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Functional Significance of Serotonin Receptor Dimerization

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Abstract

The original model of G protein activation by a single G-protein-coupled receptor (GPCR) is giving way to a new model wherein two protomers of a GPCR dimer interact with a single G protein. This article will review the evidence suggesting that 5-HT receptors form dimers/oligomers and will compare the findings with results obtained from studies with other biogenic amine receptors. Topics to be covered include the origin or biogenesis of dimer formation, potential dimer interface(s), and oligomer size (dimer versus tetramer or higher order). The functional significance will be discussed in terms of G-protein activation following ligand binding to one or two protomers in a dimeric structure, the formation of heterodimers and the development of bivalent ligands.

Keywords

Serotonin receptor; GPCR activation; homodimer; oligomer; dimer interface

Introduction

Following the discovery of G proteins, models of receptor-mediated activation of adenylate cyclase included a single receptor activating a single heterotrimeric G-protein (reviewed in Gilman 1987). This model prevailed for several decades, during which time radioligand binding studies were beginning to show biphasic competition curves with positive or negative co-operativity and western blots of solubilized G-protein-coupled receptors (GPCR) were revealing the presence of multiple bands the predicted size of dimers and higher order oligomers (reviewed in González-Maeso 2011). Reports began appearing in the literature citing the presence of GPCR dimers/oligomers identified using a variety of different methods including co-immunoprecipitation (Co-IP), resonance energy transfer (RET), fluorescence lifetime imaging (FLIM), and bimolecular fluorescence complementation (BiFC). More recently, techniques with near single molecule sensitivity have been called upon to help resolve the issue of GPCR oligomer size and the effect of ligand binding, including total internal reflected fluorescence (TIRF), fluorescence recovery after photobleaching (FRAP), and fluorescence correlation spectroscopy (FCS).

Currently, there are hundreds of reports in the literature citing specific examples of homodimers and heterodimers of a wide variety of different GPCR expressed in recombinant cell systems. GPCR dimer/oligomer formation has been reported to regulate all aspects of GPCR function including synthesis, ligand binding, G-protein coupling, and receptor trafficking (reviewed in Milligan 2008). Even so, the functional relevance and the presence of GPCR dimer/oligomers in vivo is still a subject of great debate. Concerns have been raised about the functional relevance of GPCR dimerization as monomeric receptors

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have been reported to activate G-proteins in reconstituted systems (Bayburt et al. 2007; Whorton et al. 2007) and the crystal structure of the agonist occupied beta2-adrenergic receptor (B2-AR) revealed a monomeric receptor in complex with a single G-protein (Rasmussen et al. 2011). Some of the studies providing evidence for the presence of GPCR dimers/oligomers in vivo have been criticized for employing methods requiring disruption of native membranes (Liang et al., 2003) or the use of proximity-based RET techniques (Albizu et al., 2010). However, recent studies demonstrating homodimers of photo-activated rhodopsin in association with G-transducin (Jastrzebska et al. 2013a), along with transgenic mice (Rivero-Müller 2010), heterodimer selective antibodies (Gupta et al. 2010), and novel pharmacological approaches (Teitler and Klein 2012), are adding a new dimension to the characterization of GPCR homo- and hetero-dimers in native tissues and in vivo.

This review will summarize the results from studies designed to examine the functional significance of 5-HT receptor dimer/oligomer formation with respect to their origin, dimer interface, oligomer size, and mode of activation and G-protein coupling. Where appropriate, comparisons will be made with results obtained from other biogenic amine receptors. Evidence for the presence of 5-HT dimers/oligomers in vivo will be discussed, as well as the current status of bivalent ligand development targeting 5-HT receptor dimers/oligomers.

Homodimer biogenesis

Some of the earliest evidence for the presence of 5-HT receptor dimers/oligomers comes from co-immunoprecipitation (Co-IP) and Western blot studies of the 1A, 1B and 1D receptor subtypes (Xie et al. 1999; Salim et al. 2002). In subsequent years, the combined applications of Co-IP and the more the sensitive and sophisticated RET techniques, strengthened the hypothesis in favor of homodimers of the 1A, 2A, 2C, and 4 receptor subtypes (Herrick-Davis et al. 2004; Berthouze et al. 2005; Kobe et al. 2008; Brea et al. 2009; Paila et al. 2011). In these studies, bioluminescence and fluorescence RET techniques were applied to study fluorescence-tagged receptors expressed in recombinant cell systems. Positive RET signals, suggestive of close proximity between the parent receptors to which the fluorescent probes were attached, were interpreted as evidence in favor of 5-HT receptor homodimers on the plasma membrane of living cells.

