



# GPCR heterodimerization in the reproductive system: functional regulation and implication for biodiversity

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A G protein-coupled receptor (GPCR) functions not only as a monomer or homodimer but also as a heterodimer with another GPCR. GPCR heterodimerization results in the modulation of the molecular functions of the GPCR protomer, including ligand binding affinity, signal transduction, and internalization. There has been a growing body of reports on heterodimerization of multiple GPCRs expressed in the reproductive system and the resultant functional modulation, suggesting that GPCR heterodimerization is closely associated with reproduction including the secretion of hormones and the growth and maturation of follicles and oocytes. Moreover, studies on heterodimerization among paralogs of gonadotropin-releasing hormone (GnRH) receptors of a protochordate, *Ciona intestinalis*, verified the species-specific regulation of the functions of GPCRs via multiple GnRH receptor pairs. These findings indicate that GPCR heterodimerization is also involved in creating biodiversity. In this review, we provide basic and current knowledge regarding GPCR heterodimers and their functional modulation, and explore the biological significance of GPCR heterodimerization.

**Keywords:** GPCR, heterodimer, reproduction, diversity hormones

## INTRODUCTION

The development of “omics” technologies and ensuring construction of a variety of databases provide vast information regarding primary sequences and functional domains of genes and proteins in diverse organisms, leading to annotation or prediction of biochemical and pharmacological propensities of novel genes and proteins. Even in this post-genomic era, several functions of proteins have yet to be fully elucidated or predicted. One of the most unpredictable and confounding post-translational protein functions is the heterodimerization of G protein-coupled receptors (GPCRs).

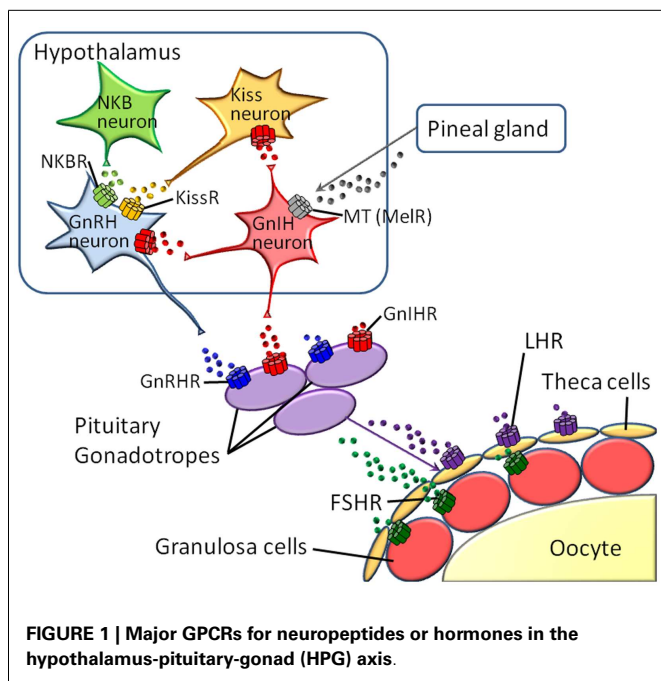
Currently, a wide range of GPCRs have been proved to function not only as monomers or homodimers but also as heterodimers formed after translation. It has been shown that GPCR heterodimerization alters or fine-tunes ligand binding, signaling, and internalization of GPCR protomers (1–9). The greatest difficulty in studies on GPCR heterodimers lies in the lack of procedures for the prediction of either GPCR protomer pairs for heterodimerization or the resultant functional alteration of GPCRs. Consequently, high-throughput analysis of GPCR heterodimers (e.g., “GPCR heterodimerome”) has not yet been accomplished. Despite this shortcoming, there have been increasing findings regarding the biological and pathological significance of GPCR heterodimerization.

Reproduction is regulated by diverse neuropeptides and hormones, with the receptors belonging to the GPCR family, e.g., melatonin, kisspeptin, neurokinin B (NKB), gonadotropin-inhibitory hormone (GnIH), gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prostanoids (10–13). In vertebrates, these hormones

and neuropeptides play crucial roles in the hypothalamus-pituitary-gonad (HPG) axis (Figure 1). Furthermore, various species-specific GPCRs for highly conserved cognate hormones or neuropeptides have been identified (14–18, Kawada et al., forthcoming). Collectively, these findings suggest that GPCR heterodimerization participates in the fine-tuning and diversification of reproductive functions. In this article, we provide an overview of GPCR heterodimerization and discuss the implication of GPCR heterodimers in reproductive functions and their diversification.

