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Mechanistic insight into how gonadotropin hormone receptor complexes direct signaling[1]

Running title

Gonadotropin hormone receptor signaling complexes

Summary sentence

How gonadotropin hormone/receptor complexes direct signaling and physiological responses via receptor-receptor and receptor-signalosome interactions.

Keywords

Gonadotropin hormones, gonadotropin hormone receptors, luteinizing hormone receptor, follicle stimulating hormone receptor, dimer, oligomer, signaling, homomer, heteromer, crosstalk.

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Abstract

Gonadotropin hormones and their receptors play a central role in the control of male and female reproduction. In recent years, there has been growing evidence surrounding the complexity of gonadotropin hormone/receptor signalling, with it increasingly apparent that the Gαs/cAMP/PKA pathway is not the sole signalling pathway that confers their biological actions. Here we review recent literature on the different receptor-receptor, receptor-scaffold and receptor-signaling molecule complexes formed and how these modulate and direct gonadotropin hormone-dependent intracellular signal activation. We will touch upon the more controversial issue of extragonadal expression of FSHR and the differential signal pathways activated in these tissues, and lastly, highlight the open questions surrounding the role these gonadotropin hormone receptor complexes, and how this will shape future research directions.

Introduction

Receptor-mediated control is essential for the coordination of most physiological processes. The gonadal actions of the gonadotropin hormone receptors (GpHRs), follicle stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) are critical for reproduction, with modulation in their expression and/or activity resulting in reproductive disorders including premature ovarian insufficiency [1-3], anovulation [4], ovarian hyperstimulation syndrome [5, 6] and polycystic ovarian syndrome [7-9], to name but a few. Unsurprisingly, the GpHR are key targets of assisted reproductive technologies, which drives the on-going interest and need for advances in our knowledge of how these receptors mediate their reproductive functions, to provide novel therapeutic strategies for targeting their specific actions in reproductive disorders.

As Class A G protein-coupled receptors, early studies showed the GpHRs to primarily couple to Gαs to mediate their physiological functions. A plethora of evidence now exists that shows that this is an overly simplistic view, with these receptors coupling to multiple G proteins, and activating alternative/additional signaling pathways through G protein-independent pathways via the molecular scaffold β-arrestin. In this review, we will introduce the canonical and non-canonical signaling pathways activated by FSHR and LHR. We will discuss how the different receptor-signaling, receptor-scaffold and receptor-receptor complexes modulate and direct gonadotropin hormone-dependent intracellular signaling. We will touch upon the more controversial issue of extragonadal expression of FSHR and the differential signal pathways activated in these tissues. Lastly, we will finish with open questions surrounding the role these GpHR complexes, and how this will shape future research directions in this field.

Canonical and non-canonical GpHR signaling.

It is widely agreed that the effects of gonadotropin hormones and their differential functions in their target cells are mediated by the activation of Gαs/adenylyl cyclase/cAMP/PKA pathway. However, it is becoming increasingly apparent that this is an over-simplified model of gonadotropin hormone/receptor actions. Other signaling cascades, which are involved in several cellular processes such as proliferation and differentiation, have now been identified as important GpHR targets (See Figure 1 for summarizing overview). For example, GpHRs have also been shown to activate Gαq/Ca^{2+}/IP_3, which translates the extracellular signal to a variety of intracellular physiological responses [10].

Gαs/cAMP/PKA pathways

Before addressing the additional/alternative signaling pathways activated by the GpHRs, we would be remiss in not summarizing the conventional/canonical pathways activated. The stimulatory G protein (Gαs) is the best documented signaling pathway activated by the GpHRs. Gαs interacts with the intracellular loops 2 (IL2) and 3 (IL3) of hormone-bound FSHR on the ERW and BBXXB motifs, resulting in adenylyl cyclase activation, cAMP production, and downstream activation of cAMP-dependent targets, such as PKA, [11]. For the LHR, Gαs also interacts with IL2/TM3 interface and IL3, however this occurs via changes in interaction patterns of R^{464} in the (E/D)R(Y/W) highly conserved motif [12, 13].

FSH is required for growth and maturation of ovarian follicles in female and for normal spermatogenesis in male [14]. FSH supports the maturation of preovulatory follicles, whereby FSH/FSHR stimulates estrogen production in granulosa cells by inducing aromatase expression in a Gαs/cAMP/PKA dependent manner. Likewise, in males FSH -dependent spermatogenesis and steroidogenesis is also Gαs/cAMP/PKA mediated, via FSH-dependent up-regulation of steroidogenic acute regulatory protein (StAR) expression [14]. Indeed, knock-out mouse models lacking either the FSH β-subunit or the FSHR result in significant reproductive defects in both sexes [15, 16]. Consistently, rare human inactivating mutations in either FSH β-subunit or FSHR lead to similar reproductive defects [17].

Depending on the physiological situation, FSH has to control distinct, sometimes opposite, integrated biological responses in its target cells, ranging from differentiation, cellular metabolism, steroidogenesis and proliferation, to apoptosis [17]. In preantral granulosa cells, FSH-dependent MAPK/ERK activation is mediated via PKA-dependent destabilization of the constitutive MEK

phospho-tyrosine phosphatase, dual specificity phosphatase 6 (DUSP6). FSHR is phosphorylated by PKA and PKC second messenger-dependent kinases, in addition to G protein-coupled receptor kinases (GRK) 2, 3, 4 and 6. Additionally, FSH-dependent activation of PKA activates cross-talk with Akt-dependent pathways to stimulate mTOR and P70S6 kinase, which subsequently leads to mRNA translation [18]. In granulosa cells, this FSH-dependent regulation of mRNA translation enhances granulosa cell proliferation, via FSH-dependent ERK mediated phosphorylation of tuberin, in a Gαs dependent-manner. Such activation of the mTOR effector, tuberin, stimulates P70S6K activity, leading to enhanced cyclin D2 expression [18]. Moreover, activation of the mTOR pathway by FSH induces expression of follicular differentiation markers, including LHR, inhibin-α, aromatase and the subunit of PKA, βII [18], showing the importance of signaling pathway crosstalk in mediating the physiological functions of FSH/FSHR.