There are several lines of evidence suggesting that GPCR dimerization occurs prior to receptor expression on the plasma membrane. The most notable example involves heterodimerization of class C $GABA_BR1$ and $GABA_BR2$ receptors, which has been demonstrated to be essential for receptor trafficking from the ER to the plasma membrane (White et al. 1998). Dimerization has been proposed as a general mechanism necessary for proper trafficking of class A GPCR to the plasma membrane (reviewed in Milligan 2010). Experimental evidence in favor of this model includes the identification of preformed, constitutive homodimers of 5-HT receptors on the plasma membrane (Herrick-Davis et al. 2004; Berthouze et al. 2005; Kobe et al. 2008; Brea et al. 2009; Paila et al. 2011), the identification of non-trafficking, mutant receptors that dimerize with and retain their wild-type counterparts within intracellular compartments (Benkirane et al. 1997; Zhu and Wess 1998) and the detection of positive BRET signals in both plasma membrane and endomembrane-enriched sub-cellular fractions prepared from HEK293 cells expressing vasopressin or β_2-adrenergic (B2-AR) receptors (Terrillon et al. 2003; Salahpour et al. 2004). While these results suggest that GPCR dimerization may occur within intracellular compartments, direct evidence demonstrating the formation of class A GPCR homodimers within the ER and Golgi of intact living cells was not provided by these studies.

Direct visualization of 5-HT receptor proximity within the ER and Golgi was provided by acceptor photobleaching RET studies of the $5\text{-}HT_{2C}$ receptor (Herrick-Davis et.al., 2006). Time-lapse confocal microscopy was used to track the migration of fluorescence-tagged 5-

HT_{2C} receptors, along with specific markers to identify the endoplasmic reticulum (ER) and Golgi compartments. At early time points post-transfection into HEK293 cells, 5-HT_{2C} receptors were observed to be co-localized with ER markers and subsequently with Golgi markers during receptor biosynthesis. Positive fluorescence RET signals between differentially tagged 5-HT_{2C} receptors were observed in the ER and Golgi compartments during receptor biosynthesis and were present once the receptors reached the plasma membrane (Herrick-Davis et al. 2006), consistent with the hypothesis that homodimer biogenesis begins in the ER.

The results obtained with 5-HT_{2C} receptors are similar to findings with adrenergic receptors using Co-IP and RET (Salahpour et al. 2004; Lopez-Gimenez et al. 2007), suggesting the presence of preformed dimeric/oligomeric complexes within the ER/Golgi compartments. In these studies, mutations that eliminated dimer formation also eliminated cell surface targeting of the receptors. Recent studies with CCR5 receptors indicate that chaperone protein binding is involved in the preassembly of homodimers along with G-proteins in the ER (Kuang et al. 2012). Taken together, these results suggest that homodimerization begins in the ER during receptor biosynthesis and plays an important role in protein folding required for exit from the endoplasmic reticulum.

Dimer interface

More than two dozen GPCR crystal structures have been reported to date. Several of the crystal structures reveal dimers/oligomers arranged in a parallel orientation of potential physiological relevance: rhodopsin (Lodowski et al. 2007; Murakami and Kouyama, 2008), B2-AR (Cherezov et al. 2007), opsin (Scheerer et al. 2008), CXCR4 (Wu et al. 2010), mu- and kappa-opioid (Manglik et al. 2012; Wu et al. 2012) and the recently solved structure for the inactive state of the beta1-adrenergic (B1-AR) receptor (Huang et al. 2013). These crystal structures reveal two potential dimer interfaces, one involving transmembrane domains 1 and 2 (TMD1/2) and helix 8 (H8) and another involving either TMD4/5 or TMD5/6. A role for H8 at the dimer interface has been confirmed by cross-linking of native cysteine residues in H8 of rhodopsin receptors endogenously expressed in native disc membranes (Knepp et al, 2012).

Recently, the first 5-HT receptor crystal structures were reported. The 5-HT_{1B} and 5-HT_{2B} crystal structures were obtained by incorporation of a thermally stabilized apocytochrome (from *E. coli*) into the third intracellular loop of each receptor (Wacker et al., 2013; Wang et al., 2013). In both cases, the crystal structures were solved for the agonist (ergotamine) bound form of the receptor. Similar to the active conformations observed for other GPCR, the crystal structures of the 1B and 2B receptors predicted the classic rotation at the intracellular ends of TMDs 3, 5, and 6. However, the two 5-HT receptors differed in the degree of rotation of TMD 6, suggesting slightly different active conformations or different degrees of activation. The degree of activation is thought to be related to the magnitude of the rearrangement of TMDs 5, 6, and 7, which open up at their intracellular interface, thereby facilitating G protein coupling and activation. The 5-HT receptor crystal structures provided detailed information regarding ligand binding selectivity and provided a structural basis for beta-arrestin biased signaling. However, little was learned about their potential dimeric/oligomeric arrangement as the receptors were aligned in anti-parallel fashion.

