR can heteromerize (Mazurkiewicz et al., 2015b; Feng et al., 2013). Heteromerization has been demonstrated using BRET (Feng et al., 2013) and fluorescent correlation spectroscopy (Mazurkiewicz et al., 2015b), moreover, heteromerization appears to alter the pharmacology of the LHR and FSHR by enhancing ligand disassociation, and negatively regulating cAMP production (Feng et al., 2013). LHR/FSHR heteromerization is only physiologically relevant to female reproduction, where co-expression of FSHR and LHR in granulosa cells primarily occurs during the peri-ovulatory period, (Jeppesen et al., 2012). A defined window of co-expression suggests a role for the heteromer in modulating the pleurotrophic actions of LHR during ovulation. Recent studies have also found that co-treatment of FSH with either hCG or LH can potentiate their respective effects on steroidogenesis

and apoptosis (Casarini et al., 2016). Integration of these findings into the physiological role of LHR and FSHR remains to be demonstrated.

4. *In vivo* role of GPCR heteromerization to health and disease

Although many GPCR heteromers have been identified, the *in vivo* physiological role(s) of many remains speculative and inferred from heterologous cell types or primary cell cultures. This is mainly due to the technical difficulties often encountered with identifying endogenous receptors. However, with recent methodological advances, such as proximity ligation assays and time-resolved FRET, several studies have made significant head-way in identifying GPCR heteromers *in vivo*, and deciphering their functional roles.

Recent work has identified two novel heteromers of dopamine D4 receptor (D4R)/ α 1B adrenergic receptors (α 1BR) and dopamine D4/ β 1 adrenergic receptors (β 1R), the formation of which is controlled by circadian rhythm and the light-dark cycle. A study by Gonzalez et al., identified D4R- α 1BR and D4R- β 1R heteromers in primary pinealocytes and pineal glands, via proximity ligation assay (González et al., 2012), and that formation of D4R-adrenergic receptor heteromers was controlled by the expression level of D4R, with increased heteromer formation at sunrise, and decreased/little heteromer formation at sunset. Pharmacologically, adrenergic receptor-D4R heteromers cross-antagonised the release of melatonin via the regulation of the synthesis and secretion of serotonin, the precursor of melatonin. Thus, low level heteromer formation correlated with increased release of melatonin at sunset, and low melatonin release and high heteromer formation at sunrise (González et al., 2012), clearly exhibiting how key physiological functions are intricately controlled at the level of heteromer formation.

Isoforms of the dopamine receptor family have been shown to heteromerize with GHSR. Via TR-FRET and FRET, dopamine D2 receptor (D2R)/GHSR heteromers were demonstrated *in vivo* within the hypothalamus (Kern et al., 2012). Moreover, D2R/GHSR heteromers amplified the anorexigenic effects of D2R. Using cabergoline, the D2R-selective agonist, a dose-dependent suppression of food intake in wild-type and ghrelin knock-out mice was observed, however, there was no effect on food intake in GHSR knock-out mice. These data illustrate that the anorexigenic effects of D2R are augmented by GHSR, presenting a possible avenue for therapeutic interventions in food intake disorders (Kern et al., 2012).

GHSR and somatostatin 5a (SST5a) receptors have been demonstrated via TR-FRET in β islet cells of the pancreas. Heteromerization of GHSR and SST5a changed the G protein-coupling

preference of GSHR to *G α i*, mediating ghrelin and somatostatin-induced inhibition of glucose-stimulated insulin secretion. Moreover, the degree of heteromerization detected was ligand-regulated, with high ghrelin and low somatostatin increasing heteromer formation, as detected via BRET (Park et al., 2012). This, in turn regulated the canonical (GSHR- *G α q* coupling) versus non-canonical (GSHR-*G α i* coupling) signaling detected. Thus, suggesting a role for the GSHR/SST5a heteromer formation and activity in fine-tuning and regulating β islet-cell mediated insulin secretion (Park et al., 2012).

A further study has also elucidated the *in vivo* roles of GSHR/GPR83 heteromers. GPR83 is an orphan GPCR expressed within hypothalamic nuclei where it controls energy balance, and co-localizes with GSHR in the arcuate nucleus. *In vitro* analysis using HEK293 cells demonstrated heteromerization of GPR83 and GSHR via BRET (Müller et al., 2013). Moreover, *in vivo* exploration of the physiological role of the GPR83/GSHR heteromers using transgenic knock-out approaches showed that GPR83 knockout mice were protected from weight gain when fed on high-fat diets, despite hyperphagia (Müller et al., 2013) demonstrating an intriguing role for GPR83 and GSHR heteromers in both feeding behaviour and energy metabolism.