While PKA is deemed to be the master regulator of transcription factors of the cAMP response element-binding protein (CREB) and activating transcription factors (ATF) family FSH-mediated PKA activation has been shown to cross talk with the MAPK/ERK cascade, to control Sertoli cell mitotic phase and the activity of nuclear targets, such as retinoic acid receptor α [19], a critical modulator of male germ cell development. Studies utilizing granulosa-cell based microarrays have found a similar gene expression profile in 108 genes in FSH versus PKA-CQR, a constitutively active mutant of PKA [20]. Furthermore, PKA has been shown to be involved in global chromatin remodeling, where H3 histone phosphorylation occurs in FSH stimulated granulosa cells [21]. This is of importance when considering the relevance for alternative pathways, such as β-arrestins, which can also target histone post-translational modifications, such as acetylation.

For LH/LHR, cAMP was also thought to be the primary LH-dependent signal which mediates the LH surge, as activation of adenylyl cyclases (AC) via the AC agonist forskolin, could mimic the ovulatory effects of LH [22]. Indeed, LH-dependent cAMP signaling has been shown to play an important role in ovulation, controlling multiple follicular functions including steroidogenesis, the stimulation of cyclooxygenase/lipooxygenase expression leading to increased prostaglandin/leukotriene synthesis; and the stimulation of plasminogen activator, which catalyzes the conversion of plasminogen to plasmin [23]. Although the Gαs pathway is important in these processes, recent studies also suggest that the Gαq pathway may also have important roles in aspects of LH-mediated ovulation [24-26], but this will be discussed in later sections. In rodents, LHR is expressed in mural granulosa and theca cells, leading to the hypothesis that paracrine signaling and intercellular communication are also essential for cumulus-oocyte complex response to the LH surge [22]. Moreover, studies have shown that heterodimerization between LHR and FSHR is associated with an attenuation of LH-dependent Gαs-signaling [27-29]. More recently, FSHR and LHR crosstalk was found to alter LH-induced Gαq/11 calcium signaling, which will also be discussed in more detail in latter sections [28].

Functional crosstalk between LH/LHR activated pathways has also been suggested for Gαs- Ras activation. In primary rat Leydig cell cultures, the cell permeable cAMP mimetic, 8 bromo-cAMP was shown toactivate Ras/MEK and ERK1/2 phosphorylation in a PKA-dependent manner [30]. Further studies have shown contradictory data whereby the activation of Ras is PKA-independent, with further studies required to fully delineate this process [31-33].

Interestingly, the two endogenous ligands of LHR, LH and human chorionic gonadotropin (hCG) have been reported to show functional diversification in the signal pathways they activate. hCG was found to be more potent in stimulating cAMP production, whereas LH was a more potent

activator of ERK and AKT phosphorylation, with differential downstream effects on steroidogenesis, apoptosis and proliferation [34]. This is of significance, as LH and hCG have traditionally believed to be biologically equivalent, and hCG is widely used in clinical practice in place of LH for stimulating the 'LH' surge and ovulation during ovarian stimulation protocols of IVF, highlighting the need for further research in this area.

Gαq/11/Ca2+/IP3/PKC pathway

In addition to the conventional Gαs/cAMP/PKA signaling, both LHR and FSHR have also been shown to couple to Gαq/11, in the presence of high concentrations of the ligand and/or receptor, in multiple cell types [24-26, 35, 36]. In isolated rat Sertoli cells, inhibition of phospholipase C diminished FSH-induced Ca^{2+} influxes in a concentration-dependent manner. Moreover, treatment with a Gαs inhibitor had no effect on this response, highlighting the direct FSH/FSHR-dependent regulation of Ca^{2+} influx in a distinct manner form the Gαs/cAMP pathway [35]. Similarly, crosstalk between PKC and cAMP/PKA pathways has been described in Sertoli cells, with the intracellular release and rapid influxes of Ca^{2+} resulting in the activation of such kinases. There is increasing evidence that PKC is an additional effector of FSH, with roles in oocyte maturation, cumulus oocyte complex expansion and modulation of progesterone production within in the ovary [37, 38]. FSHR activated intracellular Ca^{2+} release in human embryonic kidney (HEK) and virally transduced human granulosa (KGN) cells has also been linked to interaction between APPL-1 and FSHR, which will be discussed in latter sections of this review. Moreover, studies in mouse ovaries have found that the expression of PKC isoforms can change according to developmental stage,

which is suggestive of different isoforms controlling specific ovarian functions, such as follicular maturation, ovulation and luteinization, from pre-puberty to adulthood [38, 39].

For LHR, *in vivo* studies with granulosa cell specific deletions of Gαq/11 determined the physiological role of LHR-mediated Gαq/11 protein activation during ovulation [40]. This elegant study revealed that $G\alpha q^{fl/fl}$ mice were sub-fertile due to the entrapment of the oocytes in preovulatory follicles and corpora lutea. The defect in follicular rupture was concluded to be secondary to the failure of LHR to fully induce the expression of progesterone receptor in these Gαq/11 knockout animals, with ovulation reduced by approximately 50% and fertility diminished by approximately 85% [40]. Our *in vitro* studies also suggest a role of LHR-FSHR heteromeric complexes in this process, which we will be discussed in further detail in latter sections.

