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Arrestin recruitment and signaling by G protein-coupled receptor heteromers

Kendall L. Mores¹, Robert J. Cassell¹, and Richard M. van Rijn^{1,2,3}

¹Department of Medicinal Chemistry and Molecular Pharmacology, College of Pharmacy, West Lafayette, IN 47907

²Purdue Institute for Drug Discovery, West Lafayette, IN 47907

³Purdue Institute for Integrative Neuroscience, West Lafayette, IN 47907

Abstract

G protein-coupled receptors (GPCR) have a long history of being considered a prime target for drug development to treat a plethora of diseases and disorders. In fact in 1827, the first approved therapeutic in the United States was morphine, a drug that targets a GPCR, namely the mu opioid receptor. However, with the rise in biologics over the last two decades, the market share of small molecules targeting GPCRs has declined. Still, two phenomena concerning GPCR pharmacology, specifically heteromerization and biased signaling, have bolstered new interests in this particular class of drug targets. Heteromerization, the process by which two distinct GPCRs come together to form a unique signaling complex, has been demonstrated between many different GPCRs and has spurred efforts to discover heteromer selective drugs. Additionally, the discovery of biased signaling, a concept by which a GPCR can transduce intracellular signaling by favoring a specific pathway (e.g. G-protein) over another pathway (e.g. arrestin), has led to the development of signal-biased drugs with potentially fewer side effects. Our goal for this review is to highlight studies that have investigated the interplay of these two phenomena by providing an overview of the current literature describing instances where GPCR heteromers have distinct arrestin recruitment profiles when compared to the individual GPCRs, with a focus on those GPCRs expressed in the central nervous system.

Graphical Abstract

[†]**Corresponding author:** Richard M. van Rijn, Department of Medicinal Chemistry and Molecular Pharmacology, College of Pharmacy, Purdue University, West Lafayette, Indiana 47907 (Phone: 765-494-6461; rvanrijn@purdue.edu).

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Introduction

G protein-coupled receptors (GPCRs) are the largest family of membrane-bound proteins, are expressed ubiquitously throughout the human body and are activated by a diverse set of ligands including monoamines, odors, peptides, and hormones. This diversity of distribution and activation, as well as the important role that these receptors play in a variety of cellular signaling processes, ensured early on that GPCRs were considered as major targets for the discovery of drugs to treat a multitude of disorders. The first federally regulated drug in the United States, morphine, was approved in 1827 and subsequently shown to target a GPCR, specifically the μ opioid receptor (μ OR) (Kinch et al., 2014). Since the beginning of the 21st century, the rise of biologics has produced a relative decline in the market share of GPCR-based drugs (Kinch, 2015; Sriram and Insel, 2018). However, a number of novel concepts of GPCR pharmacology, including allosteric modulation, receptor heteromerization and biased signaling, have emerged (Gomes et al., 2016; Reiter et al., 2012; Wootten et al., 2013), discoveries which may allow drugs targeting GPCRs to reclaim the ground lost.

Originally, GPCRs were believed to function as single monomeric units however there has been increasing evidence that GPCRs may interact with one another to form higher order oligomeric complexes (Ferre et al., 2009; Fuxe et al., 1981). GPCRs can form either homomers (comprised of two or more identical GPCRs) or heteromers (comprised of two or more different GPCR types or subtypes). The ability of receptors to participate in receptor complexes provides cells with a low-energy opportunity to expand their repertoire of unique functional targets. To be classified as a GPCR heteromer, certain criteria must be met (Gomes et al., 2016). First, it must be demonstrated that the heteromer protomers co-localize and physically interact, and furthermore that the heteromer complex exhibits properties distinct from those of the individual protomers and that these properties should be lost upon disruption of the heteromer complex. Examples of the changes that may occur upon GPCR heteromerization include changes to G-protein signaling, such as coupling to a different G_{α} -

protein subtype, or changes to ligand efficacy (Fribourg et al., 2011; Rashid et al., 2007). Heteromerization can also alter the endocytic fate of the receptor following activation, via changes in desensitization, internalization and post-endocytic trafficking (Angers et al., 2002; Ward et al., 2013). Due to their unique pharmacology GPCR heteromers have long been considered novel drug targets that could potentially allow for the development of more efficacious drugs.