Computer modeling studies designed to investigate potential GPCR dimer/oligomer interfaces have reported that there is no energy barrier to the formation of a TMD1-H8 or a TMD4/5 interface, but predict a symmetric TMD1-H8 interface to be stronger and more stable than a TMD4/5 interface (Johnston et al. 2012; Periole et al. 2012). Similar conclusions were reached from saturation FRET studies of the B2-AR (Fung et al. 2009) and from cysteine cross-linking and FRAP studies of D2 dopamine receptors (Guo et al. 2008;

Fonseca and Lambert 2011), suggesting a preferred TMD1-H8 interface and a less stable TMD4/5 interface potentially responsible for transient association of dimers into tetramers.

Potential dimer interfaces involved in the formation of 5-HT receptor dimers/oligomers have been investigated for the 1A, 2A, 2C and 4 receptor subtypes, and appear to favor a TMD4/5 dimer interface. Potential dimer interfaces involved in 5-HT_{1A} receptor homodimers were investigated using a combination of lux-FRET and computer modeling. In this study, specific residues in TMD4 were identified by mutagenesis and reduction in FRET as being essential for maintaining the homodimer structure (Gorinski et al. 2012). Computer simulations of the 3D structure of the 5-HT_{2A} receptor are consistent with a dimer interface that can regulate the topology of the binding pocket (Bruno et al. 2011). In addition, mutagenesis studies have identified resides at the intracellular end of TMD4 as being critical for the formation of 5-HT_{2A}/mGluR2 heterodimers (Moreno et.al., 2012). In a similar manner, a cysteine residue in TMD4 of the 5-HT_4 receptor has been reported to play a role in dimer formation (Berthouze et al. 2007).

Hendrickson and colleagues used a cysteine cross-linking approach in an attempt to elucidate potential dimer interfaces responsible for the formation of 5-HT_{2C} homodimers (Mancia et al. 2008). In these studies, homology modeling based on the crystalline structure of rhodopsin provided a 5-HT_{2C} 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 in HEK293 cells was evaluated by Western blot. These experiments identified potential dimer interfaces at the extracellular end of TMD1 and also at TMD4/5 (Mancia et al. 2008). However, higher-order oligomers of 5-HT_{2C} 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-Galpha fusion proteins, it was concluded that 5-HT_{2C} receptor homodimers are quasisymmetrical at the TMD4/5 interface and asymmetrical with respect to G protein coupling (Mancia et al. 2008).

Studies using membrane impermeable cross-linking reagents with linker arms of specified length may be useful in distinguishing between TMD1/2 and TMD4/5 dimer interface models. For example, the 5-HT_{2C} receptor in intact cells is sensitive to cross-linking with the membrane impermeable cross-linker BS^3 (Herrick-Davis et.al., 2004). Treatment with BS^3 resulted in the appearance of immunoreactive bands the predicted size of dimers, but oligomers were not detected. There are only four lysine residues in the 5-HT_{2C} receptor that are exposed to the extracellular environment such that they could participate in cross-linking following treatment of intact cells with BS^3. There is one lysine residue in the N-terminus near the top of TMD1, one in extracellular loop 2 (EL2), and two in EL3. If a TMD1/2-H8 dimer interface is responsible for the formation of 5-HT_{2C} homodimers, then the lysine residue located in the N-terminus near the top of TMD1 would likely be a key player in cross-linking with BS^3. Therefore, a loss in cross-linking following mutation of this lysine residue would support a TMD1/2 dimer interface model. On the other hand, loss of cross-linking following mutation of the lysine residue in EL2 would favor a model in which TMD4/5 were in close proximity to each other in the homodimer. Removal of the lysine residues in EL3 would not be predicted to have any effect as the EL3 regions are not in close proximity in either arrangement.

Oligomer size

Currently, there is no consensus in the published literature as to the oligomer size of biogenic amine GPCRs. The issue is complicated by the technical challenges associated with distinguishing between monomers, dimers and tetramers. Many different methods have been employed to address the issue of GPCR oligomer size including RET, FLIM, TIRF, FRAP,

and FCS. These methods differ in sensitivity and the population of receptors that they examine. RET and FLIM are proximity assays while TIRF and FCS have near single molecule sensitivity. TIRF, FRAP and FCS measure the mobile fraction of receptors within the plasma membrane, while quantitative BRET measures the entire fraction of receptors throughout the cell. TIRF and FCS allow discrete regions of plasma membrane to be evaluated, as does the combined use of confocal microscopy with RET.

