# Molecular and functional insights into Gonadotrophin Hormone Receptor Dimerisation and Oligomerisation

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**Abstract**

The gonadotrophin hormones, follicle stimulating hormone and luteinising hormone, are essential for reproduction. They work in concert to control multiple aspects of gonadal function to ultimately produce meiotically competent and fertilisable gametes, provide the optimal endometrial environment and support for implantation and maintain pregnancy via progesterone production throughout the first trimester of pregnancy. These complex and multidimensional functions are mediated via the gonadotrophin hormone receptors, luteinising hormone receptor (LHR) and follicle stimulating hormone receptor (FSHR), Class A G protein-coupled receptors (GPCR), which couple to multiple G protein-dependent and independent signal pathways to control these physiological processes. Over the last two decades, a plethora of experimental evidence has shown that GPCRs can associate to form dimers and oligomers. This association provides a means of mediating the diverse functional requirements of a single receptor subtype and for the gonadotrophin hormone receptors, has been shown to alter the pharmacology and signal activation profile of these receptors. This review will detail the historical and current evidence detailing the formation of gonadotrophin hormone receptor homomers and heteromers. We will discuss the functional insight gained from *in vitro* and *in vivo* studies, and the potential impact in modulating reproductive health and disease.

## 1. Introduction

The coordinated actions of receptor-mediated processes ensure the correct functioning of all physiological and endocrine processes. This is particularly pertinent for the gonadotrophin hormone receptors (GpHRs), follicle-stimulating hormone receptor (FSHR) and luteinising hormone receptor (LHR), whose actions are essential for reproduction and fertility(1-3). Localised to specialised cellular compartments of the testes and ovaries, the GpHRs control and regulate gonadal functions; in males, regulating testosterone production and the proliferation and maturation of sperm(1), and in females, regulating gonadal steroidogenesis, follicular growth and maturation, follicle recruitment and dominant follicle selection, ovulation, and corpus luteum function(2,3). Due to the importance of GpHRs in initiating and maintaining fertility, they are key targets of assisted reproductive technologies, particularly *in vitro* fertilisation (IVF). Additionally, in recent years, the extragonadal expression of GpHRs has been reported, with proposed roles in prostate cancer(4), placental function(5,6), osteoclast activity(7,8), thermiogenesis(9) and the development of Alzheimer's disease(10). Thus, understanding the mechanisms underpinning how GpHRs function is imperative for the generation of more efficacious, targeted, effective and potentially personalised pharmacological-based therapeutic strategies for improvements in reproductive health, and also non-reproductive health and disease. This review will discuss a concept that has emerged over the last two decades as an important modality for regulating GpHR function, namely, the formation of GpHRs dimers and oligomers. We will first briefly appraise the functional roles of di/oligomerisation for the wider G protein-coupled receptors superfamily, before delving into the evidence presented for GpHR di/oligmerisation, and the impact on GpHR function. We will finish with discussing pertinent and outstanding questions in our GpHR di/oligomerisation and future perspectives for this important area of research.

## 2. Why do GpHR di/oligomerisation?

For many years, the accepted central dogma for how GPCRs functioned, was as a single receptor unit, binding hormone and mediating activation of a linear, single G protein-dependent signal pathway. However, an explosion of literature over the last 20-30 years has questioned this idea and presented evidence that this view of GPCR signalling was perhaps, unsurprisingly, too simplistic. Indeed, most GPCRs, including the GpHRs, can couple to more than 1 G protein-dependent signal pathway, may have more than 1 endogenous ligand, and can activate non-G protein-dependent pathway activation via recruitment of the molecular scaffolding protein, β-arrestin (recently reviewed in (11-13)). In addition, more recent studies have added additional layers to this complexity with internalised GPCRs, including the luteinising hormone receptor(14), and closely related the thyroid stimulating hormone receptor(15,16), able to sustain cAMP-dependent signalling from the endosome(17,18). The complex requirements of a single GPCR subtype show the diverse modalities that GPCRs need to exploit to mediate their physiological effects. A concept that has emerged as increasingly important mode of regulating GPCRs functionality, is receptor dimerisation and oligomerisation. The association of GPCRs, including the GpHRs, with self (homomerisation) or with other GPCRs (heteromerisation) has been shown to occur, providing a platform to regulate different aspects of a GPCRs lifecycle(19), and afford the ability to diversify functional responses, regulate the magnitude of signal response, the specificity of signal produced and ligand directed biased signalling(11,12,20).

Although studies have shown that GPCRs, including FSHR and LHR can di/oligomerise, most information has been derived from heterologous cell systems over-expressing receptors, therefore, the significance in health and disease remains largely unclear. Innovative approaches to study the GpHR di/oligomerisation have pushed forward our understanding of

100 the physiological relevance of Class A GPCR di/oligomerisation, showing its role and
101 relevance to regulating GpHR function, *in vivo*.