Extragonadal activation of GpHRs and differential signal pathway activation

Although controversial, there is gathering evidence that GpHRs are present in and have distinct roles in extragonadal tissues including the placenta [41], vessel smooth muscle cells [42], bone osteoclasts [43], adipocytes [44], myometrium, endometrial stromal cells and glandular epithelium [45] and monocytes [46]. The presence of FSHR in these tissues has been linked to the promotion of angiogenesis, skeletal integrity, myometrial contractility, and adipose tissue accumulation [42, 43, 47]. FSHR has been found in the epithelial cells of fetal vasculature within the chorionic villi and villous stromal cells in human placenta but is not present in trophoblast cells. Furthermore, functional studies in HUVECs showed FSH-stimulated AKT activation, but not cAMP production [47]. The stimulation of these cells with FSH also resulted in tube formation, cell migration and proliferation, nitric oxide production, cell survival and wound healing [42, 47]. FSHR was detected

in the endothelial cells of both non-pregnant and pregnant myometrium vessels [47]. Moreover, activation of the FSH/FSHR pathway activated protein Gαq/11 in endothelial cells, promoting activation of the VEGFR-2 pathway, even in the absence of VEGF, a result that could induce proliferation and migration, independent of VEGF. Functionally, FSHR has been shown to drive bone resorption via Gαi activation, atypical to the conventional Gαs signaling. FSHR-dependent Gαi2 signaling was shown to activate ERK/MAPK, Akt, and NF-kB pathways, to promote osteoclast formation, function and survival. Furthermore, blocking the Gαi2 pathway, or absence of Gαi2 results in bone unresponsiveness to FSH [48]. Both the myometrium and endometrium have been shown to express FSHR [47]. In a separate study, FSH was shown to regulate myometrial contractility at supraphysiological doses. Interestingly, cAMP was found to be activated in all cases, however, IP3 was only stimulated with high FSHR densities. Furthermore, these pathways initiated either myometrial contractility quietening (cAMP) or an activation of myometrial contractility (IP3), suggesting that FSHR densities dictate the action of FSH on the myometrium [49], suggesting a potential role for FSHR complexes that may direct FSH/FSHR-signal activation and preference. FSHR has been identified in mouse, human and chicken adipocytes, where FSH directly stimulates the adipocytes via Gαi signaling [48]. This signaling results in an upregulation of adipogenic genes, such as *Fas*, *Lpl* and *Pparg*, along with an induction of lipid biosynthesis. Interestingly, FSHR activation leads to cAMP reduction and subsequent UCP1 inactivation in differentiated brown fat cells, which is in contrast with the mechanism of action of β3AR, which causes differentiation of white to beige adipocytes via Gαs and cAMP production [48]. Although the functional roles of extragonadal FSHR are being delineated, these roles remain controversial, mainly due to issues with reproducibly detecting FSHR expression in

193 these tissue types [47, 50-52]. Thus, highlighting the requirement of further studies and more
194 sophisticated genomic approaches to address this important question.

195

196 **GpHR complexes and internalization**

197 Traditionally, receptor internalization was viewed as a mechanism to desensitize and down
198 regulate plasma membrane GPCR- G protein-dependent signaling. However, recent studies that
199 have harnessed refined live imaging microscopy-based technologies have shown this to be too
200 simplistic a view, with internalized GPCRs continuing to signal from endosomal compartments,
201 as a means to regulate signal output. For the GpHRs, the intricate link between receptor
202 internalization and compartmentalized signaling is beginning to emerge, with interesting roles of
203 molecular scaffolding and trafficking proteins revealed.

204

205 *β-Arrestins*

206 Having first been identified as a negative regulator of GPCR signaling, the role of β -arrestins as
207 a scaffold for different signaling proteins is now well recognized. The role of β-arrestin in receptor
208 trafficking is determined by the confirmation they adopt following receptor binding. As β-arrestin
209 can bind to either the transmembrane core or the C-tail of GPCRs [53, 54], and more than one
210 GRK subtype can be expressed in a given cell, with more than one Ser/Thr potentially being
211 phosphorylated, different receptors show distinct phosphorylation patterns. These patterns, or
212 phosphorylation barcodes, can be sensed through two domains of the β-arrestin molecule; the
213 phosphorylation sensor, and activation sensor [53, 54], which results in distinct conformational
214 signatures that are recognized by different downstream effectors and signaling molecules. The

internalization and recycling of FSHR is mediated by GRK phosphorylation and β-arrestin binding, in a clathrin-dependent manner [18, 53]. In the case of FSHR, a cluster of five Ser/Thr residues in its C-tail has been identified as key sites for GRK 5 and 6 phosphorylation [55]. Interestingly, while GRK 5 and 6 promote β-arrestin binding for signaling and scaffolding [56], GRK 2 is required for β-arrestin mediated FSHR desensitization [53, 57, 58]. In contrast, human, porcine and murine LHR recruit β-arrestins in a phosphorylation independent manner [59], via engagement of ADP ribosylation factor 6 (ARF6) and its intracellular loop (ICL) 3 [59, 60], via the following mechanism: in its inactive state, GDP-bound ARF6 is bound to β-arrestin and is anchored to the plasma membrane, upon ligand binding and LHR activation, ARF is activated, triggering ARF6 GDP-GTP exchange and the subsequent release of β-arrestin. The released β-arrestin binds to the LHR via ICL3, which mediates its desensitization and internalization [53, 57].

A well-documented illustration of the example of the ability of β-arrestin to interact with specific signaling partners is the temporal encoding of MAPK/ERK activation. In contrast to G protein-mediated ERK/MAPK which is rapid and transient, β-arrestin activation of this ERK/MAPK occurs at a slower rate and has a longer half-life [53]. FSHR has been shown to activate the ERK/MAPK pathway via a mechanism involving MEK-dependent β-arrestin 1 phosphorylation at Thr383, where this agonist-induced phosphorylation of β-arrestin is required for EEK/MAPK recruitment to the β-arrestin complex, and ERK/MAPK activation [53]. Additionally, LHR also exhibits a sustained ligand-induced ERK/MAPK signaling profile, which requires receptor internalization, where β-arrestin's are involved in the internalization of LHR, therefore highlighting the potential for additional scaffolding roles for this adaptor protein. Specifically, work carried out in tumor derived Leydig MA-10 cells demonstrated that activation

of tyrosine kinase Fyn, a known activator of LHR-induced ERK1/2 phosphorylation, was dependent on β-arrestin 1/2 [53, 61, 62].