With regard to determining GPCR oligomer number, the various methods described above have yielded a variety of different answers, with no apparent consensus even within the same biogenic amine receptor sub-family. For example, RET, FRAP and TIRF studies of M1, M2 and M3 muscarinic receptors have reported monomers (Hern et al. 2010), dimers (Goin and Nathanson 2006; Hern et al. 2010; Patowary et al. 2013) and tetramers (Pisterzi et al. 2010; Patowary et al. 2013). RET and FRAP studies of B1-AR and B2-AR have reported monomers (James et al. 2006; Dorsch et al. 2009), dimers (Mercier et al. 2002; Dorsch et al. 2009) and higher order oligomers (Dorsch et al. 2009; Fung et al. 2009). D1 and D2 dopamine receptors have been reported to form homodimers and higher order oligomers assayed by RET (Guo et al. 2008) and using a nuclear translocation assay (O'Dowd et.al., 2011). To complicate the issue further, FRAP and TIRF studies have suggested that B1-AR and M1 muscarinic receptors may exist in equilibrium between monomeric and dimeric states (Dorsch et al. 2009; Hern et al. 2010), while FRAP and RET have identified mixed populations of homodimers and higher order oligomers for D2 dopamine and M3 muscarinic receptors (Fonseca and Lambert 2011; Patowary et al. 2013). On the other hand, studies of 5-HT receptors suggest that they are predominantly dimeric.

While RET studies have reported that 5-HT_{1A} receptors can form higher order oligomers (Ganguly et al. 2011), the majority of studies to date have reported homodimers as the preferred signaling unit for 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_4, and 5-HT_7 receptors (Kobe et al. 2008; Brea et al. 2009; Herrick-Davis et al. 2004; Mancia et al. 2008; Pellissier et al. 2011; Smith et al. 2011). For the 5-HT_{2A} receptor, guanyl nucleotide-insensitive biphasic radioligand binding curves were observed for a series of antagonists, suggestive of negative co-operativity in a homodimeric complex (Brea et al. 2009). 5-HT_4 receptors purified as a pentameric assembly of two receptors and a single heterotrimeric G-protein, consistent with a 2:1 receptor:G-protein stoichiometry and providing evidence for 5-HT_4 receptor homodimers (Pellissier et al. 2011).

FCS and molecular brightness analysis, techniques with single molecule sensitivity, have provided some of the strongest evidence to date demonstrating the presence of GPCR homodimers in intact cells (Herrick-Davis et al. 2012). FCS measures fluctuations in fluorescence intensity from individual fluorescent molecules, or in this case fluorescence-tagged 5-HT_{2C} receptors, freely diffusing with the plasma membrane. FCS measurements were made on the plasma membrane of HEK293 cells and on primary hippocampal neurons transfected with GFP- or YFP-tagged 5-HT_{2C} receptors. The molecular brightness of fluorescence-tagged receptors was twice that of monomeric controls and equal to dimeric controls, providing conclusive demonstration of the presence of 5-HT_{2C} homodimers and also the lack of tetramers or higher order oligomers (Herrick-Davis et al. 2012).

Attempts to reconcile differences in GPCR oligomer status reported in the literature have led to the suggestion that receptor expression level may influence GPCR monomer-dimer and/or dimer-tetramer states on the plasma membrane, with low expression levels favoring monomeric forms and higher expression levels favoring association of dimers into tetramers (Hern et al. 2010; Lambert 2010; Patowary et al. 2013). FCS studies of 5-HT_{2C} receptors examined the homodimer status at the single molecule level using receptor expression levels in the nanomolar range (Herrick-Davis et al. 2012) and found no evidence for dimers

dissociating into monomers. As the FCS technique is best suited for studying proteins at low expression levels, the possibility that significantly higher expression levels may lead to receptor clustering or oligomerization in specific microdomains can't be ruled out in the FCS study.

It is recognized that palmitoylation promotes sequestration of GPCR into discrete microdomains (Kobe et al. 2008; Day and Kenworthy 2009; Woehler et al. 2009), and that the majority of class A GPCR have at least one palmitoylation site in the C-terminus following helix 8. Receptor clustering into membrane microdomains would increase local receptor concentrations, complicating the interpretation of RET-based studies that monitor protein proximity, as positive RET is suggestive of but does not demonstrate protein-protein interaction. Further complicating the issue, stochastic RET has been reported for receptors clustered in membrane microdomains (Meyer et al. 2006; Kobe et al. 2008; Woehler et al. 2009). In one study, 5-HT_{1A} receptors clustered in membrane microdomains gave RET values consistent with an oligomer number greater than 2, while non-palmitoylated mutant receptors that were excluded from microdomains gave an oligomer number of 2 (Woehler et.al., 2009). It is interesting to note that studies employing techniques with near single molecule sensitivity (TIRF and FCS) have only reported monomers or dimers, without tetramers or higher order oligomers (Hern et al. 2010; Kasai et al. 2011; Herrick-Davis et al. 2012).