A major action during the process of GPCR signaling is the recruitment of arrestins to the GPCR. The arrestins are a family of four proteins (arrestin 1–4): arrestins 1 and 4 are primarily expressed in the eye and associated with visual transduction, while the non-visual arrestins 2 and 3, also known as β -arrestin 1 and 2 (terms we will use in this review), are more widely expressed. Upon GPCR activation, receptors may undergo phosphorylation at intracellular loops and carboxyl-terminal tail by protein kinases or G-protein regulated kinases. The incorporation of phosphoryl groups to the GPCR increases the affinity of the receptor for arrestins. Subsequent arrestin recruitment to, and interaction with, the GPCR supports receptor desensitization and targets the GPCR for clathrin-mediated internalization (Moore et al., 2007). During this process, arrestins can either remain connected to the GPCR for subsequent actions, or may dissociate (Eichel et al., 2018). A debate is currently ongoing on whether arrestin recruitment can occur in the absence of G-proteins (Choi et al., 2018; Eichel et al., 2018; Grundmann et al., 2018; Gurevich and Gurevich, 2018). Increasing evidence, though, suggests that, beyond promoting receptor desensitization and internalization, the arrestins can serve as scaffolding proteins for a number of intracellular signaling cascades such as mitogen-activated protein kinases and serine/threonine kinases (DeWire et al., 2007). Recent studies have proposed that the pattern of receptor phosphorylation may act as a barcode determining which specific kinase will interact with the receptor-complex (Nobles et al., 2011; Yang et al., 2015; Zhou et al., 2017). The discovery that under certain conditions receptors can preferentially interact and signal via G-proteins or β -arrestins has given rise to efforts to produce so-called signal biased drugs i.e. drugs that induce receptor conformations that favor G-protein activation over β -arrestin recruitment or vice versa. The ultimate ambition of these efforts is to produce drugs that have fewer adverse effects while retaining efficacy.

In this review, we provide an overview of studies that have investigated and found unique pharmacology of GPCR heteromers as it relates to changes in the association of arrestin proteins to the constituent receptors. Studies investigating the effects of heteromerization on arrestin recruitment and signaling can be largely subdivided into two categories. We have framed this review to first describe studies which investigate changes in receptor trafficking properties as a proxy for heteromer-mediated arrestin recruitment. We then describe the second category, which are studies that employed primarily biophysical assays, such as Förster or Bioluminescence Resonance Energy Transfer (FRET and BRET), or commercially produced Tango and PathHunter assays to track heteromer-mediated arrestin recruitment and signaling (Dogra et al., 2016; Kopra et al., 2013) (Figure 1).

Studies using receptor trafficking to assess arrestin recruitment

Prior to the development of cellular assays that allow for the direct quantification of arrestin recruitment, visualization of changes in membrane localization of fluorescent receptors using fluorescent microscopy commonly served as a method to assess heteromer existence and heteromer-mediated arrestin recruitment (Figure 1A and B). Due to the role of arrestins as key mediators of receptor internalization, observed changes in receptor or heteromer trafficking visualized via fluorescent microscopy were used to infer differences in arrestin recruitment between individual receptors and receptor heteromers.