Interestingly, for FSHR, the expression levels of the receptor can determine signaling bias to arrestin-dependent signaling pathways. For example, studies in FSHR A189V mutant mice revealed that low receptor expression levels at the plasma membrane result in FSHR signaling biasing to activation of β-arrestin only, with no detectable cAMP production [63]. Furthermore, work in an immortalized human granulosa tumor cell line found LHR and FSHR-dependent ERK signaling. Knocking down arrestin expression resulted in FSHR mediated activation of cAMP/PKA pathway and apoptosis, which is suggestive that arrestin expression levels and arrestins themselves playing a role in dictating pathway bias and specific modulation of physiological responses. In terms of FSHR functions during follicular and Sertoli cell development, this is of significance as levels of proliferation and apoptosis may be tightly regulated via such mechanisms.

In contrast to that discussed above, G proteins and β-arrestin have been found to co-operate during persistent heterotrimeric G protein signaling from intracellular endosomal compartments. Indeed, as an example, endosomally localized parathyroid hormone receptor (PTHR) mediated cAMP production has been shown to be enhanced by β-arrestin [54, 57]. Additionally, for the human β2AR, the binding of adrenaline and noradrenaline to cells in the target tissues of sympathetic neurotransmission leads to the activation of Gαs/adenylyl cyclase/cAMP/PKA, and the phosphorylation of proteins involved in muscle-cell contraction. Additionally, it has been shown that persistent β-arrestin associations of Ser/Thr phosphorylation sites within the C-tail of the receptor enables simultaneous G protein binding to the receptor core and formation of megaplexes [64]. The existence and significance of β-arrestin-G protein cooperation has been

investigated for FSHR, where in both HEK293 and Sertoli cells, the integrative action is critical for FSH-dependent ribosomal assembly and mRNA translation. In contrast to PTHR and β2AR, FSHR is not physically involved in the formation of this signaling complex, where it may provide support for a catalytic mediated activation of β-arrestin, such as been shown for β1AR.

GAIP Interacting Protein C-terminus (GIPC) and adaptor protein, phosphotyrosine interacting with PH domain and Leucine Zipper 1(APPL1)

The initial studies that identified GAIP Interacting Protein C-terminus (GIPC) revealed it to be a member of the regulator of protein signaling family protein, which interacts with the C-terminus of GAIP, [65]. Early studies showed that GIPC bound to the C-tail of LHR, an important interaction for directing LHR endosomal localization and signaling [66]. More recent studies have shed further light on the importance of this interacting protein for directing GpHRs to the very early endosome (VEE). GIPC has been shown to re-route the fate of internalized GpHRs from early endosomes (EE) to recycling VEE, whereby enabling sustained ERK phosphorylation [66], (recently reviewed by [53]). Attempts to characterize this physically distinct VEE compartment have shown it to be devoid of the classical early endosomes (EE) markers including EE antigen 1, PI3P and Rab 5 [67], and lacking in alternative interacting/trafficking proteins screened to date [67, 68]. The localization of GpHRs to the VEE requires is dependent on the PDZ motifs localized to the receptor C-tail, which facilitates the interaction between the GpHR and the GIPC [67]. Furthermore, the targeting of these endosomes was dependent on the receptor-GIPC interaction during early endocytosis [67, 69].

The ability of FSHR to interact with GIPC may occur via the direct interaction with the adaptor protein leucine zipper motif (APPL1), a protein which is present on VEEs [69] and can directly bind to GIPC [70]. The adapter protein, APPL1, is present on VEE's highlighting the potential for a functional role for this adaptor protein within the VEE compartments. APPL1 lacks catalytic activity; however, it is composed of various membrane and protein interacting domains, functioning as an endosomal marker in addition to its ability to integrate between different trafficking and signaling pathways from the endomembrane [66, 71]. FSHR has been shown to form complexes with APPL1, via intracellular loop 1, IL1, Lys393, Leu394 and Phe399, which facilitates FSH-induced PI3K/Akt activation, IP_3 production, Ca^{2+} release and the nuclear exclusion of forkhead transcription factor 1 (FOXO1a) [18, 53, 72-74] . Given that FSHR associates with both APPL1 and FOXO1a, and that APPL1 interacts with AKT [73], an interplay between FSHR-FOXO1a complex with an active FSHR-APPL1-Akt complex, which leads to the phosphorylation of FOXO1a and the abrogation of apoptosis has been proposed [18, 73, 75]. In granulosa cells, FSH stimulation results in rapid FOXO1a phosphorylation and extrusion from the nucleus and control of lipid biosynthetic pathways [73, 76]. Interestingly, while GIPC has been shown to be essential for directing GpHRs to the VEE, APPL1 is not required for this process, but is essential for receptor recycling [53, 69]. Moreover, under conditions over-expressing FSHR, FSH-stimulated IP_3 production decreases the expression of aromatase, which is suggestive of an inhibitory role of APPL-1/IP3/Ca^{2+} on steroid hormone production [38]. However, further studies are still required to confirm the result in the presence of physiologically relevant FSHR levels, but these data do suggest that APPL-1 mediated activation of Ca^{2+} signaling is independent of cAMP production, as previously hypothesized.

303 The recycling of LHR from the VEE to the plasma membrane has been shown to be driven
304 by interaction of the internalized LHR with APPL1. The recycling of LHR to the plasma membrane
305 by APPL1 was shown to be directed and dependent LH/LHR activation of Gαs-mediated PKA-
306 dependent phosphorylation of APPL1, at Ser 410 [69], suggesting that LH signal pathway
307 activation directs its internalization and recycling. Interestingly APPL1 knockdown increased LH-
308 dependent cAMP production, but not ERK activation, suggesting the specific regulation of LH-
309 induced Gαs/cAMP production [77, 78]. This suggests that the activity of GpHRs in the VEEs is
310 highly heterogeneous, spatially restricting the cAMP microenvironment to VEE subpopulations
311 and mediating APPL1 phosphorylation, thereby enabling the rapid recycling pathways for GpHR
312 sorting [69].