Teitler and colleagues have taken a different approach to the oligomer number puzzle (Teitler and Klein 2012). They employed the pseudo-irreversible binding of risperidone to the 5-HT_7 receptor as a tool to monitor receptor oligomer number (Teitler et al. 2010). Treatment with saturating concentrations of risperidone, followed by repeated drug wash-out, completely eliminated 5-HT-mediated signaling through 5-HT_7 receptors expressed in HEK293 cells. Radioligand binding studies performed in parallel revealed that risperidone was producing its insurmountable, inactivating effect while occupying only 50% of the 5-HT_7 receptors (Knight et al. 2009; Teitler et al. 2010). Similar results were obtained for native 5-HT_7 receptors endogenously expressed in astrocytes (Smith et al. 2011). Mathematical modeling of the results produced an oligomer number of 2, providing some of the more convincing evidence to date for the presence of native receptor homodimers (Teitler and Klein 2012). Based on these results, a method has been proposed for monitoring the oligomer number of native GPCR in primary cultures and in vivo, using drug-induced inactivation followed by reactivation (Teitler and Klein 2012).

Studies examining the effect of ligand on GPCR oligomer status have produced differing results ranging from no effect, to dissociation or association of dimers into oligomers. RET studies that examined the effect of ligand on oligomer status concluded that ligand binding did not promote the dissociation of dimers into monomers or association into higher order oligomers of 5-HT_{1A} (Kobe et al. 2008; Paila et al. 2011), 5-HT_{2C} (Herrick-Davis et al. 2007) and 5-HT_4 receptors (Brea et al. 2009). FRET/FLIM studies of serotonin 5-HT_{1A} receptors (Kobe et al. 2008) and FCS studies of 5-HT_{2C} receptors (Herrick-Davis et al. 2012), along with FRAP studies of B1-AR and B2-AR (Dorsch et al. 2009) and FRET studies of M1 and M2 muscarinic receptors (Goin et al. 2006), report no effect of agonist on GPCR homodimer/oligomer status in intact cells. On the other hand, studies of 5-HT_{1A} receptors (Ganguly et al. 2011), B2-AR (Fung et al. 2009), M3 muscarinic (Alvarez-Curto et al. 2010), and D1/D2 dopamine receptors (Guo et al. 2008; O'Dowd et al. 2011) 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.

G-Protein activation

Another area of great interest in the GPCR dimerization field is the question of the functional significance in terms of G-protein activation. Based on the crystal structure of the G-protein heterotrimer and the initial crystal structures of GPCR, it appeared that two receptors would be required to provide enough surface area to form the predicted docking interface with the alpha and beta-gamma subunits of a single G-protein (Filipek et al. 2004). In spite of this observation, monomeric GPCR have been reported to activate G-proteins in reconstituted systems (Bayburt et al. 2007; Whorton et al. 2007). Consistent with these findings, the crystal structure of the agonist occupied B2-AR/G-protein complex revealed a monomeric receptor in association with a single G-protein (Rasmussen et al., 2011), as did 2D projection analysis of the B2-AR/G-protein complex following purification in lauryl-maltose-neopentyl-glycol (Westfield et al., 2011). In contrast, 3D projection analysis following purification of photo-activated rhodopsin in lauryl-maltose-neopentyl-glycol revealed a single G–protein in complex with a rhodopsin dimer (Jastrzebska et al. 2013a). Several GPCR have been solubilized as homodimers in association with a single G-protein including the leukotriene B4 BLT1 (Banères and Parello 2003), D_2-dopamine (Han et al., 2009), 5-HT_4 (Pellissier et al. 2011), and rhodopsin (Jastrzebska et al. 2011). G-protein docking simulations using the recently solved crystal structure of the B1-AR homodimer suggest that one protomer of the dimer is predominantly responsible for contacting the G-protein (Huang et al. 2013). However, 3D projection mapping of photo-activated rhodopsin shows the G-protein heterotrimer more centrally positioned under the rhodopsin dimer (Jastrzebska et al. 2013a). Both protomers of the dimer contact the G-protein, in an asymmetrical manner (Jastrzebska et al. 2013b), suggesting that the dimer is the minimal functional unit.

Studies using intact cells have reported that binding to one protomer of a homodimer is sufficient for maximal activation of mGluR1 (Hlavackova et al. 2005) and D_2-dopamine receptors (Han et al. 2009). However, this is not the case for all GPCR, as the second protomer of the dimer generally is not silent with respect to signaling. Studies have reported that binding to the second protomer in a dimer can enhance or decrease G-protein-mediated signaling (Kniazeff et al. 2004, Han et al. 2009; Pellissier et al. 2011).

The functional significance of dimerization in terms of G-protein activation by serotonin receptors has been examined for 5-HT_{2C}, 5-HT_4 and 5-HT_7 receptors. In the case of the 5-HT_{2C} receptor, a mutant (S138R) receptor was created that was incapable of binding 5-HT and had lost its basal activity and ability to activate G-proteins (Herrick-Davis et al. 2005). 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 and stimulating inositol phosphate production in response to 5-HT stimulation (Herrick-Davis et al. 2005). These results suggest that both protomers participate in G-protein signaling and that signaling does not occur if one protomer is held in an inactive conformation. Similar results were reported for the 5-HT_7 receptor using the antagonist risperidone, which binds in a pseudo-irreversible manner to one protomer of a dimer (Teitler et al. 2010). These experiments revealed that irreversible binding of risperidone to one protomer of a dimer did not interfere with 5-HT binding to the second