## GPCR PROTOMERS AS ALLOSTERIC MODULATORS

It is widely accepted that GPCRs can assume various active conformations which enable coupling with distinct G proteins and other associated proteins followed by particular signal transduction cascades (3, 8, 19, 20). Moreover, allosteric effectors interact with GPCRs at binding sites different from those for agonists or antagonists and modulate the conformations of GPCRs, leading to alterations in agonist/antagonist binding affinity or signal transduction (3, 8, 19, 20). Also of significance is that each of the active conformations responsible for individual signaling pathways is not interconvertible (3, 8, 19–21). Combined with experimental evidence that ligand binding and signaling of GPCR protomers are altered via heterodimerization, GPCR heterodimerization is believed to induce protomer-specific modulation (i.e., stabilization or destabilization) of active conformations as an endogenous allosteric modulator. This view is compatible with the fact that a single GPCR protomer acquires diverse biochemical and/or pharmacological properties via heterodimerization with different GPCR partners.



## TYPICAL FUNCTIONAL CONSEQUENCES OF GPCR HETERODIMERIZATION

Obviously, colocalization of GPCR protomers in a cell is a prerequisite for the formation of the corresponding GPCR heterodimer in native tissues. However, many early studies demonstrated functional alteration of GPCRs only after co-transfection of cultured cells with two GPCRs but not at the level of endogenous co-expression in the same cells in a particular native tissue. Consequently, the biological and physiological significance of such “*in vitro*-only” GPCR heterodimers is highly questionable. Consistent with this, the International Union of Basic and Clinical Pharmacology (IUPHAR) release the paradigm for GPCR heterodimer studies in 2007 (2). First, interaction between GPCR protomers in native tissues should be proved by at least two different experimental procedures including co-immunoprecipitation, fluorescence resonance energy transfer (FRET), or bioluminescence resonance energy transfer (BRET). Second, alteration of biochemical or pharmacological functions of GPCRs should be observed in native tissues or co-transfected cells. Third, biological roles of GPCR heterodimers should be verified using gene-knockout or gene-silenced procedures. At present, meeting all of these criteria is too difficult. Thus, IUPHAR proposed that researchers fulfill at least two of the three criteria. In the following, we focus on GPCR heterodimers which were detected in native tissues (Table 1).

G protein-coupled receptor heterodimers are classified into two groups in light of their functions: obligatory and non-obligatory GPCR heterodimers. Obligatory GPCR heterodimers require heterodimerization of GPCR protomers to serve as functional receptors, such as gamma amino butyric acid (GABA) type B receptor and taste receptors. GABAR<sub>B1</sub> alone is sequestered in the endoplasmic reticulum (ER) due to the presence of an ER retention signal, which is masked by heterodimerization with

**Table 1 | Typical functional alteration of GPCRs via heterodimerization.**

Heterodimer	Effect
GABAR <sub>B1</sub> -GABAR <sub>B2</sub>	Transition from ER to plasma membrane and function
T1R1-T1R3	Recognition of umami substances
T1R2-T1R3	Recognition of sweet substances
AT1-B2	Increase of IP <sub>3</sub> accumulation induced by angiotensin II or bradykinin
MOR-DOR	Reduction in binding affinity of Met-enkephalin Increase in binding affinity of endomorphin-1 and Leu-enkephalin Shift of coupling of G <sub>z</sub> to G <sub>i</sub>
KOR-DOR	Enhancement of signaling induced by synthetic KOR agonists
OR1-CB1	Suppression of OR-triggered ERK phosphorylation by a CB1 antagonist Suppression of CB-triggered ERK phosphorylation by a OR1 antagonist
MC3R-GHSR	Increase in cAMP production induced by melanocortin Decrease in ghrelin-induced signaling
D1-D2	Shift of coupling of G <sub>s</sub> to G <sub>q/11</sub>
MT1-GPR50	Decrease of melatonin-binding, Gi-coupling/signaling, and internalization
NK1-MOR	Alternation of internalization and resensitization profile
R1-R4	Upregulation of ERK phosphorylation via Ca <sup>2+</sup> -dependent PKC $\alpha$ activation and Ca <sup>2+</sup> -independent PKC $\zeta$ activation
R2-R4	Reduction in cAMP production via shift of coupling of G <sub>s</sub> to G <sub>i</sub>
EP1- $\beta$ 2AR	Dissociation of G <sub>s</sub> from $\beta$ 2AR induced by EP1 agonists