102 **3. Biochemical and biophysical evidence of gonadotrophin receptor dimerisation**

103 The first suggestion or inference that GpHR could associate, originated from the electron
104 microscopy studies of the 1970's and 1980's. Using ovarian theca and granulosa cells and
105 binding of labelled hCG to LHR, showed that LHR existed as 'clusters'(21,22). The proposed
106 clustering of LHR on hCG binding was suggested to stagnate the receptors, thereby
107 minimising the lateral diffusion of receptors, to negatively regulate G protein-coupling(22). In
108 more recent years, this concept has been further explored using time-resolved
109 phosphorescence anisotropy, a biophysical technique that can track the movement of LHR
110 within the plasma membrane. Utilising endogenous LHR expression in the Leydig tumour cell
111 line, MA-10 cells, and ovine luteal cells, binding of hCG to LHR, was shown to rotationally
112 immobilise LHR, the result of increased LHR-LHR interactions induced by ligand binding. This
113 suggested the presence of a ligand-induced increase in LHR clustering into specialised micro-
114 domains, and potentially association to form homomers(23,24). This 'clustering' could of
115 course be attributed to the initial steps in receptor desensitisation processes, and clustering
116 into clathrin coated pits for receptor internalisation, in line with classical Class A GPCR
117 endocytosis on ligand binding(17,18). More recent studies of LHR suggest that internalisation
118 kinetics and routing fate of LHR (and FSHR) are different to more typical Class A GPCRs,
119 such as β2 adrenergic receptor, (25,26), with LHR (and FSHR) trafficking to a newly identified
120 and smaller endosomal compartment- the very early endosome. However, how
121 di/oligomerisation of LHR (and FSHR) links with internalisation to endosomal compartments
122 remains to be determined. Interestingly, the rotational diffusion rate of LHR was different when
123 bound to LH and hCG, suggesting potential differential regulation of LHR clustering and
124 potentially homomerisation by these two hormones(23).

125

Co-immunoprecipitation of differentially epitope-tagged LHR and FSHR provided some of the first biochemical evidence supporting the formation of LHR and FSHR dimers and oligomers. Initially Western blotting of single tagged LHR showed varying molecular weight LHR species, suggesting the presence of LHR monomers, dimers and oligomers in whole cell lysates(27). To confirm that the larger dimeric and oligomeric bands were formed from interacting LHR protomers, co-immunoprecipitation of two differentially tagged-LHR was carried out, showing the presence of higher molecular forms of LHR and supporting the hypothesis that LHR does form dimeric and higher order oligomeric LHR. Interestingly, hCG dose dependently increased the number of LHR dimers and oligomers observed, but only when the LHR was stably expressed, and therefore efficiently trafficked to the membrane in its mature post-translationally modified form(27). As co-immunoprecipitations analyse whole cell fractions, it is likely that this reflects differences in plasma membrane versus intracellular endoplasmic reticulum localised LHR, and the differences in cellular localisation of LHR that stable versus transient transfection of LHR results in(27). Latter studies utilising the proximity-based resonance energy transfer technique of bioluminescence energy transfer (BRET), confirmed the association of LHR into dimers/oligomers, and demonstrated the specificity of this interaction. Interestingly, ligand treatment was shown to have no effect on LHR association via this method(28).

Direct biochemical evidence for the formation of FSHR homomers was also achieved using a combination of co-immunoprecipitation, FRET and BRET techniques. Similarly, to findings with the LHR, FSHR was shown to reside as monomers, dimers and oligomeric complexes(29,30), with FSH treatment having little effect on the relative ratios of FSHR monomers, dimers and oligomers, formed, in concordance with later BRET studies and single molecule imaging studies of LHR dimers and oligomers(31).

The subcellular location and timing of GpHR homomer formation has been identified. Using a combination of co-immunoprecipitation with subcellular fractionation and BRET techniques LHR and FSHR dimers and oligomers were shown to be localised to the plasma membrane and endoplasmic reticulum(29). Moreover, utilisation of a misfolded mutant LHR that was shown to be retained in the ER, revealed association of wild type LHR with the misfolded ER retained mutant LHR, showing that LHR dimers and oligomers were formed during the post-translational processing and modification of LHR within the ER. Analogous studies to investigate this question with FSHR, showed FSHR dimers and oligomers were also formed in the ER following translation.

## 4. GpHR di/oligomerisation and functional asymmetry

A debated and remaining question in GPCR di/oligomer field is how ligand binding within a dimer or oligomer impacts on subsequent ligand binding to additional receptor protomers within a complex. An elegant study by Urizar et al(32), provided the link between glycoprotein hormone receptor di/oligomerisation, and negative cooperativity, via BRET and competition binding assays. To explore the effect of ligand binding within a dimer on subsequent ligand bindings to additional receptor protomers within a complex, a chimeric receptor of the LHR extracellular domain fused to the transmembrane region of the TSHR was generated. When the full length, wild type TSHR was expressed alone and binding assays conducted, as expected, hCG failed to compete with TSH for binding to TSHR. However, when TSHR was co-expressed with the chimera LT receptor, hCG competed with TSH for binding, and desorption studies using radiolabelled TSH with ‘cold’ hCG suggested the presence of two binding sites per dimer, as opposed to a single binding pocket formed by the dimeric interaction of the two receptor protomers(32). This evidence suggests that GpHR dimers, and potentially oligomers are linked by strong negative cooperativity, with binding of ligand to one receptor protomer within the dimer, decreasing the binding affinity of ligand to the unbound receptor protomer. This important series of experiments demonstrate that GpHR display

strong functional asymmetry via allosteric communication between protomers within a dimer and potentially oligomer(32).