protomer, but rendered the dimer inactive with respect to G-protein activation. The studies with 5-HT_{2C} and 5-HT_7 receptors support the hypothesis that both protomers of a 5-HT receptor dimer participate in G-protein-mediated signaling. When one protomer is held in an inactive conformation, either by mutagenesis or by irreversible occupancy with antagonist, the second protomer (while still capable of binding 5-HT) is incapable of G-protein activation in response to 5-HT binding. These results favor a model where one protomer within a dimer is capable of influencing the activity of the other protomer, either by directly promoting conformational changes across a putative TMD4/5 dimer interface or via asymmetric G-protein coupling.

Elegant studies using ligand binding selective mutant receptors (5-HT_4-RASSL) demonstrated that agonist binding to one protomer of a 5-HT_4 receptor dimer 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 (Pellissier et al. 2011). In this case, the two-fold increase in activation suggests that binding to one protomer gives half-maximal activation, such that each protomer contributes equally toward the signaling process.

GPCR dimers/oligomers have been demonstrated to display asymmetry with respect to ligand binding and G-protein activation (reviewed in Maurice et al. 2011). Ligand occupancy of one protomer of a dimer can influence the conformation of the binding pocket of the second protomer (Guo et al. 2003; Vilardaga et al. 2008; Jastrzebska et al. 2013a). Additionally, the active state of a GPCR dimer appears to be asymmetric with respect to G-protein coupling (Goudet et al. 2005; Damian et al. 2006; Mancia et al. 2008; Han et al. 2009; Jastrzebska et al. 2013b), greatly expanding the signaling repertoire. Taken together, the results of these studies indicate that the relative proportion of signaling contributed by each protomer in a GPCR dimer is likely to be ligand, receptor and G-protein dependent.

Recently, evidence has been provided to support a role for antagonists in mediating functional selectivity in G-protein signaling. In an elegant series of experiments designed to examine the functional significance of 5-HT_{2A} homodimer signaling, antagonists were observed to display biphasic inhibition of radioligand binding (negative co-operativity) and differential regulation of inositol phosphate production versus arachodonic acid release in a monophasic or biphasic manner, respectively (Brea et al. 2009). For the antagonists examined in this study, the negative cooperativity observed for the inhibition of second messenger production mirrored the negative co-operativity observed for radioligand binding. These studies are among the first to provide evidence that antagonists can display functional selectivity at homodimeric GPCR, and have important implications for future drug development as many GPCR-targeted therapeutics are antagonists.

Heterodimers

Heterodimers add another layer of complexity to G-protein-mediated signaling and functional selectivity (reviewed in González-Maeso and Sealfon 2012; Wertman and Dupré 2013). For 5-HT receptors, heterodimers of 5-HT_{1A} receptors have been reported with FGFR1 (Borroto-Escuela et al. 2012), μ opioid (Cussac et al. 2012), adenosineA2a (Lukasiewicz et al. 2007), and galanin receptors (Fuxe et al. 2012). 5-HT_{2A} receptor heterodimers have been reported with mGluR2 (Gonzalez-Maeso et al. 2008; Rives et al., 2009; Moreno et al. 2011) and D2 dopamine receptors (Albizu et al. 2011; Lukasiewicz et al. 2011), and 5-HT_4-B2-AR heterodimers have been reported (Berthouze et al. 2005). The most notable heterodimer between 5-HT receptor subtypes is the 5-HT_{1A}-5-HT_7 heterodimer.

Heterodimers of 5-HT_{1A} and 5-HT_7 receptors have been reported to regulate GIRK channel activity in heterologous systems and in hippocampal neurons (Renner et al. 2012). Heterodimerization decreased 5-HT_{1A}-mediated activation of Gαi and GIRK channel activity, indicating an inhibitory role of the 5-HT_7 protomer. Interestingly, 5-HT_7 receptor production in the hippocampus was shown to decrease during postnatal development indicating that the concentration of heterodimers and their functional significance would change over time during development (Renner et al. 2012).

5-HT_{2A}-mGluR2 heterodimers have been postulated to play a role in psychosis (Gonzalez-Maeso et al. 2008). Their existence in vivo has been postulated based on their co-immunoprecipitation from human cortical tissue. In the heterocomplex, activation of the mGluR2 protomer was reported to inhibit signaling and behavioral responses to hallucinogens (Gonzalez-Maeso et al. 2008; Moreno et al. 2011). Additional evidence for the in vivo expression and functional relevance of this heterocomplex is provided by studies using mGluR2 knock-out mice. Removal of the mGluR2 receptor eliminated the well known head twitch behavioral response to hallucinogens mediated through 5-HT_{2A} receptors, implicating a role for the 5-HT_{2A}-mGluR2 heterocomplex in mediating behavioral responses to hallucinogens (Moreno et al. 2011). The potential differential regulation of 5-HT_{2A} and mGluR2 receptor expression levels, as suggested by increased 5-HT_{2A} and decreased mGluR2 in post-mortem brain tissue from schizophrenic subjects, has been suggested to provide a role for the 5-HT_{2A}-mGluR2 heterocomplex in the etiology of schizophrenia (Gonzalez-Maeso et al. 2008).