Studies investigating the effects of heteromerization on trafficking properties have reported changes in agonist-mediated internalization as well as changes in the extent of co-localization with arrestin. One such example is the heteromerization of the β_2 -adrenergic receptor (β_2 AR) with other β -adrenergic subtypes, whereby β_2 AR agonist isoproterenol-mediated internalization of the β_2 adrenergic receptor is reduced when co-expressed with the β_1 AR, or the β_3 AR, suggesting an increase in G-protein bias for isoproterenol acting at the β_1 AR- β_2 AR or β_2 AR- β_3 AR heteromers when compared with β_2 AR monomers (Breit et al., 2004; Lavoie et al., 2002). A similar situation was observed for arrestin recruitment to the δ opioid receptor (δ OR) upon activation by etorphine; studies found a reduction in etorphine-induced δ OR internalization when co-expressed with the β_2 AR or the κ OR compared to when the δ OR was expressed alone (Jordan and Devi, 1999; Jordan et al., 2001). These previous studies highlight cases in which agonist-induced receptor internalization was reduced in the setting of heteromerization, however studies focusing on the μ OR revealed other possible heteromer fates following activation. Specifically, in cells where the μ OR is co-expressed with the somatostatin receptor subtype 2A (SSTR_{2A}), stimulation with a selective agonist for one receptor (be it the μ OR or the SSTR_{2A}) led to cross-internalization of the 'unstimulated' GPCR (Pfeiffer et al., 2002). Similarly, for heteromers between the μ OR_{1D} subtype and the gastrin-releasing peptide receptor (GRPR), activation with morphine led to co-internalization of GRPR, although conversely GRP was unable to co-internalize μ OR_{1D} (Liu et al., 2011).

The creation of fluorescently labelled arrestin proteins (with, for example, green fluorescent protein [GFP]) opened up studies directly measuring changes in arrestin localization (i.e. from the cytosol to the membrane) and co-localization with fluorescently tagged receptors (Figure 1B). A study by Pfeiffer et al. used a dual approach in which they tracked receptor co-internalization and in a separate experiment visualized arrestin recruitment in cells expressing the μ OR and the neurokinin-1 (NK1) receptor. It was reported that the NK1 selective agonist substance P can co-internalize the μ OR, and inversely that the μ OR selective peptide DAMGO can co-internalize the NK1 receptor. Additionally, substance P and DAMGO respectively caused co-localization of β -arrestin 2 with the μ OR and the NK1 in early endosomes leading to slower receptor recycling and resensitization (Pfeiffer et al., 2003). Conversely, a study investigating SSTR₂-SSTR₅ heteromers found that β -arrestin 2 association with the SSTR₂ is strong when expressed alone and leads to robust SSTR₂ internalization and accumulation in endosomes. However, in cells where the SSTR₂ is co-expressed with SSTR₅, stimulation with the SSTR₂ selective agonist L-779,796 reduces the strength of the SSTR₂- β -arrestin 2 interaction resulting in a faster recycling rate of SSTR₂.

(Grant et al., 2008). SSTR agonists are used clinically to treat acromegaly and this study highlights the potential upside of selectively targeting SSTR₂-SSTR₅ heteromers to avoid SSTR₂ internalization and receptor tolerance.

In contrast to lack of SSTR₂-SSTR₅-arrestin heteroreceptor complex formation, Decaillot et al. showed that the FLAG-epitope-tagged chemokine C-X-C receptor 7 (CXCR7) exhibits very little association with β -arrestin 2-GFP when expressed alone. Yet, when CXCR7 is co-expressed with another chemokine receptor subtype, CXCR4, the resultant heteromer constitutively co-localizes with β -arrestin 2 (Decaillot et al., 2011). These findings match those observed for the μ OR- δ OR heteromer, which also displays a high level of constitutive β -arrestin 2 recruitment and enhanced ERK phosphorylation (Figure 2A) compared to basal recruitment to the individual receptors (Rozenfeld and Devi, 2007). Several research groups have identified that δ OR antagonism reduces morphine tolerance (Abdelhamid et al., 1991; Fundytus et al., 1995; Miyamoto et al., 1993; Mosberg et al., 2014; Wells et al., 2001). Interestingly co-incubation of the μ OR- δ OR heteromer with the μ OR agonist DAMGO ([D-Ala², NMe-Phe⁴, Gly-ol⁵]-enkephalin) and the δ OR antagonist TIPP ψ prevented this constitutive β -arrestin 2 recruitment (Rozenfeld and Devi, 2007). Similarly, the δ OR antagonist naltriben prevented methadone-induced endocytosis of the μ OR- δ OR heteromer (Figure 2B) (Milan-Lobo and Whistler, 2011) as well as the analgesic tolerance observed with repeated methadone administration in mice (Milan-Lobo et al., 2013). In order to target this specific attribute of μ OR- δ OR heteromer signaling, various groups have developed bivalent molecules that link a μ OR agonist with a long spacer to a δ OR antagonist specifically for this purpose (Daniels et al., 2005; Harvey et al., 2012; Olson et al., 2018). Studies using the δ OR agonist TAN-67 have suggested that it may have a preference to interact with μ OR- δ OR heteromers and by doing so reduces alcohol use in mice (van Rijn and Whistler, 2009). Interestingly, in follow up studies it was shown that TAN-67 does not strongly recruit β -arrestin (Chiang et al., 2016) and that the G $_{\alpha i}$ -protein-biased action of TAN-67 can be localized to the dorsal striatum (Robins et al., 2018b), a brain region where μ OR and δ OR are co-localized on medium spiny neurons of the indirect pathway (Banghart et al., 2015). Thus it appears that the μ OR- δ OR heteromer exhibits a complex pharmacological profile that can be targeted in different ways to accomplish diverse goals.