313 Although not strictly related to APPL1/GIPC mediated mechanisms of GpHR
314 internalization/recycling/signaling, we would be remiss in not discussing a study that showed the
315 first *in vivo* link between GpHR signaling and trafficking. Using the transgenic cytomegalovirus
316 enhancer/chicken β-actin-Epac1-camps mice, this study explored the intercellular communication
317 of the LH signal from the outer granulosa cell layer of an ovarian follicle, to the oocyte. This study
318 found that LHR persistently signals when internalized, contributing to the transmission of LH-
319 dependent signaling effects in follicle cells and the oocyte [79]. Although this made important
320 advances in showing internalized LHR persistently activate cAMP, with potential physiological
321 implications in maintaining the high cAMP required for the maintenance of oocyte meiotic arrest
322 [79], the cellular machinery mediating such persistent cAMP signaling by internalized GpHRs
323 remains to be elucidated.

324

GpHR complexes

GpHR homomers

The ability of GpHRs to self-associate and form homomers has long been documented. The physical LHR-LHR interactions or 'clustering' was first evidenced via electron microscopy images of granulosa and theca cells [80, 81]. The biochemical studies of the late 1990's and early 2000's using co-immunoprecipitation documented the formation of LHR and FSHR homomers in heterologous cell lines and interrogated the stage in receptor processing that GpHRs formed homomers and the ligand dependency of these interactions [82, 83]. More recent advances in biosensor technology and the advent of proximity-based energy transfer techniques such as bioluminescence and fluorescence resonance energy transfer (FRET/BRET) confirmed the earlier biochemical findings showing specific and interactions between LHR-LHR and FSHR-FSHR, however, these studies also showed the inherent stability and ligand-independency in LHR and FSHR homomers, contrasting with the earlier biochemical studies which suggested ligand-dependent changes in LHR homomers formed [83-85]. Additional biophysical studies using time-resolved phosphorescence anisotropy and tracking of LHR in the presence and absence of ligands also provided evidence of LHR-LHR associations [86-88]. Although these studies made important advances in understanding the nature, stability and ligand-dependency of GpHR homomers, determining the functional roles of these receptor complexes has been challenging, requiring innovative approaches and exploitation of the idiosyncrasies of gonadotropin hormone structure-function relationship.

Functional complementation – how forced receptor homomerization directs intracellular signaling pathway activation.

Functional complementation (also known as transactivation or intermolecular cooperation) has been used to dissect key aspects of GPCR oligomerization for many GPCRs [89-97], including the GpHRs. These studies provided the first insight into how LHR and FSHR homomerization directs signal specificity and magnitude. Exploiting the structural knowledge of the GpHRs, these elegant studies utilized the compartmentalized nature of GpHR ligand binding and G protein-signalling domains, with ligand binding predominately mediated by the extracellular N terminal domain and receptor/G protein signal by transmembrane (TM) domain, as evidenced by both naturally occurring and experimentally generated GpHR activating and inactivating mutations (reviewed by [10]). This enabled the generation of both LHR and FSHR mutants that were either unable to bind ligand (ligand-binding defective receptors) but were theoretically still able to couple to G proteins, or mutant receptors that were unable to perpetuate ligand-dependent G protein-signaling (signaling defective receptors) but could bind ligand. *In vitro* experiments confirmed that when these receptors were individually expressed, they could traffic to membrane but had no functional binding and/or signaling activity. Remarkably, when ligand binding defective and signaling defective receptors were co-expressed, they were able to bind hormone and generate ligand-induced G protein-dependent signal activation (reviewed by [98] and [99]). Intuitively, functional restoration and recapitulation of ligand binding and signal activation can only occur via functional complementation and generation of receptor complexes comprised of at least 1 signal- and 1 binding- defective receptor protomers with the minimum functional unit of dimers and presenting the possibility of oligomer formation.

Early experiments utilizing a naturally occurring LHR mutation containing a premature stop codon at transmembrane domain 5, identified from a patient diagnosed with Leydig cell hypoplasia [100]. Although binding of hCG was detected, this mutant LHR failed to activate hCG-dependent cAMP production. To determine if signaling could be recapitulated, a chimeric FSHR/LHR was generated (termed FLR), comprised of the extracellular domain of the FSHR and LHR transmembrane domain. FLR could therefore bind FSH and importantly only generated cAMP in response to FSH and not hCG. Remarkably, when the truncated mutant LHR was co-expressed with FLR and expressing cells treated with hCG, cAMP production was observed, showing that the mutant LHR, that bound hCG (but could not stimulate cAMP production) had trans-activated the FLR [100]. This demonstrated that not only could GpHR protomers undergo functional crosstalk, but also highlighted the homomerization of these receptors was able to recapitulate ligand-dependent signaling via the principle G protein-dependent signaling pathway, Gαs/adenylyl cyclase/cAMP.