In recent years, several heteromers have been identified with relevance to fetal/maternal health. The intriguing formation of angiotensin 1 receptor (AT1R) and bradykinin 2 receptor (B2R) heteromers were implicated in the pathophysiological development of pre-eclampsia (AbdAlla et al., 2001; Quitterer et al., 2004). The expression of AT1R-B2R heteromers were more abundant on platelets and omental vessels isolated from women with pre-eclampsia, with increased heteromer formation due to the up-regulated expression of B2R, resulting in the elevation of angiotensin II-dependent *G α q* activity.

Heteromerization can provide a mechanism underlying how the multifaceted functional roles of a single receptor subtype can be mediated. Thus, the identification of GPCR heteromers *in vitro*, and the functional and physiological validation of heteromers *in vivo* identifies potential novel therapeutic interventions and drug targets.

5. Pharmacological exploitation of GPCR heteromers

GPCR heteromers have attracted significant attention from the pharmaceutical industry due to their potential to address the non-specific effects of drugs thought to result from heteromerization, or to exploit the novel properties of heteromers in the design of new therapeutics. The unique biochemical signature of GPCR heteromers, be it a change in trafficking, pharmacology or signaling properties, provide a platform for screening and differential drug targeting. Indeed, well-characterized GPCR heteromers have already been targeted in the development for novel treatment of Parkinson's disease, addiction, schizophrenia, depression and chronic kidney disease. (Guidolin et al., 2015). The main strategies to target GPCR heteromers to date are to activate the heteromer specifically via bivalent ligands, using small molecules that target heteromer-specific allosteric binding pockets, or inhibit heteromer function via a drug combination approach, or multifunctional ligands.

5.1. Specific activation of GPCR heteromers

Drug development strategies to specifically target GPCR heteromers focus on conditions where targeting of the heteromer has beneficial activities over the homomer. To date there are few known ligands with proven heteromer-specific activity *in vivo*. The best described examples are those targeting the opioid receptors.

For example, CYM51010 is a repurposed compound thought to be a specific ligand for the μ - δ opioid receptor heteromer (Gomes et al., 2013). Interestingly this compound is a biased ligand to the β -arrestin pathway for these heteromers and activates analgesic responses similarly to morphine, yet with reduced tolerance under chronic treatment. The binding site of this compound may be an allosteric site unique to the heteromer, or at least distinct from each orthosteric site in each protomer, as the μ -opioid receptor antagonist only partially inhibits the *in vitro* activity of the heteromer. Furthermore, the *in vivo* action of CYM51010 (but not morphine), was blocked by a heteromer-specific antibody, showing specificity for the heteromer-specific actions (Gomes et al., 2013).

Bivalent ligands, in the context of GPCR homo/heteromerization, consist of a compound that is selective for one protomer linked by a spacer arm to a compound selective for its partner protomer. These are distinct from bitopic ligands that are compounds able to interact with orthosteric and allosteric sites. Although bitopic (or dualsteric) ligands have been designed based on a monomeric GPCR model, it is certainly possible that these ligands could be designed to be heteromer-specific, although this has not yet been explored for this class of compounds. Bivalent ligands are thought to target and stabilize pre-formed/constitutive heteromers rather than induce heteromerization (Berque-Bestel et al., 2008) and have been used as tools to understand the functional significance of specific GPCR heteromers. To date many have been developed towards heteromers containing the μ OR. One interesting example is a bivalent ligand that targets the heteromer of μ OR/metabotropic glutamate receptor 5 for treatment of chronic inflammatory pain. The compound identified was a μ OR agonist coupled to a metabotropic glutamate receptor 5 antagonist and also illustrated that highly defined spacer lengths were key to its activity *in vivo* (Akgün et al., 2013). In the context of endocrine systems, bivalent ligands have provided a useful tool to support the clinical and therapeutic relevance of the D2R dopamine receptor/somatostatin SST2 receptors in acromegaly. These heteromers are expressed in GH-secreting pituitary tumors and have enhanced *G α i*-signaling activity over their homomeric counterparts (Colao et al., 2007; Rocheville M1 et al., 2000). Employment of bivalent ligands, however, demonstrated that targeting both these receptors can effectively inhibit GH and prolactin secretion from GH-secreting pituitary adenomas, more effectively than their response to somatostatin receptor antagonist octreotide (Jaquet et al., 2005a, 2005b). Bivalent ligands have also been developed for the gonadotrophin hormone receptors in order to improve receptor specificity of allosteric low molecular weight compounds to these receptors. Although the study did not assess the compound in the context of LHR/FSHR heterodimers, a small molecular activator of both LHR and FSHR was made more specific to LHR (although resulted in a loss of potency) by linking it with an FSHR-specific negative allosteric modulator (Bonger et al., 2009, 2010).