Studies directly measuring arrestin recruitment

The studies described thus far have relied on antibodies and/or fluorescently tagged receptors to localize receptors with each other and with arrestin proteins. In this section we will describe studies that used BRET, FRET and bimolecular fluorescence complementation (BiFC) assays as well as specifically engineered assays such as the TANGO and PathHunter assays to study heteroreceptor complex trafficking and signaling. In the case of FRET assays, a fluorescent donor is fused to the carboxyl-terminal tail (C-tail) of one GPCR, and a fluorescent acceptor is fused to the C-tail of a different GPCR (Figure 1C). If in close enough proximity (<100Å), energy emitted following excitation of the donor can excite the acceptor, suggestive of heteromerization. BRET assays follow largely the same principal as FRET assays however the fluorescent donor component is replaced by a *Renilla* luciferase (*Rluc*), which can provide a photon source for excitation of the acceptor via the metabolism of a substrate, typically coelenterazine (Figure 1D). Differentially, BiFC assays rely on a

single fluorescent protein which is expressed as two non-fluorescent fragments, or as two luciferase fragments for Bimolecular Luminescence Complementation (BiLC), which are connected to the C-tails of the GPCRs, or a GPCR and an arrestin protein. In the case of BiFC and BiLC assays, the interacting components need to be in close proximity to allow for the two fragments to come together and irreversibly reform a functional protein (Figure 1E). In the case of the TANGO assay (Kroeze et al., 2015), the C-tail of a GPCR is fused to the C-terminal tail of the vasopressin receptor, which is a known strong β -arrestin recruiter (Tohgo et al., 2003). This vasopressin C-tail is then connected to a transcription factor via a TEV protease recognition site. The second critical component of this assay is a protease-fused β -arrestin 2 which can cleave the transcription factor from the GPCR upon recruitment, leading to transcription of a luciferase gene and subsequent bioluminescence (Figure 1F). Similar to the BiFC assay, the PathHunter assay relies on functional complementation, however in this case the C-tail of a GPCR is fused to a small pro-link peptide, which complements an inactive galactosidase enzyme fused to β -arrestin 1 or 2, and thus will provide a bioluminescent signal upon arrestin recruitment (Figure 1G).