Utilization of functional complementation approaches have also explored the signal specificity generated by LHR and FSHR homomers. Mutant FSHR comprised of the N terminal domain fused to either a GPI anchor or CD8 single transmembrane domain co-expressed with differential ligand binding defective mutant FSHR were shown to activate either cAMP or IP3, but not both second messengers [101]. This suggests that differential functional complementation dimeric and oligomeric pairs stabilize different receptor conformations with distinct activational states that communicate and direct the specificity of G protein-coupling, intracellular pathway activation and ultimately physiological responses. This idea is in keeping with recent findings reported for other GPCRs, including the rhodopsin receptor and M2 muscarinic acetylcholine receptor receptors [102]. Our studies utilizing LHR functional complementation mutants also

support this idea, providing insight into how modulating the functional role of each LHR protomer via altering the ratio of ligand binding defective and signaling defective receptors within an oligomer can regulate ligand-directed signal output. Via single molecule imaging of LHR using the super resolution imaging approach of photoactivated dye, localization microscopy (PD-PALM), we quantified the number of LHR monomer, dimers and oligomers at the plasma membrane [97, 103]. Using HEK293 cells stably expressing wild type mouse LHR, or co-expressing ligand binding defective LHR (LHR^{B-}) and signaling defective LHR (LHR^{S-}), we observed that approximately 40% of both WT LHR and LHR^{B-}/LHR^{S-} formed homomers. Analysis of the types of LHR complexes observed showed that wild type LHR preferentially formed dimers, with a small number of lower order and higher order wild type LHR complexes. However, LHR^{B-}/LHR^{S-} formed lower levels of dimers with increased formation in lower order trimers and tetramers. Although there were no ligand-dependent changes in the total number of wild type or functional complementation LHR homomers, nor the type of homomeric complexes formed, distinct differences between WT LHR and LHR functional complementation mutants in LHR and hCG-dependent G protein-dependent signaling were observed. LH and hCG-dependent Gαs activation, as assessed by BRET and cre-luciferase assays showed equal ligand-dependent activation by the WT LHR and LHR functional complementation mutants. However, differences between the WT LHR and LHR functional complementation mutants were observed in the ability of LH and hCG to activate Gαq-dependent Ca^{2+} and IP_1 pathways, with LH-dependent Gαq activation (but not hCG) impaired in the functional complementation model [97, 103] This suggests that for full LH-dependent Gαq activation, an element of cis or unidirectional activation of LHR is required. A similar finding was observed with the related thyroid stimulating hormone receptor (TSHR) dimer, which showed that Gαs activation required ligand binding at a single

414 protomer within the dimer, however, for Gαq activation ligand occupation of both receptor
415 protomers was required [104].

416 Altering the composition and ratio of LHR^{B-}:LHR^{S-} revealed that cells with an excess of
417 cell surface LHR^{B-}:LHR^{S-} amplified both Gαs and Gαq-dependent signaling. Interestingly, the
418 difference in Gαs and Gαq signals observed corresponded with an enrichment in LHR^{B-} receptor
419 protomers in both the trimeric and tetrameric complexes. Together this suggests that modulating
420 the composition and functional role of a protomer engaged in an oligomeric complex can fine tune
421 the amplitude of G protein-dependent signaling responses generated.

422 Studies interrogating the mechanism of functional complementation have shown that not
423 all binding and signal defective mutant LHR and FSHR pairs can undergo functional
424 complementation when co-expressed [105, 106]. This suggests that for functional
425 complementation to occur, a more nuanced structural specificity in receptor mutant pairing that
426 promotes and facilitates inter-protomer communication is required. A critical factor appears to be
427 the location of the point mutation in the binding defective receptor mutants, as the N terminal
428 extracellular region of the GpHRs are comprised of leucine rich repeats (LRR), which are essential
429 for mediating ligand binding and appear to be a key factor in the facilitation of receptor
430 transactivation. As such, binding defective mutant LHRs with mutations that are localized to the
431 LRR regions 1-3 could undergo functional complementation when co-expressed with signal
432 defective LHR. However, if binding defective mutations were located to LRR regions 4-8,
433 functional complementation failed to occur [105, 106]. This is most likely due to the proximity of
434 LRR 4-8 to the hinge region, a crucial region which communicates ligand binding to the TM
435 region, to enable signal activation. The hinge region also contains a suppressor of TM activation,
436 which constrains the unliganded receptor in an inactive conformation. On ligand binding, this

437 constraint is relaxed, however mutations in LRR4-8 may interfere with the conformational changes
438 that occur which enable TM activation and therefore prevent receptor transactivation occurring.

439 Although our current knowledge of the physiological significance of GpHR
440 homomerization remains limited, our previous work has begun to shed some light on this. Also
441 utilizing a functional complementation approach, we showed that LHR homomerization was
442 sufficient to mediate LHR functions in male mice, *in vivo*. Employing the LHR knockout (LuRKO)
443 mice [107], and a BAC transgenic approach to ensure targeted co-expression of ligand binding
444 defective and signaling defective mutant LHRs, we showed that co-expressing LHR binding and
445 signaling defective mutants could rescue the hypogonadal phenotype and fertility of male LuRKO
446 mice [108]. Moreover, serum testosterone levels in the functional complementation mouse line
447 were equivalent to wildtype litter mates. Although, serum LH was slightly increased when
448 compared to wild type littermates, as a result of the increased hypothalamic-pituitary drive to
449 initiate and maintain LH-dependent testosterone production. This study provided the first evidence
450 of the physiological relevance of GpHR (and Class A GPCR) homomerization, which until this
451 point had been speculation only [108]. A subsequent *in vitro* study has critically debated our
452 observations, suggesting that our results were due to the idiosyncrasies of the BAC transgenics
453 [109]. However, the control experiments from our study conclusively showed that expressing the
454 single binding or signaling mutant LHRs failed to rescue the hypogonadal phenotype of the
455 LuRKO mice [108], providing confidence in LHR functional complementation occurring in male
456 mice *in vivo*. Interestingly, co-expression of LHR^{B-} and LHR^{S-} in female LuRKO mice has no
457 effect on the hypogonadal phenotype of the LuRKO animals (Rivero Muller *et al.*, unpublished
458 data). This may reflect the low levels of LHR^{B-} and LHR^{S-} expression in female mice, and the
459 inability to induce the dynamic and cellular compartmentalized changes in LHR expression that

460 are required during the ovarian cycle. It may also additional reflect the inability of functional
461 complementation to mediate the multiple signaling and functional requirements of LHR in
462 females, including LH-dependent Gαq activation, as previously discussed.