Compelling evidence for the presence and functional regulation of heterodimers in vivo comes from studies employing a μ-δ opioid heterodimer selective monoclonal antibody (Gupta et al. 2010). The antibody selectively labeled opioid receptors in the brain of wild-type mice but not in μ, δ, or μ-δ null mice. Chronic, but not acute, treatment with morphine was shown to increase the expression of μ-δ heterodimer complexes in brain regions involved in pain transmission such as the rostral ventral medulla. Occupancy of the δ protomer in the heterocomplex has been reported to restore the analgesic potency of morphine which declines over time with chronic administration (reviewed in Rozenfeld and Devi 2011), implicating the potential for bivalent ligands as novel therapeutics in pain management.

Bivalent ligands for 5-HT receptors

Evidence supporting the existence of receptor homodimers and heterodimers provides impetus for exploring the realm of bivalent ligands as novel therapeutics. Bivalent ligands for 5-HT receptors have been synthesized and tested in binding and functional assays with varying results. To date, bivalent ligands have been designed for the 1A, 1B, 1D, 2A, and 4 receptor subtypes.

A bivalent or dimeric version of the 5-HT_{1A} selective agonist 8-OH-DPAT was synthesized and tested for activity in a GTPγS binding assay (Pauwels et al. 1998). The dimeric ligand had the same potency as the monomeric ligand, but displayed greater efficacy. In addition, the efficacy of the dimeric ligand was unaltered by increasing GDP concentrations, which decreased the efficacy of the monomeric ligand (Pauwels et al. 1998). These results indicate that increased efficacy can be obtained with a bivalent form of the ligand and suggest that the bivalent ligand stabilized a different conformation of the 5-HT_{1A} receptor homodimer than the monomeric ligand.

Dimeric forms of 5-HT were synthesized and found to retain full agonist activity with increased potency at 1B/1D receptors (Halazy et al. 1996). An increase in selectivity for 1B/1D over 1A receptors was observed and was related to the length of the spacer arm used to

bridge the receptor dimer (Halazy et al. 1996). Sumatriptan, a drug used to treat migraine, has been synthesized in bivalent form and found to have increased potency over the monomeric form of the drug in stimulating GTPγS binding in recombinant cells and in mediating contraction of the rabbit saphenous vien (Perez et al. 1998). The dimeric form also displayed a 10-fold increase in binding affinity and selectivity for 1B/1D over 1E/1F receptor subtypes (Dupuis et al. 1999). Interestingly, the 5-HT_{1F} receptor selective agonist, LY-334370, was observed to change receptor selectivity when in the dimeric form, as it displayed high affinity and selectivity for 1D (0.3nM) over 1F receptors (>10uM), while retaining its agonist properties (Choi et al. 2008).

For 5-HT_{2A} receptors, bivalent ligand development has not yet provided any leads in terms of enhancing the pharmacological properties over the monomeric version of the ligand. Progress has been made in terms of determining the optimum linker arm length for bridging the gap between receptor protomers. Dimeric versions of the antagonist M-100907 were found to retain their antagonist properties at the 5-HT_{2A} receptor (Shashack et al. 2011). While antagonist potency varied with linker arm length, 12 – 18 atom linkers were optimum for producing the most potent bivalent ligands, the potency was not enhanced over monomeric forms of the ligand (Shashack et al. 2011). These results call to mind studies with 5-HT_{2C} and 5-HT_7 receptor homodimers discussed above in the section on G-protein activation. If 5-HT_{2A} receptor homodimers behave in a similar fashion to 5-HT_{2C} and 5-HT_7 receptor homodimers, wherein inactivation of one protomer of the dimer silences the signaling properties of the second protomer (Herrick-Davis et al. 2006; Teitler et al. 2010), then bivalent antagonist ligands may not have improved potency or efficacy over monomeric versions of the ligand. However, bivalency may provide a means to improve selectivity.

The 5-HT_{2A} partial agonists, pergolide and terguride, displayed decreased binding affinity and lost their agonist characteristics when converted into their homobivalent forms (Kren et al. 2004). In a similar fashion, ML10302, a 5-HT_4 selective partial agonist, lost its agonist properties upon conversion into its homo-bivalent form (Russo et al. 2007). 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-HT_4 receptor homodimer. In contrast to the 12-18 atom linker length found to be optimal for the 5-HT_{2A} bivalent ligand ML10302 (Shashack et al. 2011), spacer arms of 20 - 24 atoms were found to be optimal for a series 5-HT_4 receptor bivalent ligands (reviewed in Lezoualc'h et al. 2009). At present, additional studies are required to generate bivalent ligands with improved pharmacological profiles for the potential treatment of 5-HT-related disorders.