As stated in the introduction, in order to be defined as a functional heteromer, the heteroreceptor complex needs to have a distinct function compared to the individual protomers. One way to interpret a functional difference would be a change in signaling bias for the heteroreceptor complex. Using BRET technology, several studies were able to demonstrate enhanced arrestin recruitment to heteromers. For example, BRET was used to show that norepinephrine only induces β -arrestin 2 recruitment to the α_{1A} -adrenergic receptor when co-expressed with the CXCR2, but not when expressed by itself (Mustafa et al., 2012). Similarly, using BiFC and BRET assays it was shown that the angiotensin II receptor type 2 (AT₂R), which normally does not recruit β -arrestin 2 upon angiotensin activation, may enter into close proximity with β -arrestin 2 when co-expressed with the angiotensin II receptor type 1 (AT₁R), presumably through formation of AT₁R-AT₂R heteromers. Surprisingly, while angiotensin II induces strong β -arrestin 2 recruitment to the AT₁R-AT₂R heteromer, there was no report of increased internalization (Porrello et al., 2011). BRET assays were also used to show that AT₁R stimulation with the β -arrestin 2-biased AT₁R agonists [Sar¹,Ile⁴,Ile⁸]-angiotensin II and TRV120023 leads to arrestin-mediated internalization of co-expressed bradykinin B₂ receptors and β_2 -adrenergic receptors, respectively (Toth et al., 2017; Wilson et al., 2013). A strategy combining BiFC and BRET (called complemented donor-acceptor resonance energy transfer) using AT₁R and α_{2C} -adrenergic receptors fused to fragments of a split Venus fluorescent protein, and a *Rluc*-fused- β -arrestin 2 was utilized to demonstrate that co-stimulation with angiotensin II and norepinephrine increased β -arrestin 2 recruitment to the heteromer. Interestingly, the AT₁R- α_{2C} AR heteromer gained a unique ability to interact with G_{α_s}-proteins (Bellot et al., 2015), instead of the canonical G_{α_q}-protein and G_{α_i}-protein signal pathways of the AT₁R and α_{2C} AR, respectively. Clearly, heteromers can exhibit altered biased signaling profiles from their individual receptors in several different ways; biased to or away from arrestin recruitment and/or switching to different G_α-proteins, providing avenues to develop drugs that benefit from promoting or attenuating these pharmacological attributes of the heteromer.

BRET assays have further been utilized to identify instances where heteromerization can lead to a reduction in agonist-induced arrestin recruitment. This is the case for putative

heteromers between the atypical chemokine 3 receptor and the arginine vasopressin receptor 1A (Albee et al., 2018) as well as between the melatonin MT₁ receptor and the orphan GPR50 (Levoye et al., 2006).

The phenomena of reduced or enhanced arrestin recruitment and internalization of receptor heteromer complexes raises intriguing questions. For example, to what degree do drugs that favor the formation of heteroreceptor complexes promote or impede heteromer stability? Also would chronic use of drugs that promote or discourage arrestin recruitment to the heteromer alter the competitive dynamic between homomer and heteromeric function? The study by Milan-Lobo et al, as mentioned earlier may at least provide an answer to the latter question; repeated use of a methadone/naltrexone cocktail inhibited μ OR- δ OR heteromer internalization, leading to an artificially high μ OR- δ OR heteromer/ μ OR homomer ratio resulting in analgesic tolerance for methadone (Milan-Lobo et al., 2013).

Arrestin-dependent heteromer-selective kinase signaling in neurons

The striatum appears to be a hotspot for heteromers with unique arrestin pharmacology; besides the potential presence of μ OR- δ OR heteroreceptor complexes mentioned in the previous section the adenosine and dopamine receptors have also been documented to form heteromers *in vitro* as well as *in vivo* in the striatum (Fuxe et al., 2014). Using BRET² (Bertrand et al., 2002) technology it was reported that the adenosine A_{2A} receptor (A_{2A}R) agonist CGS21680 cross-promotes β -arrestin 2 recruitment to the dopamine D₂ receptor (D₂R), an effect which becomes even stronger when co-stimulated with the D₂R agonist quinpirole (Figure 2C) thereby impacting receptor internalization and accelerating β -arrestin 2-mediated AKT phosphorylation (Figure 2A) (Borrito-Escuela et al., 2011). β -arrestin 2-biased signaling of the A_{2A}R-D₂R heteroreceptor complex has been linked to the antipsychotic action of certain D₂R agonists (Beaulieu et al., 2005; Sahlholm et al., 2018). Using mouse striatal derived neuronal cell cultures Bagher et al found that the D₂R antagonist haloperidol when incubated with the cannabinoid 1 receptor (CB₁R) could reduce β -arrestin 1 recruitment to the CB₁R (Figure 2D). Putative CB₁R-D₂R heteroreceptor complex signaling appeared to change in signaling bias towards G_{αs}-proteins (whereas the CB₁R and D₂R canonically signal via G_{αi}-proteins), resulting in increased cAMP production (Bagher et al., 2016). Signaling of the CB₁R-D₂R heteromer has been suggested to have an inhibitory effect on D₂R agonist-mediated hyperlocomotion (Marcellino et al., 2008), supporting the importance of understanding heteroreceptor complex signaling to develop therapeutics for locomotor disorders.