463

464 *GpHR heteromerization*

465 Within the dominant follicle, there is a unique window when LHR and FSHR are co-expressed
466 within granulosa cells, posing the question of whether LHR and FSHR can heteromerize, and
467 importantly if they do associate, the functional and physiological roles of such heteromers. The
468 first biophysical evidence of LHR/FSHR heteromerization came from BRET experiments showing
469 that LHR and FSHR could co-associate [90] and functional complementation experiments
470 suggested crosstalk between LHR and FSHR. Later studies utilized BRET and fluorescence
471 correlation spectroscopy to additionally support the physical association of LHR and FSHR [27,
472 110]. However, the functional importance of FSHR /LHR heteromers was first demonstrated from
473 the interesting finding that co-expression of LHR and FSHR negatively regulated both LH and
474 FSH-dependent Gαs-dependent cAMP activation [111]. Our follow-on studies investigating the
475 effect of co-expressing FSHR and LHR on Gαq-dependent signaling showed that expression of
476 LHR alone produced a transient Ca^{2+} mobilization. However, co-expression of LHR/FSHR
477 produced a more sustained Ca^{2+} response, that was dependent on activation of Gαq -and on influx
478 of extracellular Ca^{2+}. As previously discussed, LH-dependent Gαq activation has been shown to
479 be important for mediating key facets of ovulation, it was therefore important to establish the
480 existence of the sustained Ca^{2+} response in a physiologically relevant cell type. Using human
481 granulosa lutein cells, which endogenously co-express LHR and FSHR, the presence of a sustained

Ca^{2+} response in this physiologically relevant cell type was confirmed. Moreover, the sustained Ca^{2+} response was also found to be sensitive to extracellular calcium channel blockers, suggesting the requirement for extracellular Ca^{2+} to mediate the sustained LH-dependent Ca^{2+} response. To determine the nature and composition of the LHR/FSHR heteromers mediating this switch from transient to sustained LH-dependent Ca^{2+} signaling, we carried out super resolution imaging of LHR/FSHR heteromers in HEK293 cells using PD-PALM. In contrast to our previous data with LHR homomers, an LH-dependent increase in the number of LHR/FSHR heteromers formed was observed. Moreover, analysis of these complexes revealed the specific enrichment in LHR/FSHR heterotetramers. These finding suggests that cell surface re-organization of LHR/FSHR heteromers, via enrichment of the heterotetramers, mediated the switch from transient to sustained LH-dependent calcium signaling. Other studies have also observed functional crosstalk between LH/hCG and FSH, with FSH potentiating of the respective effects of LH and hCG on apoptosis and steroidogenesis. These findings provide a tantalizing hint at the potential physiological roles LHR/FSHR heteromers. However, understanding how the LHR/FSHR heteromers switch in signaling regulates physiological processes in vivo, remains to be determined.

GpHR- Growth Factor receptor cross talk

Although a little beyond the scope of this review, we would be remiss in not briefly discussing the growing evidence for functional cross talk between the GpHRs and growth factors receptors and the physiological relevance of these interactions. Crosstalk between LHR and the epidermal growth factor receptor (EGFR) in the peri-ovulatory follicle has been well characterized, with important roles determined in mediating key ovulatory processes. Transactivation between LHR and EGFR is initiated by the rapid LH-dependent upregulation of EGF-like peptide expression, in

a PKA-dependent manner. This functional cross-talk initiates a series of complex EGFR mediated intracellular cascades to ultimately induce oocyte meiotic resumption (we direct you to these comprehensive reviews for more thorough updates on this [112-114]). Recent evidence also suggests a role of this EGF/EGFR signaling network in the control of maternal transcript translation in the quiescent oocyte, a process that is integral to oocyte developmental competency. Thus, demonstrating the importance of LHR-EGFR pathway cross talk [115-117].

The role of lipid rafts in directing GpHR complex- G protein-dependent signaling

Single particle tracking studies have provided evidence of LHR localization to specialized membrane microdomains. Both rat and human LHR have been shown to undergo hCG-dependent reorganization into lipid rafts, with microdomains smaller than unliganded rLHR and hLHR [86-88]. Interestingly, when the microdomains were disrupted using the cholesterol depleting agent, methyl-β-cyclodextrin, a decrease in hCG-dependent cAMP accumulation was observed but not a total abrogation, suggesting that the ligand-dependent membrane organization of LHR into lipid rafts is not essential for Gαs coupling. Additionally, constitutively active mutant (CAM) LHRs were also shown to localize to lipid rafts [118], with domains approximately the same size as ligand-bound LHR, showing localization to lipid rafts was dependent on active conformation of the receptor. Interestingly, disruption of the lipid rafts via methyl-β-cyclodextrin also had no effect on the constitutive cAMP production of the CAM LHR, also supporting that LHR localization to lipid rafts is not essential for Gαs activation [118]. Once localized to the microdomains, LHR showed decreased lateral diffusion within the membrane. Recent studies have suggested a role for LHR aggregates within lipid rafts during desensitization, with movement of LHR back to the bulk

plasma membrane when recovered [119], suggesting a role of these microdomains in regulating signal duration. However, the relationship between GpHR raft location and activation of alternate G protein-dependent pathways, such as Gαq, and G protein-independent signaling pathways remains largely unknown. But given the changing lipid environment during the ovarian cycle and the dynamic and changing functional roles of LH/hCG/LHR during folliculogenesis, ovulation and the luteal phase, these microdomains may be one factor that facilitates the diverse signal requirements of LHR to mediate these physiological processes.