*GABAR*, GABA receptor; *T1R*, taste receptor; *AT1*, angiotensin receptor 1; *B2* bradykinin receptor 2; *MOR*,  $\mu$ -opioid receptor; *DOR*,  $\delta$ -opioid receptor; *KOR*,  $\kappa$ -opioid receptor; *OR1*, orexin receptor 1; *CB1*, cannabinoid receptor 1; *MC3R*, melanocortin receptor 3; *GHSR*, ghrelin receptor; *D1*, dopamine receptor 1; *MT1*, melatonin receptor 1; *NK1*, tachykinin receptor 1; *Ci-GnRHR*, *Ciona intestinalis* GnRH receptor; *EP1*, prostaglandin E2 receptor 1.

GABAR<sub>B2</sub> (22–24). Moreover, the GABAR<sub>B1</sub> protomer harbors a ligand-binding site, whereas the GABAR<sub>B2</sub> protomer merely couples to G proteins (22–24). Therefore, the GABAR<sub>B1</sub>-GABAR<sub>B2</sub> heterodimer serves as an authentic GABA receptor. Taste receptors also exhibit heterodimerization-dependent pharmacological profiles. The heterodimer between T1R1 and T1R3 is exclusively responsive to umami taste, while the T1R2-T1R3 heterodimer is a specific receptor for sweet taste-inducing molecules (25–27).

In contrast, non-obligatory GPCR heterodimers are composed of the functional GPCR protomers and modulate the biochemical or pharmacological activities of the protomers (**Table 1**). Non-obligatory GPCR heterodimers account for the major population and exhibit diverse modulatory functions. In human embryonic kidney (HEK) 293 cells expressing the angiotensin II receptor (AT1)-bradykinin receptor (B2) heterodimer, angiotensin II triggered inositol triphosphate (IP<sub>3</sub>) accumulation much more potently and effectively than it did in the cells expressing AT1 alone, whereas IP<sub>3</sub> accumulation by bradykinin was slightly weaker in cells expressing the AT1–B2 heterodimer than in the cells expressing only B2 (28). This enhancement was also detected *in vivo*, where AT1 and B2 were shown to form a heterodimer in smooth muscle, omental vessel, and platelets (28, 29).

The opioid receptor family is composed of three subtypes, namely,  $\mu$ -,  $\delta$ -, and  $\kappa$ -opioid receptors (MOR, DOR, and KOR), all of which mediate inhibition of cAMP production with different ligand-selectivity (30). Co-expression of MOR and DOR in HEK293 cells resulted in a 10-fold reduction in binding affinity of a synthetic MOR-selective agonist, DAMGO (31). Moreover, the MOR–DOR heterodimer differs in rank order of affinities for endogenous peptide ligands; Met-enkephalin, possessing the highest affinity for MOR among endogenous opioid peptides, exhibited twofold lower affinity to the MOR–DOR heterodimer, while the affinity of endomorphin-1 and Leu-enkephalin to the heterodimer was increased two to threefold, compared to MOR (31). Moreover, heterodimerization of MOR and DOR predominantly induced activation of a pertussis toxin-insensitive G protein, G<sub>z</sub> in COS-7 cells, while monomeric or homodimeric MOR and DOR were coupled to a pertussis toxin-sensitive G protein, G<sub>i</sub> (32). This is consistent with findings that the binding of ligands to the MOR–DOR heterodimer followed by signal transduction is resistant to pertussis toxin (32). A KOR-selective agonist, U69593, exhibited as potent and efficacious activities at the heterodimer as at KOR, whereas 6'-GNTI was a sixfold more potent agonist for the KOR–DOR heterodimer than for the KOR homodimer (33). More recently, *N*-naphthoyl- $\beta$ -naltrexamine (NNTA), a potent antagonist for MOR, was shown to manifest a prominent agonistic activity at MOR–DOR (34). In the mouse tail-flick assay, intrathecal NNTA elicited 100-fold greater antinociception, compared to intracerebroventricular administration (34). These heterodimerization-based pharmacological alterations are expected to provide crucial clues to understand why various *in vivo* pharmacological profiles are inconsistent with those from *in vitro* studies using cells expressing each opioid receptor alone and to develop more specific clinical agents for opioid receptors.