A 2016 study of κ opioid receptor (κ OR) - neurotensin 1 receptor (NTSR1) heteromers, that were detected in striatal neurons, revealed that this heteromer favorably recruits β -arrestin and promotes persistent ERK activation (Figure 2A). Preventing heteromerization via mutagenesis of a κ OR residue important in heteromer formation reversed the signaling bias back to G_{αi}-protein. The authors also found that co-stimulation of HEK293 cells expressing κ OR and NTS1R with dynorphinA(1–13) and neurotensin(8–13) attenuated β -arrestin recruitment and shortened ERK activation dynamics (Liu et al., 2016), thereby highlighting the promise of pharmacologically targeting heteromers.

In the ventral tegmental area, from which neurons can project to the striatum, and which is involved in drug seeking, Navarro et al reported the existence of a heteromer between the orexin OX_1 receptor (OX_1R) and the corticotropin-releasing factor CRF_1 receptor. Co-incubation with both CRF and OX_1R agonists led to a reduction in β -arrestin 2 recruitment, as measured by BRET, as well as a decreased ERK activation (Figure 2A), both of which could be restored by disruption of the heteromer (Navarro et al., 2015). Interestingly, cocaine also restored β -arrestin 2 recruitment and ERK activation; using sequential resonance energy transfer (SRET) this response was linked to CRF_1R - OX_1R heteromers interacting with the σ_1 receptor, a membrane receptor with two transmembrane domains which serves as one of the targets of cocaine (Navarro et al., 2015).

A 2018 study by Koshimizu et al. found evidence for heteromers between the μ OR and the vasopressin V1b receptor (V1bR) in the rostral ventral medulla. BRET assays were used to additionally show that the μ OR-V1bR heteromers displays strong constitutive β -arrestin 2 recruitment. Agonist stimulation of cells co-expressing the μ OR and V1bR further led to robust phosphorylation of ERK, which, as mentioned, is a MAP kinase known to scaffold with β -arrestin (Figure 2A) (Koshimizu et al., 2018). In another recent study, Ang et al. used proximity ligations, FRET and BiFC technology to support the existence of heteromers between the free fatty acid receptors 2 and 3 (FFAR2-FFAR3). Next, using a TANGO assay, they were able to show that in cells co-expressing FFAR2 and FFAR3, either a FFAR2 or FFAR3 agonist produced more robust β -arrestin recruitment to the FFAR2-FFAR3 heteromer, specifically the FFAR3 unit, when compared to singular FFAR3 expression (Ang et al., 2018). They also noticed enhanced p38 phosphorylation upon receptor stimulation in cells expressing both FFARs (Figure 2A). This is noteworthy as p38 has been reported to scaffold with β -arrestin and contribute to the dysphoric effects of κ OR agonists (Bruchas et al., 2007; DeWire et al., 2007; Land et al., 2009). It is currently unknown if the κ OR- β -arrestin-p38 signaling cascade relies on κ OR heteromerization with another GPCR.