Summary

To date, there are many studies reporting the presence of 5-HT receptor homodimers of the 1A, 1B, 1D, 2A, 2C, 4, and 7 receptor subtypes in heterologous expression systems (Xie et al. 1999; Salim et al. 2002; Herrick-Davis et al. 2004; Berthouze et al. 2005; Kobe et al. 2008; Mancia et al. 2008; Brea et al. 2009; Woehler et al. 2009; Teitler et al. 2010; Ganguly et al 2011; Paila et al. 2011; Pellissier et al. 2011). Pharmacological studies of the 5-HT_7 receptor, using a novel inactivation and reactivation approach, provide the most compelling evidence for the existence of homodimers of native 5-HT receptors in vivo (Smith et al. 2011; Teitler and Klein 2012). Studies with 5-HT_{2C} receptors suggest that homodimerization begins during receptor biosynthesis within the ER and is a naturally occurring step in receptor maturation and processing (Herrick-Davis et al. 2006). Receptor dimerization may be a prerequisite for normal receptor trafficking and expression on the plasma membrane, as it may be necessary for passing ER quality control check-points that determine functionality. It is also possible that dimerization in the ER is a prerequisite for

trafficking to the plasma membrane as dimers may represent the minimal structural/ functional signaling unit.

Studies designed to explore potential dimer interfaces of biogenic amine GPCR suggest a TMD1/2-H8 dimer interface to be more stable, and a TMD4/5 interface potentially responsible for transient association of dimers into tetramers (Fonseca and Lambert 2011; Johnston et al. 2012; Periole et al. 2012; Huang et al. 2013). However, while evidence suggests that 5-HT receptors are predominantly dimeric (Kobe et al. 2008; Brea et al. 2009; Teitler et al. 2010; Pellissier et al. 2011; Herrick-Davis et al. 2012), studies seem to implicate a role for a TMD4/5 interface in the formation of 5-HT receptor homodimers (Berthouze et al. 2007; Mancia et al. 2008; Bruno et.al., 2011; Gorinski et al. 2012; Moreno et al. 2012).

The results of experiments with 5-HT_{2C} and 5-HT_4 receptors suggest a ligand/dimer/G-protein stoichiometry of 2:1:1, consistent with a model in which a receptor homodimer binds two molecules of ligand and one G-protein (Herrick-Davis et al. 2005; Pellissier et al. 2011). Several reports suggest that if the dimer is the basic signaling unit for biogenic amine receptors, then a TMD1/2-H8 interface may be preferred (Johnston et al. 2012; Periole et al. 2012; Huang et al. 2013), and G-protein docking studies to the dimer suggest that one protomer of the dimer is largely responsible for making contact with the G-protein alpha sub-unit. However, many studies have demonstrated that the second protomer in a dimer is not silent and contributes to the signaling process (Kniazeff et al. 2004, Guo et al. 2005; Han et al. 2009). Several studies have also provided evidence of cross-talk between protomers of a homodimer. This could occur through asymmetric coupling to the G-protein or through a direct interaction of the protomers across a TMD4/5 or TMD5/6 interface (reviewed in Maurice et al. 2011). Studies with 5-HT_{2C}, 5-HT_4, and 5-HT_7 receptors indicate that both protomers of a homodimer play a role in signaling (Herrick-Davis et al. 2005; Teitler et al. 2010; Pellissier et al. 2011). When one protomer is forced into an inactive conformation the dimeric complex is incapable of signaling through G-proteins. When both protomers of the dimer are G-protein competent, binding to one protomer can elicit a response, but binding to both protomers produces maximal G-protein activation.

Heterodimerization opens up a new avenue for differential regulation of signaling, either by enhancement or inhibition of the original pathways activated by the cognate homodimers or by activation of new pathways. For example, heterodimers of 5-HT_{1A}-5-HT_7 (Renner et al. 2012) and 5-HT_{2A}-mGluR2 receptors (Moreno et al. 2011) have been shown to display different functional characteristics than their respective homodimer counterparts. Changes in expression level of one partner of a heterodimer either during development, aging, chronic drug treatment or disease progression, would have profound effects on the relative concentration and thus physiological consequence of heterodimer signaling. This has important implications for the pharmacological intervention of physiological processes regulated by GPCR heterodimers and for diseases caused by altered GPCR expression and or function.

Studies with bivalent ligands targeting 5-HT receptors have been in progress for the past 15 years. So far, the results of the studies described herein indicate that simultaneous occupancy of both protomers of a GPCR homodimer with a bivalent ligand may enhance ligand selectivity, potency or efficacy, or may even change the pharmacological profile of the ligand from a partial agonist to an antagonist. 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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