When an orexin receptor OR1 was co-expressed with a cannabinoid receptor CB1 in HEK293 cells, addition of a CB1-specific antagonist, SR-141716A, resulted in the suppression of orexin-triggered phosphorylation of ERK1/2 (35). Likewise, an OR1-specific antagonist, SB-674042, attenuated the ERK phosphorylation activated by a CB1 agonist, WIN55212-2 (35). These data verify the regulatory mechanism by which one GPCR protomer bound to an antagonist modulates the pharmacological profile of another GPCR protomer through heterodimerization.

Melanocortin receptor 3 (MC3R) and ghrelin receptor (GHSR) were found to be co-expressed in a number of neurons in the arcuate nucleus (36, 37). Co-transfection of MC3R and GHSR into COS-7 cells enhanced melanocortin-induced intracellular cAMP accumulation, compared with activation of MC3R in the absence of GHSR, whereas both agonist-independent basal and ghrelin-induced signaling of GHSR were diminished (36). These findings reveal mutual opposite signal modulation by each protomer and suggest that the molecular mechanism underlying a certain agonist-independent active conformation of a protomer is also involved in the regulation of the signaling functionalities of its partner GPCR in a heterodimer. Since MC3R and GHSR play pivotal roles in the orexigenic system, the MC3R–GHSR heterodimer is involved in hypothalamic body weight regulation.

There is increasing evidence for a pathological relevance of GPCR heterodimer. AT1–B2 heterodimer is highly likely to be functionally correlated with preeclampsia. The AT1–B2 heterodimer was more abundant on platelets of preeclamptic women than on platelets of normotensive pregnant women (29). Such increase in the number of heterodimers is due to enhanced expression of B2, as the expression level of B2 was elevated four to fivefold on platelets of preeclamptic women compared to platelets of normotensive pregnant women, whereas expression of AT1 was unchanged (29). Moreover, mobilization of intracellular calcium ions induced by angiotensin II was up-regulated 1.7- to 1.9-fold in platelets of preeclamptic women, compared to normotensive pregnant women (28, 29).

Heterodimerization between dopamine receptor subtypes, D1 and D2, has shown to be implicated in depression. The D1–D2 heterodimer was detected at higher levels in the post-mortem striatum of the patients compared to in normal subjects using co-immunoprecipitation and D1–D2 heteromer-selective antibodies (38). Moreover, dissociation of the D1–D2 heterodimer by an interfering peptide that disrupts the heteromer resulted in substantially reduced immobility in the forced swim test without affecting locomotor activity, and decreased escape failures in learned helplessness tests in rats (38). It should be noted that the heterodimerization between D1 and D2 leads to a drastic shift of G protein coupling; D1 and D2 monomer/homomer are coupled to G<sub>s</sub> and G<sub>i</sub>, respectively, while Gq/11 is a major G protein-coupled to the D1–D2 heterodimer (39).

More recently, MOR–DOR heterodimer was found to play pivotal roles in the opioid system. An interaction-disrupting peptide fragment for the MOR–DOR heterodimer enhanced morphine analgesia and reduced anti-nociceptive tolerance to morphine in mice (40).

## HETERODIMERS AMONG REPRODUCTION – ASSOCIATED GPCRS

### MELATONIN RECEPTOR

Melatonin participates in reproductive functions via upregulation of the synthesis and secretion of GnIH in the hypothalamus of mammals and birds (10, 11). Moreover, melatonin receptors were also shown to be expressed in gonads (41), and melatonin significantly decreases testosterone secretion from LH/FSH-stimulated testes of European starlings before breeding (42). Two class A (rhodopsin-like) GPCRs for melatonin, MT1 and MT2, have been

identified in mammals (1, 43). A human orphan GPCR, GPR50, sharing the highest sequence homology with MT1 and MT2, was shown to form a heterodimer with both receptors in HEK293 cells (1, 43). Moreover, heterodimerization of GPR50 with MT1 resulted in a marked reduction of the ability of MT1 to bind to ligands and to couple to G proteins, resulting in decreased in Gi protein-coupled intracellular signaling and  $\beta$ -arrestin – assisted internalization in HEK293 cells, whereas functions of MT2 were not affected (1, 43). These data indicate that GPR50 antagonizes MT1 but not MT2 via heterodimerization. In addition, this is the first report on the functional role of an orphan receptor as a protomer of a GPCR heterodimer.