Current hurdles and future directions

In their original form, BRET, FRET and BiFC assays are limited to studying the interaction or proximity of two proteins of interest. However, if one wanted to expand their investigation to include a third protein of interest i.e. arrestin this would require transfer of a BRET or FRET donor/acceptor from one of the GPCRs to the arrestin protein, thus preventing a detailed view of the entire heteroreceptor complex. Promisingly, with the advent of novel adaptations of these fluorescent imaging techniques, particularly fragmentation of luciferase and fluorescent proteins and SRET (also referred to as BiLC, or complemented donor-acceptor resonance energy transfer), it has become more feasible to study heteromer-mediated arrestin recruitment and further downstream scaffolding/signaling events (Bellot et al., 2015; Navarro et al., 2015; Sahlholm et al., 2018). With strategic planning it should be feasible to study interactions between up to six different proteins by splitting *Rluc* (for sequential BRET-FRET) or CFP (for sequential FRET-FRET), splitting the YFP BRET acceptor, and splitting a dsRED FRET acceptor (Carriba et al., 2008; Vidi and Watts, 2009). Going beyond three proteins is especially important in light of the existence of two distinct β -arrestin isoforms. The majority of the described studies in this review were focused on β -arrestin 2; the κ OR-NTS1R study that did investigate both isoforms did not show a different

profile for the heteromer when recruiting β -arrestin 1 or 2. Still, it is important to realize that studies have shown that β -arrestin 1 can have properties and functions that are distinct from β -arrestin 2 (Robins et al., 2018a; Zurkovsky et al., 2017). Indeed, a study of heteromerization between thyrotropin hormone receptor (TRHR) subtypes 1 and 2 found that TRHR2 was able to recruit β -arrestin 1 only when co-expressed with TRHR1, but not when expressed alone (under which circumstances it would exclusively recruit β -arrestin 2) (Hanyaloglu et al., 2002). As described by Vidi and Watts, using multicolor BiFC it should be feasible to fuse β -arrestin 1 to the N-terminal fragment of split YFP and to fuse β -arrestin 2 to the N-terminal fragment of split CFP (Vidi and Watts, 2009). Fusing a GPCR with the C-terminal fragment of CFP provides insight into β -arrestin isoform recruitment bias to the receptor. Still, this approach is not perfectly suited to the study of heteromers as it is limited to scenarios where only the untagged heteromer protomer is stimulated (i.e. it is not possible to differentiate heteromer from homomer-mediated arrestin recruitment when using an agonist for the tagged receptor).

A potential pitfall with these techniques is the reliance on using recombinant receptors, which are often altered by fusing a large protein to the C-tail of the receptor, potentially impacting native trafficking patterns. The Jockers group has reported an intramolecular β -arrestin 2 BRET sensor in which β -arrestin 2 is fused on opposite sides to *Rluc* and GFP constructs that circumvents the issue of receptor modification (Kamal et al., 2009). Using this approach, it was demonstrated that agonist stimulation of either the melatonin MT₂ receptor or the serotonin 5-HT_{2C} receptor leads to an enhanced BRET signal in cells co-expressing the two GPCRs (Kamal et al., 2015), suggestive of increased β -arrestin recruitment to the MT₂-5-HT_{2C} heteromer (Figure 2E).

As a whole, the presented studies demonstrate that GPCR heteromers have the ability to uniquely contribute to physiological processes, and that arrestin recruitment to heteromer complexes may be either beneficial (activation of specific kinases) or adverse (analgesic tolerance). Thus therapeutically one goal would be to create drugs that either promote or disrupt the arrestin pharmacology of the heteromer. Using PathHunter β -arrestin assays the Devi group was able to identify a μ OR- δ OR selective molecule, CYM51010, and very recently a new bivalent molecule D24M was developed which also appears to be selective for the μ OR- δ OR heteromer. Encouragingly, both molecules displayed beneficial features: CYM51010 injected animals developed analgesic tolerance at a much slower pace than morphine, whereas mice injected with D24M experienced reduced opioid withdrawal symptoms (Gomes et al., 2013; Olson et al., 2018). As new technologies continue to emerge, and new heteromer selective molecules are developed, our ability to study and understand the importance of heteromer-mediated arrestin recruitment and signaling will continue to improve.