In subsequent follow-up studies the mode of communication between receptors within a dimer was explored. The transmembrane membrane was shown to mediate the allosteric communication between GpHR protomers in dimers(33), in line with previous reports and now accepted mode of allosteric communication between Class C GPCR dimers(12). Interestingly, homomers comprised of constitutively active receptors failed to display any evidence of negative cooperativity with symmetry in ligand binding observed(33). A question that remains is the link between negative cooperativity and preference of signal pathway activated and/or magnitude of signal response observed. For the related TSHR, it has been proposed that single receptor occupancy within a dimer is sufficient for Gs activation, but occupation of both receptor protomers is required for Gq activation (and can also mediate Gs activation(34). How negative cooperativity regulates ligand binding and subsequent G protein-signalling within an oligomer remains to be determined.

## 5. Functional complementation of GpHR - roles *in vitro* and *in vivo*

To explore the functional significance of GpHR dimers and oligomers, the phenomenon of functional complementation (also known as transactivation or intermolecular cooperation) has been exploited. This experimental approach utilises the relatively compartmentalised nature of GPCR ligand binding and signal activation and has used to explore the impact of di/oligomerisation on the functions of number of GPCRs, including the GpHRs (reviewed by (35)). The structural properties of GpHRs make them a particularly good experimental model for utilising functional complementation to study di/oligomerisation, as ligand binding is largely mediated by the large extracellular N terminus domain of the receptors, and G protein coupling for signal propagation, by transmembrane domains 5-7, as evidenced by the naturally and

experimentally induced activating and inactivating mutations of the GpHRs. Using these discrete functional properties, mutant GpHRs have been generated that are either 'binding defective'- that cannot not bind to ligand but are theoretically still able to couple of G proteins, or 'signal defective' that can bind ligand but cannot couple to G protein(s) to generate intracellular signals, that if expressed by themselves are functionally inactive. Remarkably, when co-expressed, these mutant receptors undergo functional complementation, or trans-activation, with the 'signal defective' receptor binding ligand, and the 'binding defective' receptor coupling to G protein. The functioning of these receptors can only occur via interaction of at least 1 signal- and 1 binding- defective receptor protomers within a complex, and thus restoration of functional response is via the formation of dimers and oligomers(35). This experimental paradigm has therefore proved a valuable and highly utilised model for studying the functional consequences of GpHR di/oligomerisation.

*In vitro aspects*

The ability of GpHRs to undergo functional complementation was first reported by Osuga et al(36). Using a mutant LHR that had previously been identified from a patient with Leydig cell hypoplasia that was shown to possess a premature stop codon at transmembrane domain 5, producing a truncated LHR, they showed that expression of this TM6/7 deleted LHR could bind hCG, but couldn't activate cAMP production. To explore aspects of functional complementation, a chimeric FSHR/LHR, termed FLR, was generated, comprised of the FSHR extracellular domain and TM region of LHR, which generated cAMP in response to FSH (and not hCG)(36). Remarkably, when the FLR was co-expressed with the truncated mutant LHR and treated with hCG, cAMP production was observed, showing that the mutant LHR, that could bind hCG, but not activate cAMP production, had trans-activated the FLR. This showed that GpHRs could undergo functional complementation, via intermolecular communication and cross talk between these two receptor species(36), and most likely via the formation of dimers/oligomers.

To explore the minimum TM domain requirements for transactivation to occur and how receptor protomers engaged in transactivation communicate, further deletions of the LHR were undertaken. It was found that co-expression of the LHR extracellular region fused to transmembrane domain 1 (TM1), with FLR was sufficient to mediate hCG-dependent cAMP(36). Interestingly, fusion of the LHR extracellular domain to the single membrane spanning CD8 peptide failed to mediate hCG dependent cAMP production, suggesting the requirement of transmembrane mediated inter-protomer communication for transactivation to occur(36). In contrast to this, other studies have shown that the extracellular domains of the GpHRs fused to CD8 or GPI to anchor to the extracellular region of the receptor to the plasma membrane were sufficient to mediate transactivation(37,38), and suggest a 'kiss and run' type interaction of the extracellular domain of one protomer directly interacting and activating neighbouring receptors. The latter view contrasts with our knowledge on how other GPCRs undergoing transactivation communicate and with more recent evidence in the GpHR field, whereby activation of neighbouring receptor protomers occurs via intermolecular communication via the TM bundles(12,28,30,33,39). Our own studies that have utilised a combination of single molecule imaging and molecular modelling would also suggest that dimerisation, and indeed oligomerisation of LHR undergoing functional complementation is via intermolecular communication between the TM bundles(31), showing the importance of inter protomer TM bundle cross talk in directing the functions of GpHR dimers and oligomers.