### TACHYKININ RECEPTOR

Tachykinins (TKs) are vertebrate and ascidian multifunctional brain/gut peptides involved in smooth muscle contraction, vasodilation, nociception, inflammation, neurodegeneration, and neuroprotection in a neuropeptidergic endocrine, paracrine fashion (44–48). The mammalian TK family consists of four major peptides: Substance P (SP), Neurokinin A (NKA), NKB, and Hemokinin-1/Endokinins (HK-1/EKs) (EK is a human homolog of mouse and rat HK-1). TK receptors belong to the class A GPCR family. Three subtypes of TK receptors, namely NK1, NK2, and NK3, have been identified in mammals, and several submammalian orthologs have been cloned or suggested by genomic database search. In the ascidian, *Ciona intestinalis*, authentic TK, and its cognate receptor, Ci-TK-I and Ci-TK-R were identified (49, 50). Recently, there are accumulating reports on reproductive roles of TKs as well as the expression of TKs and TK receptors in genital organs of mammals (46, 48, 49, 51–54). In *C. intestinalis*, Ci-TK-I enhances oocyte growth from the vitellogenic stage to the post-vitellogenic stage via upregulation of gene expression and enzymatic activity of several proteases such as cathepsin D, carboxypeptidase B1, and chymotrypsin (55–57). Over the past few years, there has been increasing evidence that NKB plays a central role in the direct enhancement of GnRH synthesis and release in the hypothalamus of mammals, eventually leading to the recognition of novel regulatory function in sexual maturation and reproduction [(58–65)].

Only one tachykinin receptor-relevant heterodimer has thus far been identified. NK1 and an opioid receptor subtype, MOR, were shown to co-exist in pain-processing brain regions, including trigeminal dorsal horn neurons, and to heterodimerize in co-transfected HEK293 cells (66). NK1–MOR heterodimerization altered their internalization and resensitization profile, while ligand binding and signaling intensities of the protomers were not affected. In cells expressing NK1–MOR heterodimer, both DAMGO and SP induced the recruitment of  $\beta$ -arrestin to the plasma membrane and internalization of NK1–MOR heterodimers with  $\beta$ -arrestin into the same endosomal compartment (66). Recent studies also verified that other tachykinin receptors, such as NK3, are co-localized with various GPCRs including kisspeptin receptors and opioid receptors (59). Consequently, tachykinin receptors are expected to form heterodimers with a wide variety of GPCRs, which, in turn, are potentially involved in the molecular mechanisms underlying novel reproductive functions.

### GnRH RECEPTOR

Gonadotropin-releasing hormones are hypothalamic decapeptides that regulate the HPG axis to control reproduction by releasing gonadotropins, FSH, and LH from the pituitary in vertebrates (Figure 1). The endogenous receptors, GnRHRs, which belong to the Class A GPCR family, have also been shown to possess species-specific paralogs forms in vertebrates. Type I GnRHRs, which completely lack a C-terminal tail region, are restricted to humans, rodents, and cows (14–16, 67, 68). Type II GnRHRs, which bear a C-terminal tail, are widely distributed throughout almost all vertebrates, whereas the type II *gnrhr* gene is silenced due to a deletion of functional domains or interruption of full-length translation by the presence of a stop codon in humans, chimpanzees, cows, and sheep (14–16, 67, 68). To date, no convincing evidence for heterodimerization of GnRHRs in native tissues has been provided.