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Non-standard abbreviations

A_{2A}R	Adenosine A _{2A} receptor
AT₁R	Angiotensin II type 1 receptor
AT₂R	Angiotensin II type 2 receptor
β₁AR	β ₁ adrenergic receptor
β₂AR	β ₂ adrenergic; receptor
β₃AR	β ₃ adrenergic receptor
BiFC	Bimolecular Fluorescence Complementation
BiLC	Bimolecular Luminescence Complementation
BRET	Bioluminescence Resonance Energy Transfer
CB₁R	Cannabinoid 1 receptor
CRF₁R	Corticotropin-releasing factor 1 receptor
CXCR2	Chemokine C-X-C receptor 2
CXCR4	Chemokine C-X-C receptor 4
CXCR7	Chemokine C-X-C receptor 7
DAMGO	[D-Ala ² , NMe-Phe ⁴ , Gly-ol ⁵]-enkephalin
δOR	δ opioid receptor
D₂R	Dopamine D ₂ receptor
FFAR2	free fatty acid receptor 2
FFAR3	Free fatty acid receptor 3
FRET	Forster Resonance Energy Transfer
GPCR	G protein-coupled receptor
GFP	Green fluorescent protein
κOR	κ opioid receptor
μOR	μ opioid receptor
NK1R	Neurokinin-1 receptor
NTSR1	Neurotensin receptor 1
OX₁R	Orexin OX ₁ receptor
Rluc	<i>Renilla</i> luciferase

SRET	Sequential Resonance Energy Transfer
SSTR₂	Somatostatin receptor type 2
SSTR₅	Somatostatin receptor type 5
TRHR1	Thyrotropin hormone receptor 1
TRHR2	Thyrotropin hormone receptor 2
V1bR	Vasopressin V1b receptor
YFP	Yellow fluorescent protein

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Highlights

- GPCR heteromers exhibit unique pharmacology in relation to arrestin recruitment
- Compared to the individual GPCRs some heteromers have decreased arrestin recruitment
- Compared to the individual GPCRs some heteromers have increased arrestin recruitment
- Heteromer-mediated arrestin recruitment can alter MAPK signaling
- Heteromer-mediated arrestin signaling may play a role in analgesia and locomotion

Figure 1. Cellular techniques and assays used to measure heteromer-mediated arrestin recruitment.

A. Heteromer co-localization between receptor pairs measured using a GFP-tagged receptor and a mCherry-conjugated antibody tagged receptor. **B.** Cross-recruitment of GFP-tagged arrestin to the heteromeric protomer visualized using a mCherry-conjugated antibody. **C.** Arrestin recruitment measured using FRET between a GFP-tagged arrestin and a C-terminally mCherry-tagged receptor. **D.** Arrestin recruitment measured using BRET between a Rluc-tagged arrestin and a C-terminally YFP-tagged receptor. **E.** Arrestin recruitment measured using BiFC, by the reconstitution of a split Venus fluorescence protein fused to a receptor and an arrestin protein. **F.** TANGO assay measuring arrestin recruitment by utilizing a protease-linked arrestin protein which upon recruitment will cleave a transcription factor from the receptor C-tail leading to luciferase expression. **G.** DiscoverX PathHunter assay measuring arrestin recruitment through the reconstitution of a bioluminescent β -galactosidase component formed between complementary constructs present on both receptor and arrestin proteins.

Figure 2. Examples of heteromerization leading to unique arrestin recruitment pharmacology.

A. Different heteromers can increase or decrease activation of MAPK proteins p38, ERK and AKT in an arrestin-dependent manner. **B.** Constitutive recruitment of arrestin to the μ OR- δ OR heteromer is attenuated when occupied with a μ OR agonist and a δ OR antagonist. **C.** Enhancement of arrestin recruitment by agonism of the heteromer protomer; the quinpirole-mediated recruitment of arrestin to the D₂ receptor is enhanced upon activation of the A_{2A} receptor by CGS. **D.** Reduction of arrestin recruitment by antagonism of the heteromer protomer; the ACEA-mediated recruitment of arrestin to the CB₁ receptor is reduced by the antagonism of the D_{2L} receptor by haloperidol. **E.** Enhancement of arrestin recruitment by co-expression of heteromer pairs; the ligand-mediated recruitment of arrestin to the MT₂ and 5HT_{2C} receptors by their native ligands is enhanced in the context of a heteromer.