Studies have shown that not all binding and signal defective mutant LHR and FSHR can undergo functional complementation(38,40), suggesting a specificity in pairing, and structural requirements for inter-protomer communication and ultimately transactivation to occur. The leucine rich repeats (LRR) of the extracellular region of LHR are essential for mediating ligand binding and appear to be a key factor in the facilitation of transactivation. It was found that binding defective mutant LHR with mutations that are localised to the LRR regions 1-3 were

able to undergo functional complementation when co-expressed with signal defective LHR. However, if binding defective mutations were located to LRR regions 4-8, they were unable to undergo functional complementation(38,40). This most likely reflects the close proximity of LRR 4-8 to the hinge region of the LHR, which is crucial for transducing ligand binding to the TM region for signal activation. Additionally, as the hinge region also contains a suppressor of TM activation to constrain the unbound receptor in an inactive conformation, which on ligand binding is relaxed, mutations in LRR4-8, may interfere with the conformational changes that occur to allow TM activation, and thus transactivation fails to occur.

Signal specificity of both LHR and FSHR has also been demonstrated using functional complementation. Co-expression of CD8 or GPI-anchored FSHR with differential signal defective mutant FSHR were shown to preferentially activate both cAMP and IP3, only cAMP, or only IP3(41), suggesting differential activational states and receptor conformations that mediate the specificity of G protein-coupling and subsequent intracellular pathway activation within dimers and oligomers. Our *in vivo* and *in vitro* studies studies utilising LHR transactivation mutations also support this idea(31), further details of which will be further discussed in latter sections.

We would be remiss in admitting more atypical examples of GpHR transactivation within heteromers that has been observed. LHR has been shown to transactivate the epidermal growth factor receptor (EGFR), leading to the downregulation of ovarian aromatase expression during the LH surge during the ovarian cycle(42). In two different mouse models with inactivating mutations of the EGFR, it was shown that the down-regulation of the Cyp19a1 via hCG (used to mimic the LH surge), was markedly impaired but not completely abolished, as it requires efficient signalling via transactivation of the EGFR. The *in vivo* of LHR-mediated transactivation of the EGFR in ovulation has also been determined(43-45).

*In vivo aspects*

Most studies investigating the functional consequences of GPCR di/oligomerisation to date have primarily utilised *in vitro* approaches, which rely upon the over-expression of GPCRs in heterologous cell lines. As a result, the physiological consequence of GPCR di/oligomerisation remains poorly understood and our understanding is lacking in the physiological necessity and functional relevance of GPCR di/oligomerisation *in vivo*. A study that made significant inroads into proving the physiological relevance of Class A GPCR di/oligomerisation, utilised a functional complementation approach and the LHR as a model GPCR, to determine if LHR di/oligomerisation was sufficient to mediate LHR functions *in vivo*. Using the LHR knockout (LuRKO) mice, and a BAC transgenic approach, targeted co-expression of ligand binding defective LHR (C22A mutation, $LHR^{B-}$) with signal defective mutant LHR (a deletion of transmembrane domains 6 and 7, $LHR^{S-}$) could rescue the hypogonadal phenotype of male LuRKO mice(46). Testes size and serum testosterone levels were equivalent to that of wild type litter mates. Importantly, the infertile phenotype of LuRKO animals was also reversed, with litter frequency and sizes from mating studies in LuRKO mice co-expressing $LHR^{B-}$ and $LHR^{S-}$ equivalent to wild type littermates(46). Serum LH was slightly raised in comparison to wild type littermates, showing the increased hypothalamic-pituitary drive to the testes to initiate and maintain LH-dependent testosterone production. This seminal study showed the first *in vivo*, physiological evidence for GPCR di/oligomerisation, and importantly, that di/oligomerisation was a functionally relevant mode of LHR activation/signal propagation *in vivo(46)*. Although there has been a subsequent *in vitro* study that critically debated whether the observations of this *in vivo* study were due to idiosyncrasies of the BAC transgenic method for introducing the $LHR^{B-}$ and $LHR^{S-}$ mutants into the LuRKO mouse background(47), the control experiments from this study conclusively showed that the expression of single $LHR^{B-}$ or $LHR^{S-}$ mutants failed to rescue the infertile and hypogonadal phenotype of these mice(46). Thus, confirming the specificity of the BAC approach, and confidence in LHR functional

complementation occurring in male mice *in vivo*. Interestingly, co-expression of $LHR^{B-}$ and $LHR^{S-}$ in female LuRKO mice, failed to rescue the infertile and hypogonadal phenotype of these animals. This may reflect the low levels of $LHR^{B-}$ and $LHR^{S-}$ expression female mice, and the inability to induce sufficient LHR expression during the ovarian cycle. It may also additional reflect the inability of functional complementation to mediate the multiple signalling and functional requirements of LHR in females, as previously discussed. Further insights as to why the latter proposition may be the case, will be discussed in the next section.

## 6. Single molecule imaging of gonadotrophin receptor dimerisheteroation

The advances in technology development have resulted in an explosion of single molecule imaging and single particle tracking techniques. These advances have given unprecedented insight into the nature and composition of GpHR homomers and heteromers, and the link with signal activation.