Gonadotropin-releasing hormones have also been identified in a wide range of invertebrates that lack a pituitary (Kawada et al., forthcoming). To date, seven GnRH peptides (tGnRH-3 to -8 and Ci-GnRH-X) and four GnRH receptor subtypes (Ci-GnRHR-1 to -4) have been identified in *C. intestinalis* (69–71). Molecular phylogenetic analysis indicates that Ci-GnRHR2 (R2), R3, and R4 are *Ciona*-specific paralogs of R1 generated via gene duplication (70, 72). Only R1 activated  $IP_3$  generation followed by intracellular  $Ca^{2+}$  mobilization in response to tGnRH-6, whereas R2 and R3 exclusively stimulate cAMP production in response to multiple tGnRHs; tGnRH-6, -7, and -8 exhibited near-equipotent cAMP production via R2, which was 100-fold more potent than tGnRH-3, -4, and -5. tGnRH-3 and -5 specifically triggered R3-mediated cAMP production (70, 73–75). R4 is devoid of binding to any tGnRHs or of activating any signaling pathways (70). Recently, we have shown that the orphan paralog, R4, is responsible for the fine-tuning of the GnRHERgic signaling via heterodimerization with R1. The R1–R4 heterodimer elicited a 10-fold more potent  $Ca^{2+}$  mobilization than R1 monomer/homodimer in a tGnRH-6-selective manner, while cAMP production by R1 was not modulated via heterodimerization with R4 (73). The R1–R4 heterodimer potentiated translocation of both  $Ca^{2+}$ -dependent PKC $\alpha$  by tGnRH-6 and  $Ca^{2+}$ -independent PKC $\zeta$  by tGnRH-5 and -6, eventually leading to upregulation of ERK phosphorylation, compared with R1 alone (73). These results provide evidence that the species-specific GnRHR orphan paralog, R4, serves as an endogenous modulator for the fine-tuning of the activation of PKC subtype-selective signal transduction via heterodimerization with R1. R4 was also shown to heterodimerize with R2 specifically in test cells of vitellogenic oocytes (74). Of particular interest is that the R2–R4 heterodimer in HEK293 cells decreased cAMP production in a non-ligand selective manner via a shift from activation of Gs protein to Gi protein by R2, compared with R2 monomer/homodimer (74). Considering that R1–R4 elicited a 10-fold more potent  $Ca^{2+}$  mobilization than R1 monomer/homodimer in a ligand selective manner but did not affect cAMP production, these results indicate that R4 regulates differential GnRH signaling cascades via heterodimerization with R1 and R2 as an endogenous allosteric modulator. Collectively, these studies suggest that heterodimerization among GnRHR paralogs, including the species-specific orphan receptor subtype, is involved in rigorous and diversified GnRHERgic signaling in a protochordate lacking an HPG axis.

## LH RECEPTOR

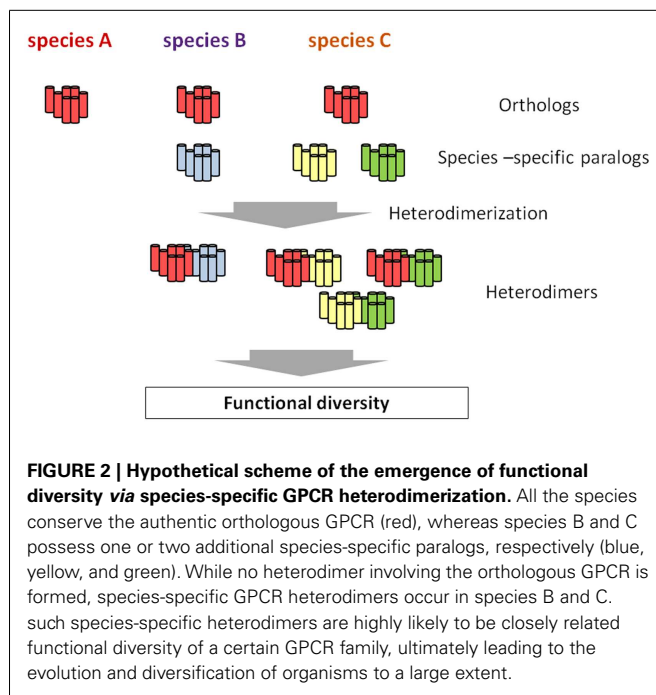
Luteinizing hormone is a central pituitary peptide hormone responsible for gonadal maturation (Figure 1). A single GPCR for LH has been identified in mammals. Although no LH receptor-containing GPCR heterodimer has been detected, studies on LH homodimers suggests that LH can also serve as a multifunctional protomer in various GPCR heterodimers. Co-expression of a ligand-binding-deficient LH receptor mutant and a signaling-deficient LH receptor mutant resulted in the restoration of normal gonadal and genital function in transgenic mice, indicating that LH receptor functions as a dimer *in vivo* (76).