5.2. Specific pharmacological inhibition of GPCR heteromer function

GPCR heteromers may play a role in the off-target effect of certain drugs and/or have a demonstrated role in human disease, thus selective inhibition of these complexes has been achieved via combination therapy or bi/multifunctional ligands. A combination therapy approach relevant for endocrine systems is currently in Phase 2 clinical studies for chronic kidney disease. This is based on a therapy to target chemokine receptor CCR2/angiotensin II type 1A receptor heteromers, repurposing two known antagonists for CCR2 and angiotensin 1A receptor, termed DMX-200 (Ayoub et al., 2015).

Bifunctional or multifunctional peptide ligands could represent an alternate strategy to block action of specific heteromers. These peptide ligands possess agonist activity to one GPCR and

antagonistic properties to another. For example, bifunctional ligands that are agonistic at δ and/or μ -opioid receptors yet antagonistic at pro-nociceptive cholecystokinin receptor (Agnes et al., 2006) or substance P neurokinin-1 receptor, are thought to create a potent analgesic with fewer side effects than morphine (Yamamoto et al., 2008), however it is unknown whether such compounds target heteromers of these receptors.

6. Perspectives

How individual cells integrate and decode multiple signals from an array of distinct receptors remains an outstanding question. The evidence to date strongly supports a significant role for GPCR heteromerization in the cellular signaling network, and the downstream significance of these associations in both health and disease. The number of reports of GPCR heteromers across diverse physiological and pathophysiological systems has dramatically increased, particularly as the technology to identify and characterise these heteromers—from the single molecule to its *in vivo* function—have been developed. Although this field of GPCR biology has driven continuous debate on the significance of such interactions, the communication of a standardized framework to study GPCR heteromers (Gomes et al., 2016; Largent-Milnes et al., 2013) and tools to demonstrate *in vivo* significance will contribute to our evolving models of GPCR signaling.

Current challenges are to understand how to effectively exploit this complexity, although certain strategies are showing real promise, as discussed above. Furthermore, what is the extent of GPCR heteromerization and how does it contribute to the overall signal crosstalk in cells that express hundreds of distinct GPCRs exposed to multiple ligands? This latter question highlights a functional requirement for crosstalk via heteromerization of GPCRs that contain more than two GPCRs, considering not only oligomeric composition, but also that a heteromer may contain more than two different GPCRs. There is increasing evidence that for certain GPCRs heterotetramers may be the functional form, e.g. D2R–A2A and D2–D3 dopamine receptors, containing equivalent ratios of each GPCR, i.e. association of two homodimers (Casadó et al., 2009). This is thought to provide a platform for coupling of distinct G proteins, and certainly this is consistent with the idea that functionally asymmetric GPCR oligomers could regulate G protein signal strength (Jonas et al., 2015). The idea that more than two GPCRs form heteromers has been demonstrated for chemokine receptors (heteromer of CCR2–CCR5–CXCR4) (Sohy et al., 2009). Such complex associations could be highly significant for endocrine systems where extensive signal crosstalk is exposed to a highly dynamic extracellular environment. Despite the recent technical advances, a missing piece of the GPCR signaling puzzle is crystal structure information of a heteromeric GPCR complex. The explosion of available crystal structures of GPCRs has transformed drug design strategies for the GPCR monomer model, and although crystal structures of homodimers (Geng et al., 2016; Fotiadis et al., 2006) and homo-oligomers (Huang et al., 2013) have been identified, heteromeric structures would provide unprecedented information, not only on interfaces, but unique heteromer-specific targeting sites for structure-based drug discovery. With the generation of antibodies that target GPCR heteromers and the use of nanobodies in GPCR research (DeVree et al., 2016; Irannejad et al., 2013; Maussang et al., 2013; Miao and McCammon, 2016; Nygaard et al., 2013; Rasmussen et al., 2011; Ring et al., 2013; Staus et al., 2016), there is a real technical possibility of creating tools to aid stabilisation of such structures for crystallization. Likewise there is increase interested in immunotherapy pathways, particularly in cancer, and whilst there are no current approved GPCR antibody drugs, antibodies specific to GPCR heteromers have been generated

(Gomes et al., 2014).

Overall, GPCR heteromers are becoming increasingly accepted as part of the GPCR signalosome, which is integrated in to an even more complex cellular network. As the signal wires mediated by GPCR heteromers are untangled, this will provide new insights and therapeutic avenues in to the roles of these GPCR complexes in endocrine systems.

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