Early insights into the membrane organisation of LHR were provided by single particle tracking studies, which presented evidence of rat LHR localising to small specialised membrane microdomains, or lipid rafts, following binding of hCG(48). These microdomains were approximately 3 times smaller than unbound receptor, and importantly, when disrupted using the cholesterol depleting agent, methyl-β-cyclodextrin, showed a decreased hCG-dependent cAMP production(48). As a total abrogation of hCG-dependent cAMP production was not observed, it suggests that localisation of hCG-bound LHR to lipid rafts is not essential for Gs coupling. In support of this, although constitutively active mutant LHR also localised to lipid rafts, and these LHR microdomains were approximately the same size as hCG-bound LHR, cAMP production was not affected by raft disruption using methyl-β-cyclodextrin(49). That said, the relationship between raft location and coupling of LHR to alternate G protein-dependent and independent pathways remains unknown. Additionally, whether ligand-specific

differences in raft localisation and/or size of these microdomains exist. This may be particularly pertinent given the changing lipid environment, and LH/hCG/LHR signal requirements of the ovarian follicle during folliculogenesis and corpus luteum function.

A recent study utilising a combination of precision FRET and fluorescent correlation spectroscopy with photon counting histogram methods, has explored the nature and composition of FSHR homomers within the plasma membrane. Using a fusion of a C tail-truncated human FSHR, the C terminus tail of the LHR and fluorescent proteins compatible with FRET to the proximal end of the C-tail, FSHR was shown to traffick to and reside in the plasma membrane almost exclusively as homodimers(50). These findings contrast with previous crystal structure analysis of the FSHR ectodomain and hinge region, which proposed that FSHR exists as trimers(51). However, the crystal structures lack the vital interactions of the extracellular and transmembrane domain, due to complexities of generating stable crystals with intact transmembrane domains. Additionally, the functional and physiological significance of different FSHR complexes remains to be determined.

Our recent studies have provided significant insight into how the composition and functional role of each protomer within an LHR oligomer can regulate signal amplitude. Utilising a functional complementation approach, and the same functional complementation $LHR^{B-}$ and $LHR^{S-}$ from our previously discussed *in vivo* study, we employed the super resolution imaging approach of photoactivated dye, localisation microscopy (PD-PALM), to quantitate the number of LHR monomer, dimers and oligomers at the plasma membrane, and determine the nature of these complexes. Using heterologous cell lines that stably expressed either the wild type mouse LHR, or co-expressing $LHR^{B-}$ and $LHR^{S-}$ mutant receptors, we observed that approximately 40% of LHR formed homomers, with remaining 60% residing as monomers(31). Sub-analysis of the types of associated LHR complexes that were observed revealed that the

predominant associated form of wild type LHR was dimers, with a small number of lower order and higher order wild type LHR complexes. In contrast, in cells co-expressing $LHR^{B-}$ and $LHR^{S}$, approximately half the number of dimers were observed, with a concomitant increase in lower order trimers and tetramers(31). Interestingly, treatment with LH or hCG had no effect on the number of associated wild type or functional complementation LHR, nor the types of oligomeric complexes formed, in line with previous findings from BRET analysis(28). Assessment of LH and hCG-dependent Gs and Gq-association in wild type LHR and functional complementation models, revealed that LH-dependent Gq activation was impaired in the functional complementation model, suggesting that for full LH-dependent Gq activation, an element of cis or unidirectional activation of LHR is required(31). This may shed light as to why the female functional complementation mutant mice were infertile, as previously studies have shown LH-dependent Gq activation is required for ovulation(52). Generation of cell lines with varying cell surface ratios of $LHR^{B-}$:$LHR^{S-}$ revealed that cells with an excess of $LHR^{S-}$:$LHR^{B-}$ resulted in amplification of Gs and Gq-dependent signals. Interestingly, the difference in Gs and Gq signal observed, corresponded with an enrichment of $LHR^{B-}$ receptor protomers in both trimers and tetramers, suggesting, that modulation of specific the composition and functional role adopted by a protomer engaged in an oligomeric complex, can fine tune the amplitude of signal response generated.

## 7. Gonadotrophin receptor heteromers

The formation of LHR/FSHR heteromers has long been a debated topic. With specific relevance to female reproductive physiology, LHR and FSHR are co-expressed within granulosa cells of the dominant pre-ovulatory follicle, suggesting a functional role for LHR-FSHR crosstalk in mediating/facilitating ovulation. As such, heteromerisation of LHR/FSHR was first demonstrated by BRET(53), and subsequently by fluorescent correlation spectroscopy(50), using heterologous cell models, co-expressing LHR and FSHR. Importantly, heteromerisation of LHR/FSHR was shown to alter the pharmacology of FSHR

and LHR, enhancing dissociation of bound ligands, and negatively impacting on Gs-dependent signal pathway activation(53). As LHR is known to signal via both Gs and Gq-dependent pathways, with the latter particularly important for mediating LH-dependent ovulatory events(52), studies by our group further investigated the impact of LHR/FSHR on LH-dependent Gq activation. We found a change from a transient to more sustained calcium signal, that was both dependent on Gq activation and influx of extracellular calcium(54). Importantly, the presence of a sustained calcium response was also confirmed in human granulosa lutein cells, which endogenously co-express LHR and FSHR, which was also found to be sensitive to extracellular calcium channel blockers. PD-PALM studies revealed an LH-dependent increase in LHR/FSHR heteromerisation, with specific enrichment in heterotetramers, suggesting that modulation of LHR/FSHR heteromers mediated the switch from transient to sustained LH-dependent calcium signalling(54). Investigation of cross-talk between LH/hCG and FSH has shown that co-treatment of FSH with either LH or hCG results in potentiation of their respective effects on apoptosis and steroidogenesis(55), giving an insight into the potential physiological roles of LHR/FSHR heteromers. However, understanding the exact physiological role of LHR/FSHR heteromers *in vivo*, remains to be determined.