## PROSTAGLANDIN RECEPTOR

Prostanoids consist of prostaglandin (PG) D, PGE<sub>2</sub>, PGF<sub>2α</sub>, PGI<sub>2</sub>, and thromboxane A<sub>2</sub> and are responsible for a variety of actions in various tissues including the relaxation and contraction of various types of smooth muscles, pain transmission, fever generation, and sleep induction (77). Numerous studies have also proved that ovulation, corpus luteum development and regression are mediated by PGs (78–80). To date, eight GPCRs for PGs have been identified in mammals. Heterodimerization of a PGE<sub>2</sub> receptor, EP1, with β<sub>2</sub> adrenergic receptor (β<sub>2</sub>AR) caused considerable reduction in cAMP production by β<sub>2</sub>AR *via* enhancement of the dissociation of G<sub>s</sub> protein from β<sub>2</sub>AR in the presence of endogenous or synthetic EP1 agonists in primary cultures of airway smooth muscle or COS-7 cells (81). Of importance in the functional regulation by the GPCR heterodimer is that EP1 have a direct inhibitory effects on bronchodilatory signaling but rather modulates the function of the β<sub>2</sub>AR. These findings strongly suggest that the heterodimerization of β<sub>2</sub>AR with EP1 causes the β<sub>2</sub>-agonist resistance found in asthma (81).

## EFFECTS OF GPCR HETERODIMERIZATION ON DIVERSIFICATION OF ANIMAL SPECIES AND BIOLOGICAL FUNCTION

G protein-coupled receptors are largely categorized into two groups. The first group consists of GPCRs conserved as authentic “homologs” in various species, and the second one includes species-specific GPCRs. The latter is further classified into GPCRs for species-specific ligands and subtypes of GPCRs for highly conserved ligands in various species. For instance, *C. intestinalis* GnRH receptors consists of four GPCRs as stated above: R1, R2, R3, and R4. Phylogenetic tree and biochemical analyses proved that R1 is structurally and functionally homologous to vertebrate GnRH receptors, whereas R2, R3, and R4 are *C. intestinalis*-specific paralogs that occurred via gene duplication in the *Ciona* evolutionary lineage (70, Kawada et al., forthcoming). Likewise, species-specific GnRHR-III has been identified in teleost species (14, 15), and lamprey has also three GnRHRs which are genetically independent of teleost GnRHR subtypes (16). Such species-specific GPCR paralogs are thought to determine the functional diversity and physiological regulatory systems, because GPCR paralogs can form species-specific GPCR heterodimers, which, if expressed in the same cells, control the unique expansion and fine-tuning of GPCR-mediated signaling pathways (Figure 2), as shown for *C. intestinalis* GnRHRs (73, 74). In other words, heterodimerization involving species-specific GPCRs is highly likely to contribute to the evolution and diversification of organisms to a large extent.



## CONCLUSION AND PERSPECTIVES

To date, GPCR heterodimerization has attracted keen attentions in light of biochemical and pharmacological features of GPCRs and the development of drugs with high selectivity, given that GPCR heterodimerization has been explored almost exclusively in mammals, except for *C. intestinalis* GnRHRs. Nevertheless, recent studies in various fields suggest that GPCR heterodimerization plays crucial roles in the regulation of the HPG axis and the evolution and diversification of reproductive functions. In this regard, of special interest is whether kisspeptin receptors or GnIH receptors heterodimerize with any GPCRs. Moreover, heterodimerization involving species-specific GPCR paralogs is expected to be responsible for the emergence of unique physiological functions in the respective organisms. Accordingly, combined with the fact that GPCRs form corresponding heterodimers after translation, investigation of GPCR heterodimerization in non-mammalian organisms will provide novel insight into the generation of biodiversity directed by a post-translational protein interaction.

In keeping with this issue, of particular interest is the clarification of the *in vivo* functional correlation between GPCR heterodimerization and biological events. Real-time imaging of GPCR heterodimerization could enable the visualization of biological functions of GPCR heterodimers of interest. Although this experimental strategy is unlikely to be applied to mammals, organisms equipped with transparent or semi-transparent skins, including ascidian, medaka, or zebrafish, are useful for live-imaging of GPCR heterodimers (50). Such studies are currently in progress in our laboratory.

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