Perspectives and conclusions

In recent years, GpHRs have emerged as being capable of activating multiple complex signaling pathways, in both gonadal and non-gonadal tissues. Whilst great steps have been taken in our understanding of the pathways activated, there are several important unanswered questions that remain. Firstly, the link between dimerization and trafficking is still unknown. It is clear that GpHR trafficking and recycling control and direct GpHR signaling, yet the endocytotic processing and fate of differential GpHR homomeric and heteromeric complexes and the impact on intracellular signaling remains unknown. With recent developments in single molecule imaging, a more precise understanding of the mechanisms of receptor activation, internalization and oligomerization, will follow, and with the advent of techniques such as cryo-EM, an understanding of the distinct conformations adopted by the receptor upon association with different ligands, allosteric modulators, and effector proteins will also unfold. While extensive work is being carried out in these areas, the majority of studies have been restricted to heterologous cell lines engineered to express the GpHRs, which is far from ideal. Unpicking the link between the complexes formed and the functional roles *in vivo* remains a major caveat to the current research. Likewise,

understanding the roles for these diverse signaling pathways in modulating the functions of non-reproductive GpHRs and importantly in infertility remains to be determined. Elucidating such links may help to identify small molecule analogues which can manipulate complexes formed to direct signaling and provide more efficacious, alternative approaches to the current therapeutic treatment strategies. Lastly, it is critical that we continue assembling the complex signaling network and modules that are activated by GpHRs to understand and reveal the mechanisms which control the preferential activation of distinct pathways and understand the crosstalk between the different signaling cascades triggered upon GpHR activation. In understanding this, we will gain important insights into how the modulation of these pathways governs transcriptional, translational and posttranslational processes. Integrating new advances at both the cellular and molecular levels in GpHR activation with in vivo models and clinical medicine will facilitate the ability to pharmacologically target receptor signaling with high fidelity, therefore ultimately leading to the development of efficacious and specific therapeutics for infertility and beyond.

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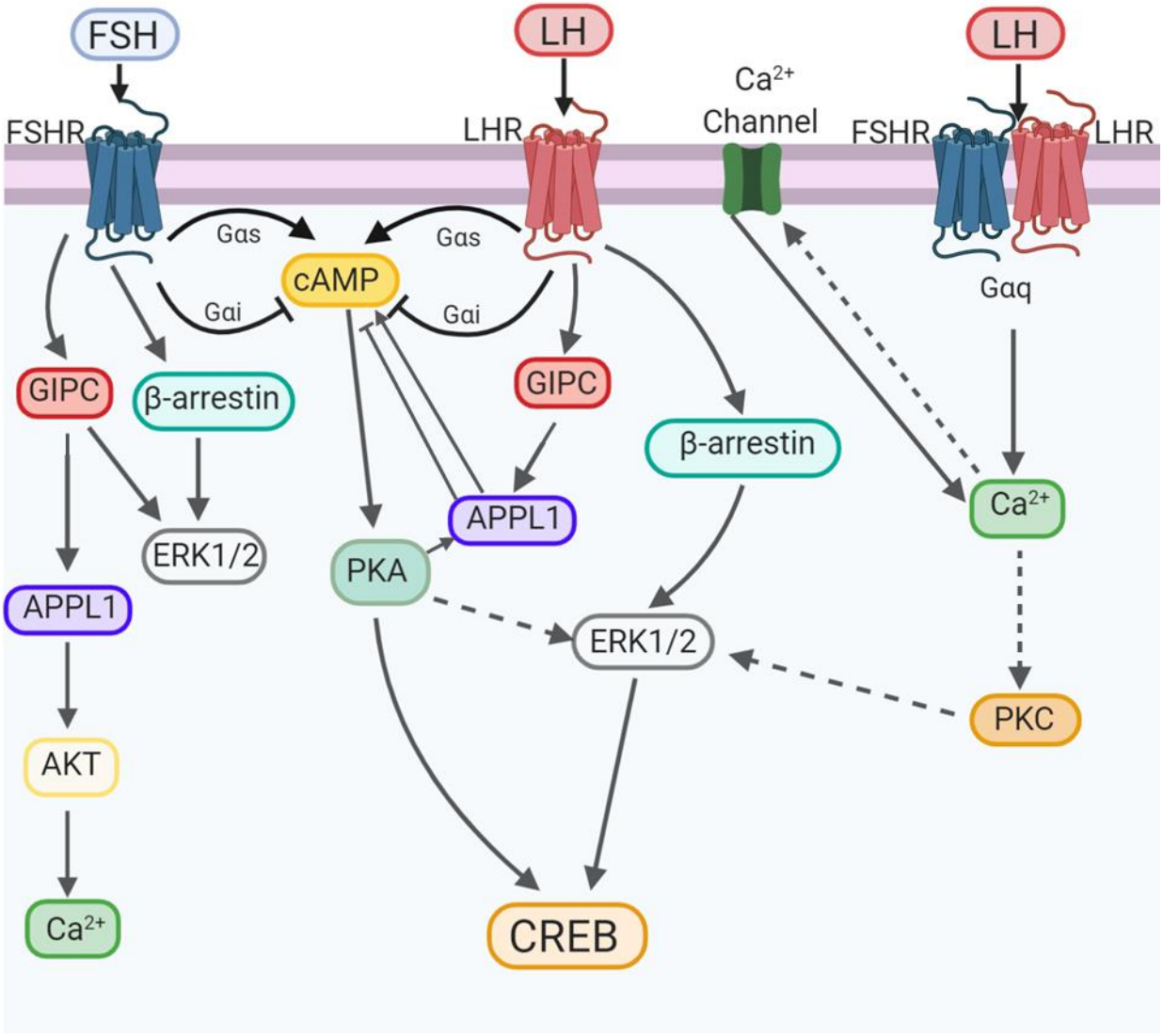
Figure 1. Simplified schematic of GpHR complex signaling. FSHR and LHR G protein-dependent and independent pathway activation through receptor-receptor interactions, receptor-G signaling protein interactions and receptor-adapter/scaffolding protein interactions. Dashed lines represent currently hypothesized/unresolved mechanisms of pathway activation and/or crosstalk, blunt ended arrows, inhibitory pathways and spear-headed arrows, activated pathways.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Gαq
GIPC
β-arrestin
GIPC
β-arrestin
APPL1
Ca2+
ERK1/2
PKA
APPL1
ERK1/2
PKC
AKT
Ca2+
CREB

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