## 8. Perspectives and conclusions

It is unquestionable that GpHRs can associate to form homomers and heteromers. The formation of homomers and heteromers has been shown to impact on aspects of GpHR pharmacology, including ligand binding, signal specificity and signal magnitude. Despite the overwhelming experimental evidence detailing the functional relevance of GpHR di/oligomerisation, limitations in probing FSHR and LHR *in vivo* coupled with the technical complexities, e.g., the lack of specific FSHR and LHR antibodies, mean that our knowledge of the physiological roles of these receptor complexes remains limited. However, with the relative ease and decreasing cost of gene modification techniques such as CRISPR, future research

programs in this area will enhance our knowledge to gain insight into the physiological role(s) of GpHR homomers and heteromers.

With technological advances, come questions. The intriguing finding from the single molecule imaging studies of LHR that 60% of LHR at the plasma membrane appears to be monomers(31), suggests a distinct functional role for monomers in mediating/regulating LHR functions. Indeed, deciphering how different 'flavours' of GpHR monomers, homomers and heteromers regulate gonadotrophin hormone functions will provide much needed insight into how these distinct receptor complexes regulate and fine-tune GpHR functions *in vitro* and importantly, *in vivo*. Identifying the unique 'signatures' of GpHR complexes at the cell surface, the drivers which control the formation of these complexes, and the resulting cellular responses, will provide invaluable insight into how GpHR di/oligomerisation impacts reproductive health, and potential dysregulation in disease. Such advances will provide the opportunity for novel and potentially personalised pharmacological treatment strategies for treatment reproductive pathologies, and may lead to advances for the improvement in the success rate of assisted reproductive technologies.

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## References


1. Jonas KC, Oduwole OO, Peltoketo H, Rulli SB, Huhtaniemi IT. Mouse models of altered gonadotrophin action: insight into male reproductive disorders. Reproduction 2014; 148:R63-70
2. Fowler PA, Huhtaniemi IT. The ovarian gonadotropin receptors in health and disease. Rev Endocr Metab Disord 2002; 3:55-63
3. Hillier SG. Gonadotropic control of ovarian follicular growth and development. Mol Cell Endocrinol 2001; 179:39-46
4. Ben-Josef E, Yang SY, Ji TH, Bidart JM, Garde SV, Chopra DP, Porter AT, Tang DG. Hormone-refractory prostate cancer cells express functional follicle-stimulating hormone receptor (FSHR). J Urol 1999; 161:970-976
5. Stilley JAW, Segaloff DL. Deletion of fetoplacental Fshr inhibits fetal vessel angiogenesis in the mouse placenta. Mol Cell Endocrinol 2018;
6. Stilley JA, Christensen DE, Dahlem KB, Guan R, Santillan DA, England SK, Al-Hendy A, Kirby PA, Segaloff DL. FSH receptor (FSHR) expression in human extragonadal reproductive tissues and the developing placenta, and the impact of its deletion on pregnancy in mice. Biol Reprod 2014; 91:74
7. Zhu LL, Blair H, Cao J, Yuen T, Latif R, Guo L, Tourkova IL, Li J, Davies TF, Sun L, Bian Z, Rosen C, Zallone A, New MI, Zaidi M. Blocking antibody to the β-subunit of FSH prevents bone loss by inhibiting bone resorption and stimulating bone synthesis. Proc Natl Acad Sci U S A 2012; 109:14574-14579
8. Zhu LL, Tourkova I, Yuen T, Robinson LJ, Bian Z, Zaidi M, Blair HC. Blocking FSH action attenuates osteoclastogenesis. Biochem Biophys Res Commun 2012; 422:54-58
9. Liu P, Ji Y, Yuen T, Rendina-Ruedy E, DeMambro VE, Dhawan S, Abu-Amer W, Izadmehr S, Zhou B, Shin AC, Latif R, Thangeswaran P, Gupta A, Li J, Shnayder V, Robinson ST, Yu YE, Zhang X, Yang F, Lu P, Zhou Y, Zhu LL, Oberlin DJ, Davies TF,

Reagan MR, Brown A, Kumar TR, Epstein S, Iqbal J, Avadhani NG, New MI, Molina H, van Klinken JB, Guo EX, Buettner C, Haider S, Bian Z, Sun L, Rosen CJ, Zaidi M. Blocking FSH induces thermogenic adipose tissue and reduces body fat. Nature 2017; 546:107-112

**10.** Lin J, Li X, Yuan F, Lin L, Cook CL, Rao CV, Lei Z. Genetic ablation of luteinizing hormone receptor improves the amyloid pathology in a mouse model of Alzheimer disease. J Neuropathol Exp Neurol 2010; 69:253-261

**11.** Rivero-Müller A, Jonas KC, Hanyaloglu AC, Huhtaniemi I. Di/oligomerization of GPCRs-mechanisms and functional significance. Prog Mol Biol Transl Sci 2013; 117:163-185

**12.** Wang W, Qiao Y, Li Z. New Insights into Modes of GPCR Activation. Trends Pharmacol Sci 2018; 39:367-386

**13.** Farran B. An update on the physiological and therapeutic relevance of GPCR oligomers. Pharmacol Res 2017; 117:303-327

**14.** Lyga S, Volpe S, Werthmann RC, Götz K, Sungkaworn T, Lohse MJ, Calebiro D. Persistent cAMP Signaling by Internalized LH Receptors in Ovarian Follicles. Endocrinology 2016; 157:1613-1621

**15.** Werthmann RC, Volpe S, Lohse MJ, Calebiro D. Persistent cAMP signaling by internalized TSH receptors occurs in thyroid but not in HEK293 cells. FASEB J 2012; 26:2043-2048

**16.** Boutin A, Allen MD, Neumann S, Gershengorn MC. Persistent signaling by thyrotropin-releasing hormone receptors correlates with G-protein and receptor levels. FASEB J 2012; 26:3473-3482

**17.** Hanyaloglu AC. Advances in Membrane Trafficking and Endosomal Signaling of G Protein-Coupled Receptors. Int Rev Cell Mol Biol 2018; 339:93-131

**18.** Jean-Alphonse F, Hanyaloglu AC. Regulation of GPCR signal networks via membrane trafficking. Mol Cell Endocrinol 2011; 331:205-214

**19.** Terrillon S, Bouvier M. Roles of G-protein-coupled receptor dimerization. EMBO Rep 2004; 5:30-34

**20.** Jonas KC, Huhtaniemi I, Hanyaloglu AC. Single-molecule resolution of G protein-coupled receptor (GPCR) complexes. Methods Cell Biol 2016; 132:55-72

**21.** Amsterdam A, Berkowitz A, Nimrod A, Kohen F. Aggregation of luteinizing hormone receptors in granulosa cells: a possible mechanism of desensitization to the hormone. Proc Natl Acad Sci U S A 1980; 77:3440-3444

**22.** Brawer J, Richard M, Farookhi R. Pattern of human chorionic gonadotropin binding in the polycystic ovary. Am J Obstet Gynecol 1989; 161:474-480

**23.** Roess DA, Brady CJ, Barisas BG. Biological function of the LH receptor is associated with slow receptor rotational diffusion. Biochim Biophys Acta 2000; 1464:242-250

**24.** Roess DA, Horvat RD, Munnelly H, Barisas BG. Luteinizing hormone receptors are self-associated in the plasma membrane. Endocrinology 2000; 141:4518-4523

**25.** Sposini S, Jean-Alphonse FG, Ayoub MA, Oqua A, West C, Lavery S, Brosens JJ, Reiter E, Hanyaloglu AC. Integration of GPCR Signaling and Sorting from Very Early Endosomes via Opposing APPL1 Mechanisms. Cell Rep 2017; 21:2855-2867

**26.** Jean-Alphonse F, Bowersox S, Chen S, Beard G, Puthenveedu MA, Hanyaloglu AC. Spatially restricted G protein-coupled receptor activity via divergent endocytic compartments. J Biol Chem 2014; 289:3960-3977

**27.** Tao YX, Johnson NB, Segaloff DL. Constitutive and agonist-dependent self-association of the cell surface human lutropin receptor. J Biol Chem 2004; 279:5904-5914

**28.** Guan R, Feng X, Wu X, Zhang M, Zhang X, Hébert TE, Segaloff DL. Bioluminescence resonance energy transfer studies reveal constitutive dimerization of the human lutropin receptor and a lack of correlation between receptor activation and the propensity for dimerization. J Biol Chem 2009; 284:7483-7494

**29.** Thomas RM, Nechamen CA, Mazurkiewicz JE, Muda M, Palmer S, Dias JA. Follicle-stimulating hormone receptor forms oligomers and shows evidence of carboxyl-terminal proteolytic processing. Endocrinology 2007; 148:1987-1995

**30.** Zhang M, Feng X, Guan R, Hébert TE, Segaloff DL. A cell surface inactive mutant of the human lutropin receptor (hLHR) attenuates signaling of wild-type or constitutively active receptors via heterodimerization. Cell Signal 2009; 21:1663-1671

**31.** Jonas KC, Fanelli F, Huhtaniemi IT, Hanyaloglu AC. Single molecule analysis of functionally asymmetric G protein-coupled receptor (GPCR) oligomers reveals diverse spatial and structural assemblies. J Biol Chem 2015; 290:3875-3892

**32.** Urizar E, Montanelli L, Loy T, Bonomi M, Swillens S, Gales C, Bouvier M, Smits G, Vassart G, Costagliola S. Glycoprotein hormone receptors: link between receptor homodimerization and negative cooperativity. EMBO J 2005; 24:1954-1964

**33.** Zoenen M, Urizar E, Swillens S, Vassart G, Costagliola S. Evidence for activity-regulated hormone-binding cooperativity across glycoprotein hormone receptor homomers. Nat Commun 2012; 3:1007

**34.** Allen MD, Neumann S, Gershengorn MC. Occupancy of both sites on the thyrotropin (TSH) receptor dimer is necessary for phosphoinositide signaling. FASEB J 2011; 25:3687-3694

**35.** Jonas KC, Rivero-Müller A, Huhtaniemi IT, Hanyaloglu AC. G protein-coupled receptor transactivation: from molecules to mice. Methods Cell Biol 2013; 117:433-450

**36.** Osuga Y, Hayashi M, Kudo M, Conti M, Kobilka B, Hsueh AJ. Co-expression of defective luteinizing hormone receptor fragments partially reconstitutes ligand-induced signal generation. J Biol Chem 1997; 272:25006-25012

**37.** Lee C, Ji I, Ji TH. Distinct mechanisms of cAMP induction by constitutively activating LH receptor and wild-type LH receptor activated by hCG. Endocrine 2004; 25:111-115

**38.** Lee C, Ji I, Ryu K, Song Y, Conn PM, Ji TH. Two defective heterozygous luteinizing hormone receptors can rescue hormone action. J Biol Chem 2002; 277:15795-15800

**39.** Guan R, Wu X, Feng X, Zhang M, Hébert TE, Segaloff DL. Structural determinants underlying constitutive dimerization of unoccupied human follitropin receptors. Cell Signal 2010; 22:247-256

**40.** Ji I, Lee C, Song Y, Conn PM, Ji TH. Cis- and trans-activation of hormone receptors: the LH receptor. Mol Endocrinol 2002; 16:1299-1308

**41.** Ji I, Lee C, Jeoung M, Koo Y, Sievert GA, Ji TH. Trans-activation of mutant follicle-stimulating hormone receptors selectively generates only one of two hormone signals. Mol Endocrinol 2004; 18:968-978

**42.** Andric N, Thomas M, Ascoli M. Transactivation of the epidermal growth factor receptor is involved in the lutropin receptor-mediated down-regulation of ovarian aromatase expression in vivo. Mol Endocrinol 2010; 24:552-560

**43.** Hsieh M, Thao K, Conti M. Genetic dissection of epidermal growth factor receptor signaling during luteinizing hormone-induced oocyte maturation. PLoS One 2011; 6:e21574

**44.** Panigone S, Hsieh M, Fu M, Persani L, Conti M. Luteinizing hormone signaling in preovulatory follicles involves early activation of the epidermal growth factor receptor pathway. Mol Endocrinol 2008; 22:924-936

**45.** Hsieh M, Lee D, Panigone S, Horner K, Chen R, Theologis A, Lee DC, Threadgill DW, Conti M. Luteinizing hormone-dependent activation of the epidermal growth factor network is essential for ovulation. Mol Cell Biol 2007; 27:1914-1924

**46.** Rivero-Müller A, Chou YY, Ji I, Lajic S, Hanyaloglu AC, Jonas K, Rahman N, Ji TH, Huhtaniemi I. Rescue of defective G protein-coupled receptor function in vivo by intermolecular cooperation. Proc Natl Acad Sci U S A 2010; 107:2319-2324

**47.** Zhang M, Guan R, Segaloff DL. Revisiting and questioning functional rescue between dimerized LH receptor mutants. Mol Endocrinol 2012; 26:655-668

**48.** Smith SM, Lei Y, Liu J, Cahill ME, Hagen GM, Barisas BG, Roess DA. Luteinizing hormone receptors translocate to plasma membrane microdomains after binding of human chorionic gonadotropin. Endocrinology 2006; 147:1789-1795

**49.** Lei Y, Hagen GM, Smith SM, Liu J, Barisas G, Roess DA. Constitutively-active human LH receptors are self-associated and located in rafts. Mol Cell Endocrinol 2007; 260-262:65-72

**50.** Mazurkiewicz JE, Herrick-Davis K, Barroso M, Ulloa-Aguirre A, Lindau-Shepard B, Thomas RM, Dias JA. Single-molecule analyses of fully functional fluorescent protein-tagged follitropin receptor reveal homodimerization and specific heterodimerization with lutropin receptor. Biol Reprod 2015; 92:100

**51.** Jiang X, Fischer D, Chen X, McKenna SD, Liu H, Sriraman V, Yu HN, Goutopoulos A, Arkinstall S, He X. Evidence for Follicle-stimulating Hormone Receptor as a Functional Trimer. J Biol Chem 2014; 289:14273-14282

**52.** Breen SM, Andric N, Ping T, Xie F, Offermans S, Gossen JA, Ascoli M. Ovulation involves the luteinizing hormone-dependent activation of G(q/11) in granulosa cells. Mol Endocrinol 2013; 27:1483-1491

**53.** Feng X, Zhang M, Guan R, Segaloff DL. Heterodimerization between the lutropin and follitropin receptors is associated with an attenuation of hormone-dependent signaling. Endocrinology 2013; 154:3925-3930

**54.** Jonas KC, Chen S, Virta M, Mora J, Franks S, Huhtaniemi I, Hanyaloglu AC. Temporal reprogramming of calcium signalling via crosstalk of gonadotrophin receptors that associate as functionally asymmetric heteromers. Sci Rep 2018; 8:2239

**55.** Casarini L, Riccetti L, De Pascali F, Nicoli A, Tagliavini S, Trenti T, La Sala GB, Simoni M. Follicle-stimulating hormone potentiates the steroidogenic activity of chorionic gonadotropin and the anti-apoptotic activity of luteinizing hormone in human granulosa-lutein cells in vitro. Mol Cell Endocrinol 2016; 422:103-114